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TRACE METALS IN BLOOD AND URINE AS POTENTIAL MARKERS OF BONE BREAKDOWN IN PATIENTS WITH BONE METASTASES

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TRACE METALS IN BLOOD AND URINE AS POTENTIAL MARKERS OF BONE BREAKDOWN IN PATIENTS WITH BONE METASTASES

by

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ABSTRACT

TRACE METALS IN BLOOD AND URINE AS POTENTIAL MARKERS OF BONE BREAKDOWN IN PATIENTS WITH BONE METASTASES.

Fiona Jane Roberts

In the western world cancer is the second most important cause of death, after heart disease, accounting for 20-25% of all mortalities, and is today probably one of the most feared diseases. Approximately one third of all cancer patients will develop skeletal metastases (secondary bone cancer). Early diagnosis and effective monitoring during treatment is therefore essential in terms of making any impact on survival rates and developing new cancer therapies.

Unfortunately, the current methods for diagnosing and measuring bone metastases, such as bone scans and urinary hydroxyproline determinations, lack sensitivity and specificity. The urinary pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPYD), have recently been identified as sensitive and specific markers of bone breakdown. However the analysis of the pyridinium crosslinks using high performance liquid chromatography (HPLC) has proved far from ideal for routine clinical assessment. The results from studies to critically evaluate this method are presented and particular problems encountered when the crosslinks are extracted from the urine samples are discussed. The tedious, time consuming and cumbersome sample preparation procedure are also shown to adversely effect the robustness and reproducibility of the method. The recent introduction of an immunoassay method potentially overcomes many of the inherent problems with the HPLC analysis. This enzyme linked immunoasorbant assay (ELISA) is evaluated and found to compare favourably with the HPLC method, offering several distinct advantages. The method is quick, simple, robust, demonstrates good accuracy and precision, is less prone to interferences and can be easily introduced into clinical laboratories on a routine basis. In addition it also minimises sample preparation time. However, there is still a requirement for alternative and better biochemical markers to measure bone breakdown.

It is well known that bone is an active, living tissue and that bone metabolism and remodelling are tightly coupled processes, where the rate of bone formation equals the rate of bone resorption in healthy bone. When an imbalance occurs, this leads to unhealthy bone and ultimately a clinical disease of the skeleton. Some trace metals, e.g lead, accumulate in the bone and since the development of bone metastases results in extensive bone breakdown, the subsequent release of these metals into the blood and urine may potentially serve as markers. In this work inductively coupled plasma-mass spectrometry (ICP-MS) has been used and methods developed to determine such metals in clinical matrices.

The development of a simple dilution method is described for use in preliminary trials to measure the blood lead levels and other trace metal profiles, in patients with bone metastases. The blood lead results attained agree closely with a certified reference material, and the method is shown to remain under analytical control. The trial results are presented and discussed with reference to further and more detailed investigations. The selection criteria for other suitable elements such as Al, Ba, Cd, Ce, Pb, Sr, and Zr in blood and urine, along with an assessment of the analytical and clinical praticalities of the methodology which must be considered for subsequent full clinical trials is also discussed following a critical evaluation.

Finally the results obtained in a extended clinical trial are presented. The crosslink levels (serving as the reference marker), measured by ELISA, were compared with the trace metal levels (Cd, Pb and Sr) in blood and urine samples, measured by ICP-MS, in order to assess their diagnostic potential and effectiveness in monitoring treatment. The blood lead levels were found to offer the greatest potential, correlating well with the DPYD values in the majority of cases. The blood strontium levels also showed some promise. However the blood cadmium and the urinary trace metal levels proved less suitable. The results attained in this feasibility study support a more detailed clinical investigation, on a much larger scale, and over a longer period of time. The need to incorporate a full statistical evaluation of all factors that can influence the final results is highlighted.

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Relevant scientific seminars and conferences were regularly attended at which work was often presented, external institutions were visited for consultation purposes and several papers prepared for publication.

Signed.....

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LIST OF FIGURES

N.B. Unless otherwise indicated all error limits in figures are one standard deviation.

ABBREVIATIONS

AAS	Atomic absorption spectrometry.
ACP	Aldol condensation product.
AH	Aldol histidine.
AP	Alkaline phosphatase.
B	Blood.
B-Cd	Blood cadmium.
B-Pb	Blood lead.
B-Sr	Blood strontium.
CCMP	Capacitively coupled microwave plasma.
C-PCP	C terminal procollagen peptide.
CSF	Cerebral spinal fluid.
CT	Computed tomography.
DA	Deoxy analogue of OHP found in skin.
DC	Delves cup.
DHLNL	Dihydroxylysylnorleucine.
DHNL	Dihydroxynorleucine.
DNA	Dirobosonucleic acid.
DPYD	Deoxypyridinoline.
EDTA	Ethylenediaminetetraacetic acid.
ELISA	Enzyme linked immunoasorbant assay.
ETAAS	Electrothermal atomic absorption spectrometry.
FI	Flow injection.
GGHYL	Gluco galactosyl hydroxylysine.
GHYL	Galactosyl hydroxylysine.
GLA	Gamma carboxyglutamic acid.
HEX-HYL	Hexosyl hydroxylysine.
HEX-LYS	Hexosyl lysine.
HFBA	Heptafluorobutyric acid.
HG	Hydride generation.
h-HLNL	Histidinohydroxylysylnorleucine.
HHMD	Histidinohydroxymesodesmosine.
his	Histidine.
HLNL	Hydroxylysylnorleucine.
HNL	Hydroxynorleucine.
HPLC	High performance liquid chromatography.
HYL	Hydroxylysine.
HYP	Hydroxyproline.
ICP	Inductively coupled plasma.
ICP-AES	Inductively coupled plasma-atomic emission spectrometry.
ICP-MS	Inductively coupled plasma-mass spectrometry.
ICTP	The carboxy terminal telopeptide.
ID	Isotope dilution.
INTP	The amino terminal telopeptide.
LP	Lysylpyridinoline.
LYS	Lysine.
MIP	Microwave induced plasma.

MRI	Magnetic resonance imaging.
MS	Mass spectrometry.
NAA	Neutron activation analysis.
N-PCP	N terminal procollagen peptide.
NSAID	Non steroidal anti-inflammatory drug.
OA	Osteoarthritis.
OC	Osteocalcin.
ODS	Octadecylsilane.
OHP	Hydroxypyridinoline.
OSA	Octane sulphonic acid.
P	Plasma.
PAP	Prostate acid phosphatase.
PCA	Penicillamine.
PFPA	Pentafluoroproprionic acid.
PIXE	Proton induced X-ray emission spectrometry.
PSA	Prostate specific antigen.
PYD	Pyridinoline.
RA	Rheumatoid arthritis.
RIA	Radioimmunoassay.
S	Serum.
SDS	Sodium decyl sulphate.
STPF	Stabilised temperature platform furnace.
TFA	Trifluoroacetic acid.
THGA	Transversely heated graphite atomiser.
TRAP	Tartrate resistant acid phosphatase.
U	Urine.
U-Cd	Urinary cadmium.
U-Pb	Urinary lead.
U-Sr	Urinary strontium.

CHAPTER 1

INTRODUCTION.

CHAPTER 1: INTRODUCTION

1.1: BASIC BIOLOGY OF BONE AND CARTILAGE.

Bone is a specialised connective tissue that, together with cartilage, makes up the skeleton. These tissues serve three main functions: a) mechanical support and site of muscle attachment for locomotion, b) protection for vital organs and bone marrow and c) a metabolic reserve of ions for the entire organism, especially calcium and phosphate.

There are two types of bone: compact and cancellous bone. Compact or cortical bone is solid and composed of numerous cylindrical structures (Haversian systems) and is a major component of long bones and of the outer sheet of predominantly cancellous bones, such as vertebrae. Cancellous bone, also known as spongy or trabecula bone, is much less dense than cortical bone, but can still provide considerable structural support. It is also more metabolically active than cortical bone. Bone is made up of two fundamental components:- cells (osteoblasts and osteoclasts) and an intercellular matrix (1,2).

1.1.1: Principle bone cells (Osteoblasts and Osteoclasts).

The two principle cell types are osteoblasts and osteoclasts, which have evolved to regulate the growth and turnover of bone and mediate ion fluxes between the bone and blood. The osteoblasts are cells that form new bone. They are derived from precursor cells in the blood, and are capable of forming the bone matrix, which in a well regulated process ultimately becomes fully mineralised bone. They are also capable of synthesising the various structural proteins and growth factors. Osteoblasts migrate to areas where bone has been eroded by the osteoclasts, laying down new bone in the cavities.

Osteoclasts are bone resorbing cells which originate in the bone marrow. Precursor cells from the blood stream collect at bone resorption sites and fuse to form multinucleated osteoclasts. The osteoclasts can erode any region of the mineralised organic matrix, but in particular damaged sites since, as with other supporting materials, bone suffers from wear and tear (1).

1.1.2: The bone matrix.

The bone matrix is composed in part of organic materials, which consists predominantly of type I collagen (85-95%), non collagenous constituents or components of proteins and carbohydrates, and proteoglycans which are specific to bone and dental hard connective tissues (1,3). The rest consists of inorganic salts, which confer on bone its hardness and much of its rigidity. Major ions which compose the mineral part of bone include calcium, phosphate, hydroxyl and carbonates, less numerous ions are those of citrate, magnesium, sodium, potassium, fluoride, chloride, iron, zinc, copper, aluminium, lead, strontium, silicon and boron, many of these being present only in trace quantities (1)

1.1.2.1: Collagen.

Collagen is a protein and is the major structural component of the body (1,2). The structure and biosynthesis of collagen is very complex (4), plus there are several genetically distinct types of collagen, each type specifically tailored to the particular function of each body tissue (5). The special properties that enable collagen to act as the major supporting framework of the body are largely dependant on the high structural stability of the collagen fibres. Collagen fulfils this function by means of its unique molecular configuration, the highly specific alignment of the molecules during

extracelluar aggregation and finally by the formation of covalent crosslinks (4-7). These crosslinks are particularly important in conferring on the fibres the high tensile strength and resistance to chemical attack necessary for their function (4-7). Both bifuntional and trifunctional crosslinks are formed and it is known these crosslinks in bone collagen change with maturation (8,9).

Many of the non-collagenous proteins originate from plasma or other non-bone sources. It has been suggested that they act as cementing material in which the collagen fibres are embedded, hence preserving the structure and orientation of the fibres which ultimately appears to contribute to the overall structure and functional quality of the bone. The matrix is calcified by means of the deposition of hydroxyapatite crystals along the collagen fibres and as a result more than 95% of the body's calcium resides in bone (1).

1.1.3: Bone remodelling.

Bone remodelling is the removal of bone by the osteoclasts and the deposition of new bone by osteoblasts. It converts woven bone into mature bone and it is involved in bone growth and changes in bone shape, the adjustment of the bone to stress, bone repair and calcium ion regulation in the body (1,2). Bone is a highly dynamic connective tissue with the capacity for continuous remodelling. The activities of bone formation and bone resorbing cells are regulated by local and systemic humoral factors, such as parathyroid hormone, cytokines, 1,25-dihydroxy vitamin D_3, the eicosanoids, growth factors, bacterial products and mechanical stress. (1,2,10,11).

Since bone is a living tissue, the process of bone synthesis and bone breakdown are going

on simultaneously and the status of the bone represents the net result of a balance between these two processes. The osteoclasts invade the bone surface and erode it dissolving the mineral and matrix, over a period of about 7 - 10 days, followed by the osteoblasts which arrive to build new bone, laying down collagen and minerals over a period of about three months (1,10,12).

In healthy individuals this rejuvenating process of formation and resorption are tightly coupled processes. However when an imbalance occurs, as a result of any dysfunction of either the local or systemic regulatory systems, pathological changes in the rate of bone formation and resorption will occur, which will lead to unhealthy bone and ultimately a clinical disease of the skeleton (13,14), such as osteoporosis (15), Paget's disease (16) and malignancy (17,18).

1.1.4: Measurement of bone turnover using biochemical markers.

Biochemical markers are by far the most commonly used parameters to obtain an insight into the process of bone turnover. The principle advantages of these biochemical markers compared to the other procedures currently in use, for example, bone density, bone biopsy and calcium kinetic studies, are that they are non-invasive, and therefore have the potential to be used routinely. In addition they reflect the activity of the whole skeleton, including cortical, subcortical and trabeculae bone, which have different remodelling rates in normal and abnormal states, and hence can readily detect acute changes in skeletal metabolism. However it should be noted that such markers will not detect a specific defect of the cellular activity of one compartment of bone if the summated turnover of the skeleton is unchanged.

Due to these advantages, interest has now focused on the use of biochemical markers in the evaluation and management of patients with a number of diseases. The need for more sensitive and specific circulatory and urinary markers that reflect the metabolic activities in bone, has resulted in an explosion of research interests and activities within this area. It is important that any marker should be able to distinguish accelerated bone loss associated with disease from the normal bone turnover. Many of the available markers used to measure bone formation and resorption (19-23), will be discussed in more detail in Sections 1.4, 1.5 and 1.6.

Currently available biomarkers such as alkaline phosphatase and pyridinium crosslinks, have considerably enhanced the possibility of monitoring changes in bone turnover, particularly bone loss. Nevertheless there is still no "ideal" marker and although biochemical markers are useful for monitoring the severity of a certain disease, the effect of treatment, and may potentially be useful in predicting bone loss, it is still impossible to make a diagnosis based solely on the level of a specific biochemical marker. To date there is insufficient evidence to suggest that one marker is better than another, or one is better than another for a particular type of metabolic disease. Only further detailed studies will ultimately determine which markers or combination of markers are best suited to a particular application (19-23).

1.2: BASIC FACTS ABOUT CANCER.

1.2.1: What is cancer.

Cancer is a general term used to refer to malignant tumours, that is tumours capable of progressive growth and distant spread, resulting in the development of secondary tumours, known as metastases. Cancer is probably the most feared disease of our time. It is second only to cardiac disease as a leading cause of death in the western world, and in spite of major progress in cancer treatment, about half of the patients die of their disease.

The development of cancer (carcinogenesis) is a gradual, multistep process. It is fundamentally a genetic disease at the cellular level, initially resulting from acquired changes or mutations in the genetic makeup of a particular cell or group of cells (24,25) which fail to respond to the regulatory factors controlling normal cell growth and division. Consequently the cells continue to grow and divide, yielding an ever increasing mass of cancer cells (Figure 1.1). Unless unchecked, the cancer cells invade surrounding normal tissues, enter the circulation, and spread throughout the body, *i.e.* metastasise (Figure 1.2) eventually interfering with the function of normal cells, tissues and organs, and progressively leading to the death of the patient (26-30). Such loss of growth control usually requires the accumulation of damage to several different cellular regulatory mechanisms (24,25), so most cancers tend to develop late in life. Further details can be found in a range of general texts on cancer, such as those by Oppenheimer (26) and Cooper (27).

Figure 1.1: Schematic diagram showing clonal selection and tumour progression. The development of a malignant neoplasm occurs by a series of steps, each of which involves mutations and selection for more rapidly growing cells within the tumour cell population (27).

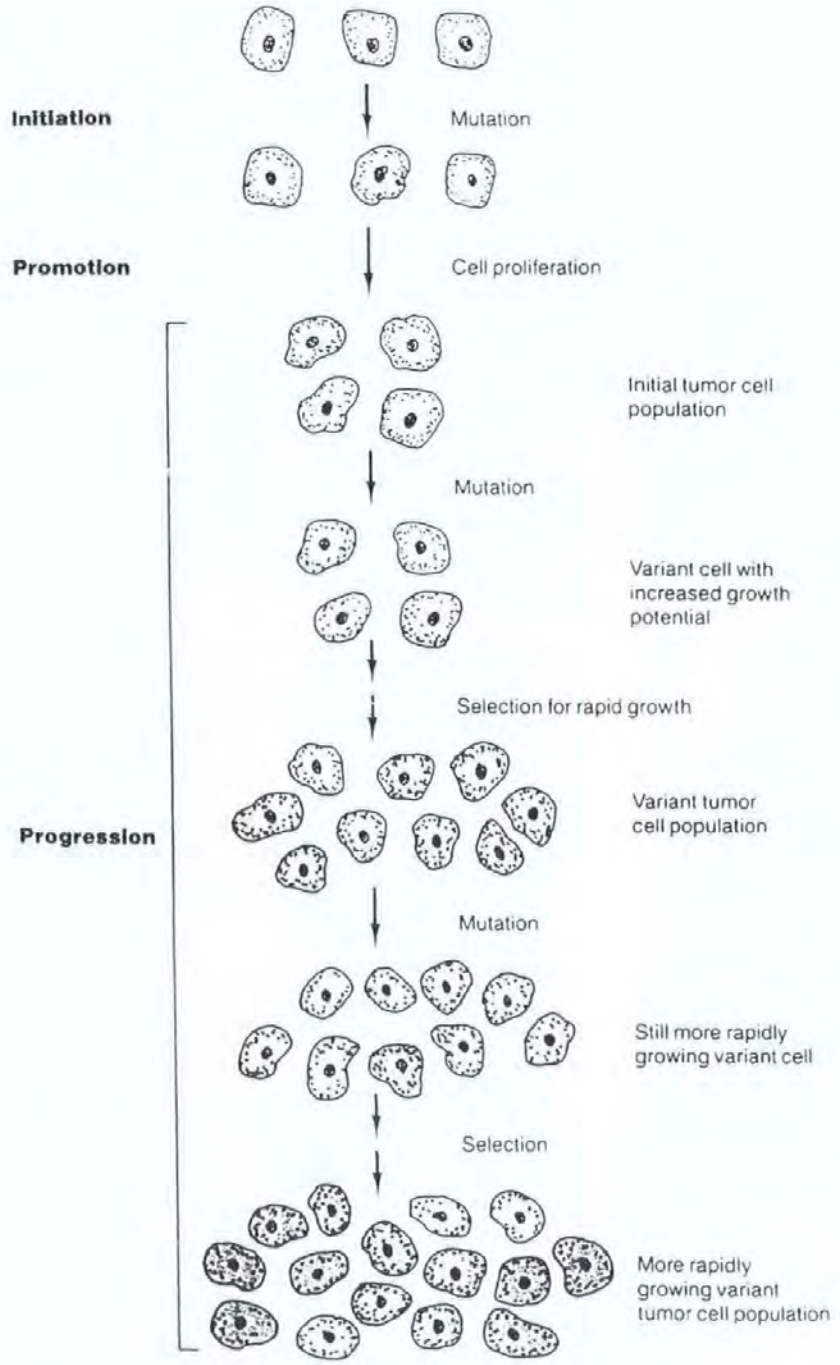
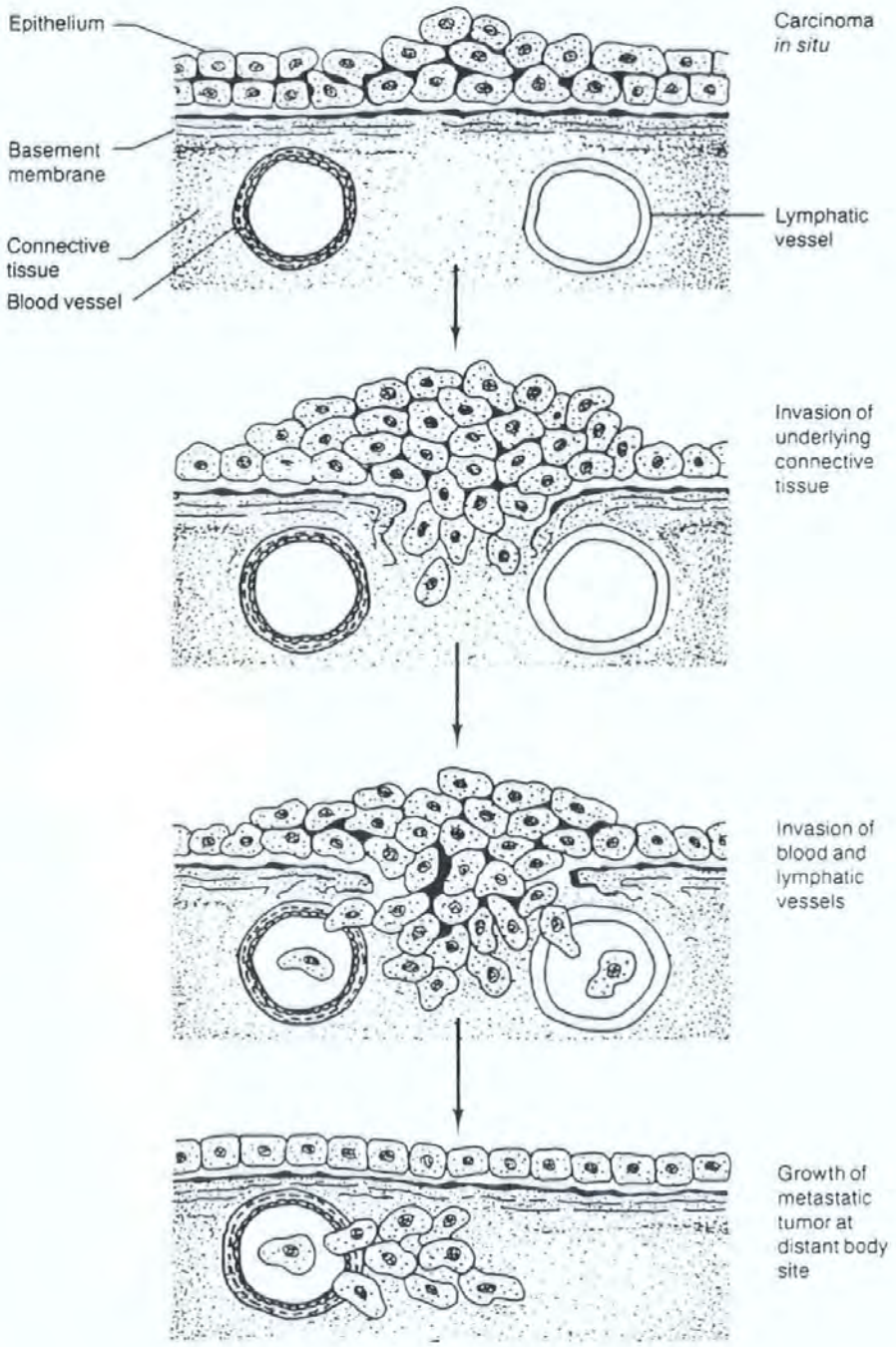


Figure 1.2: A simple diagram showing the stages of invasion and metastasis. Cancer cells first invade underlying normal tissue and eventually reach and penetrate blood and lymphatic vessels. The cancer cells can then be carried throughout the body, leading to the establishment of metastic tumours at distant body sites (27).



1.2.2: The prevalence of cancer in todays society.

The prevalence of cancer in current society is largely a consequence of the elimination of other diseases that constituted major killers in the past. The general improvements in public health, as a result of, sanitation, nutrition and personal hygiene, combined with the triumphs of medical science with respect to developments in vaccines and antibiotics, has virtually eliminated infectious diseases, such as influenza, pneumonia and tuberculosis. This has also increased life expectancy in western society to over 70 years, and hence the focus is now on new health problems, such as cancer and heart disease. Figure 1.3 identifies the ten most common cancers overall in the UK (31), and Figures 1.4 and 1.5 identify the ten most common cancers for males and females respectively (32,33). Further statistical details concerning the incidence of cancer in the UK can be found in Appendix 1.1 (32,33).

Although much feared, cancer is not the primary cause of death. Heart disease accounts for 35% of all deaths, followed by cancer, accounting for approximately 20-25% of deaths in the western world. Other causes of death such as accidents, murders and AIDS each account for less than 5% of all deaths each year (27). Figure 1.6 gives the ten commonest causes of cancer deaths, overall in the UK (34), and Figures 1.7 and 1.8 give a breakdown of the ten cancers which cause the most deaths in the UK for males and females respectively (35,36). Further statistical details on mortality rates for males and females can be found in Appendix 1.2 (35,36).

Figure 1.3: Ten commonest human cancers, UK 1988 (31).

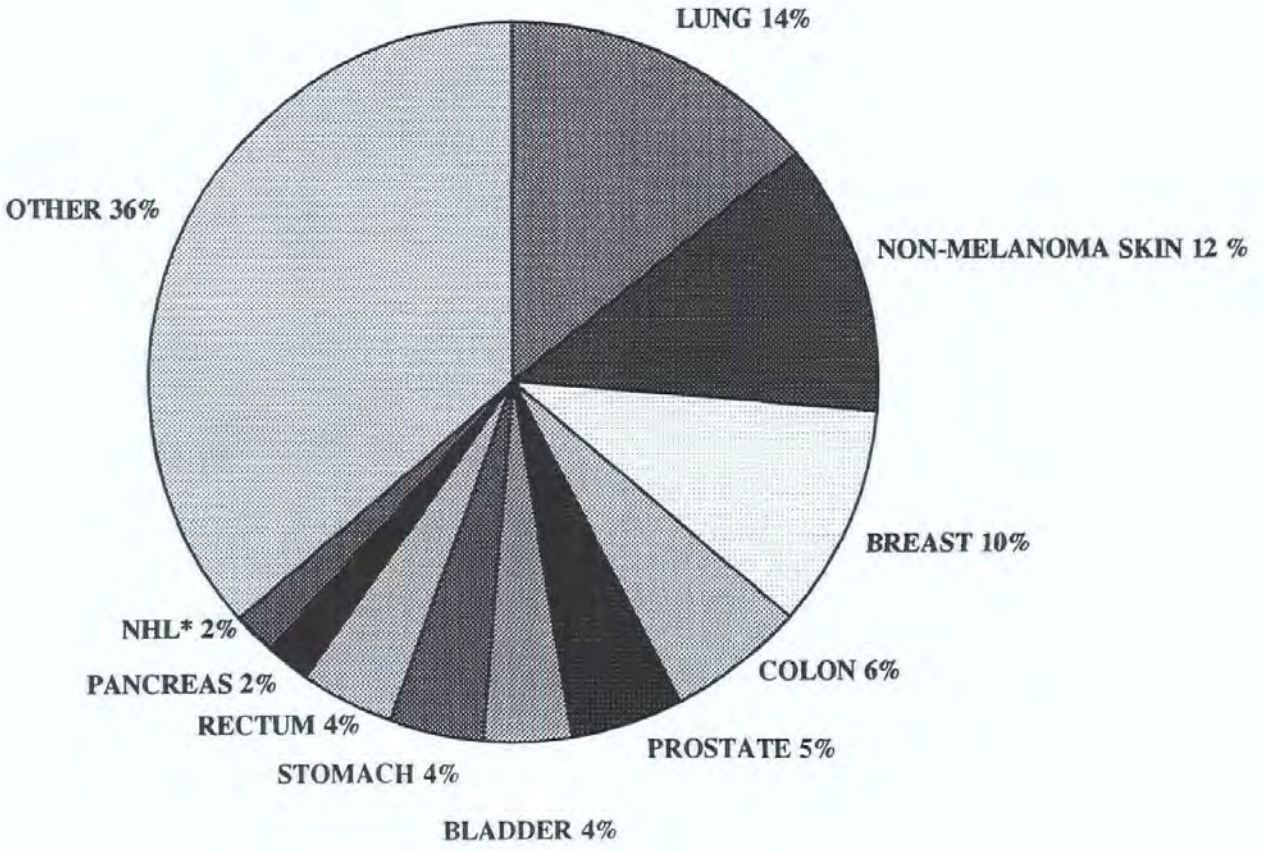


Figure 1.4: Ten commonest cancers for men, UK 1988 (32).

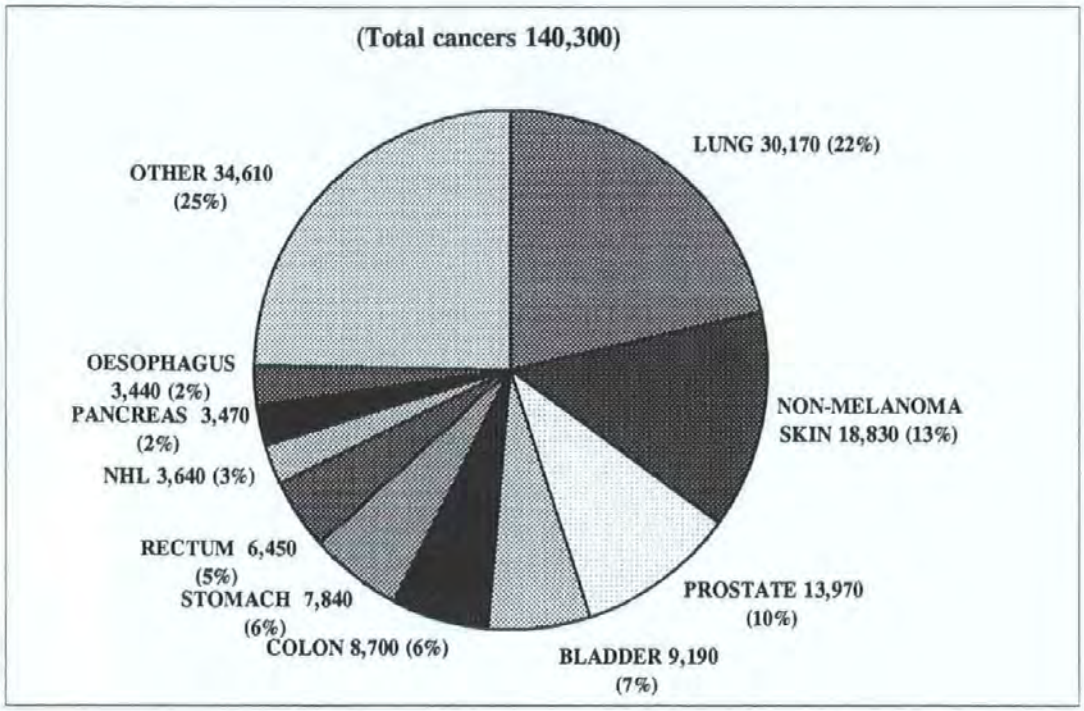


Figure 1.5: Ten commonest cancers for females, UK 1988 (33).

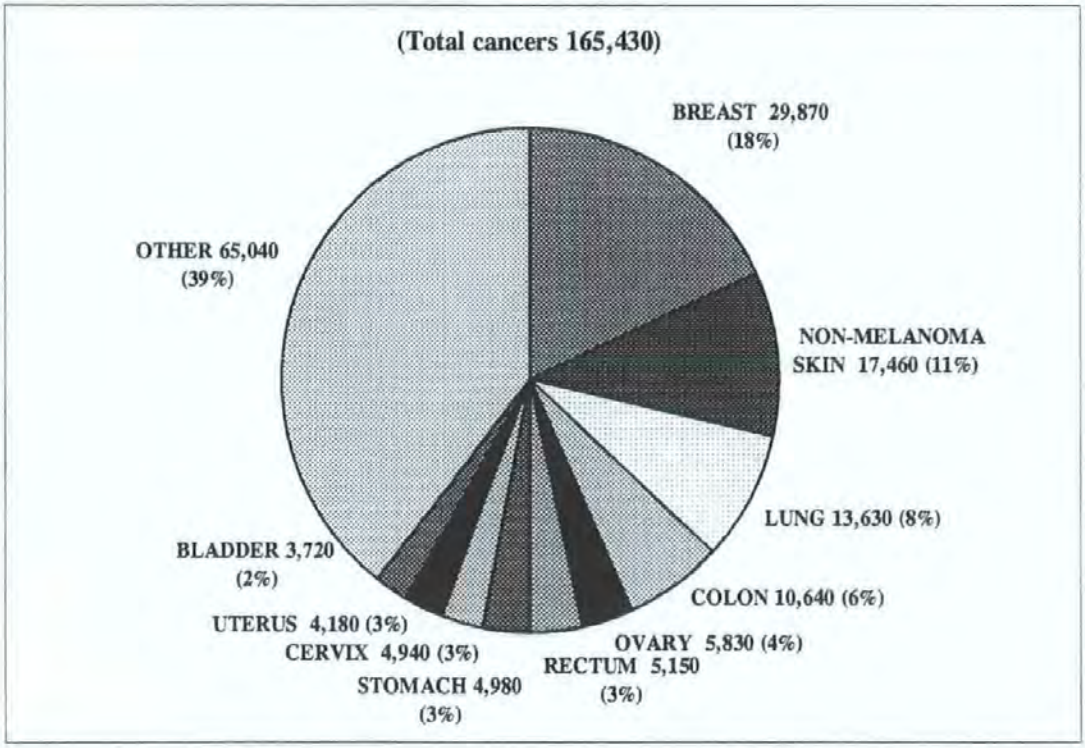


Figure 1.6: Ten commonest causes of human cancer deaths, UK 1994 (34).

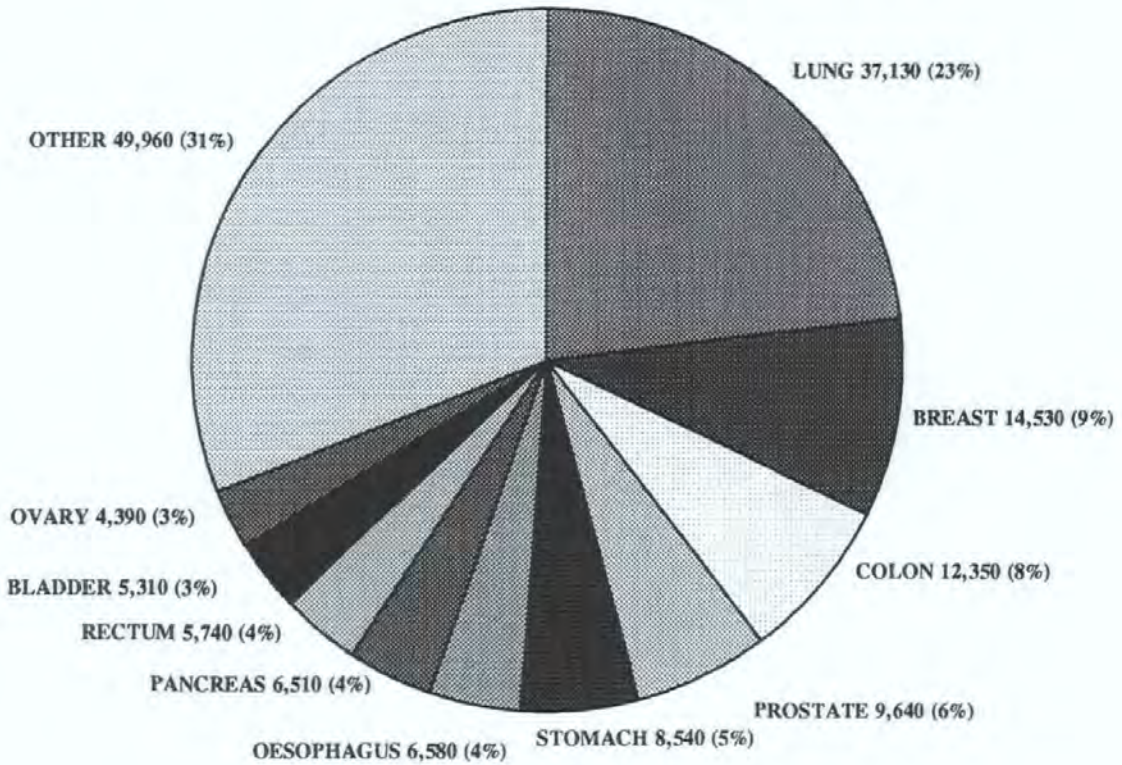


Figure 1.7: Ten commonest causes of cancer death in men, UK 1994 (35).

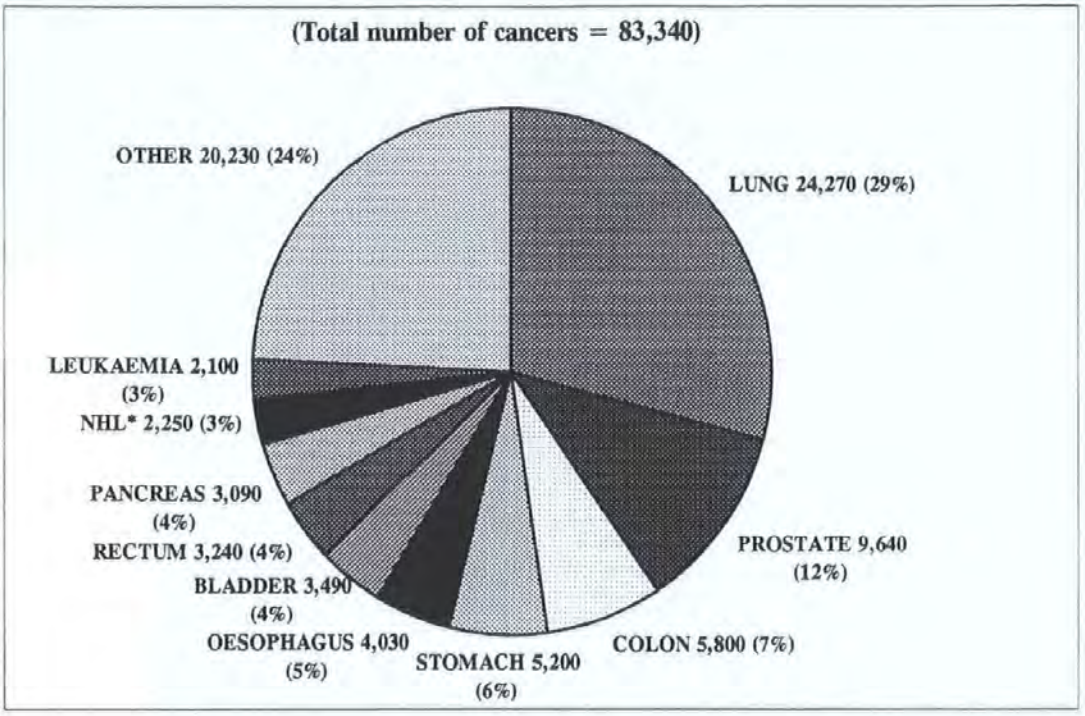
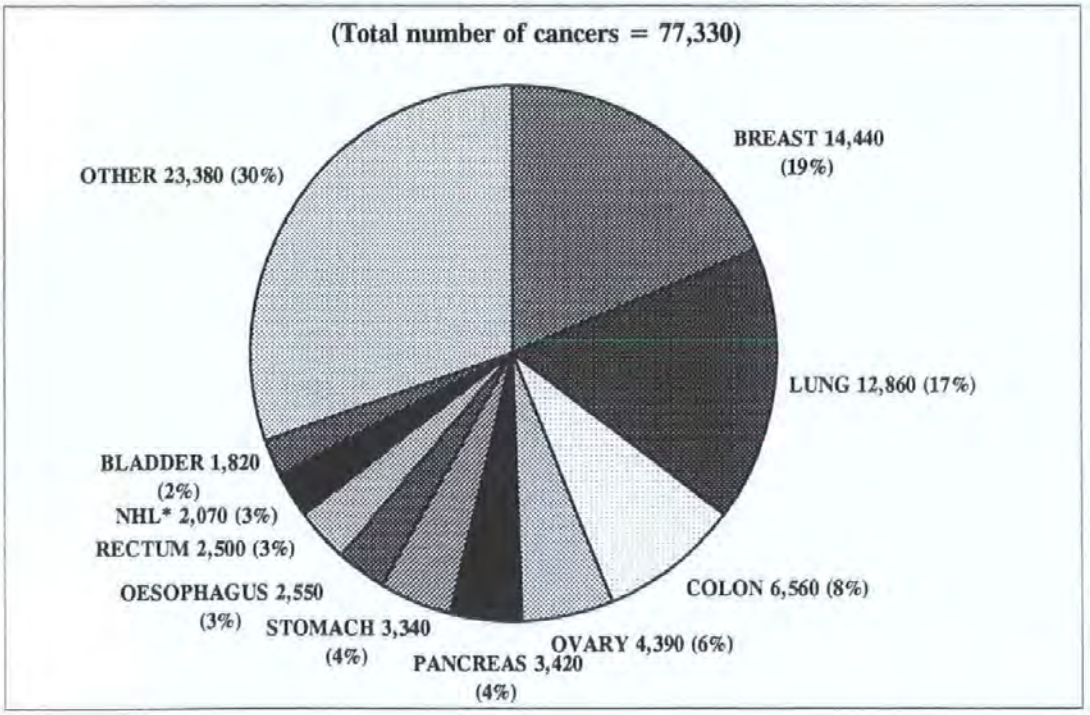


Figure 1.8: Ten commonest causes of cancer death in women, UK 1994 (36).



1.2.3: Causes of cancer.

The cause of cancer remains largely unknown, however a variety of factors have been implicated. Substances that cause cancer are known as carcinogens. Many carcinogens react with the DNA, to induce mutations (37). These carcinogens are called initiating agents, since the induction of critical mutations is generally thought to be the initial event leading to cancer development. Such carcinogens include ionising radiation and some chemicals. Other chemicals contribute to the development of cancer not by inducing mutations but by stimulating cell proliferation. These chemicals are called promoting agents, for example hormones and those found in the diet (27,38-39). It has been estimated that up to 80% of human cancers may be attributable to such environmental risk factors, which are summarised in Table 1.1 (26,27,40-53).

Cancer is generally not considered to be a hereditary disease, however, there are a number of ways in which susceptibility to cancer can be genetically transmitted (27,54). These inherited cancers are extremely rare and account for only a small fraction of total cancer incidence.

1.2.4: Treatment of cancer.

Substantial progress has been made in the treatment of cancer, but in most cases current therapies ultimately fail, and about 50% of patients with cancer eventually die of their disease. Due to the progressive nature of the development of tumours, early detection and diagnosis is critical to the outcome of cancer (26,27). Cancer is generally treated by surgery, radiation and chemotherapy (26,27,55).

Table 1.1: Summary of environmental risk factors (carcinogens).

Risk factor	Causes\implications	References
Smoking	Causes about 30% of all cancer deaths. Directly responsible for the majority of lung cancers (80-90%). Also implicated in the development of other cancers, e.g. oral, pharynx, larynx, oesophagus, bladder, kidney and pancreas.	27,40-41
Alcohol	Excessive consumption has been associated with increased risks of cancer to the oral cavity, larynx, pharynx and oesophagus. In addition excess drinking results in cirrhosis leading to an increased incidence in liver cancer.	27
Ultra violet radiation	A major cause of the very common but usually curable skin cancer. Also responsible for the more serious skin cancer, melanoma.	27,42
Ionising radiation (x-rays\decay of radioative particles	Different kinds of radiation vary both in their ability to penetrate tissue and in the amount of biological damage they cause. In general increased levels of leukaemia, bone, and bronchus cancers have been linked to radiation exposure.	27,42
Diet	Many potential carcinogen are found in food, but attempts to specify dietary agents that effect cancer incidence have yielded controversial and contradictory results. Hence no conclusive evidence exists. Table 1.2 shows a number of dietary components which have been linked to increased or decreased cancer risks.	27,42-49
Viruses	Several viruses are known to be associated with human cancers (refer to Table 1.3). It has been estimated that viruses can contribute to over 20% of human cancers worldwide.	27,42,50-53

Table 1.1: Continued.

Risk factor	Causes\implications	References
Occupational carcinogens	Generally easy to identify, since a high incidence of a particular type of cancer becomes apparent in a specific group of workers (see Table 1.4). Once occupational carcinogens are recognised, appropriate action can be taken. Occupational exposure probably accounts for approx. 5% of cancer mortality.	27,42,43-47
Pollution	A number of potential carcinogens have been introduced into the environment as industrial pollutants, but are present at such low amounts, it is unlikely they have any major effect on cancer incidence. However the effects of long term exposure to low levels is currently unknown.	27,42-43,47
Carcinogenic medicines	Some medicines may increase the risk of cancer (1%) as a side effect of their actions on cells in patients receiving treatment. These drugs are banned unless their therapeutic benefit outweighs their possible dangers. Hormones, e.g. oestrogen (linked to increases in cervical and breast cancer). Immunosuppressive drugs (linked to increases in lymphomas and reticulosarcoma). Steroid contraception and anabolic steroids (androgens), linked with increases in liver cancer.	27,47

Table 1.2: Dietary factors and cancer risk (27).

Dietary Component	Effect on Cancer Risk
High fat	Increased risk of colon and possibly breast cancer
High calorie	Obesity resulting in increased risk of endometrial and possibly breast cancer
Cured, smoked and pickled food	Increased risk of stomach cancer
Aflatoxin	Increased risk of liver cancer
Vitamin A or ß-carotene	Decreased risk of lung and other epithelial cancers
Vitamin C	Decreased risk of stomach cancer
Vitamin E and selenium	Deficiencies associated with increased cancer risk
Fibre	Decreased risk of colon cancer
Cruciferous vegetables	Decreased cancer risk

Table 1.3: Viruses associated with human cancers (27).

Virus	Type of Cancer
Hepatitis B virus (HBV)	Hepatocellular carcinoma
Human papillomaviruses (HPV)	Cervical and other anogenital carcinomas, squamous cell skin carcinoma
Epstein-Barr virus (EBV)	Burkitt's and other B-cell lymphomas, nasopharyngeal carcinoma
Human T-cell lymphotrophic virus (HTLV-1)	Adult T-cell leukaemia
Human immunodeficiency virus (HIV)	Lymphomas, Kaposi's sarcoma, anogenital carcinomas

Table 1.4: Carcinogens from occupational exposure (27).

Carcinogen	Occupational Exposure	Cancer Risk
4-aminobiphenyl	Chemical and dye workers	Bladder
Arsenic	Mining, pesticide workers	Lung, skin and liver
Asbestos	Construction workers	Lung
Auramine	Dye workers	Bladder
Benzene	Leather, petroleum, rubber and chemical workers	Leukaemia
Benzidine	Chemical, dye and rubber workers	Bladder
Bis(chloromethyl) ether	Chemical workers	Lung
Chromium	Metal workers, electroplaters	Lung
Isopropyl alcohol	Manufacturing by strong acid process	Nasal
Leather dust	Boot and shoe manufacturing and repair	Nasal and bladder
Mustard gas	Mustard gas workers	Lung, larynx and nasal
Napthylamine	Chemical, dye and rubber workers	Bladder
Nickel dust	Nickel refining	Nasal and lung
Radon	Underground mining	Lung
Soots, tars and oils	Coal, gas and petroleum workers	Lung, skin and bladder
Vinyl chloride	Rubber workers, polyvinyl chloride manufacturing	Liver
Wood dusts	Furniture manufacturing	Nasal

However the success of these treatments varies considerably according to the kind of cancer and how early it is detected. The success of the treatment of most cancers is usually measured as the fraction of patients who survive for five years without evidence of disease. The survival rates for the ten most common adult cancers for males and females are shown in Figures 1.9 and 1.10 respectively (56). The survival rates are of course substantially influenced by the time at which the cancer is detected and treatment is initiated. More detailed statistics can be found in Appendix 1.3 (56-59).

The limiting factor in cancer treatment is metastasis. Localised cancers can usually be effectively treated by surgery or radiotherapy. Once invasion of surrounding normal tissue has occurred, the effectiveness of surgery as a treatment depends on removing all of the tissue that contains cancer cells. Once the cancer has metastasised to distant body sites surgery is no longer effective and must be combined with chemotherapy to treat the disseminated disease. In general, treatment of the most common kinds of cancer is ineffective once metastasis has occurred. In some cancers, metastasis has occurred by the time of diagnosis in more than 50% of patients. The chances of survival once metastasis has occurred are dramatically reduced, for example the five year survival rate for breast cancer is over 90% if the cancer is detected early enough but declines to 20% once it has metastasised (27).

A variety of chemotherapeutic drugs are used in an attempt to kill the cancer cells. Unfortunately, the chemotherapeutic drugs currently available are not specific for cancer cells. Since cancer cells closely resemble normal cells, the fundamental problem in cancer treatment is selectively interfering with the growth of cancer cells without adverse side

Figure 1.9: Male 5 year relative % survival rates, England and Wales, 1981 (56).

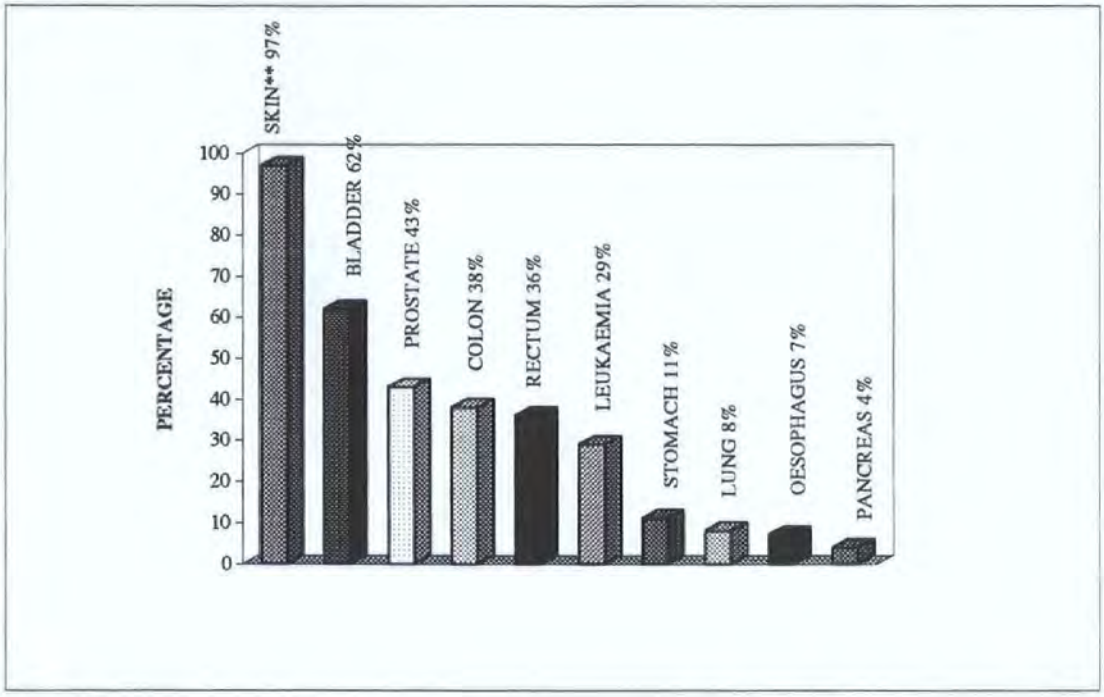
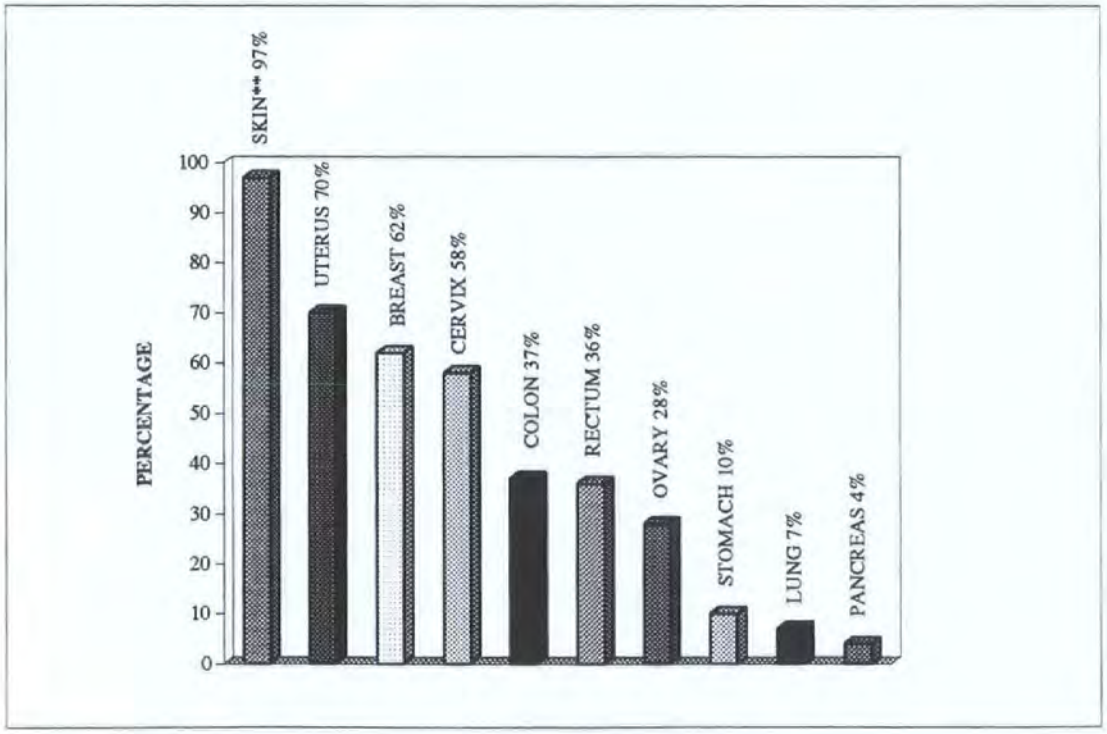


Figure 1.10: Female 5 year relative % survival rates, England and Wales, 1981 (56).



effects to the normal cells. This toxicity to the normal cell population limits the dosage of therapeutic drugs that can be tolerated by the patient, thus limiting the effectiveness of chemotherapy.

1.3: BONE METASTASES (SECONDARY BONE CANCER).

Metastasis is one of the most serious and deadly aspects of cancer. The ability of malignant tumours to spread throughout the body rather than remaining confined to their site of origin is responsible for most cancer deaths. The skeletal system is the third most common site of metastasis in terms of both frequency and clinical affects (60,61). Primary cancers of the bone are rare, but secondary cancers or metastases in bone are common. Bone metastases are particularly common in patients with advanced cancers of the breast, prostate, bronchus, kidney and thyroid (20, 62-66).

1.3.1: Distribution and route of spread.

Irrespective of the tissue of origin of the primary cancer, the distribution of bone metastases is predominantly in the axial skeleton. It is rich in red marrow with a large capillary network and sluggish blood flow, which may be suitable for tumour growth. The lumbar spine is most commonly affected, followed by pelvis, ribs, sternum, femur, humeral shaft and shin (15). There are four known routes that may result in skeletal metastases (67):-

i) Direct invasion:- Soft tissue tumours may involve adjacent bone by direct extensions into underlying bone structures, although this is often not called "true" metastic disease.

ii) Lymphatic spread:- Deposits may occur in regionally draining lymph nodes which can have secondarily affects in adjacent bone structures, for example carcinoma of the cervix can lead to pelvic malignancy.

iii) Haematogenous spread:- more commonly via venous involvement rather than arterial invasion.

iv) Interspinal fluid:- cerebrospinal fluid is an additional pathway for the dissemination of secondaries from intercranial tumours.

1.3.2: Disruption to normal bone remodelling.

As discussed in Section 1.1.3, bone is a metabolically active tissue continuously being remodelled. The normal physiological process can be disturbed by the direct local and\or distant systemic effects of tumour cells. Recent research has indicated that many of the factors that may be involved in the regulation of bone remodelling (13), and produced by normal cells in the bone environment, can also be produced in excessive amounts by tumour cells. Thus metastic malignant cells could directly interfere with the function of normal bone cells and so disrupt the bone architecture. (15,67-69).

1.3.3: Diagnosis of skeletal metastases.

Bone metastases can result in intense pain, interference with the surrounding neural and muscular structure causing impaired mobility, pathological fractures, spinal cord compression, hypercalcemia and suppression of bone marrow function (20,21,70). The assessment of metastic disease of the skeleton has been and remains a difficult problem, despite the variety of techniques available (71,72).

1.3.3.1: Plain radiography.

Plain radiography indicates net results of bone resorption and repair, providing structural information on the damage from malignant disease (73,74). However the sensitivity of this technique is relatively low and substantial damage has to occur before radiographs become abnormal. To be detected, lesions must be greater than 10-15 mm in diameter with a loss of about 50% of the bone mineral content (75).

Although this technique is not as sensitive as bone scanning (refer to Section 1.3.3.2) for the detection of bone metastases, it is more specific (76). Radiographs are generally used to compliment bone scans to guard against erroneous results and to assess the localised bone pain and any pathological fractures.

1.3.3.2: Radionuclide bone scanning.

This technique is far more sensitive than conventional radiology for detecting lesions, since it detects functional changes which occur much earlier than structural changes (77,78). Radionuclide bone scanning remains unchallenged in its role of investigating skeletal pathology, because of its high sensitivity for lesion detection and its ability to rapidly evaluate the whole skeleton, despite the introduction and advancements in imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) (79).

The bone scan uses technetium - 99 (^{99} Tc) diphosphonate to reflect the skeletal activity (80). It is believed to work via the chemisorption of the phosphorus group onto calcium found in the hydroxyapatite in bone, *i.e.* the diphosphonate molecule is absorbed onto the

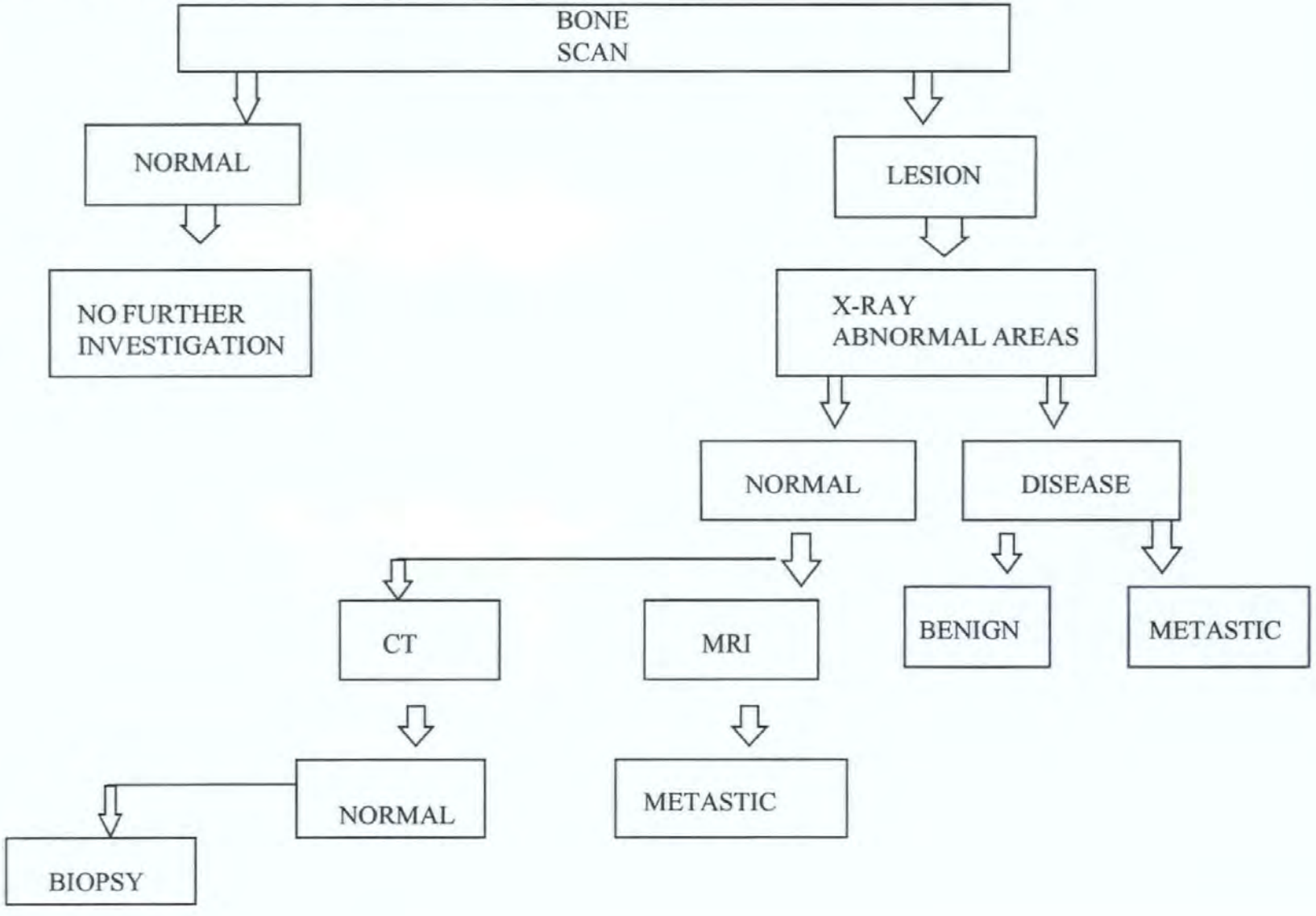
surface of the bone. This reflects the metabolic reaction of bone to a disease process (80,81). Bone scans are performed by acquiring multiple images 3-4 hours after an intravenous injection of the ^{99}Tc-diphosphonate. If a lesion is identified, due to bone scanning's lack of specificity further investigation is required (Figure 1.11). Appropriate plain x-rays are taken on areas of abnormal uptake to further study the structural nature of the problem. If the radiograph is normal and clinical metastases are likely, CT or MRI of the area may also be used for diagnosis. If clinically relevant a bone biopsy may be necessary to resolve the issue.

Extensive skeletal metastases may produce a diffuse increased uptake of the ^{99}Tc-diphosphonate, resulting in super scans, which are particularly common in prostatic and breast cancers (77). Since these cancers account for a large proportion of skeletal metastic cases, this clearly presents a major problem. The occurance of such super scans leads to a misreading of the presence of metastic bone disease, and hence a misdiagnosis and treatment of the patient.

1.3.3.3: Computed Tomography (CT).

Computed tomography is a more sensitive radiographic technique, but more readily applied to elucidate specific lesions, rather than more regular use in assessing the skeleton. It is more sensitive than radiography in diagnosing spinal metastases. It is also used in cases where bone scanning gives negative results or suggests degenerative disease, especially if there are persisting clinical symptoms, such as bone pain. It is likely to be used more frequently in future to monitor response (82).

Figure 1.11: Schematic diagram of the procedures used in the diagnosis of skeletal metastases (77).



1.3.3.4: Magnetic Resonance Imaging (MRI).

Magnetic resonance imaging is a relatively new technique to the clinical management of patients with bone metastases (83-85). Current evidence suggests MRI is a highly sensitive method for detecting tumours in bone marrow, and in many cases can be more sensitive than either bone scanning, or CT for lesion detection (86).

It is impractical to image more than a limited area of the skeleton with CT, but larger sections of the skeleton can be assessed with MRI (87). MRI is also an excellent non invasive technique for visualising the spinal cord, and hence offers several advantages over other techniques for assessing spinal cord compression (87,88). It will no doubt be increasingly used for diagnosis and follow up of bone metastases, however its use is limited by cost and availability.

1.3.4: Assessment of response to treatment.

There are many treatments available for bone metastases, such as radiotherapy (89), endocrine manipulation (90) and chemotherapy (91-96), all of which may produce significant clinical improvement. However, as with the diagnosis of bone metastases, monitoring the response to treatment is also difficult to measure objectively. This is due to both the indirect observation of regression of cancer in bone, as well as the insensitivity of current assessment methods (64). A number of methods are available, none however are ideal, each having a number of advantages and disadvantages (71,87,97).

1.3.4.1: Plain radiography.

Metastic bone destruction results from the invasion of malignant cells from the bone marrow cavity. These cells stimulate osteoclasts to resorb bone and disturb the normal coupling between osteoblast and osteoclast function (14). When bone resorption predominates, area of lysis will be visible on plain radiographs and conversely, areas of sclerosis indicates increased osteoblastic activity (98). It is generally accepted that sclerosis of lytic metastases with no radiological evidence of new lesions constitutes tumour regression. However, some patients have a mixture of sclerotic and lytic lesions before starting therapy, making interpretation of serial radiographs difficult. Even when radiological evidence of a response to successful treatment is achieved, it is often not evident for 6 months or more. Thus, this method is basically too insensitive to study the response to therapy.

1.3.4.2: Radionuclide bone scanning.

The use of bone scanning in the assessment of therapy is limited. Despite bone scanning's greater sensitivity when compared to plain radiology, it is less specific (76). An increase or decrease in the intensity and the number of lesions on a bone scan (*i.e.* hot spots) does not necessarily mean progression of the disease or a response to treatment respectively. A number of interpretations are possible.

Following successful therapy for metastic disease the increased production of immature new bone, and hence the cause of the hot spot, eventually ceases and isotope uptake gradually falls. However the healing process initially causes an increase in uptake *i.e.* the flare response (87,99), and scans performed during this period may be incorrectly

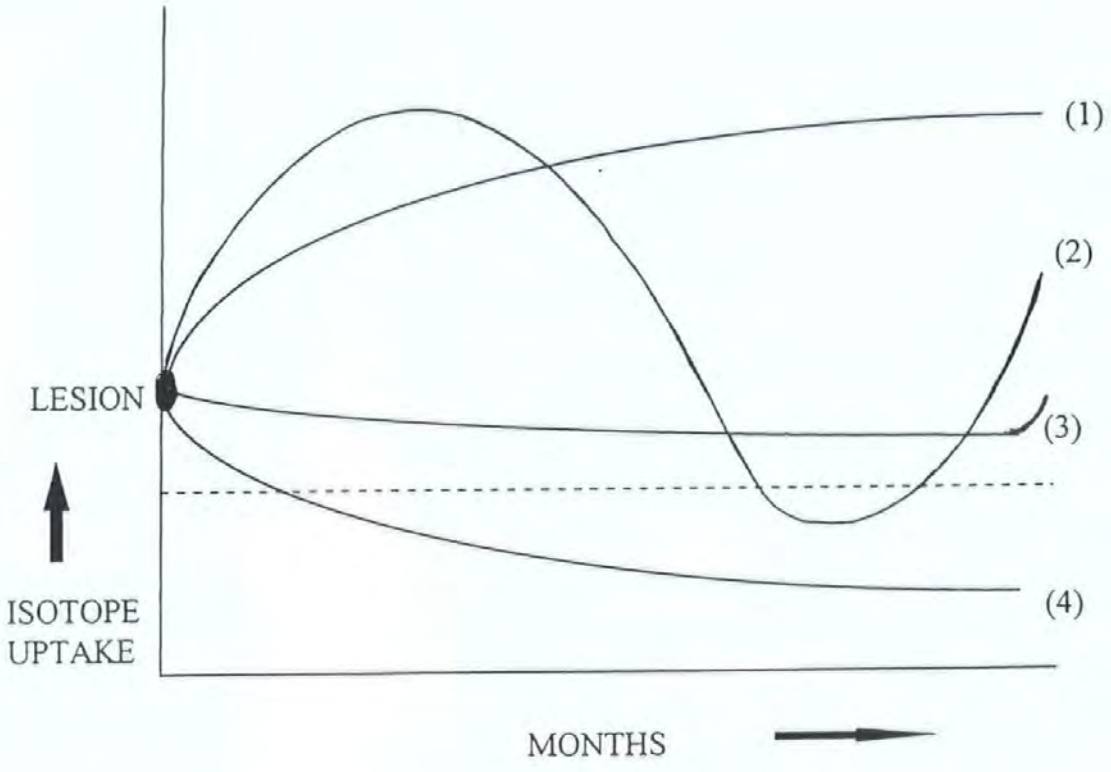
interpreted as progression of disease (100). Conversely a reduction in isotope uptake is occasionally seen in rapidly progressive disease when the overwhelming destruction allows little chance for new bone formation. Such appearances are easily mistaken for improvement (101). These different patterns of response are illustrated in Figure 1.12. Providing these limitations are taken into consideration, bone scanning may be useful in the assessment of response of bone metastases to treatment in some patients (102,103).

1.3.4.3: Computed tomography (CT).

This technique offers three dimensional information and high quality images. The bone density discrimination is far superior to that possible with plain radiographs, providing excellent bone to soft tissue resolution. Bone destruction can be identified easily hence can be used not only for diagnosis but also in the assessment of disease spread (87).

Metastic involvement of the skeleton results in a gross structural change and usually involves both cortical and trabeculae bone. Detection of minor changes in mineralisation is technically difficult but this is probably not relevant in the context of metastic disease, as only major changes in the size of a lesion or in mineralisation are accepted as a response to treatment. Quantitative assessment of bone mineralisation has been used to monitor healing of bone metastases following radiotherapy (104), and systemic therapy (63,105).

Figure 1.12: Diagram summarising the possible results from bone scanning when assessing the response to treatment (87).



(1) Progress of disease.

(2) "Flare" response to successful therapy and subsequent relapse.

(3) Typical response to successful therapy and subsequent relapse.

(4) Rapidly progressive lytic disease.

1.3.4.4: Magnetic Resonance Imaging (MRI).

The use of MRI for monitoring response to treatment is at present very limited, since there is limited machine availability. However MRI may have a use or role for detailed evaluation, for example of specific lesions prior to surgery.

1.3.4.5: Biochemical monitoring.

There is no specific marker to monitor the progress of metastic disease in the skeleton. However, metastic involvement of bone disturbs bone cell function and perturbs a variety of biochemical parameters. Major changes in bone cell activity are seen within the first few weeks of starting effective treatment, reflecting the changes in rates of formation and resorption that occur (106,107).

Although plain radiography is at the moment the "gold" standard for assessing the effects of treatment, it is clear that reliable alternatives are necessary. Modern imaging techniques will undoubtedly be refined, although such techniques are likely to remain expensive, time consuming and confined to specialist centres. The monitoring of bone metabolism shows much promise and appears to be the most likely method to replace and\or compliment plain radiography. The tests are relatively straightforward and could be readily available to most centres. Biochemical monitoring therefore appears to be a good alternative to plain radiography and preliminary clinical studies have shown biochemical markers provide an indication of response to therapy long before radiological changes can be expected (108-111). These markers have the potential to improve patient management for the evaluation of specific treatments and will be discussed in more detail in Sections 1.4, 1.5 and 1.6.

1.3.4.6: Tumour markers.

The development of reliable tumour markers would provide the most direct method to assess the response to therapy in specific cancers. Such monitoring has had a major beneficial affect on the management of testicular and ovarian cancer (87). In prostatic cancer, prostate acid phosphatase [PAP] (112) and prostate specific antigen [PSA] can be monitored, the latter tending to be a more reliable marker (113). Unfortunately to date no such marker is routinely available for breast cancer (87).

1.3.4.7: Subjective assessment.

The assessment of response to therapy purely on analgesic use is very difficult to measure objectively and in general is not accepted as a true marker of response. Nevertheless the use of pain scores, records of analgesic consumption and mobility (*i.e.* performance status) enables an approximate measurement of pain and provides useful corroboration to more objective responses (87,114). Table 1.5 illustrates the type of symptomatic assessment sheet given to patients.

Table 1.5: Example of a symptomatic assessment form given to patients (87).

Parameter	**Description**	**Score**
Pain	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4
	Intolerable	5
Analgesic use	None	0
	Simple analgesic or NSAID	1
	Simple analgesic + NSAID	2
	Moderate analgesic (eg Dihydrocodeine)	3
	Opiates (<40mg morphine daily)	4
	Opiates (>40mg morphine daily)	5
Mobility	None	0
	Vigorous exercise\activity impaired	1
	Climbing stairs\walking\bending impaired	2
	Difficulty with dressing\washing	3
	Difficulty with all activities	4
	Totally dependant and bedbound	5
Performance status	Normal	0
	Light work possible	1
	Up and about >50% of the day	2
	Confined to bed >50% of the day	3
	Completely bed bound	4

NSAID = non-steroidal anti-inflammatory drug

1.4: BIOCHEMICAL MARKERS OF BONE FORMATION.

At present, all markers used to monitor bone formation (Table 1.6) are the products of osteoblasts. Alkaline phosphatase is a marker of enzyme activity of osteoblasts, whereas oestocalcin and procollagen are produced and released by osteoblasts during bone formation (Figure 1.13).

1.4.1: Serum alkaline phosphatase.

Alkaline phosphatase (AP) is probably the best known marker for bone formation and in fact for many years has been the only available indicator of bone formation. It is one of the most frequently performed assays in clinical chemistry (115).

Serum alkaline phosphatase (AP) consists of bone, liver, intestinal, placental and kidney alkaline phosphatase isoenzymes. Bone and liver AP are predominant in human serum and are the clinically most relevant isoenzymes. Bone AP is present on the plasma membrane of osteoblasts and bone matrix vesicles. An increase in the serum bone AP reflects osteoblastic proliferation or an increased activity, usually stimulated by bone stress, strain, weakness, injury or disease (116).

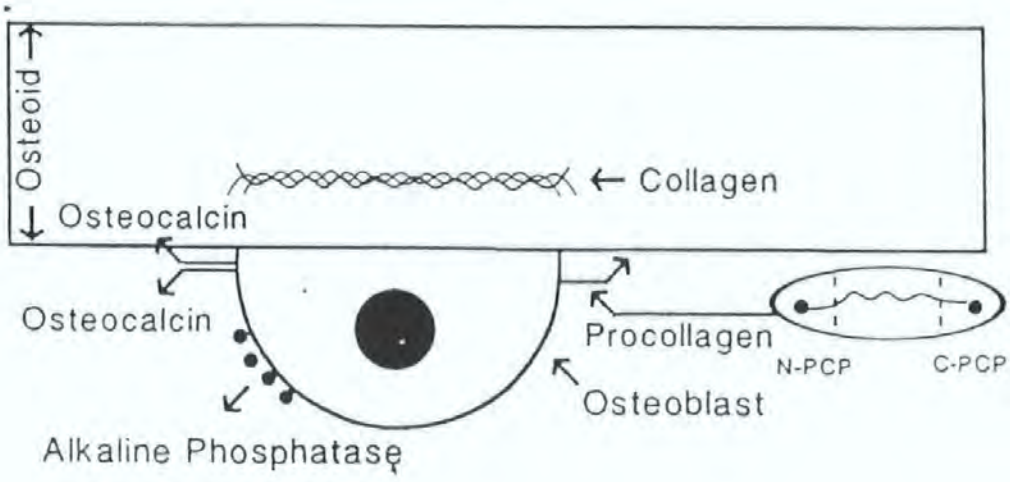
Unlike other bone formation markers AP has little diurnal variation and can be obtained from serum at anytime during a twenty four hour period for a valid assessment of bone formation. Alkaline phosphatase is not cleared by renal excretion and can therefore be used in patients with kidney failure.

Table 1.6: Comparison of bone formation markers (23).

	Ideal	Bone AP	OC	Procollagen
Specificity	Bone	Bone	Bone	Type I collagen
Only increases with increased bone formation	Yes	No	No	No
Renal excretion	No	No	Yes	No
Diurnal variation	No	No	Yes	Yes
Metabolised in liver	No	No	No	Yes
Discriminates between normal and high bone formation	Yes	Yes	Yes	Yes
Discriminates between normal and osteoporotics	Yes	No	No	No

AP = Alkaline phosphatase, OC = Osteocalcin

Figure 1.13: Model depicting the three markers of bone formation.



As illustrated, alkaline phosphatase is an ectoenzyme of the osteoblast that is shed in proportion to bone formation. Osteocalcin is synthesised exclusively by osteoblasts. Due to its affinity for hydroxyapatite, approximately 50% of the osteocalcin is deposited in bone, and the other 50% under normal conditions, finds its way into the circulation. Procollagen peptide is cleaved at both the N-terminal and the C-terminal ends inside the cell. The collagen molecule is secreted and eventually deposited in bone matrix. The procollagen peptides are also secreted from the cell theoretically in proportion to the amount of collagen synthesised (23).

In skeletal metastic disease, raised levels of AP reflect new bone formation and generally correlate well with hydroxyproline excretion (bone resorption marker) and bone scan activity (117). The highest values are often seen with osteoblastic metastases or in response to healing (71), but increased levels are not observed until extensive metastases are present, and not if pure osteolytic metastases are present (107,117). In prostate cancer, AP is a sensitive marker of response with raised levels falling to normal a few months after therapy (118).

The presence of the other isoenzymes has however resulted in this assay lacking sufficient specificity and sensitivity. In many instances the elevation is mild and its clinical interpretation unclear. Correctly identifying the contribution that the bone AP makes to the total serum AP levels is necessary to accurately monitor osteoblastic activity and hence serve as a marker of bone formation. In an attempt to improve the specificity and sensitivity of serum AP measurements, techniques have recently been developed to differentiate between the bone and liver isoenzymes (82,119-120).

Promising results have been achieved in recent clinical trials of these new assays involving electrophoretic (121), immunoradiometric (122) and lectin precipitation methods (123) to measure the bone AP isoenzyme in cancer patients with bone metastases. The improved specificity and consequent enhancement in sensitivity has demonstrated potential use for the clinical diagnosis and monitoring of response to treatment. With the development of these bone specific AP methods, this marker can now be used to monitor bone formation in patients with elevated total serum AP due to liver disease. Bone AP can be used in any clinical situation to monitor bone formation except in patients being

treated with 1,25-dihydroxy vitamin D, since this will increase osteoblast AP and in severe cases of osteomalacia, where AP can be markedly increased without an increase in bone formation (23).

1.4.2: Osteocalcin.

Osteocalcin (OC) also known as bone GLA-protein, is the most abundant non collagenous protein (25%) found in bone (124) which contains three residues of gamma-carboxyglutamic acid (GLA), a unique calcium binding amino acid. It is specific for bone tissue and dentine. Its precise function in the physiology of bone remains unknown, but it is synthesised predominantly in the bone matrix by osteoblasts and binds strongly to hydroxyapatite. However a fraction is released into the circulation, where it can be measured by radioimmunoassay (125). The metabolic pathway involved in the clearance of serum OC is also unknown, although the serum OC levels vary reciprocally with the glomeruli filtration rate and a large quantity of OC fragments are detectable in the urine (126). Since it is cleared by the kidney, patients with renal failure exhibit higher serum OC levels but with no concomitant increase in bone formation.

The main advantage of serum OC is its relatively high discriminating power, and providing the marked diurnal variation of serum OC levels is taken into account, it can be used as a sensitive marker for several metabolic bone diseases for example, osteoporosis, hyperparathyroidism and hyperthyroidism. In such cases it correlates well with histomorphometric measurements of bone turnover (127). Levels of serum OC, reflecting new bone formation also correlate well with AP activity and the mineralisation

rate (128). However, as with AP, there are some situations where the serum OC levels do not reflect bone formation, for example in Paget's disease (23,119).

In patients with skeletal metastases increased levels of OC are not consistently attained, and hence are of limited value. However the measurement of OC levels in the therapeutic management of cancer patients with bone metastases has indicated that increased levels of OC can be considered a biological marker of recovered osteoblastic activity during therapeutically induced stabilisation or agreation of skeletal metastases (129). However the clinical significance of OC as a marker of the response of bone metastases should be carefully considered with regard to the direct hormonal effect on bone metabolism, for example in monitoring endocrine treatment in prostate cancer patients with bone metastases (130).

1.4.3: Procollagen peptide.

Type I collagen is the most abundant collagen in bone, accounting for over 90% of total bone protein. Similar to other fibrillular collagen, type I collagen is synthesised and secreted as procollagen, a precursor molecule characterised by amino- and carboxy-terminal extension peptides (5). Prior to the aggregation of individual collagen molecules into fibrils, these terminal propeptides are cleared proteolytically, and released into body fluids. Since collagen and collagen propeptides are generated in a stoichiometric manner, the levels of circulating procollagen type I propeptides can be used as a quantitative marker of new type I collagen synthesis, and hence a measure of the rate of bone formation.

Due to the lack of clinical data concerning this marker, its full clinical potential has yet to be evaluated. So far, the procollagen appears to act as a good and specific marker of cancellous bone formation in some metabolic disease, correlating well with bone histomorphometry measurements (131). Other studies have shown that the serum levels of the procollagen peptide are elevated in conditions of increased bone formation, such as Paget's disease, hyperthyroidism and normal adolescent growth. A decrease has been observed in conditions of decreased bone formation, such as osteogenic imperfecta. However it appears unable to distinguish between normal adult and patient osteoporosis and osteomalacia. Also, as with serum OC, a diurnal variation for the procollagen peptide exists. (23).

Procollagen peptides appear to be the least discriminating of the bone formation markers, probably attributed to the fact there are other sites of type I collagen synthesis, for example the skin which contribute to the circulating levels of procollagen peptides.

1.5: BIOCHEMICAL MARKERS OF BONE RESORPTION.

In general, bone resorption markers (Table 1.7) can be separated into two groups. The first are markers of osteoclast function, for example tartrate resistant acid phosphatase. Whereas the second group consists of breakdown products of bone matrix degradation, such as hydroxyproline, galactosyl hydroxylysine, pyridinium crosslinks and crosslinked telopeptides of type I collagen (Figure 1.14).

1.5.1: Tartrate resistant acid phosphatase.

Acid phosphatase is a lysomal enzyme primarily present in bone, prostate, platelets, erythrocytes and the spleen, although it is the prostatic acid phosphatase that is most commonly in clinical use (132). Bone acid phosphatase is released during bone resorption and is resistant to L,(+)-tartrate, whereas the prostatic isoenzyme is inhibited. Acid phosphatase circulates in blood at much lower concentrations compared to alkaline phosphatases and shows a higher activity in serum than in plasma because of the release of platelet phosphatase during the clotting process.

Hence osteoclasts, the cells responsible for bone resorption, can be differentiated from other bone cells in histological sections by the presence of tartrate resistant acid phosphatase (TRAP). Although electrophoretic methods can separate these different isoenzymes, they generally lack sensitivity and specificity. The more recently developed immunoassay methods, appear to offer some improvement (133). Studies have shown that the TRAP levels rise with progressive metastic disease although with treatment a fall in levels is observed, correlating with a response to endocrine therapy (134). Other studies

Table 1.7: Comparison of bone resorption markers (23).

	Ideal	TRAP	HYP	GHYL	PYD\ DPYD	Telo-peptide
Source	Only bone	Mostly bone	Many tissues	Mainly bone	Mainly bone	Mainly bone
Released only during collagen breakdown	Yes	No	No	Yes*	Yes*	Yes*
Metabolised in liver	No	No	Yes	No*	No*	No*
Affected by diet	No	No	Yes	No*	No*	No*
Discriminates between normal and high bone resorption	Yes	Yes	Yes	Yes	Yes	Yes*
Discriminates between normal and osteoporotics	Yes	No	No	Yes	Yes	Yes*

* These tissues have not been fully established

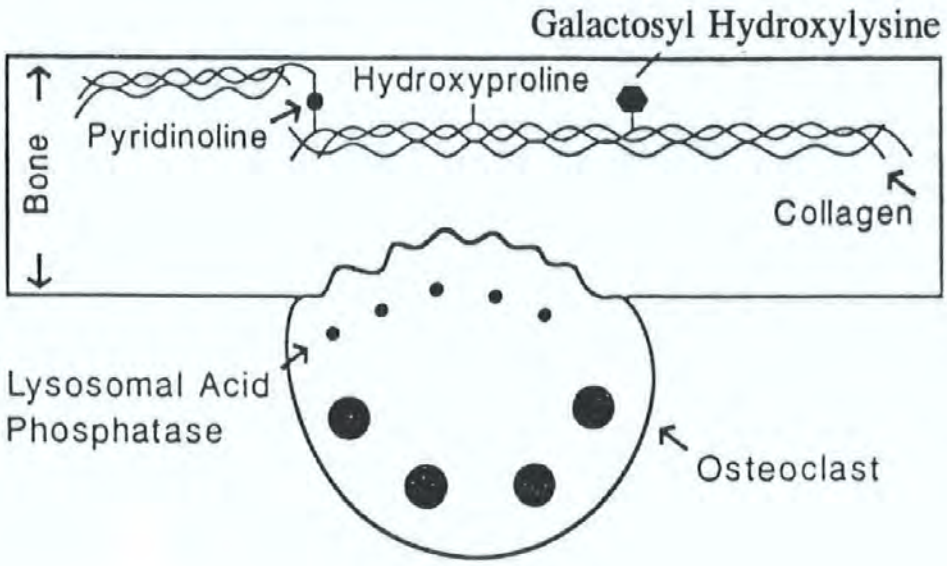
TRAP = Tartrate resistant acid phosphatase,

HYP = Hydroxyproline

GHYL = Galactosyl hydroxylysine

PYD\DPYD = Pyridinoline\deoxypyridinoline

Figure 1.14: Model depicting the markers of bone resorption.



Perhaps the most abundant enzyme in osteoclasts is lysomal acid phosphatase, which is secreted into the space forming the interface between bone and the ruffled border of the osteoclasts. The acid phosphatase finds its way into the circulation, and its concentration is proportional to the amount of bone resorbed. Hydroxyproline is largely found in collagen, and thus its excretion into urine tends to reflect bone resorption. Galactosyl hydroxylysine is much more abundant in bone collagen than in type I collagen from other sources, making the excretion of this breakdown product of collagen a marker of bone resorption. Pyridinoline and deoxypyridinoline are collagen crosslinks, and because they are formed extracelluarly and are not metabolised, their excretion in urine is proportional to bone resorption (23).

have shown that levels are elevated in early bone metastases from any site as well as many non malignant diseases, such as Paget's disease (107).

1.5.2: Hydroxyproline.

Hydroxyproline is found mainly in collagen, representing approximately 13% of the amino acid content of the molecule. Free hydroxyproline is released during the degradation of collagen and cannot be re-utilised in collagen synthesis, hence most of the endogenous hydroxyproline present in biological fluids is derived from the degradation of various forms of collagen (135). As half of human collagen resides in bone, where its turnover is faster than in the soft tissues, excretion of hydroxyproline in urine is regarded as a marker of bone resorption (135).

However other proteins are known to contain significant amounts of hydroxyproline, such as elastin (4) and a subcomponent of the compliment system (C1q), which can account for up to 40% of the urinary hydroxyproline (136). This shows that the relationship of urinary hydroxyproline to the metabolism of collagen is much more complex. About 90% of hydroxyproline released by the breakdown of collagen in tissues and especially during bone resorption, is degraded to the free amino acid. This circulates in the plasma, is filtered in the kidney, and is then almost entirely reabsorbed. It is eventually completely oxidised in the liver and is degraded to carbon dioxide and urea (137). The remaining 10% of hydroxyproline released by the breakdown of collagen circulates in the peptide bound form and these hydroxyproline containing peptides are filtered and excreted in the urine without any further metabolism. Thus, total urinary hydroxyproline represents only about 10% of total collagen.

Most studies appear to measure hydrolysed urine samples and hence the total urinary hydroxyproline content. As a consequence of its origin in tissue and its metabolism patterns, urinary hydroxyproline is poorly correlated with bone resorption as assessed by calcium kinetics and bone histomorphometry, except in the case of Paget's disease (19). However reasonable correlation with radioisotopic tracer methods in osteoporosis have also been reported (138). In metastic bone cancer hydroxyproline excretion can be at least as sensitive as bone scans, but its accuracy is dependant on the type of metastasis, *i.e.* whether it is predominantly osteoblastic or osteolytic (107).

The total pool of urinary hydroxyproline is derived, not only from endogenous sources, but also from collagenous proteins in the diet, especially meat and gelatin. Patients are therefore usually instructed to adhere to a meat and gelatin free diet prior to urine collection. This is difficult to control and hence false results are possible. It has now been accepted that since hydroxyproline is strongly influenced by diet, age and soft tissue destruction, it lacks sufficient specificity, sensitivity and discriminatory powers to be considered a useful and reliable marker in most metabolic diseases including skeletal metastases (87).

1.5.3: Hydroxylysine Glycosides.

Hydroxylysine, like hydroxyproline is an amino acid found primarily in collagen and collagen like peptides. Hydroxylysine is not re-utilised for collagen synthesis, and although it is less abundant than hydroxyproline, it can be used as a potential marker of collagen degradation. The amino acid undergoes further modification by glycosylation, giving rise to galactosyl hydroxylysine (GHYL) and glucosylgalactosyl hydroxylysine

(GGHYL) (139). Although the galactosylated hydroxylysines are found in all collagens, the ratio of GHYL to GGHYL appears to be tissue specific, with bone having a higher proportion of GHYL than other tissues (140,141).

Hydroxylysine glycosides are final products of collagen degradation (142) and are excreted into the urine unchanged in the free form (143). Unlike hydroxyproline, the hydroxylysine glycosides do not undergo any major liver metabolism (139-140,144) and are not influenced by diet. Hence these glycosides fulfil the requirements for a specific marker of bone resorption. The hydroxylysine glycosides measured by HPLC (145) have demonstrated good potential and superiority over hydroxyproline measurements in a number of clinical studies (146-148).

1.5.4: Pyridinium crosslinks.

The pyridinium crosslinks are generally accepted as the best available biochemical markers of bone resorption demonstrating excellent sensitivity and specificity. These crosslinks will be discussed in detail in Section 1.6.

1.5.5: Telopeptides of type I collagen.

During bone resorption only about 40% of the crosslinks are released as free pyridinium crosslinks. The remaining 60% is in the form of peptide-attached crosslinks (149). It has been established that type I collagen has two crosslink forming sites: the first is the amino terminal peptide region and the second the carboxyl terminal region of the molecule (150). During the course of extensive studies on the pyridinium crosslinks, particularly

since the development of immunological methods of analysis, assays have simultaneously been developed for the telopeptides of type I collagen.

1.5.5.1: The aminoterminal telopeptide (INTP).

The aminoterminal telopeptide (INTP) has been shown to be the most abundant crosslink telopeptide found in urine and proved to be the source of about 60% of the bound deoxypyridinoline found in urine (150). A recently developed immunoassay for INTP was developed against a peptide purified from adolescent human urine and has been shown to correlate with both urinary hydroxyproline and pyridinoline measured by HPLC (151). Furthermore this assay reliably measured bone resorption, in response to acute increases and decreases induced by thyroid hormone and bisphosphonate treatment respectively. These changes indicated better accuracy when compared to pyridinoline or hydroxyproline (152). A similar study has shown a decrease in INTP linked with the successful treatment of osteoporotic patients given adendronate treatment (153). This assay has also been used to study the bone growth rates in children (154).

1.5.5.2: The carboxyterminal telopeptide (ICTP).

An immunoassay to measure ICTP has been developed from a peptide prepared by the proteolytic digestion of intact demineralised human bone (155). This assay is used to measure serum levels of the peptide, and thus is different to the INTP assay which uses urine (151,155). The ICTP assay has been shown to correlate with bone resorption measured histomorphologically (156), and by calcium kinetic studies (157), indicating that ICTP reflects bone resorption.

Some clinical studies have been conducted recently although the results have been variable. Raised levels of ICTP levels were seen in cancers characterised by osteolytic lesions (158). ICTP has also been used to assess genetic influences on bone turnover (159). Another clinical study has shown that ICTP discriminates just as well as the urinary pyridinium crosslinks between normal and osteoporotic women. However ICTP offers the advantage in that since it is measured in serum, bone resorption and formation measurements on the same sample are possible, an important factor since bone loss is due to an uncoupling between the two processes (160). Serum is also generally easier and quicker to collect.

In another clinical study however, postmenopausal osteoporotic women showed that although ICTP reflected bone metabolism, it was not as sensitive when used as a marker for the changes in bone resorption induced by hormone replacement therapy. In addition it did not correspond with other measures of bone resorption during anabolic steroid therapy. Both these therapies are well known and established with respect to decreasing bone resorption in osteoporosis (161). A further limitation is that the ICTP exhibits a clear circadian rhythm (162,163).

Both the INTP and ICTP assays measure the telopeptides bound to pyridinoline and deoxypyridinoline. There has been a suggestion that these assays may demonstrate a degree of cross reactivity with peptides linked to other crosslinking molecules of type I collagen (164). A recent ELISA method claims not to suffer from this problem, since the assay is based on a different binding site mechanism (164), and this new assay has been used in clinical assessments to successfully monitor treatment in a number of metabolic

diseases (165). However both these telopeptides of type I collagen are relatively new, and further evaluation into the chemistry involved and their clinical significance as biochemical markers of bone resorption is needed and currently ongoing.

1.6: THE PYRIDINIUM CROSSLINKS.

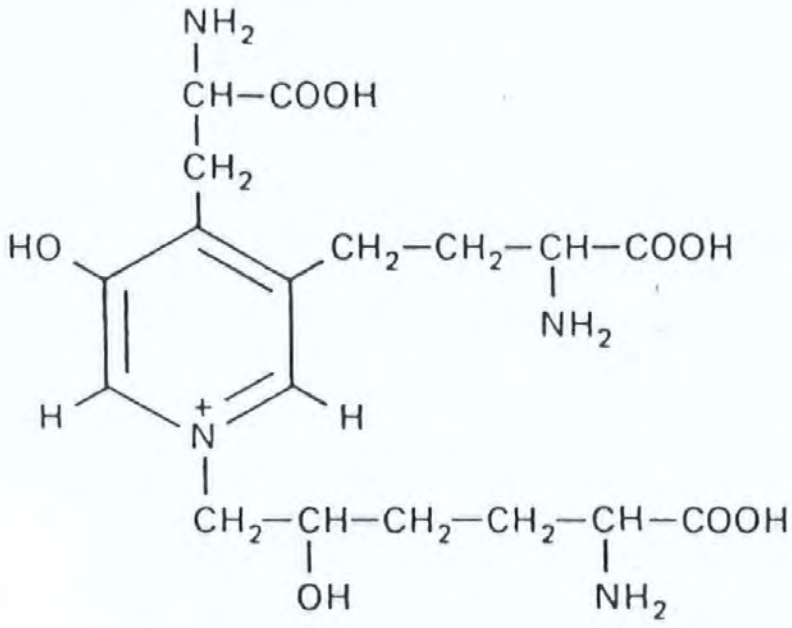
The two best known and documented crosslinks are pyridinoline (hydroxypyridinoline) and deoxypyridinoline (lysyl pyridinoline). Pyridinoline (PYD) was first discovered and isolated in 1977 in bovine achilles tendon (166). It is a 3-hydroxypyridinium derivative with three amino acid side chains (166-168) [Figure 1.15] and is present in cartilage and bone, but it is not found in skin (169). During the many studies on PYD, a new novel fluorescent compound believed to be another pyridinium derivative was discovered in 1982 in bovine femur (170). This compound was very similar to PYD but did not have the aliphatic hydroxy group attached. This compound was subsequently named deoxypyridinoline - DPYD (168,170-172) [Figure 1.15]. A glycosylated form of the crosslinks also exists (168).

1.6.1: Basic mechanism of formation.

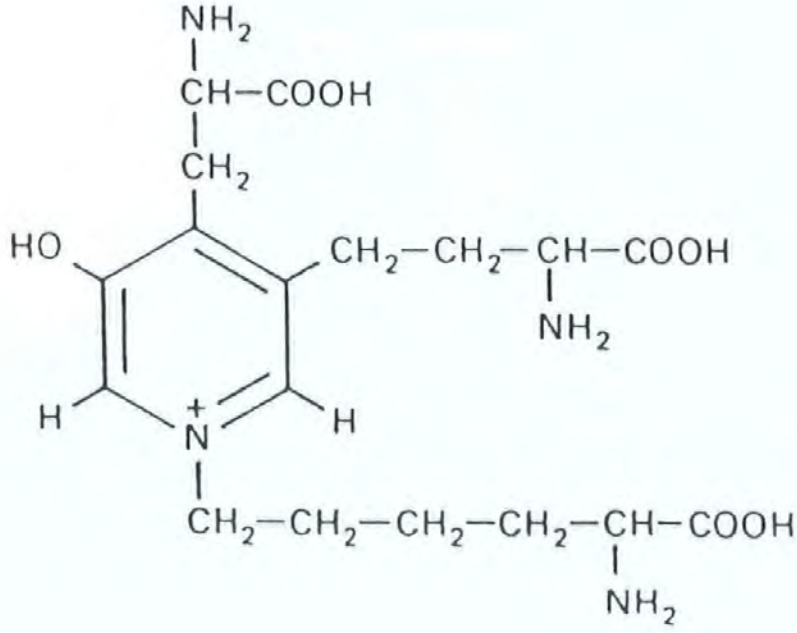
The pyridinium crosslinks are products of a unique series of reactions during the maturation process of collagen fibrils. Collagen crosslinks are initially formed from lysine and hydroxylysine residues by two major pathways yielding two major groups of crosslinks, *i.e.* those initiated by the enzyme lysyl oxidase and those derived from non enzymatically glycosylated lysine and hydroxylysine residues (7). These pathways are summarised in Figure 1.16.

Since the pyridinium crosslinks were discovered, many mechanisms of formation have been postulated. Initially it was thought these crosslinks were formed by the condensation of one hydroxylysine and two hydroxylysine-derived aldehydes (173). Later studies indicated that the crosslinks were formed by interaction of two di-functional keto amines.

Figure 1.15: Structure of the pyridinium crosslinks.

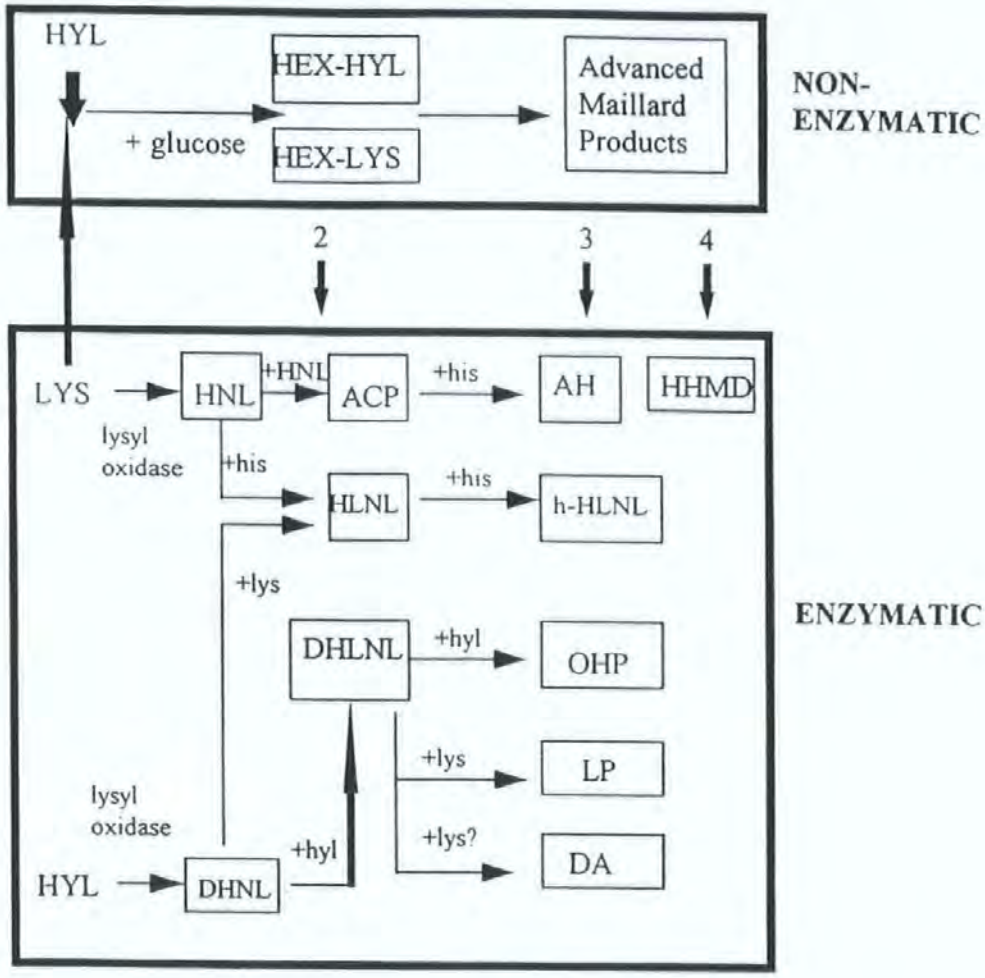


PYRIDINOLINE



DEOXYPYRIDINOLINE

Figure 1.16: Schematic diagram showing the reactions of the peptides lysine and hydroxylysine in collagen crosslink biosynthesis (7).



HYL	hydroxylysine	LYS	lysine
HEX-HYL	hexosyl-hydroxylysine	HEX-LYS	hexosyl-lysine
ACP	aldol condensation product	his	histidine
AH	aldol histidine	h-HLNL	histidino HLNL
OHP	hydroxypyridinium	LP	lysylpyridinium
HNL	hydroxynorleucine	DHNL	dihydroxynorleucine
HLNL	hydroxylysinonorleucine	DHLNL	dihydroxylysylnorleucine
HHMD	histidinohydroxymenodesmosine		
DA	deoxy analogue of OHP found in skin		

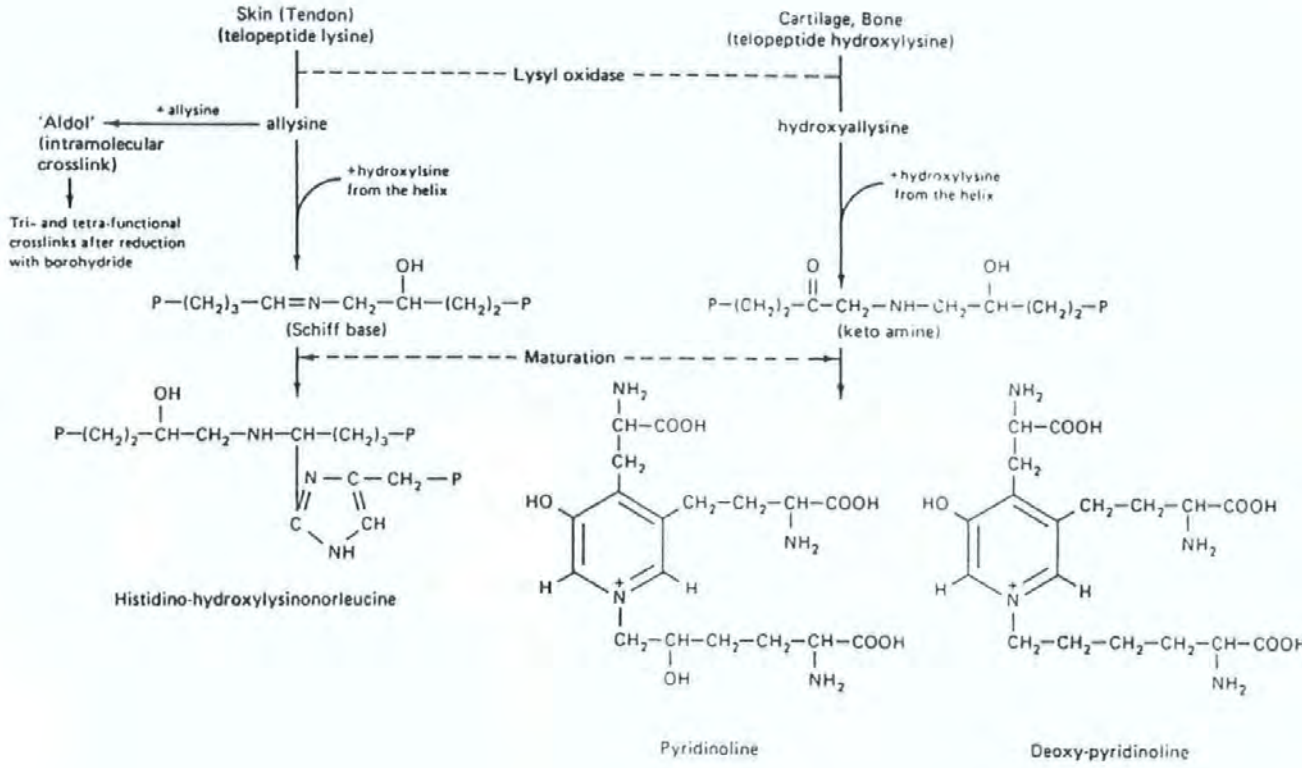
Nonenzymatic glycosylation results in the formation of hexosyl-lysine and hexosyl-hydroxylysine, some of these early difunctional products (the Schiff bases and their Amadori rearrangement products) may undergo transformations to advanced Maillard products. Lysine and hydroxylysine are converted to aldehyde moieties (DHNL and HNL) by the enzyme lysyl oxidase, they undergo further reactions to form di-, tri-, and tetrafunctional crosslinks. The numbers of residues in the enzymatically initiated crosslinks are indicated by arrows.

This explains why the reducible keto-amine crosslinks disappear from the skeletal connective tissues with age and indicates that the pyridinium crosslinks are mature, non-reducible crosslinks of collagen.

The electrochemical behaviour of PYD also confirms it is a non-reducible crosslink (174). This also provides a novel mechanism for lateral crosslinking within and between fibrils which may account for some of the unique physical properties of hard tissue collagen (175,176). This also rules out the possibility that PYD is an artefact produced during the acid hydrolysis of collagen (177). However recent studies have shown that the pyridinium crosslinks are in fact formed by interaction of two hydroxylysine residues, forming the Schiff base which then undergoes an Amadori rearrangement to form the more stable keto amine (7,178,179). This keto amine reacts with either another hydroxylysine residue to give pyridinoline (PYD), or with a lysine residue to give the deoxypyridinoline (DPYD) crosslink [Figure 1.17] (7). Studies have also shown that the keto amine may react with lysine to give a second non-fluorescent deoxy analogue, prominent in skin (180).

Many of the lysyl oxidase crosslinks may also be present in a glycosylated form if they are derived from a hydroxylysine residue containing the O-glycoside linkage, an enzymatically mediated reaction that occurs intracellularly. These types of glycosylated crosslinks, which feature O-linked galactosyl or glucosyl-galactosyl residues bound to the hydroxyl moieties, are distinct from the sugar-derived crosslinks (7).

Figure 1.17: Diagram showing the tissue specificity and maturation changes in crosslinking. The structures of the principle crosslinks in mature tissue are also shown (5).



Clear evidence now indicates the existence of PYD in collagen *in vivo* (181-183). These crosslinks play a major role in the stabilisation of collagen fibres (184,185) and through the course of maturation it has been established that the crosslink concentration correlates well with an increase in the mechanical stiffness and tensile strength of muscles (186).

1.6.2: General properties of the pyridinium crosslinks and their use as biochemical markers.

Pyridinoline (PYD) has the wider tissue distribution being prevalent in cartilage and tendon (187,188) whereas DPYD was initially thought to be found exclusively in bone and dentine. However significant amounts have also been detected in soft tissues, such as the aorta and ligaments (Table 1.8), although due to their slow turnover these tissues make a negligible contribution to the urinary output (189).

The ratio of PYD:DPYD is 3-4:1 in bone and >10:1 in cartilage (187). The urinary isolation of the crosslinks (190) has shown that the ratio of PYD:DPYD in urine is usually similar to that found in bone indicating that bone resorption is the major source of urinary pyridinium crosslinks and that the values for the two crosslinks in urine are usually highly correlated, as determined by radioisotopic (191) and histomorphometric (192) measurements.

Studies have shown that the urinary crosslinks exist in both free and bound forms (149), and a significant linear relationship between free and total forms of both crosslinks suggests the possibility of measuring the crosslinks in the free form. Further, it has been shown, that in general good predictability of 24 hour urine concentration can be obtained

Table 1.8: Distribution of the pyridinium crosslinks in human tissues (189).

Tissue	Pyridinium crosslink content of human tissue		
	n	PYD	DPYD
Articular cartilage	15	1.47+/-0.23	not detected
Cortical bone	15	0.35+/-0.09	0.08+/-0.02
Trabecula bone	7	0.26+/-0.08	0.06+/-0.01
Aorta	14	0.31+/-0.07	0.07+/-0.01
Intervertebral disc	25	1.14+/-0.11	not detected
Ligaments	10	0.47+/-0.35	0.05+/-0.03

The data for pyridinoline (PYD) and deoxypyridinoline (DPYD) are shown as residues\molecules of collagen +/- SEM.

based on a early morning void urine samples. This makes collection and measurement of the crosslinks easier, adding to the appeal of using these crosslinks as a marker (193). Both the free and conjugated forms of PYD and DPYD have demonstrated excellent chemical stability whilst stored at -20°C, and freeze thawing several times has shown no affect on the concentration of the crosslinks (194).

The cyclic pyridinium structures are stable and fluorescent, their typical fluorescence forming the basis of their detection in most assays (refer to section 1.6.3). However it should be borne in mind that these crosslinks are also photosensitive (195-197), and this should be taken into consideration during the collection and analysis of the crosslinks.

Age related changes in urinary PYD and DPYD crosslinks have been noted (187,198) and further studies have shown that urinary crosslink values reflect age related changes in bone resorption. Urinary crosslink excretion is increased in childhood (0-19 years) and in the early postmenopausal period, *i.e.* 50-59 years (199,200). In this early postmenopausal period, the urinary crosslink levels are significantly higher for females than males (201). Since the urinary levels in children is higher than in adults the crosslinks can be useful in monitoring growth in children (202,203).

The crosslinks are not influenced by diet (204) nor by kidney function (205). The physiological variations in the urinary excretion of total PYD crosslinks of collagen expressed relative to creatinine are minor compared to changes resulting from bone disease.

Bone resorption shows a circadian rhythm in human subjects (206,207), and appears to be unaffected by growth and ageing as well as being independent of the absolute level of bone turnover (208). This fact must also be borne in mind upon measurement of the crosslinks.

Immobilisation induces decalcification of bone, and in the early stages bone matrix is absorbed without any activation of osteoclasts, resulting in rapid decalcification of vertebral and cortical bones, without any discernable changes in anatomical structure (209). In short term immobilisation, TRAP and AP tend not to change, suggesting that the functional activity of osteoclasts and osteoblasts were unaltered. However urinary PYD and DPYD levels initially increased then declined, suggesting resorption of bone matrix initially, which reduced without any involvement of oestocyclic activity (210).

1.6.3: Analysis of the pyridinium crosslinks.

The current method of choice and until recently the only way the two crosslinks could be measured with any degree of success is high performance liquid chromatography (HPLC). Due to the various problems associated with the HPLC technique, research has been conducted into immunological methods to measure the pyridinium crosslinks. However it is only within the last few years that this approach has shown any sign of success. The analysis of the pyridinium crosslinks is discussed in greater detail in Chapters 3 and 4.

1.6.4: The use of pyridinium crosslinks as biochemical markers to measure bone resorption in metabolic bone diseases.

Since the discovery of the pyridinium crosslinks it was realised that collagen crosslinking compounds could act as biochemical markers of polymeric collagen and their quantification would provide an unambiguous and clinically useful measure of degradation of extracelluar collagen. Consequently these crosslinks offer potential as sensitive and specific markers of bone resorption. A large number of studies have been conducted over the last few years, too vast to be included here, but details are provided in a number of recent reviews (189,211-216). The major clinical areas of interest (except skeletal metastases) in which these crosslinks have been used are summarised in Table 1.7.

In comparison to other markers, clinical studies have shown that the pyridinium crosslinks, in particular deoxypyridinoline (DPYD), are highly correlated with the hydroxylysine glycosides, demonstrating similar accuracy and discriminatory powers with respect to distinguishing between subjects with altered bone resorption from normal subjects (147,217). However the crosslinks have the advantage that unlike the hydroxylysine glycosides, the pyridinium crosslinks are naturally fluorescent, and therefore do not need to undergo a derivatisation step prior to HPLC analysis (247). The crosslinks also show greater accuracy and a higher discriminating power than hydroxyproline (147,201).

The analytical and biological variability of the urinary crosslink measurements have affected its clinical utility as a biochemical marker of bone turnover (249). More recently the crosslinks have been identified and measured in serum (248), which should

Table 1.9: Summary of clinical studies using the pyridinium crosslinks as markers of bone resorption.

Clinical condition	Description	Ref.
Arthritis	Most clinical studies have found that the crosslink levels are increased in both rheumatoid arthritis (RA) and osteoarthritis (OA). PYD levels are particularly elevated whereas the DPYD levels are only slightly elevated or not significantly different from control levels. This corresponds to the fact that PYD is prevalent in cartilage and bone whereas DPYD is bone specific.	217-224
	One study however shows conflicting results in which the PYD levels in articular cartilage were unaffected in both RA and OA patients.	225
	Successful treatment with gold and D-penicillamine results in a decrease in the crosslink levels. However treatment with corticosteroids results in an increase in the crosslink levels, probably due to the induction of bone resorption.	218, 221
	The pyridinium crosslinks, particularly PYD can provide useful information on the stage, activity and level of bone involvement as well as the efficacy of drug therapy in arthritic disease	
Growth hormone deficient adults	The effect of growth hormone therapy resulted in an increase in the crosslink and osteocalcin levels, indicating an increase in the bone resorption and formation rate respectively.	226
Hyperpara-thyroidism	The crosslink levels were significantly higher in patients compared to controls. Patients who were treated surgically showed levels comparable to the controls and significantly lower than untreated patients.	227

Table 1.9: Continued.

Clinical condition	Description	Ref.
Hyperthyroid-ism	It is believed that thyroid replacement therapy used to treat hyperthyroidism, may cause or increase the risk of osteoporosis. This study showed that the crosslink levels increased in untreated patients and in postmenopausal woman taking sufficient medication, compared to the controls. However premenopausal woman appear to have a lower risk to osteoporosis when receiving similar treatment, since the crosslink levels were virtually the same as the controls.	228
Inherited connective disorders eg. Ehlers-Danlos syndrome (EDS), Marfan syndrome (MS) and Funnel chest (FC)	Some abnormalities in the crosslink excretion levels were observed in these disorders, however firm conclusions were not drawn until the results of further investigations were available.	229
Malnutrition (children)	The crosslink levels were lower in malnourished children compared to recovered children, indicating a decrease in the cartilage and bone turnover rate. The study of crosslink levels could therefore be used to assess the therapeutic intervention designed to alleviate stunted growth.	230
Menopause	The crosslinks increase at the menopause but a reduction in these levels is observed with successful hormone replacement therapy.	231-235
Osteoporosis	The pyridinium crosslink levels are higher in osteoporotic patients compared to controls.	236-240

Table 1.9: Continued.

Clinical condition	Description	Ref.
Osteoporosis (continued)	The use of the pyridinium crosslinks has also allowed a greater insight and understanding into the mechanisms of osteoporosis. The crosslinks demonstrated that postmenopausal osteoporosis does not have an increased responsiveness to parathyroid hormone, hence the higher bone turnover associated with osteoporosis is due to other systemic of local factors regulating bone resorption.	241
	Osteoporotic patients show abnormalities in the circadian rhythm of bone resorption. A higher resorption rate exists which would account for the increased bone loss seen in osteoporotic patients.	242
Paget's Disease	Patients with active Paget's Disease of bone showed higher crosslink levels compared to controls and these levels decreased with successful treatment with bisphosphonate pamidronate.	243
	Another study evaluated a wide range of bone resorption and formation markers and concluded that when disease activity was high most of the bone turnover markers were increased. However when disease activity was low only serum bone alkaline phosphatase and urinary pyridinium crosslinks improved the detection of the disease	244
Ullrich-Turner Syndrome	Pyridinium crosslinks levels were increased compared to controls, whereas hydroxyproline showed little difference. The higher crosslink excretion reflects enhanced bone resorption activity.	245
Vitamin D deficiency	Crosslink levels were higher in patients with a deficiency compared to controls.	246

demonstrate lower intra individual variability and will hopefully be a more suitable and reliable marker.

1.6.5: The use of pyridinium crosslinks as markers of bone breakdown in patients with skeletal metastases.

Many clinical studies have shown that the crosslinks maybe useful in the assessment of neoplastic disease. Raised levels have been observed in patients known to have bone metastases when compared to controls, and these levels fall with subsequent successful treatment (250-256).

However the diagnostic ability of the crosslinks is not so clear. In some studies levels were elevated in some patients with no known or diagnosed bone metastases (250,253,257). Only further investigation and long term follow up studies will determine whether these observations were false positives or that the crosslinks offer a possible early diagnosis method. A number of other studies have shown that the crosslinks diagnostic ability was low (257).

As expected the crosslink levels have been shown to be higher in in-patients compared to ambulatory outpatients with cancer (253), which is attributable to the greater degree of immobilisation of in-patients. Crosslinks have also been used to assess the activity of malignant haematological disorders by measuring bone resorption, increased levels being attributed to the leukaemic infiltration of the bone marrow space (258).

1.7: TRACE METALS IN THE HUMAN BODY.

The human body is composed of a vast number of elements which are categorised according to their concentration and biological significance (259-261). Elements are denoted as major or minor, the latter being subdivided into trace ($< 100\mu g/g$) or ultra-trace ($< 10ng/g$) in terms of concentration. In spite of the low levels of trace and ultra trace elements they are of great importance for the health of living organisms, and in this context a distinction is made between essential and non-essential elements.

In addition to C, Ca, Cl, H, K, Mg, N, Na, O, P, and S which are essential major elements, the trace elements Co, Cr, Cu, F, Fe, I, Mn, Mo, Se, V and Zn are also considered as essential (261). Figure 1.18 shows schematically the typical health response profile for an essential element. Death, or at best, bare survival results if the element is withdrawn from the diet. As the organism is exposed to increasing amounts of the element, the response improves until a plateau is reached indicating full health. In excess the element produces undesirable side effects and gradually results in a deterioration of health until death finally occurs (262). The non-essential elements include all other elements in a living organism, although it is accepted that for many of these elements their "essential" character may not as yet have been proved. Tables 1.10 and 1.11 summarises the physiological concentrations of those elements generally considered to be essential and non-essential (263). The determination of essential and non-essential trace elements in biological samples is important with respect to understanding health and disease, *i.e.* metabolism, physiology, nutrition, toxicology, for reaching a diagnosis and for selecting the appropriate treatment for a disease (259,263-268).

Figure 1.18: Idealised curve showing the response of an organism to increasing doses of an essential element (260).

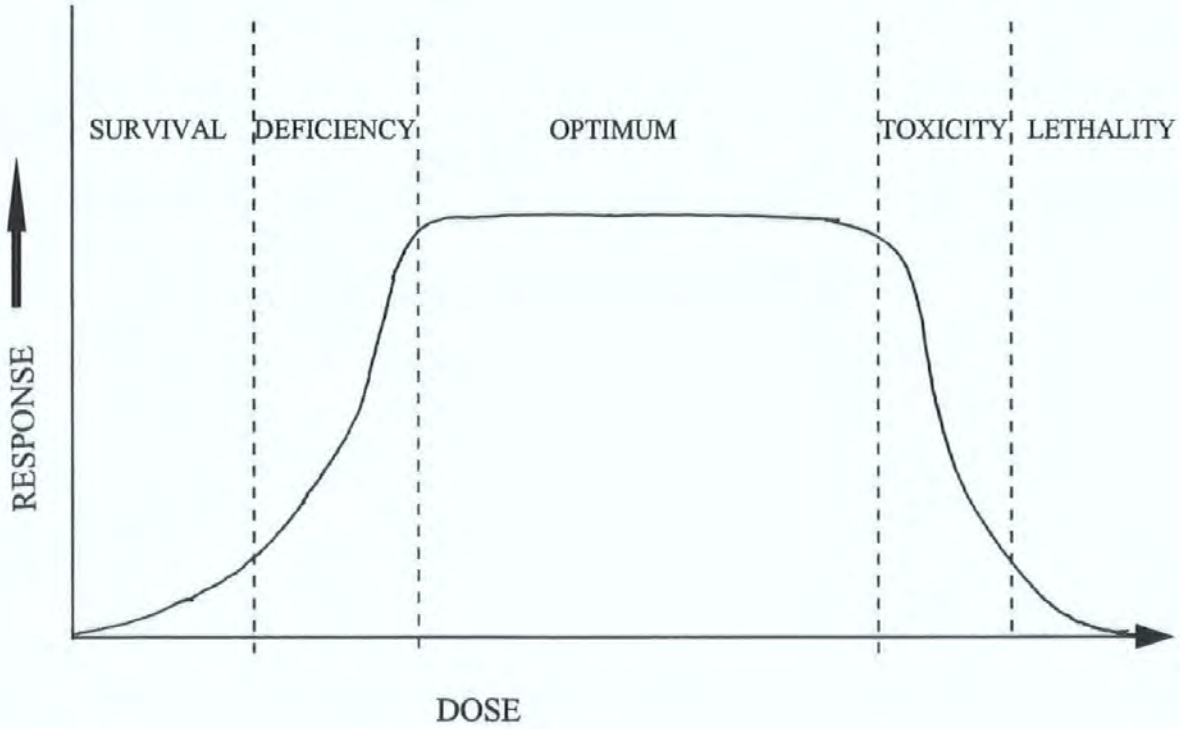


Table 1.10: Physiological concentrations of essential trace elements in body fluids (263).

Element	Matrix	Reference concentration for health controls μ\nmol l^{-1}	Reference concentration for health controls $\mu g\ l^{-1}$
Zinc (Zn)	P\S	11-24	720-1570
	U	4.5-9.0	294-558
Iron (Fe)	S	11-36	614-2010
	U	0.2-1.0	11-56
Copper (Cu)	S	3.0-11[a]	191-700
	S	12.0-26[b]	763-1652
	S	27-40[c]	1716-2542
	U	<0.8	<51
Manganese (Mn)	B	73-110	4-12
	S	9-24	0.5-1.3
	U	2-27	0.1-1.5
Selenium (Se)	B	1.2-2.2[d]	95-174
	B	1.0-2.0[e]	79-158
	S	1.1-1.9	87-150
	U	0.1-0.7	7.9-55
Chromium (Cr)	B\S	<20	<1.0
	U	<20	<1.0
Molybdium (Mo)	S	2-12	0.2-1.2
Cobalt (Co)	B	<17	<1.0
	U	<17	<1.0
Vanadium (V)	B	<6	<0.3
	U	<6	<0.3

[a] Neonates.
[b] Children over 6 months and adults.
[c] Healthy pregnant women.
[d] Adults.
[e] Children: Se concentrations is age.

B: Blood.
P: Plasma.
S: Serum.
U: Urine.

N.B. Urine values assuming 24 h collection.

Table 1.11: Concentrations of non-essential elements in body tissues (263).

Element (unit)	Matrix	Concentrations in tissues\fluids		Concentrations in tissues\fluids	
		Reference levels		Therapeutic levels	
		μmol\nmol l^{-1}	$\mu g\ l^{-1}$	μmol l^{-1}	$\mu g\ l^{-1}$
Aluminium (Al) (μmol l^{-1})	S	<0.4	<11	1.8-22	48-593
	U	<2.0	<54	>15	405
	CSF	0.15-0.19	4-5	0.22-1.26	6-34
Gold (Au) (μmol l^{-1})	S	<0.01	1.97	5-40	985-7879
Bismith (Bi) (μmol l^{-1})	S	<0.05	<10	0.2-14	42-2926
	U	<0.05	<10	0.2-14	42-2926
Gallium (Ga) (μmol l^{-1})	S	<1	70	up to 70	976
	U	<1	70		
Platinium (Pt) (μmol l^{-1})	B	<0.005	<1	0.1-50	20-9755
	S	<0.005	<1	0.1-50	20-9755
Silver (Ag) (μmol l^{-1})	B	<0.01	<1	-	-
Lead (Pb)* (μmol l^{-1})	B	0.2-1.0[a]	41-207	-	-
	B	0.2-1.2[b]	41-249	-	-
	U	0.05-04[c]	10.83	-	-
Cadmium (Cd)* (nmol l^{-1})	B	1.8-27[d]	0.2-3	-	-
	B	1.8-54[c]	0.2-6	-	-
	U	<27	<3	-	-
Beryllium (Be)* (nmol l^{-1})	U	44-100	0.4-0.9	-	-
Nickel (Ni)* (nmol l^{-1})	S	19-14	1.1-0.8	-	-
	U	7.68	0.4-4.0	-	-

Table 1.11: Continued.

Element (unit)	Matrix	Concentrations in tissues\fluids		Concentrations in tissues\fluids	
		Reference levels		Therapeutic levels	
		μmol\nmol l^{-1}	$\mu g\ l^{-1}$	μmol l^{-1}	$\mu g\ l^{-1}$
Asenic (As)* (nmol l^{-1})	B	7-125	0.5-9.4	-	-
	U	40-700[e]	3-53	-	-
Mercury (Hg)* (nmol l^{-1})	B	<30	<6	-	-
	U	<50	<10	-	-
Antimony (Sb)* (nmol l^{-1})	B	8	1.0	-	-
Tellurium (Te)* (nmol l^{-1})	B	2	0.26	-	-
Thallium (Tl)* (nmol l^{-1})	U	<1.0	<0.2	-	-

* Known toxic trace elements: concentrations are expressed for exposed healthy controls.

[a] For women.
[b] Children and men.
[c] Men.
[d] non smokers.
[e] Smokers.

B: Blood.
CSF: Cerebral spinal fluid.
P: Plasma.
S: Serum.
U: Urine.

N.B. Urine values assuming 24 h collection.

The potential for metals to be toxic has traditionally been regarded as a function of dose and potency of the metal itself. More recently however, it has become clear that several metals participate in biotransformation reactions and consequently metabolism may have important implications in terms of toxicity to humans, pharmacology and occupational health (269). In estimating metal levels for biomonitoring purposes adequate knowledge of the metabolic patterns is needed to establish relationships between external exposure and internal dose (270). As well as the type of metal present, speciation of the metals and their metabolites is also extremely important with respect to understanding metabolism and toxicology (271).

1.7.1. Measurement of trace metals in biological samples.

Analytical techniques have changed within a matter of a few years to such a profound extent that degrees of sensitivity and precision can now be attained that could not have been dreamt of one or two decades ago. Consequently the analysis of trace and ultra trace metals affecting human health and disease is now possible (261,272,273).

The performance characteristics of the analytical method, such as detection limits, accuracy, precision, speed etc, depend on a number of factors including the element concerned, the concentration of the element in the sample of interest, the sample matrix, (*i.e.* blood, serum, plasma, urine, organs and tissues such as hair, liver, kidney or lungs), on the homogeneity of the sample and the number of samples to be analysed.

The main techniques used for trace metal analysis in biological samples are neutron activation analysis (NAA) (274-277), electrothermal atomic absorption spectrometry

(ETAAS), (263,273,276,278-282), inductively coupled plasma-atomic emission spectrometry (ICP-AES) (273,276,283-285) and inductively coupled plasma-mass spectrometry (ICP-MS) (273,276,277,285-286).

The normal reference values for trace metals in various biological samples has varied considerably over the years. This has been attributed to the changes in analytical technology, allowing more accurate and sensitive methodology to be used (287), coupled to a greater understanding of the importance of quality control measures (288-292), sampling, collection and storage etc (282,293-302). A number of reviews provide details of the normal reference values for many elements in various biological samples (303-308).

1.7.2. Trace metals in bone.

Bone is a complex material and knowledge of its composition is of fundamental importance in the application and interpretation of elemental analysis. Bone is an active tissue comprising of an organic part (30%) and an inorganic part (70%) (309). About 98% of the elements found in bone are calcium, phosphate, carbonate, sodium and magnesium. There are smaller amounts of other elements, although often their role in normal bone function and in bone pathology is not fully established. It is known that bone acts as a reservoir for some potentially toxic elements, such as lead, but other elements may be important in bone metabolism (309).

A number of studies have been conducted to evaluate the elements present in bone and their concentration levels (309-313). However large variations exist, which have been

attributed to both the different analytical techniques and procedures used, as well as the type of bone sample analysed. Since cortical and trabecula bone differ structurally, there may well be a difference between the two types biochemically (309).

The measurement of metals in bone, especially trace elements is of particular interest because they are laid down over a period of time, being consistently and slowly renewed and replaced. Consequently the trace element content of bone can serve as useful indicators of dietary habits and nutrition (313), the assessment of deliberate or accidental long term exposure\pollution (314,315), as well as disease. In terms of the latter, the determination of metal levels in bone may help with the diagnosis of the skeletal state, assess the efficacy of the treatment, and supply data to help gain a better understanding of bone physiology and pathology (316-319).

1.8.AIMS OF INVESTIGATION:

1.8.1: Objectives:-

The principle objective of this study was to determine, using serial blood and urine samples taken from patients who regularly visit the oncology outpatient department (Freedom Fields Hospital, Plymouth), whether there is a rise in trace metal levels in patients who develop bone metastases, and additionally if there is a subsequent decrease in levels with successful treatment. A comparison is also made with existing methods for measuring bone breakdown and detecting bone secondaries, especially with respect to the specificity and sensitivity of these tests. A look at the predictive value of trace metal levels has also been conducted, in order to identify a potential increase in levels prior to bone secondaries being clinically apparent or identified by bone scans.

A secondary objective of this work was to gain an insight into bone metabolism, trace metal metabolism and the behaviour of bone metastases. The study hopes to promote the use of inductively coupled plasma-mass spectrometry for clinical applications. This development, along with the information obtained about trace metal and bone metabolism, may provide a number of opportunities for further research into associated topics, and in addition provide another technique to measure bone breakdown.

It is anticipated that by providing an additional technique for measuring bone breakdown this work may provide a further method of predicting, diagnosing and following up bone secondaries. Such a method would be of value in reducing the frequency of bone scans, which are currently usually used to detect bone metastases. It would also help to clarify

confusing scans, so called "super scans" when diffuse skeletal involvement produces a normal looking scan. Thus this work could have relevance to other conditions in which bone breakdown is a feature, such as osteoporosis, and may also be relevant to the study of the toxicology of trace metals, providing information about metabolism with respect to bone.

1.8.2. Plan of investigation:-

Patients with a histological diagnosis of cancer seen in the outpatients department, were selected to participate in the trial according to the inclusion and exclusion criteria:-

A) INCLUSION CRITERIA:-

i) Histological diagnosis of cancer.
ii) Informed consent obtained.
iii) Prognosis greater than 3 months.
iv) Adequate performance status.

B) EXCLUSION CRITERIA:-

i) Evidence of other significant bone disease, for example Osteoporosis, Vitamin D deficiency, Primary Hyperparathyroidism, Paget's disease of bone.
ii) Current therapy with drugs affecting bone metabolism
iii) Abnormal diet.

Once suitable patients had been selected and consented to participate in the trial, blood and urine samples were taken at each hospital visit. A bone scan was performed every 3 months or if clinically indicated, as were plain X-rays if clinically dictated. The selected patients are grouped as follows:-

i) Patients with cancer with bone metastases but not commenced on treatment.

ii) Patients with cancer and bone metastases, already commenced on treatment.

iii) Patients with cancer but no evidence of bone metastases (control group I).

iv) Patients with no known forms of cancer (control group II).

The group of patients without bone metastases at the outset of the study were divided into those who developed metastases during the course of the trial and those who were free of evidence of bone secondaries.

The trace metal levels in the blood and urine samples were determined using ICP-MS, and compared to the levels of urinary pyridinium crosslinks, measured using an ion pair reverse phase HPLC method and an ELISA method.

The results are finally compared and correlated with other clinical indices for example alkaline phosphatase and PSA levels, bone scan and plain X-ray results, as well as the patients clinical performance. The study also assesses the variables: age, sex, treatment, primary diagnosis and bed rest on the trace metal levels and urinary pyridinium crosslink levels measured.

CHAPTER 2

INSTRUMENTATION.

CHAPTER 2: INSTRUMENTATION

2.1: INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY

2.1.1 Historical background.

The use of mass-to-charge ratio to identify ions, the basic principle of mass spectrometry (MS), dates back to the beginning of the century (320), and is now widely used in chemical analysis (321). Flames, spark and arc discharge ion sources, suffer from interferences, low temperatures, contamination and sample introduction problems (322), and as a consequence the electrode-less ion sources, such as micro-wave induced plasmas (MIP) and inductively coupled plasmas (ICP) have beome the more favoured ion sources (323). Both plasmas overcome the contamination problems and are capable of very high temperatures, however with MIP's, the mean average temperature can be low and thus poor volatilisation and dissociation of many species results in strong matrix effects (322,323). Nevertheless, such a plasma has been used with some sucess as an ion source for mass spectrometry (324). The ICP ion source (325,326) as well as offering sufficiently high temperatures, also allows the rapid and complete introduction of the sample into the plasma, resulting in very efficient sample volatilisation, dissociation and excitation. This source had been used widely in emission spectrometry (327) and has also become the more accepted and widely used ion source for mass spectrometry (322).

Inductively coupled plasma-mass spectrometry (ICP-MS) was first expressed as a concept back in 1970, however the problem of coupling an ICP which operated at atmospheric pressure with a MS which operated in a vacuum, delayed the release of a commercial

instrument until 1983. Collaboration between Houk *et al* in the USA and Gray *et al* in the UK (328), along with the work by Douglas and French (324), resulted in overcoming these inherent problems and led to the development of the system and interface now employed in commercial ICP-MS instruments (322,323,329-335).

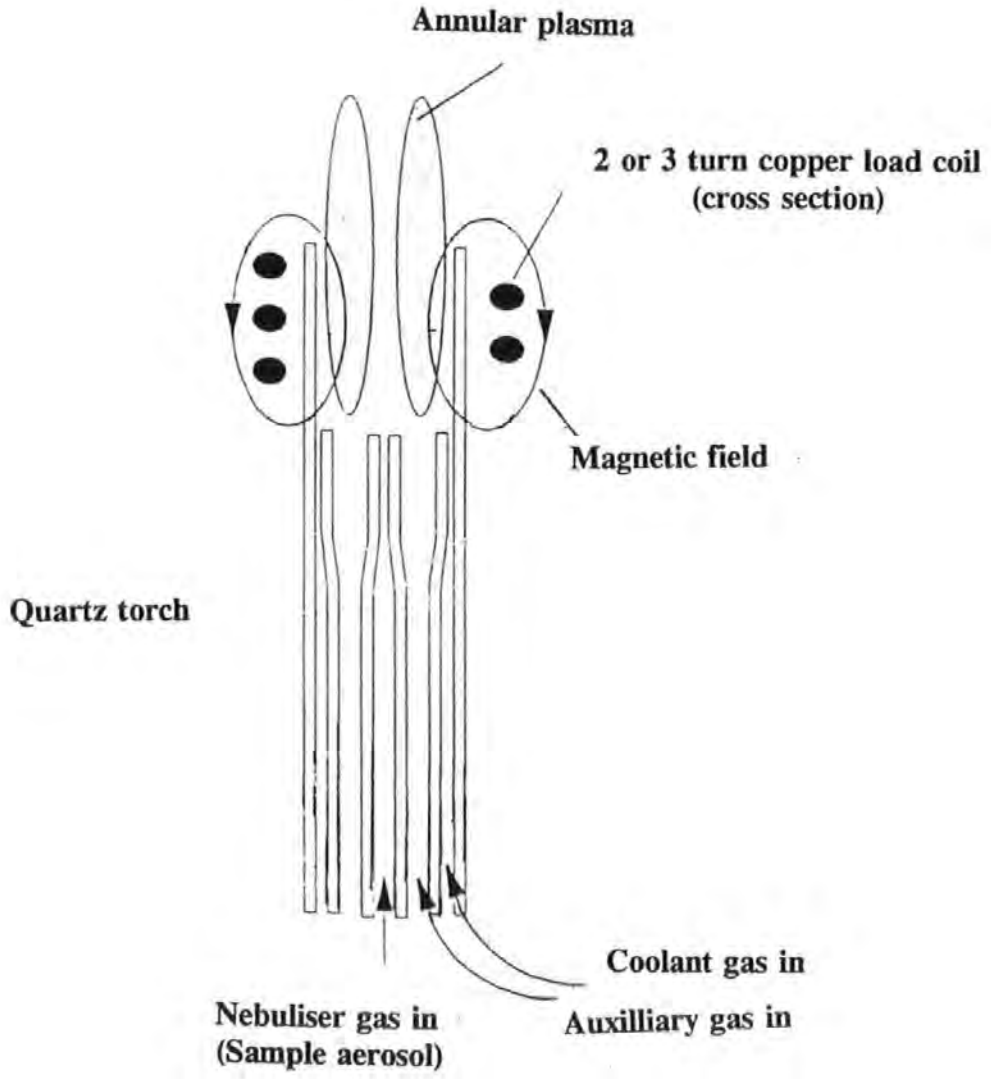
The combination of an inductively coupled plasma (ICP) as a high temperature ion source with a quadrupole mass spectrometer (MS) as a high sensitivity detector has produced a versatile analytical technique capable of elemental and isotopic analyses of samples. ICP-MS offers excellent detection limits for many elements (0.01-0.1 μgl^{-1}), a broad linear range (at least 4 orders of magnitude) and a multi element capability allowing fully quantitative measurements for elements across the mass range. ICP-MS can also offer a high sample throughput, simple spectra (compared to optical emission spectroscopy), relative freedom from chemical interferences and information about isotope ratios.

ICP-MS can therefore be used for quantitative isotope determination, isotope dilution analysis, rapid spectral scanning due to the peak hopping capability of the mass spectrometer and use of multi channel analysis and semi-quantitative determinations to within a factor of 2 or 3 in a wide range of samples (322,323,334-336).

2.1.2: Plasma Generation.

The ion source is generated by coupling the energy from a radiofrequency generator to an appropriate gas (usually argon) flowing through a quartz glass torch (Figure 2.1). The RF energy is transferred to the plasma via a water cooled copper coil. The incident power is usually between 1-2kW, at a frequency of 27.12 MHz. The plasma is formed

Figure 2.1: Schematic diagram of an inductively coupled plasma.



when the gas is seeded with electrons from a spark from a Tesla coil. These electrons are accelerated in the magnetic field causing ionisation of the gas. Further collisions between gas atoms results in a self sustaining plasma reaching a temperature of 6,000 to 10,000K (337). This process occurs almost instantaneously.

The nebuliser or injector gas, punches a hole at the base of the plasma, producing a cooler central channel. A coolant gas flow protects the quartz glass torch walls, centres and stabilises the plasma and an auxiliary gas flow ensures the hot plasma is kept clear of the tip of the nebuliser gas flow, preventing the central capillary injector tube from melting (Figure 2.1).

2.1.3: Sample introduction.

The ICP requires the sample to be introduced into the nebuliser gas flow as a gas, vapour, aerosol of fine droplets or as finely ground dispersed solid particles (333). Sample introduction systems can therefore be achieved by a variety of methods, such as nebulisation, hydride generation, flow injection, laser ablation and electrothermal atomisation (322,323,335-338). The most common method for introducing a sample is as a gas-supported aerosol from a pneumatic nebuliser, producing a wide range of droplet sizes which are discharged into a spray chamber. The larger droplets are filtered to waste in this spray chamber, since they cause signal fluctuations, plasma instability and can eventually extinguish the plasma. The smaller droplets (approximately 8μm or less), accounting for only 1-2% of the sample introduced, remains supported in the gas stream and are allowed to pass through into the plasma. Upon entering the plasma the sample is desolvated and vaporised and the constituent analytes are disassociated, excited and

ionised. This standard sample introduction system is shown in Figure 2.2.

2.1.4: Ion extraction.

A portion of the ionised gas from the tail flame of the ICP enters the ion extraction interface (Figure 2.3) and impinges onto a water-cooled sampling cone, typically made from nickel. Gas from the ICP is extruded through a small aperture (approx. 1.0mm diameter) drilled in this cone and enters the first (expansion) stage. The gas pressure in this first stage is maintained at 1-3 torr. The resultant sample beam expands rapidly, causing a drop in temperature essentially freezing the ion population and hence inhibiting reactions occurring in the sampled plasma gas. This results in the formation of a freely expanding region, known as the zone of silence or "free jet". The temperature at the centre of the free jet is approximately 200K and this helps to cool the skimmer cone. The zone of silence is bounded by shock waves known as "barrel shock" and the "Mach disc" (Figure 2.4), which are caused by collisions between fast moving atoms from the jet and the surrounding gas. Beyond the "Mach disc", the flow becomes subsonic and the extracted gas mixes with surrounding gas. To avoid losses of ions due to scattering and collisions from these shock waves surrounding the zone of silence, the tip of the skimmer cone (a second sharper nickel cone, approximately 0.75 mm diameter) is mounted upstream of the "Mach disc". This ensures that a small percentage of the gas from within the "barrel shock" region is sampled (Figure 2.4). The pressure behind the skimmer cone is usually below 5×10^{-4} torr. A slide valve is situated behind the expansion stage and this isolates the high vacuum of the mass spectrometer from the expansion stage, when the instrument is not in use (334,341,342).

Figure 2.2: Schematic diagram of the ICP-MS sample introduction system (339).

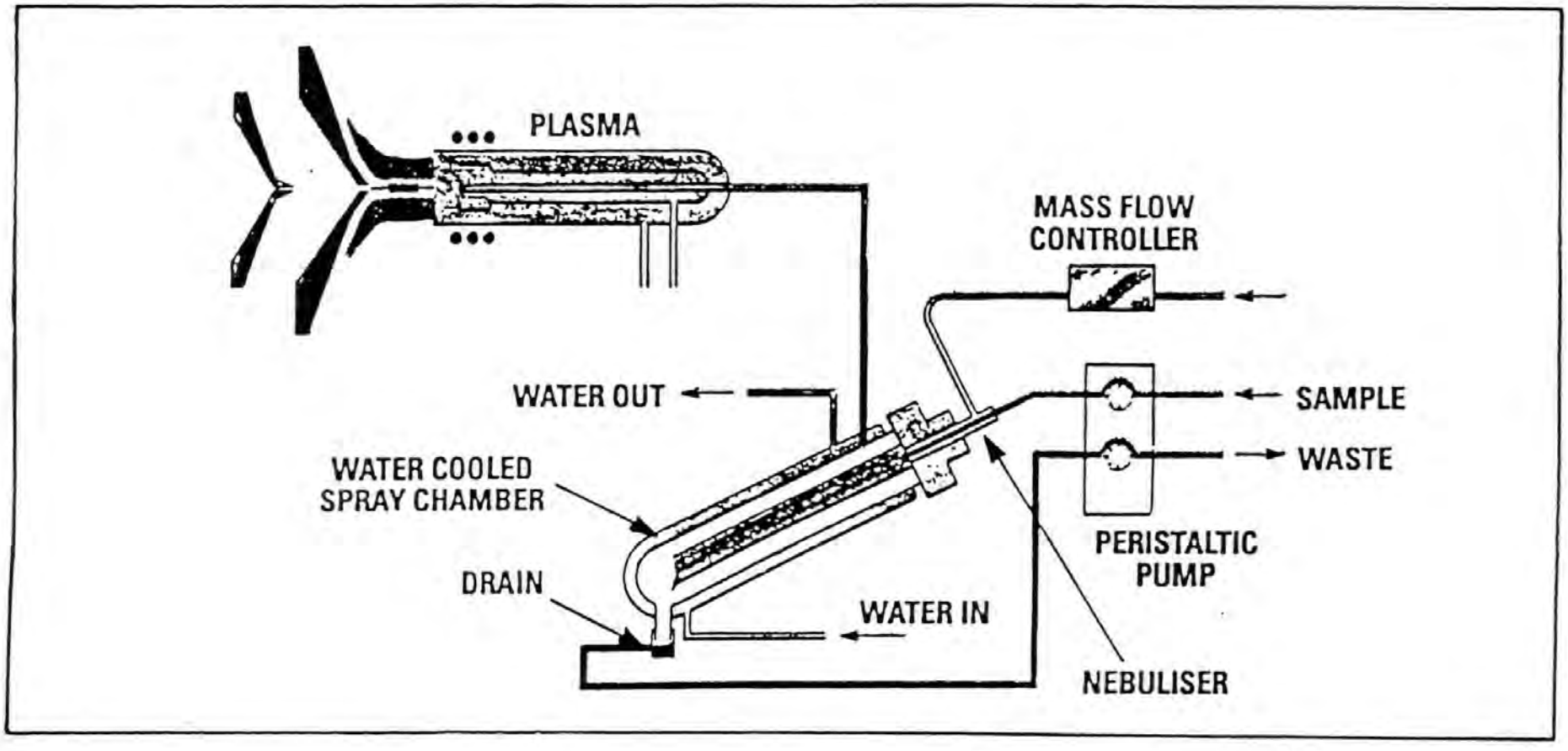


Figure 2.3: Diagram showing a typical ICP-MS ion extraction interface (340).

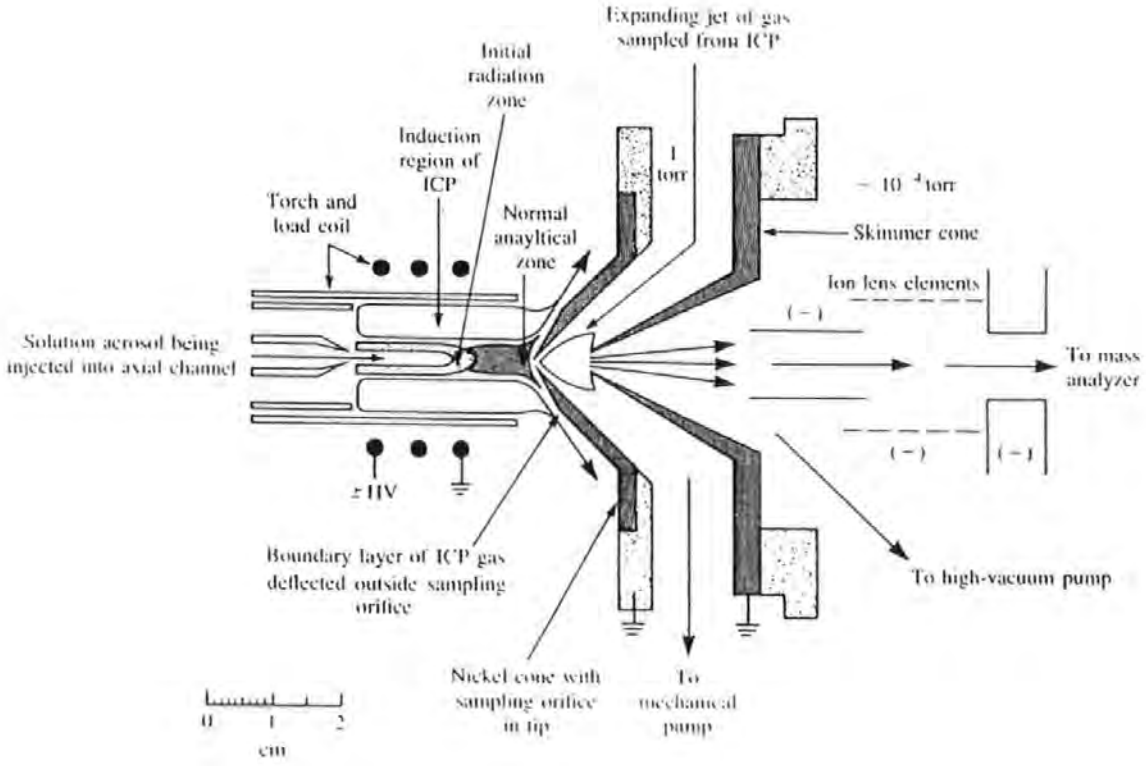
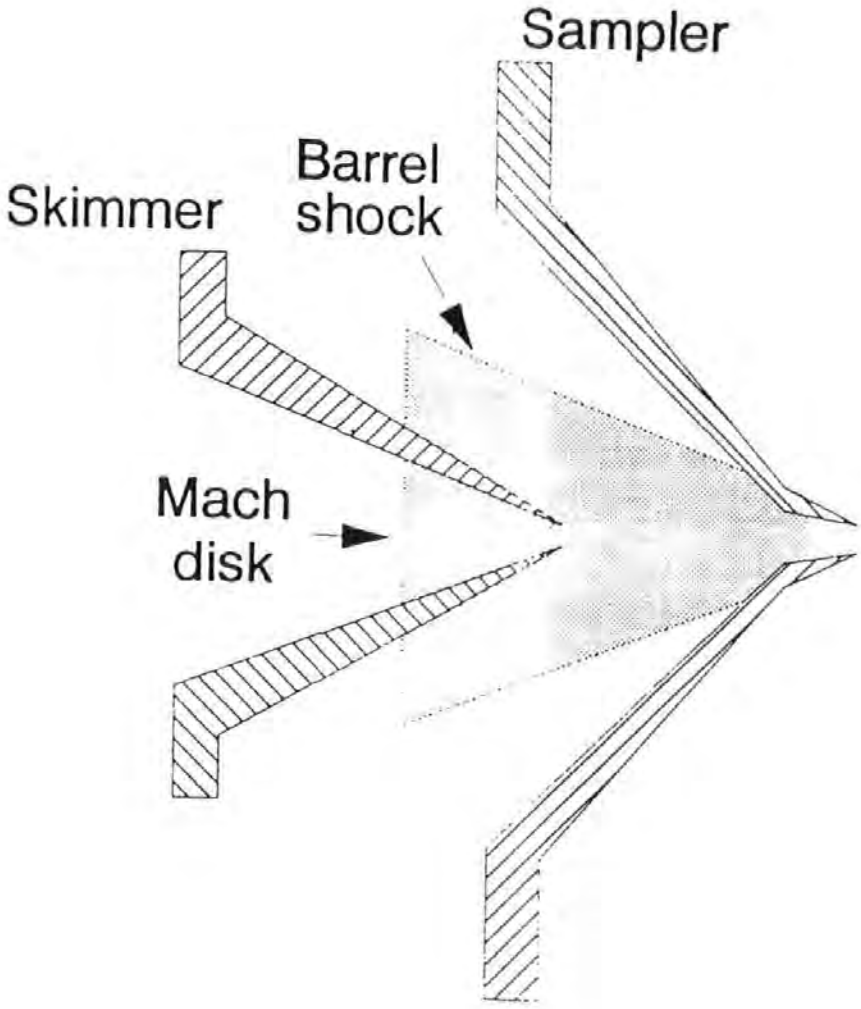


Figure 2.4: Diagram of the supersonic expansion formed in the expansion chamber, showing the barrel shock and position of the Mach disc (323).



2.1.5: Ion focusing and detection.

A series of electrostatic lenses separate ions from neutral species, focuses them and transmits them to the third vacuum stage and into the quadrupole mass spectrometer where ions are separated with respect to their mass-to-charge ratio (m\z) and are detected with a channel electron multiplier, placed just off axis to avoid being struck by residual photons (343-345). The electron multiplier pulse is then passed on to a suitable amplifier and discriminator and then onto a data handling system. A schematic diagram of a typical ICP-MS instrument is shown in Figure 2.5.

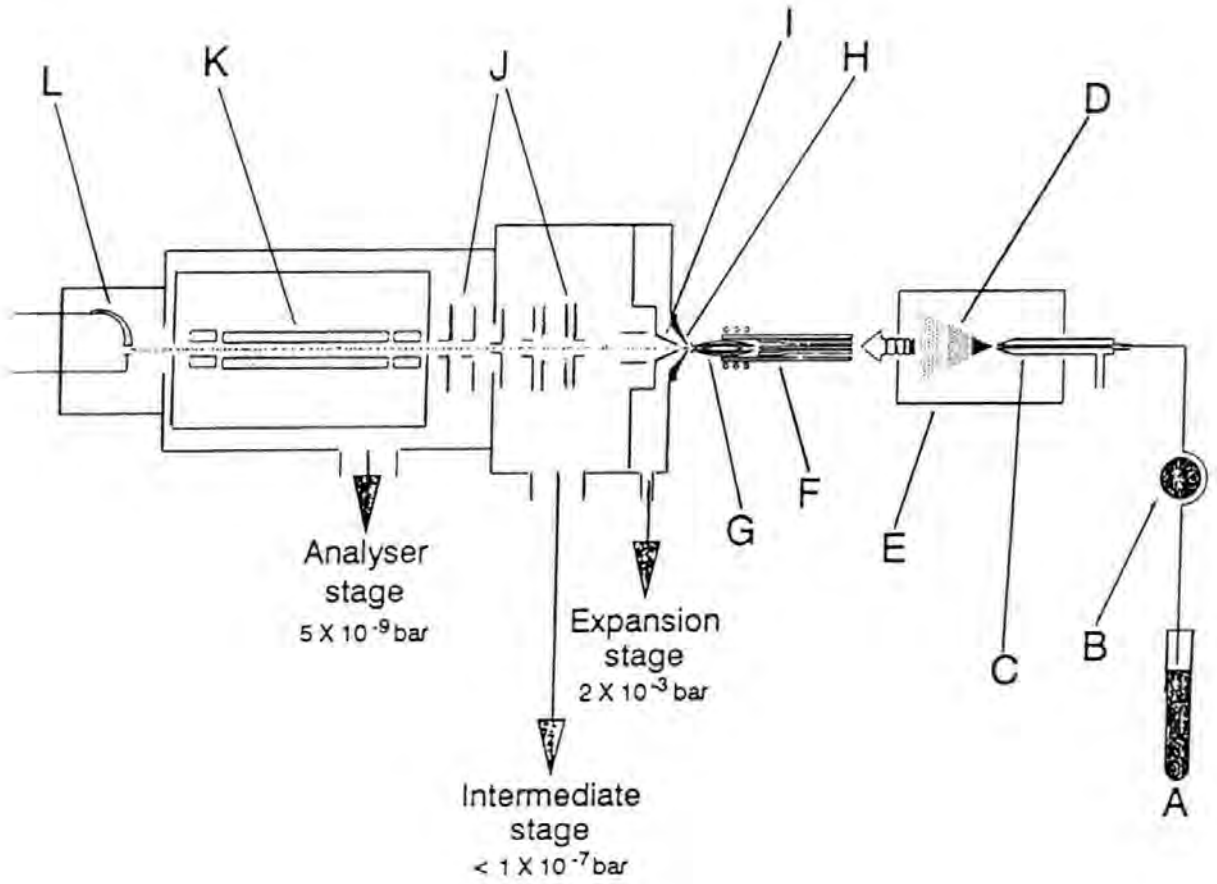
2.1.6: Interferences.

ICP-MS does have some limitations. It is expensive to purchase and maintain. It may be prone to long term stability and sensitivity problems as a result of environmental temperature fluctuations and therefore benefits from being kept in a controlled laboratory environment (air conditioned and clean room facilities). However the single largest shortcoming of the technique is that in a large number of applications it suffers from spectral interferences and non-spectral interferences (323,334,335,346,347).

2.1.6.1: Spectral interferences.

Spectroscopic interferences are caused by atomic or polyatomic ions that have the same nominal mass as the analyte of interest. Isobaric interferences are caused by the overlap of isotopes of different elements, which cannot be resolved by a commercial quadrupole mass analyser. Isobaric interferences are generally easy to predict and overcome simply by using an alternative isotope of the element of interest.

Figure 2.5: Schematic diagram of a commercial ICP-MS instrument (323).



Key: A, sample; B, peristaltic pump; C, pneumatic nebuliser; D, aerosol; E, spray chamber; F, ICP torch; G, plasma; H, sampling cone; I, skimmer cone; J, ion lenses; K, quadrupole mass filter; L, electron multiplier.

When an element is introduced into the plasma, the monatomic singly charged analyte ion (M^+) is not the only species observed in the final mass spectrum. Doubly charged ion formation is controlled by the ionisation energy and the condition of the plasma (348). Elements with a lower second ionisation energy than the first ionisation energy of argon (15.76eV) will be partly doubly ionised (M^{2+}). Their formation results in a loss of sensitivity for the singly charged species due to the small loss of signal and generates a number of isotopic overlaps at one half of the mass of the parent element.

Elements that have a high MO bond strength, result in the formation of oxide (MO^+) and hydroxide ions (MOH^+), collectively known as refractory oxide ions. The intensities of these species can be reduced to 1-2% by adjusting instrumental settings for MO^+:M^+ even for the most refractory elements. The RF forward power and the nebuliser flow rate exerting the greatest influence (349,350).

In addition to oxide formation, polyatomic ions originating from the plasma (Ar), from the matrix (O, H and often C, N, P, S and Cl) and from the air surrounding the plasma (C, N and O) exist. These ions result from the short lived combination of two or more atomic species, for example, ArO^+. They are the cause of serious interference effects, particularly below m\z 80 (351).

In general the majority of spectroscopic interference effects can largely be overcome by a careful choice of operational conditions (352-354), or by reducing the amount of water (355) or solvent reaching the plasma (356-359), or by introducing the sample in a dry form using electrothermal vaporisation (360-363), direct sample insertion (364) or laser

ablation (365). Alternatively interferences can be overcome by eliminating the interference generating constituents prior to measurement (366-369), or by adding alternative gases to replace wholly or partially the usual argon plasma gas and thus changing the conditions of the plasma (370-374).

2.1.6.2: Non-spectral interferences.

Unlike spectroscopic interferences, where the analytical signal is enhanced by another element or polyatomic species with the same nominal mass, non-spectroscopic interference is characterised by a reduction or enhancement in analyte signal due to factors exerting an influence on sample transport, ionisation in the plasma, ion extraction, or ion throughput in the resultant ion beam (346,347).

Sample introduction and transport is affected by the nebuliser design and nebuliser gas flow, and the viscosity, surface tension, density, evaporation rate and vapour pressure of the sample. It is therefore critical to matrix match samples and standards with respect to the solvent. The sample matrix can also affect the atomisation, excitation and ionisation characteristics by altering the plasma temperature (375). The nature and concentration of the sample matrix also has a direct bearing on the severity of these effects (376).

The presence of excess heavy, easily ionisable elements in the matrix creates the most serious non spectral interferences (377-380). Suppression of the analyte signal and long term stability problems can also result from the physical deposition of material on the sampler and skimmer cones and the subsequent restriction of the orifice (381,382).

Non-spectral interferences can generally be overcome or at least minimised by internal standardisation (377,383-385), standard additions, isotope dilution (386) or sample preparation techniques (346,347), for example liquid chromatography (387) and on line preconcentration (388). The careful setting of the instrument parameters will also help (389).

2.1.7: Biological\clinical applications of inductively coupled plasma-mass spectrometry (ICP-MS):

In the clinical environment, knowledge of concentrations of both biologically essential and trace elements in body fluids is very important. In clinical analyses one must be able to measure accurately small changes in concentrations that can be significant with respect to disease, deficiency and toxicology. The ability to determine rapidly and simply several trace elements, some at the μgl^{-1} level, in body fluids such as blood and urine is essential. Simultaneous multi-element methods are preferred to sequential single element methods of analysis, simply because of the additional advantages offered, such as providing more information per sample, and thus conserving both time and sample (390). The collection of samples is also of utmost importance: direct multi element analysis would simplify sample handling and reduce the risks of pre-analysis contamination. The advantages inductively coupled plasma-mass spectrometry (ICP-MS) can offer, such as speed, sensitivity and the ability to determine isotope ratios can satisfy many of these requirements and hence lend itself well to clinical studies at the ultra trace level. However the analysis of biological materials is prone to a number of additional interferences (391).

The analysis of biological materials often results in a dramatic increase in the number of potential spectral overlaps, generally as a result of the acids used for sample digestion and\or by the large variety of matrix elements present (392). Destruction of the matrix with one or more acids, such as HCl, $HClO_4$ and H_2SO_4 increase the chance of spectral overlap. Whenever possible only HNO_3 should be used in sample preparation, because H, N and O do not add any additional spectral interferences to those already existing in a pure water spectrum (351). Other matrix elements, representative of most biological materials, such as C, Ca, Cl, K, Na, P and S, also give rise to spectral overlap. The most important polyatomic ions in biological materials are listed in Table 2.1. Numerous methods have been developed to overcome many of these additional spectral interferences, examples of which have been detailed widely in the literature (391). The recent introduction of high resolution ICP-MS instruments (393) can also reduce the problems from spectral interferences associated with biological samples (394).

Additional non-spectral interferences are usually caused by high protein content and high dissolved solids, which can lead to clogging of pneumatic nebulisers, the injector tube of the torch and the entrance aperture of the sampling cone (381,382). The high salt concentrations can also lead to suppression or enhancement of the ion signal (395,396). A decrease in these matrix effects is possible using flow injection (397,398), which reduces the sample loading, or dilutes the matrix (399-401).

Despite these interferences inductively coupled plasma-mass spectrometry has demonstrated its suitability for the determination of trace amounts of inorganic elements in a variety of clinical and biological samples (286,402-410), and in applications relating

Table 2.1: Main spectral interferences encountered in the analysis of biological materials by ICP-MS (392).

Mass	Analyte	Abundance (%)	Interfering ions
44	Ca	2.1	$^{12}C^{16}O_2$
45	Sc	100	$^{12}C^{16}O_2H$, $^{13}C^{16}O_2$
46	Ti	8.0	$^{23}Na_2$
47	Ti	7.3	$^{31}P^{16}O$, $^{23}Na_2H$
48	Ti	73.8	$^{31}P^{16}OH$, $^{31}P^{17}0$, $^{32}S^{16}O$, $^{36}Ar^{12}C$
49	Ti	5.5	$^{35}Cl^{14}N$, $^{31}P^{18}O$, $^{31}P^{16}OH_2$, $^{32}S^{16}OH$, $^{33}S^{16}O$, $^{36}Ar^{13}C$
50	Ti	5.4	$^{34}S^{16}O$, $^{32}S^{16}OH_2$, $^{32}S^{18}O$
51	V	99.7	$^{35}Cl^{16}O$, $^{37}Cl^{14}N$
52	Cr	83.8	$^{35}Cl^{16}OH$, $^{35}Cl^{17}O$, $^{36}S^{16}O$, $^{40}Ar^{12}C$
53	Cr	9.5	$^{35}Cl^{16}O$, $^{40}Ar^{13}C$
54	Fe	5.8	$^{37}Cl^{16}OH$, $^{37}Cl^{17}O$
55	Mn	100	$^{37}Cl^{18}O$, $^{39}K^{16}O$
56	Fe	91.7	$^{40}Ca^{16}O$
57	Fe	2.2	$^{40}Ca^{16}OH$, $^{40}Ca^{17}O$
58	Ni	68.3	$^{42}Ca^{16}O$, $^{40}Ca^{18}O$
59	Co	100	$^{43}Ca^{16}O$, $^{42}Ca^{16}OH$
60	Ni	26.1	$^{44}Ca^{16}O$, $^{12}C^{16}O_3$, $^{43}Ca^{16}OH$
62	Ni	3.6	$^{23}Na_2{}^{16}O$
63	Cu	69.2	$^{31}P^{16}O_2$, $^{40}Ar^{23}Na$
64	Zn	48.6	$^{48}Ca^{16}O$, $^{31}P^{16}O$, $^{31}P^{16}O_2H$, $^{31}P^{16}O^{17}OH$, $^{32}S^{16}O_2$

Table 2.1: Continued.

Mass	Analyte	Abundance (%)	Interfering ions
65	Cu	30.8	$^{31}P^{16}O^{18}O$, $^{32}S^{16}O_2H$, $^{33}S^{16}O_2$
66	Zn	27.9	$^{34}S^{16}O_2$, $^{33}S^{16}O_2H$, $^{32}S^{16}O^{18}O$
67	Zn	4.1	$^{35}Cl^{16}O_2$, $^{34}S^{16}O_2H$, $^{32}S^{16}O^{18}OH$
68	Zn	18.8	$^{36}S^{16}O_2$, $^{34}S^{16}O^{18}O$
70	Ge	20.5	$^{35}Cl_2$
71	Ga	39.9	$^{40}Ar^{31}P$
72	Ge	27.4	$^{40}Ca^{16}O_2$, $^{40}Ar^{32}S$
75	As	100	$^{40}Ar^{35}Cl$
77	Se	7.6	$^{40}Ar^{37}Cl$
79	Br	50.7	$^{31}P^{16}O_3$, $^{40}Ar^{39}K$, $^{40}Ar^{23}Na^{16}O$
81	Br	49.3	$^{33}S^{16}O_3$, $^{40}Ar^{41}K$
82	Se	9.2	$^{34}S^{16}O_3$
94	Mo	9.3	$^{39}K_2{}^{16}O$
95	Mo	15.9	$^{40}Ar^{39}K^{16}O$
96	Mo	16.7	$^{39}K^{41}K^{16}O$
97	Mo	9.6	$^{40}Ar^{41}K^{16}O$

to human nutrition and toxicology (411,412). Isotopic ratios have been used to monitor metabolism *in vivo* and to study the bioavailability of elements (413-415), and isotope dilution methods have been developed for clinical studies (416,417). In addition, the coupling of ICP-MS as a detector, to a liquid chromatography system, has demonstrated vast potential in many applications (418), including the analysis of metals (418,419) and their species (418,420-422) in a number of clinical studies.

2.1.8: Experimental Instrumentation.

The ICP-MS results in this study were obtained using a PlasmaQuad II (VG Elemental, Winsford, Cheshire, U.K.) equipped with a Gilson Miniplus 2 peristaltic pump, an Ebdon nebuliser, water cooled Scott type double pass spray chamber and a Fassel demountable torch, with a 2mm injector. Details of the typical operating conditions are summarised in Table 2.2.

Table 2.2: Typical ICP-MS operating conditions.

Rf Power	Forward 1.4 - 1.5 kW Reflective 5W
Gas flows	Auxiliary 0.5 - 1.0 l min^{-1} Coolant 15 l min^{-1} Injector 0.95 l min^{-1}
Peristaltic pump	1 - 2 ml min^{-1}
Sampler cone	Nickel - 1.0mm orifice
Skimmer cone	Nickel - 0.75mm orifice
Expansion stage	3.3 x 10^{0} mbar
Intermediate stage	$< 1.0 \times 10^{-4}$ mbar
Analyser stage	1.0 x 10^{-6} mbar

2.2: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

2.2.1: Basic principles.

Chromatography is a technique for the separation of components in a mixture and their subsequent identification and quantification, based on their chemical and physical properties. In high performance liquid chromatography (HPLC) rapid separation and quantitation of many compounds is possible, at trace levels, using only small sample volumes. The components are partitioned between a mobile liquid phase (eluent) and a microparticulate stationary phase with a large surface area. The sample, dissolved in a solvent is injected into a flowing stream of eluent and pumped to the column where the components of the mixture are separated by interaction with the stationary phase. Separation occurs because each component has a different partition rate between the mobile and stationary phase. Components with the highest affinity for the column packing material are retained on the column longer. This differential elution is the basis for HPLC separation. As components elute from the column transient signals for each component are detected typically using a flow through cell. A schematic diagram of the basic HPLC components is shown in Figure 2.6 and further details concerning the theory and principles can be found in general text books on liquid chromatography (423-426).

High performance liquid chromatography is a highly versatile analytical technique. Several different modes are available [Table 2.3] (423-426), and a number of methods for detection are possible, such as electrochemical (amperometric), conductivity, fluorescence, photodiode array, radioactive monitoring, refractive index, UV\Visible using both fixed and variable wavelength (423-426). However, detection based on the use

Figure 2.6: Schematic diagram showing the basic HPLC system.

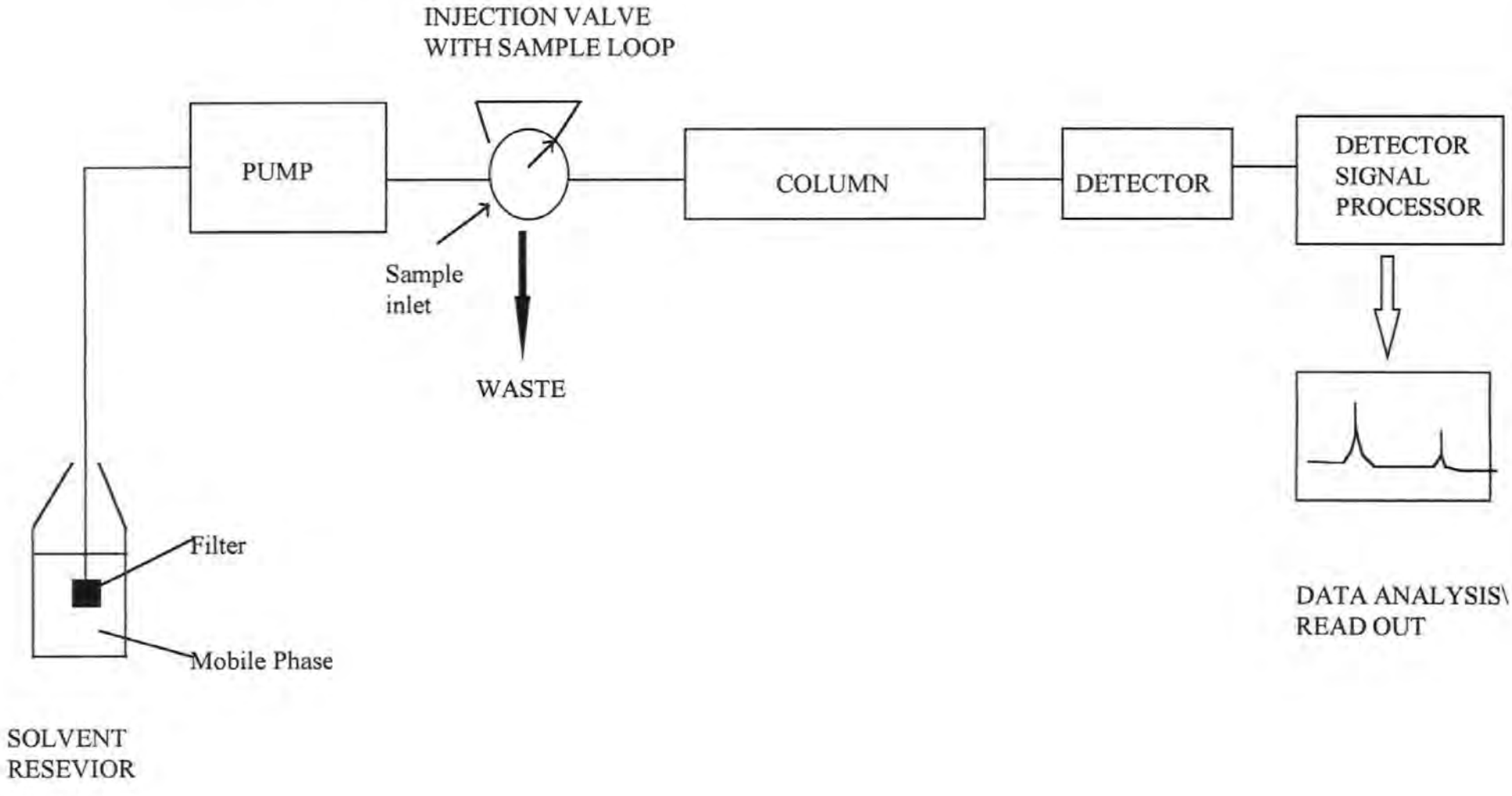


Table 2.3: Summary of the various forms of HPLC available.

Mode of chromatography\ separation	Description
Adsorption (normal phase)	Stationary phase is a relatively high polar material with a high specific surface area, e.g silica, alumina. The mobile phase is relatively non-polar, e.g hexane.
Adsorption (reverse phase)	Reverse of above: *i.e.* The stationary phase is very non-polar, and the mobile phase is relatively polar.
Liquid-Liquid partition	A liquid that is insoluble in the mobile phase is absorbed onto the porous support material. Can be used in either normal or reverse phase modes.
Bonded phase	The stationary phase is not applied to a porous particle in liquid film form but is covalently bonded by chemical reaction. Can be operated either in normal or reverse phase modes.
Ion-exchange	The stationary phase contains ionic groups which interact with the ionic groups of the sample molecules.
Ion-pair	Also useful for separating ionic compounds and overcomes certain problems inherent in the ion exchange method. Ionic sample molecules are "masked" by a suitable counter ion.
Ion	Developed as a means for separating the ions of strong acids and bases. It is a special case of ion-exchange chromatography utilising different equipment.
Size-exclusion	Gel permeation: for organic solvents. Gel filtration: for aqueous solutions. In either mode the molecules are separated by size.
Affinity	Highly specific biochemical interactions provide the means of separation. The stationary phase contains specific groups of molecules which can only absorb the sample if certain steric and charge-related conditions are satisfied.

of atomic spectrometry or mass spectrometry (including ICP-AES and ICP-MS) are of increasing importance, especially for speciation studies (418).

2.2.1.1: Reverse phase chromatography.

Reverse phase chromatography can be used in either adsorption or partition modes. Here the stationary phase is less polar than the mobile phase. This form of chromatography is probably the most frequently used and certainly one of the most versatile modes of chromatography available, since a wide variety of samples can be analysed. The most frequently used stationary phases are the chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms. Alternative stationary phases include C_8 and shorter alkyl chains, cyclohexyl and phenyl groups. The most frequently used mobile phases are based on water\methanol and water\acetonitrile systems. In this mode of chromatography the interactions of the solute and solvent in the mobile phase are of great importance with respect to retention and obtaining satisfactory separations (423-426).

2.2.1.2: Ion pair chromatography.

Ion pair chromatograpy is a subset of reverse phase chromatography which can be applied to ionised or ionisable species on the reverse phase column. The separation of charged compounds or ones that can be ionised present problems, such as poor peak shape and inadequate resolution, on a reverse phase system using simple solvent mixtures. Modifications to either the mobile phase (*i.e.* sample ionisation) or the surface of the column can overcome these problems. The use of ion pairing reagents is just one way such modifications can be achieved.

There are two basic theories which describe the role or performance of the ion pairing reagent. Firstly the ion pairing reagent can be considered as a counter charged organic molecule, added to the mobile phase. They form an "ion-pair" with the analyte in question in the solution, such that it becomes one long non-polar pseudomolecule with a masked charge couple in the centre. The pseudomolecule then partitions with the bonded phases as if the charges do not exist. Hence the pseudomolecule is now retained on the column, and a separation is possible.

Alternatively the ion pairing reagent reacts first, interacting with the bonded phase forming a non bonded phase ion exchange column. This modified bonded phase column then interacts with the analytes in the solution through a mixed partition\ion exchange mode of action (424,426).

2.2.2: Chromatographic parameters.

In order to determine and compare the performance of different chromatographic columns, there are several fundamental parameters and factors that can be derived from the chromatogram, some of which are outlined below. More detailed descriptions of these parameters are given in chromatography texts such as those by Hamilton (423) and Meyers (426).

Phase preference can be expressed by the **distribution or partition coefficient**, denoted by K. This gives the ratio of the concentration of the solute in the stationary and mobile phase:-

$$K = \frac{C_s}{C_m}$$

where C_s and C_m are the molar concentrations of a solute in the stationary and mobile phase respectively. The various components of the mixture in a chromatographic separation must have different distribution coefficients if the mixture is to be separated. The time taken for a component to elute to its maximum concentration is known as the **retention time**, denoted by t_R. Two compounds can be separated if they have different retention times. Figure 2.7 defines the retention time where t_0 is the dead time or retention time of an unretained solute, and t_R is the net retention time:-

$$t_R = t_0 + t'_R$$

t_0 is identical for all eluted substances and represents the mobile phase residence time whereas t'_R is the stationary phase residence time which is different for each separated compound.

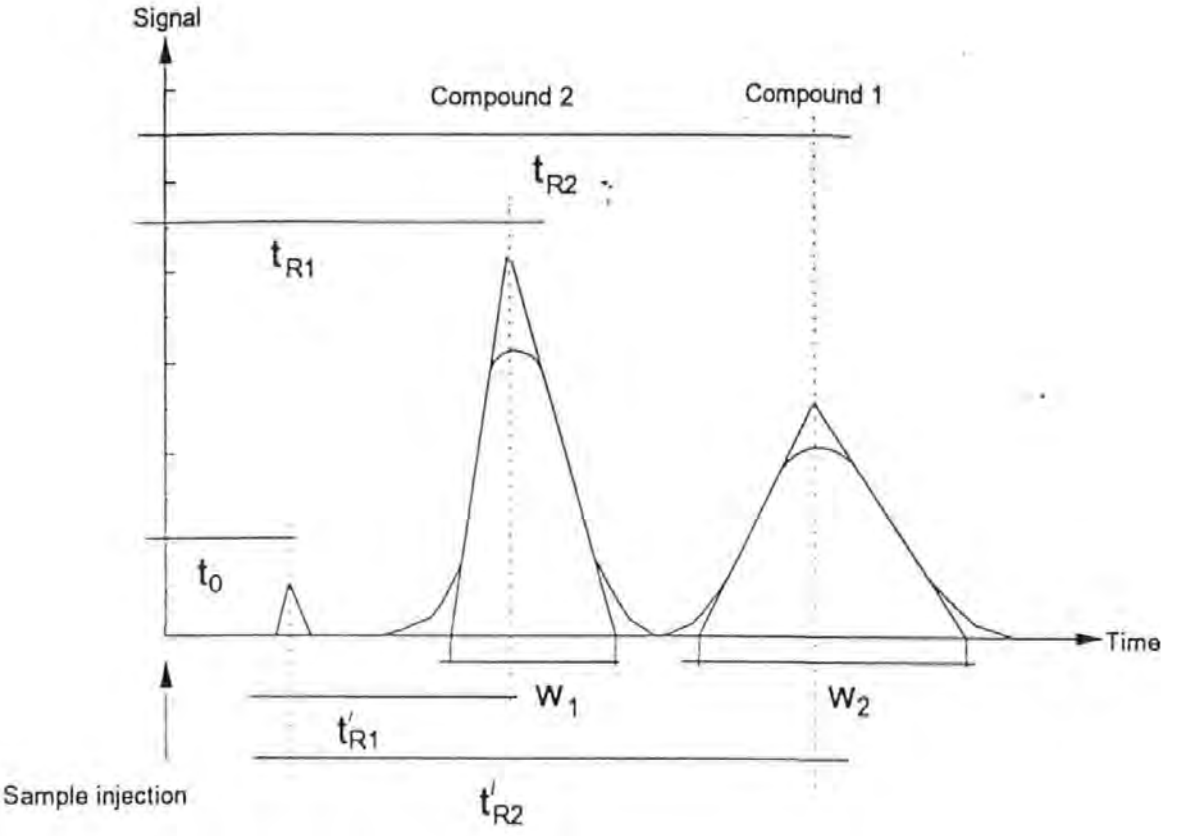
A chromatographic column may be considered as a series of narrow layers known as **theoretical plates**. The ability of a column to minimise band broadening is termed the column efficiency and can be expressed as:-

$$N = 16\left(\frac{t_R}{W}\right)^2$$

where N = the number of theoretical plates, t_R is the peak retention time, and W is the peak width.

Resolution, R_s is a measure of the ability of a column to resolve two solutes. It is experimentally defined as the difference in retention times of the two peaks, divided by the average peak width:-

Figure 2.7: Illustration of paramaters for retention time and resolution.



Key:-

t_0 = dead time or retention time of an unretained solute

t_R = net retention time

t'_R = stationary phase residence time

W_1 = peak width for compound 1

W_2 = peak width for compound 2

$$R_s = \frac{2(t_R^1 - t_R^2)}{W_1 + W_2}$$

where t_R^1 and t_R^2 are the retention times, and W_1 and W_2 are the base widths of the two peaks. This is illustrated in Figure 2.7. When R_s equals unity there is overlap of the two peaks but it is clear that two components are present. At R_s= 1.5 the overlap is considerably reduced and values such as this are considered suitable for the majority of analyses. The difference between the degrees of retention of the different solutes can also be described as the **selectivity factor**, α, which is always calculated so that the value is greater than, or equal to unity. If the value is unity, the components cannot be separated by that particular set of chromatographic conditions. The selectivity factor can be defined as:-

$$\alpha = \frac{K_B}{K_A}$$

where K_B is the partition coefficient for the more strongly retained solute and K_A the partition coefficient for the less strongly retained solute, which therefore moves more rapidly through the column.

2.2.3: Experimental instrumentation.

All chromatographic results in this study were obtained using ion pair-reverse phase-high performance liqiud chromatography (IP-RP-HPLC). The chromatographic system used consisted of a Varian 9001 isocratic pump coupled to a Varian 9070 fluorescence detector [xenon lamp] (Varian Ltd, Walton-on-Thames, Surrey, UK). Samples were loaded using a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 20 μl sample loop (onto a

5μm Spherisorb ODS-2 analyte column (100mm x 4.6mm i.d.), protected by a 5μm Spherisorb ODS-2 guard column (20mm x 4.6mm i.d), [MATRIX cartridge system, Jones Chromatography, Hengoed, Mid Galmorgan, UK]. The results were processed and the data analysis handled by a Varian LC Star Workstation, version 4.01 (Varian Ltd, Walton-on-Thames, Surrey, UK).

2.3 IMMUNOASSAYS

2.3.1 General theory and principles.

Immunoassays are major diagnostic tools used in the field of clinical chemistry. An immunoassay is an analytical technique that uses antibodies or antibody-related reagents, *i.e.* an immunological reaction, for the determination of sample components. The specific binding, characteristic of these reagents, allows the development of methods that are highly selective and which can often be used with complex matrices, such as blood and urine, with little or no sample preparation. More details concerning antigen-antibody interactions employed in immunoassays can be found in general text books on immunology (427-429). Immunoassays use readily detectable labels such as radioisotopes or enzymes, which helps to provide many of these methods with extremely low limits of detection (below 1 ng\ml). Such properties, plus the relatively low cost involved with most of these techniques, make an immunoassay the method of choice for many clinical applications.

Although fluorescent or chemiluminescent markers are tending to replace radioisotopes for labelling, enzyme labels are by far the most common type of immunoassay used at the present time. The enzyme linked immunosorbant assay (ELISA) is one of many enzyme immunoassay methods (EIA) available (430,431). Enzyme linked immunosorbant assays provide a technique with the advantages of radioisotopic immunoassays (RIA), such as good sensitivity, simplicity and ease of handling multiple samples, but with none of the disadvantages associated with the handling of radioactive isotopes. Like RIA, ELISA can be used to assay quantitatively and qualitatively for either antigens or

antibodies. Three major variations exist: indirect, sandwich and competitive ELISA (430,431).

2.3.2 Metra Biosystems Assay Kits.

Metra Biosystems Ltd, Oxford, UK, have made two ELISA based test kits commercially available for the measurement of urinary pyridinium crosslinks. Pyrilinks™ measures the mature collagen crosslinks pyridinoline (PYD) and deoxypyridinoline (DPYD) whilst Pyrilinks-D™ measures only deoxypyridinoline. Pyrilinks™ uses a polyclonal antibody to determine free PYD and DPYD present in urine from the breakdown of mature collagen (432,433). Pyrilinks-D™ uses a highly specific monoclonal antibody to determine free DPYD present in urine from the breakdown of bone collagen (434).

For the purpose of the clinical trial presented in this thesis, only the bone specific crosslink deoxypyridinoline is measured, using the Pyrilinks-D™ kits. The basic principle of the assay is based on a competitive enzyme immunoassay in a microtitre plate format (434). The DPYD antibody (rabbit or mouse anti-deoxypyridinoline monoclonal antibody) is coated onto the microtitre plates. The samples, standards and controls are diluted 1 in 10 with a buffer solution (phosphate buffered saline {PBS} and Tween {polyoxyethylene (20) sorbitan monolaurate}, 0.15M NaCl, 10M sodium phosphate, pH 7.0, and 0.05% Tween 20), then added to individual wells on the plate. Any DPYD present binds to the immobilised DPYD antibodies. An enzyme conjugate (DPYD-alkaline phosphatase) is added to the reaction mixture and left to incubate for 120 +\- 5 minutes, in the dark between 2-8 °C. During this incubation period the DPYD in the sample competes with the conjugated DPYD-alkaline phosphatase for the antibody bound to the plate. The high

immunological specificity of the DPYD antibody, combines with the catalytic ability of the alkaline phosphatase enzyme to bind with any DPYD molecules previously bound, creating an antibody-antigen-antibody sandwich. After washing away any unbound conjugate with a PBS-Tween solution, p-nitrophenyl phosphate is added to each well, resulting in a colour forming reaction to occur with the DPYD during an incubation period of 60 +\- 5 minutes at room temperature in the dark. The colour development is stopped by the addition of sodium hydroxide (1N) and the optical density is measured at 405nm using a microplate reader (Titertek Multiskan Plus Mk II, Labsystems Life Science International (UK) Ltd, Basingstoke, Hampshire, UK). The amount of substrate converted is proportional to the concentration of bound DPYD (435). The data analysis was performed using a Multiskan Mk II programme V1.5 (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK) and a curve fitting programme, Softmax V2.34 (Metra Biosystems Ltd, (UK), Oxford, UK).

CHAPTER 3

ANALYSIS OF THE PYRIDINIUM CROSSLINKS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

CHAPTER 3: ANALYSIS OF THE PYRIDINIUM CROSSLINKS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

3.1: INTRODUCTION.

The powerful separating powers of HPLC, combined with the excellent sensitivity and specificity of fluorometric detection, capitalising on the natural fluorescent properties of both pyridinium crosslinks, has resulted in the development of a number of reverse phase high performance liquid chromatography methods, as summarised in Table 3.1. The HPLC analysis of the two crosslinks usually involves the measurement of the total crosslink levels and since the crosslinks exist in both a free and bound form (149), an acid hydrolysis step is required to convert the bound into the free form. The crosslinks are then separated from the hydrolysate by partition chromatography using a cellulose column (446). These HPLC methods therefore involve a multi stage sample preparation procedure, and although this is simple and effective, it is time consuming, cumbersome and tedious, as well as being prone to a large number of variables which result in poor recoveries and irreproducible results. Some sources of variation have been identified (444), however the fact that these methods often lack suitable standards and sufficient robustness for routine clinical analysis has been the "achilles heel" in using the pyridinium crosslinks as biochemical markers to measure bone breakdown.

To make this technique more useful and attractive for routine clinical use, automated sample preparation systems have been developed (447-449). The recent development and commercial availability of an internal standard - acetyl pyridinoline (450) should also improve the precision of the HPLC assay. However certain precautions must still be

Table 3.1: Summary of HPLC methods to measure the pyridinium crosslinks.

Type of chromatography (Sample type)	Conditions	Refs.
Molecular Sieve (urine)	Columns: a)Bio-Gel P2, 100-200 mesh, 2.5 x 90 cm. b) Bio-Gel P2, 200-400 mesh, 2.5 x 40 cm Mobile phase: a) 1M $CaCl_2$, 0.05M Tris/HCl, pH 7.5. b) 0.1M acetic acid	175
Ion-exchange (urine)	Column: glass packed with 10μm Separon HEMA-BIO 100 SB (sulphobutyl), 15cm x 3mm Mobile phase: 0.3M sodium sulphate, pH 1.8 Flow rate: 0.3 ml min^{-1} λ_{ex} 297nm, λ_{em} 389nm	436
Gradient ion pair reverse phase (urine)	Column: Altex Ultrasphere ODS C18, 5μm, 250 x 4.6mm Mobile phase: 1) Solvent A: 0.1 % v\v trifluoroacetic acid (TFA) in water. Solvent B: Acetonitrile titrated with trifluoroacetic acid (TFA) to give the same UV absorbance at 220nm as solvent A. 2) Solvent A: 0.01M n-heptofluorbutyric acid (HFBA) in 5% v\v acetonitrile, Solvent B: 0.01 M HFBA in acetonitrile. Gradient: various tested using both systems. Flow rate: 1.0 ml min^{-1} λ_{ex} 297nm, λ_{em} 380nm	437

Table 3.1: Continued.

Type of chromatography (Sample type)	Conditions	Refs.
Gradient ion pair reverse phase (urine)	Column: Hypersil ODS, 5μm, 250 x 4.6mm Mobile phase: Solvent A: 20mM NH_4Cl, pH 3.5, containing 5 mM octanesulphonic acid (OSA). Solvent B: 75% acetonitrile and 25% solvent A. Gradient: 10% solvent B increasing to 40% solvent B in 30mins, increasing to 70% for 15mins, followed by 10% solvent B re-equilibration. Flow rate: 1.0 ml min^{-1} λ_{ex} 295nm, λ_{em} 400nm	438
Isocratic ion pair reverse phase (urine)	Column: Excil 100 ODS, 5μm, 100 x 4.6mm Mobile phase: 25mM sodium formate, 5mM OSA and 1mM EDTA, pH 3.25 in 20%v\v methanol Flow rate: 1.5 ml min^{-1} λ_{ex} 295nm, λ_{em} 400nm	439
Isocratic ion pair reverse phase (urine)	Supelco LC-18-DB, 33 x 4.6mm, 100Å Mobile phase: Solvent A: 0.01M HFBA in 2mM NH_4Cl. Solvent B: 75% acetonitrile, 25% solvent A. Ratio used A:B = 88:12 Flow rate: 1.0 ml min^{-1} λ_{ex} 295nm, λ_{em} 395nm	440

Table 3.1: Continued.

Type of chromatography (Sample type)	Conditions	Refs.
Isocratic ion pair reverse phase (urine)	Column: Altex Ultrasphere ODS, 5μm, 250 x 4.6mm Mobile phase: 0.01mol l^{-1} HFBA:acetonitrile (91:9) Flow rate: 0.8 ml min^{-1} λ_{ex} 297nm, λ_{em} 380nm	441
Isocratic ion pair reverse phase (tissue)	Column: Intersil ODS-2,5μm, 250 x 4.6mm Mobile phase: 0.1M sodium phosphate, pH 3.5 and acetonitrile (75:25 v\v) containing 1g sodium dodecyl sulphate (SDS) and 25mg EDTA per litre. Flow rate: 1.0 ml min^{-1} λ_{ex} 295nm, λ_{em} 395nm	442
Isocratic ion pair reverse phase (serum)	Column: Excil 100 ODS-2,5μm, 100 x 2.1mm Mobile phase: 10mM pentafluoropropionic acid (PFPA) in water. Flow rate: 0.15 ml min^{-1} λ_{ex} 290nm, λ_{em} 400nm	443
Isocratic ion pair reverse phase (urine)	Column: Supelco LC18-DB, 3μm, 33 x 4.6mm Mobile phase: Solvent A: 10mM HFBA, pH 2.5, Solvent B: 75% v\v acetonitrile, 25% v\v solvent A Ratio solvent A:B 90:10. Flow rate: 1.0 ml min^{-1} λ_{ex} 295nm, λ_{em} 395nm	444

Table 3.1: Continued.

Type of chromatography (Sample type)	Conditions	Refs.
Isocratic ion pair reverse phase (urine, serum and plasma)	Column: Phenomenex ODS, 5μm, 250 x 3.2mm Mobile phase: 0.02M HFBA:acetonitrile (77:23 v\v) Flow rate: 0.5 ml min^{-1} λ_{ex} 295nm, λ_{em} 400nm	445

taken into consideration (451). For example studies have highlighted interference problems using the HPLC method to measure the crosslinks in urine samples taken from patients prescribed sulfasalazine for rheumatoid arthritis (452). Despite the recent development of other methods to measure the crosslinks in serum (443,445) the HPLC measurement of the crosslinks in urine samples is still the accepted method despite its inherent problems.

This chapter describes the development and modifications to the HPLC methodologies in order to achieve good "in-house" chromatographic results for the pyridinium crosslinks. Details concerning the evaluation into the mobile phase and sample preparation procedures are also described.

3.2: EXPERIMENTAL.

3.2.1: Reagents and chemicals.

Analar or HPLC grades of hydrochloric acid, sodium hydroxide, sodium formate, glacial acetic acid and formic acid were obtained from Merck (Poole, Dorset, U.K.). Methanol, acetonitrile (HPLC grade) and butan-1-ol (analytical grade) were obtained from Rathburns Chemical Ltd (Walkerburn, Scotland). Heptafluorobutyric acid (HFBA), 1-octanesulphonic acid (OSA), ethylenediaminetetraacetic acid (EDTA) and cellulose (CF1 and CF11) were obtained from Aldrich (Gillingham, Dorset, UK).

The pyridinium crosslink standard solutions were prepared from bovine pelvic bone (supplied by Dr. Matthew Collinson, Radiotherapy Research Unit, Freedom Fields

Hospital, Plymouth) and human femoral bone (supplied by Dr. Norman Roberts, Department of Clinical Chemistry, University of Liverpool). These solutions were standardised against a commercial pyridinium crosslink standard (Metra Biosystems Ltd, Oxford, U.K) and a pyridinium crosslink standard prepared from human tibial bone, (supplied by Dr. Ian James, St. Bartholomew's Hospital, London, U.K). All water used throughout the study was deionised using a MilliQ purification system (Millipore, Bedford, MA, USA) or an Elgastat Maxima purification system (Elga Ltd, High Wycombe, Bucks., UK) at a resistivity of 18 MΩ. The instrumentation used was as described in Chapter 2 (Section 2.2.3).

3.2.2: Preparation of standards.

The two pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPYD) were extracted from bovine pelvic bone and human femoral bone (439). The bone samples were decalcified in 8% v\v formic acid in 10% v\v formal saline solution for several weeks. After freeze drying, 112g of bone was refluxed in 1500ml of 6M hydrochloric acid for 16hrs at 116°C, and then reduced to 500ml by distillation. The hydrolysate was then mixed with glacial acetic acid (500ml), a slurry of 50g of CF11 cellulose powder in 500ml butanolic eluent (butan-1-ol:glacial acetic acid:water, 4:1:1), and 2L of butan-1-ol, and stirred at room temperature for 30 minutes. The mixture was filtered and washed with 5L of butanolic eluent to remove the bulk of the fluorescent hydrolysis components. Pyridinoline (PYD) and deoxypyridinoline (DPYD) were eluted with 1L of MilliQ water. The aqueous fraction was freeze dried and resuspended in 10mls of 0.05M hydrochloric acid to provide a stock solution of crosslink standard.

3.2.3: Urine sample preparation.

Urine samples were prepared by a modified procedure described by James *et al* in 1990 (439). Aliquots of urine (1ml) were hydrolysed with hydrochloric acid (1ml) at 116°C for 16hrs. Hydrolysates (0.5ml) were mixed with glacial acetic acid (0.5ml), 5% w\v CF1 cellulose powder in butanolic eluent (0.5ml) and butan-1-ol (2ml) in glass vials. Columns were prepared using 5ml Gilson pipette tips plugged with glass wool filled to a level of 3cm with 5% w\v CF1 cellulose in butanolic eluent. The cellulose was allowed to settle and then washed with 5ml of butanolic eluent. Samples were loaded onto the column, and the glass vials rinsed with butanolic eluent (3 x 5ml) and the washings applied to the column. Interfering fluorophores were eluted with butanolic eluent (15ml) and the crosslinks were eluted with MilliQ water (12ml) and collected in 15ml plastic conical centrifuge tubes (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK). After centrifugation (2000rpm, 5min), the upper butanolic layer was removed using a Pasteur pipette. The lower aqueous layer containing the crosslinks was freeze dried and the samples were stored at 4°C.

3.3: RESULTS AND DISCUSSION.

3.3.1: Evaluation of the chromatographic conditions.

For the purpose of this study a simple isocratic reverse phase ion pair HPLC method was selected (439). The mobile phase consisted of 25mM sodium formate, 5mM octanesulphonic acid (OSA), 1mM EDTA, in 20% v\v methanol, with the pH adjusted to 3.25 using 6M hydrochloric acid. A flow rate of 1.5 mls min^{-1} was used. Samples were resuspended with 200 μl of a loading buffer (25mM sodium formate) prior to injection.

3.3.1.1: Separation of the PYD and DPYD crosslinks.

Figure 3.1 shows the chromatogram obtained for the St. Bartholomew's crosslink standard, analysed under the conditions given. The retention times for both PYD and DPYD were less than 5 minutes and the peak shapes were good, although baseline resolution between the two analyte peaks was not complete. However the ODS column used in this study was from a different manufacturer, which may account for this incomplete resolution. Analysis of the St. Bartholomew's standard and the Metra commercial crosslink standard, using slightly modified conditions, (17.5% v\v methanol instead of 20% v\v, and a pH of 3.4 instead of 3.25), resulted in better separation with slightly longer, although comparable retention times (Figure 3.2).

Figure 3.3 compares the chromatograms for the St. Bartholomew's standard (A), a urine sample (B), and a 50:50 co-injection of the sample and standard (C). The results show that the urine sample preparation procedure selectively extracted the crosslinks PYD and DPYD, and that the urine sample also had an unknown peak (UNK-2), which was different from the unknown peak in the bone standard (UNK-1). Unfortunately a slight shoulder on the PYD peak in the standard and some peak splitting in the real sample was also apparent. Both i) a clean up of the column, and ii) replacement with a new column, resulted in little or no obvious improvement and as a consequence of these observations further method development was necessary.

Figure 3.1: A typical chromatogram showing the St. Bartholomew's crosslink standard, analysed under the original conditions.

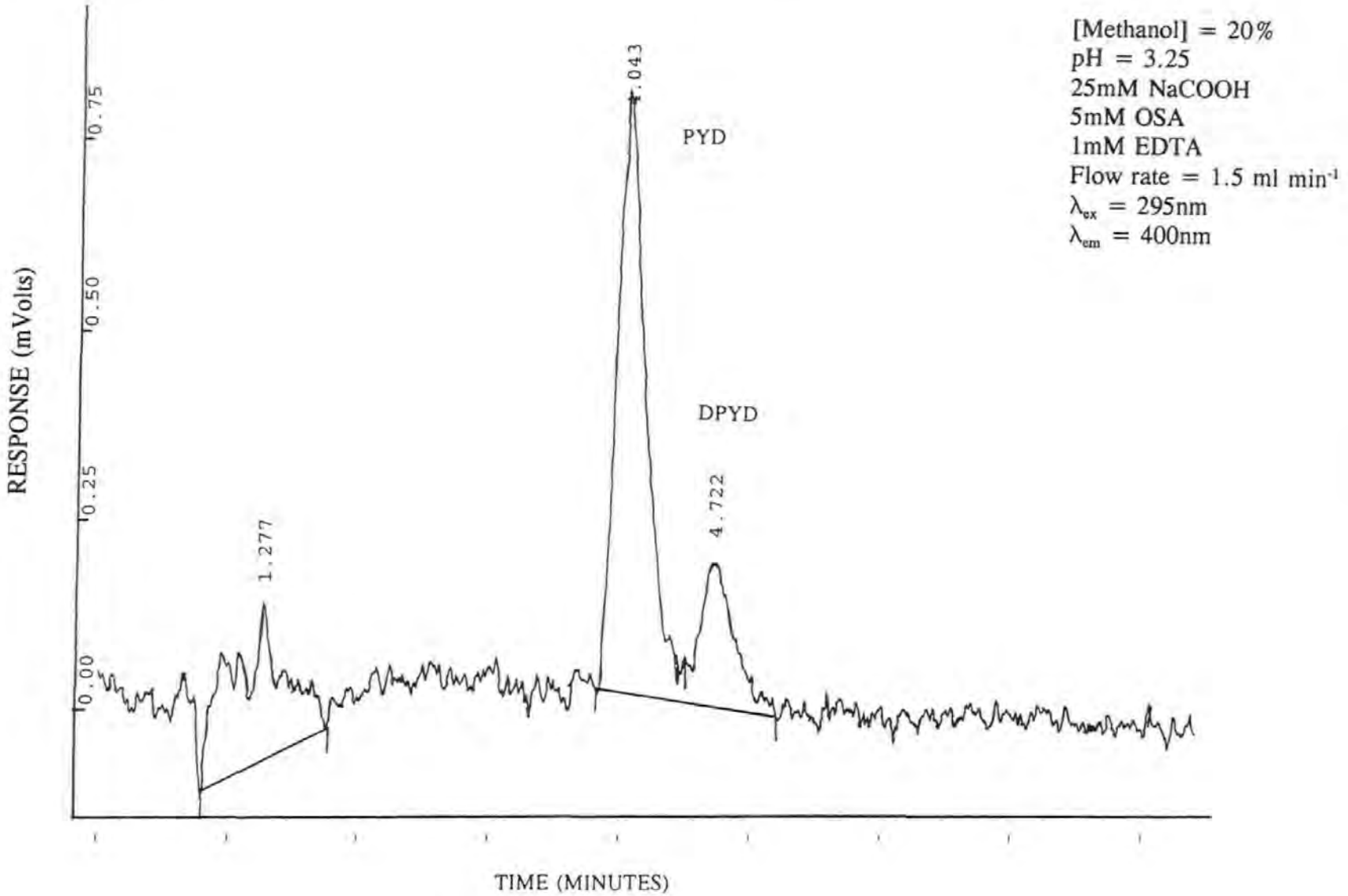


Figure 3.2: A typical chromatogram showing the St. Bartholomew's standard (A) and the Metra commercial crosslink standard (B), analysed under modified conditions.

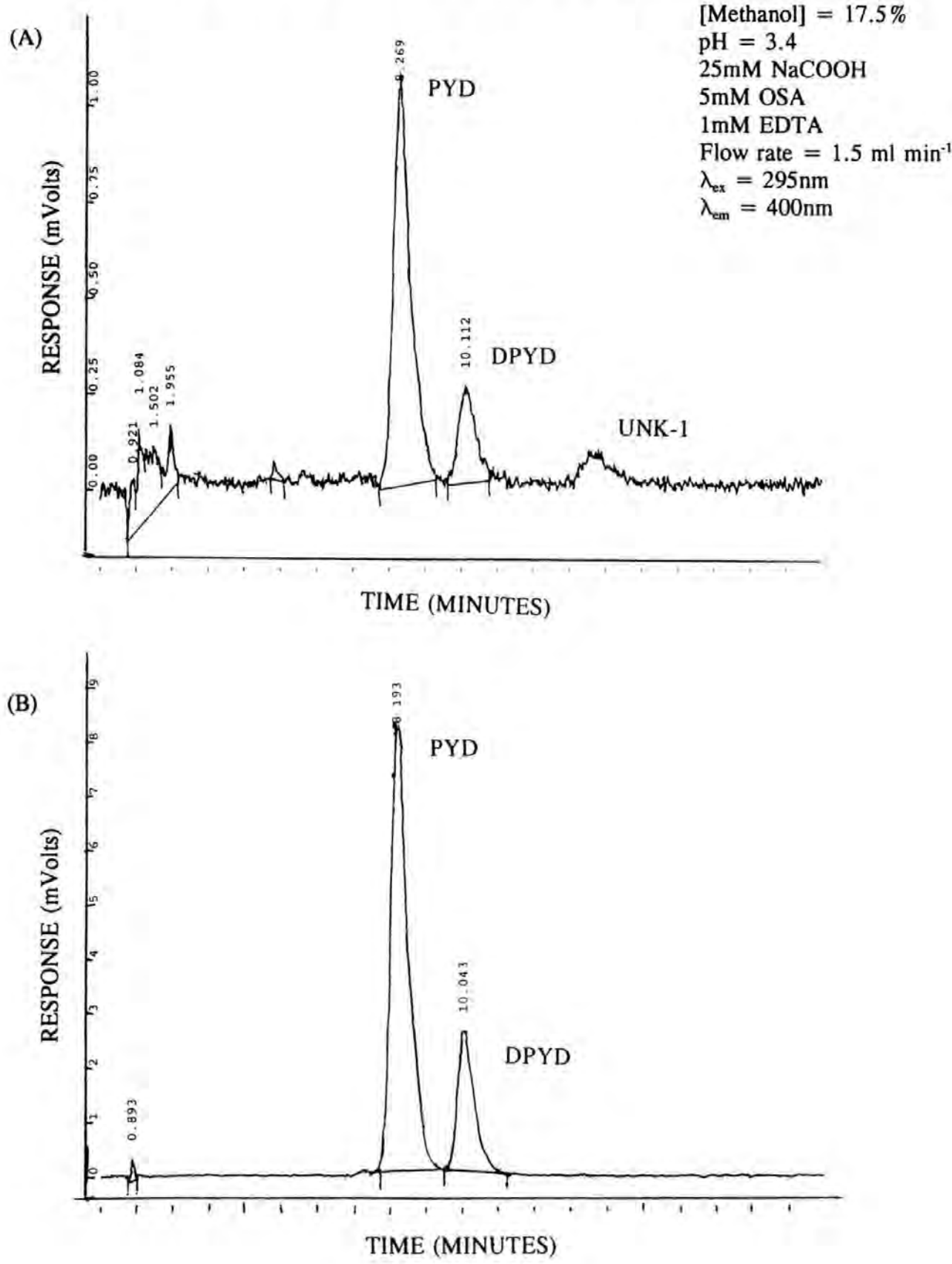
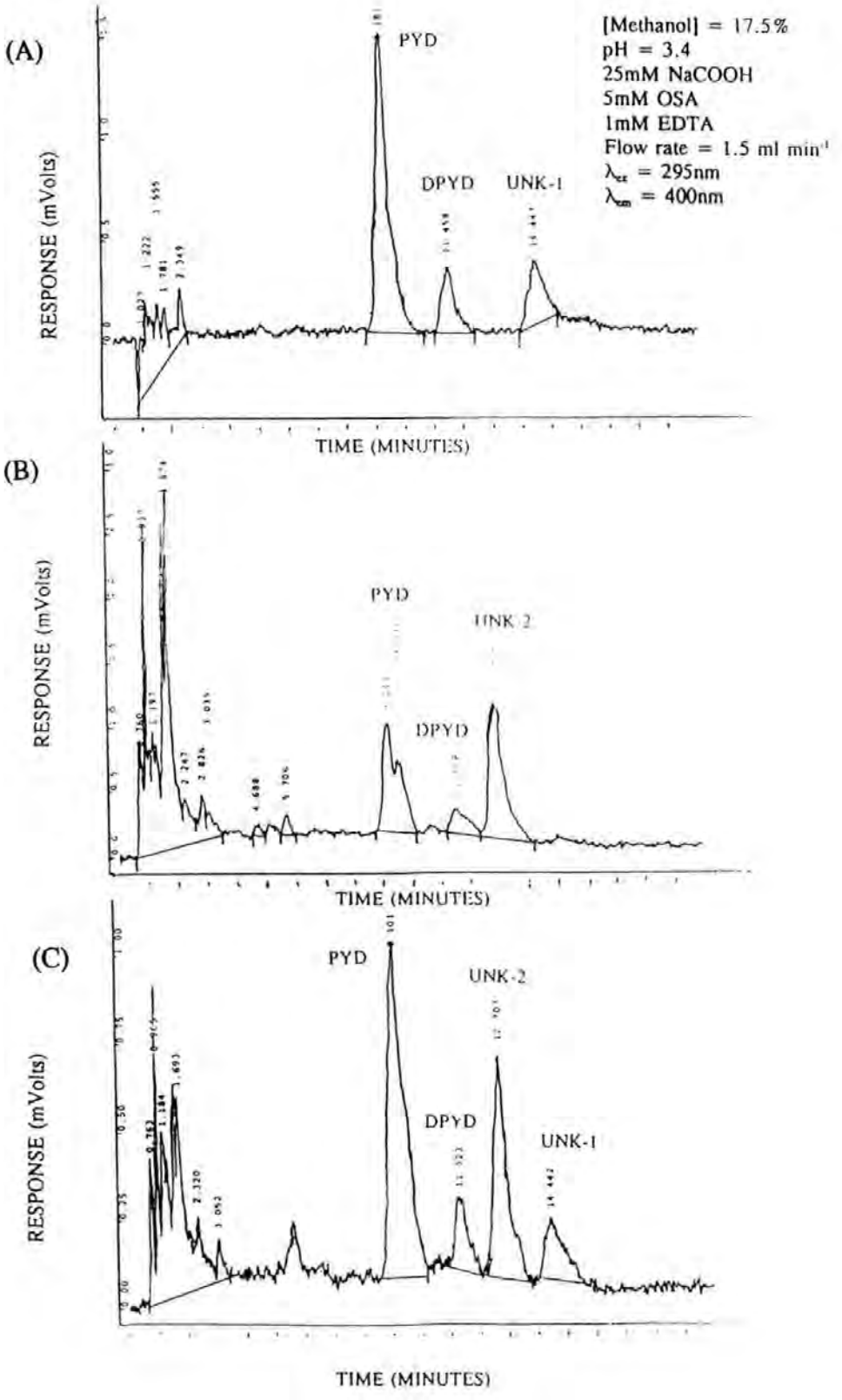


Figure 3.3: Chromatograms comparing the St. Bartholomew's crosslink standard (A), with crosslinks extracted from a urine sample (B), and a co-injection of the sample and standard (C).

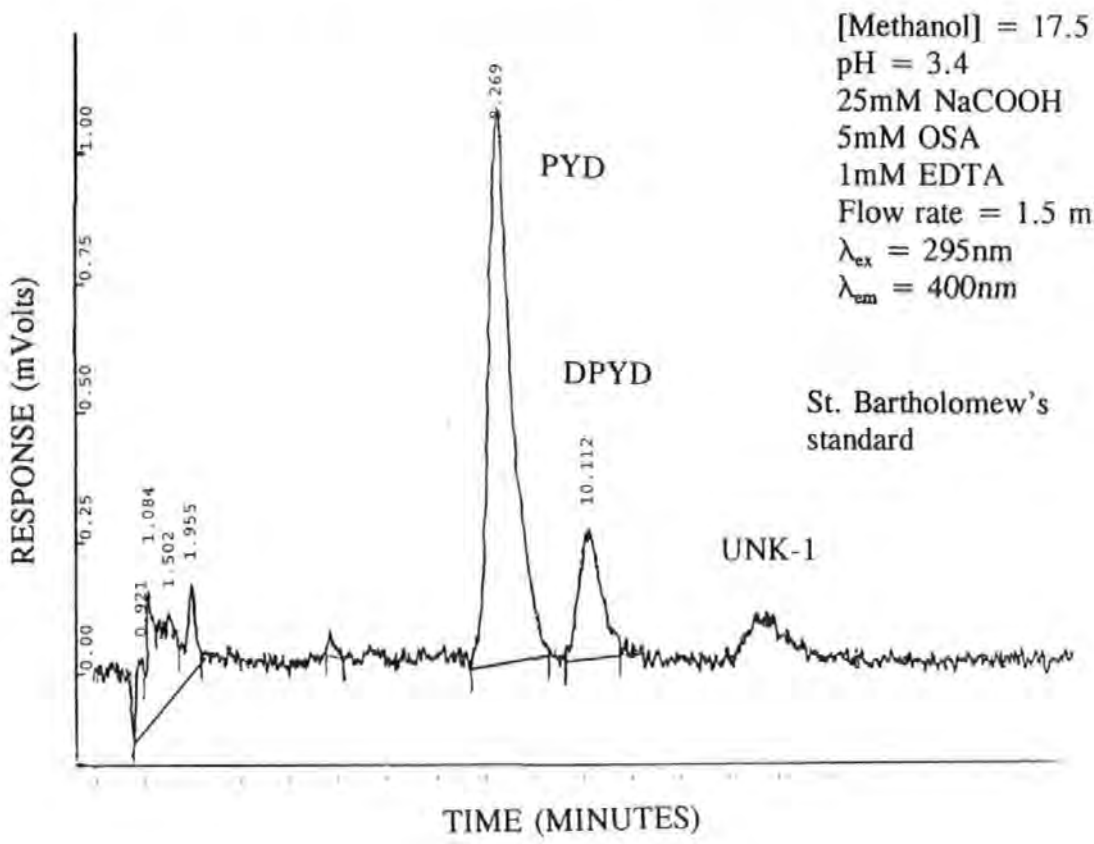


3.3.1.2: Analysis of calibration standards and detector response.

Evaluation of the methodology was further continued by analysing the "home" prepared crosslink standard, as described in Section 3.2.2., using the modified chromatographic conditions (*i.e.* 17.5% methanol and pH 3.4). Two calibration solutions were prepared from the pyridinium crosslink standard (bovine) stock solution. A 1 in 40 dilution and 1 in 4 dilution of the stock solution gave calibration standards 1 and 2 respectively. Both of these calibration standards compared well with the St. Bartholomew's standard (Figure 3.4 and Figure 3.5), indicating that the two analyte peaks were originally extracted cleanly, and that their retention times correlated well, thus confirming the two crosslinks identity. However the PYD:DPYD ratio was clearly different (*i.e.* 26:1). This could be attributed to variances between different species. Unfortunately the analysis of the standard made from human femoral bone (Figure 3.6) which should give a PYD:DPYD ratio of 3-4:1 (187), similar to the St. Bartholomew's standard (human tibial bone), clearly showed some DPYD was lost during the extraction process. Despite this, the two crosslinks were well resolved and therefore these diluted solutions could be used as calibration standards. These results did however suggest that the extraction procedure was the major source of error.

The standardisation of these two calibration solutions against the St. Bartholomew's standard, provided additional evidence to support further method development (Table 3.2). Excellent % RSD's were attained for both solutions ($< 3\%$ for PYD and $< 10\%$ for DPYD), indicating that the chromatography was capable of supporting qualitative and quantitative studies. However upon the analysis of real samples, where the crosslinks were extracted from a pooled urine sample, highly variable quantitative results were

Figure 3.4: A typical chromatogram of Calibration solution 1, prepared from bovine pelvic bone, compared to the St. Bartholomew's crosslink standard.



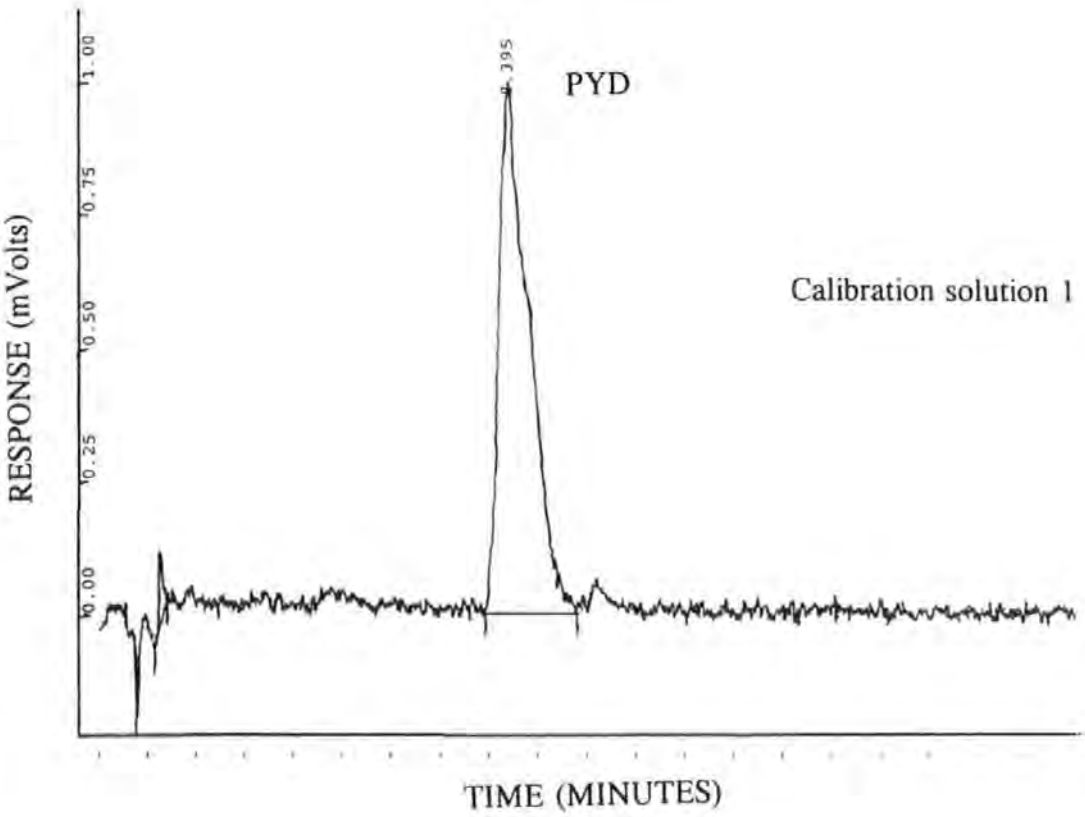
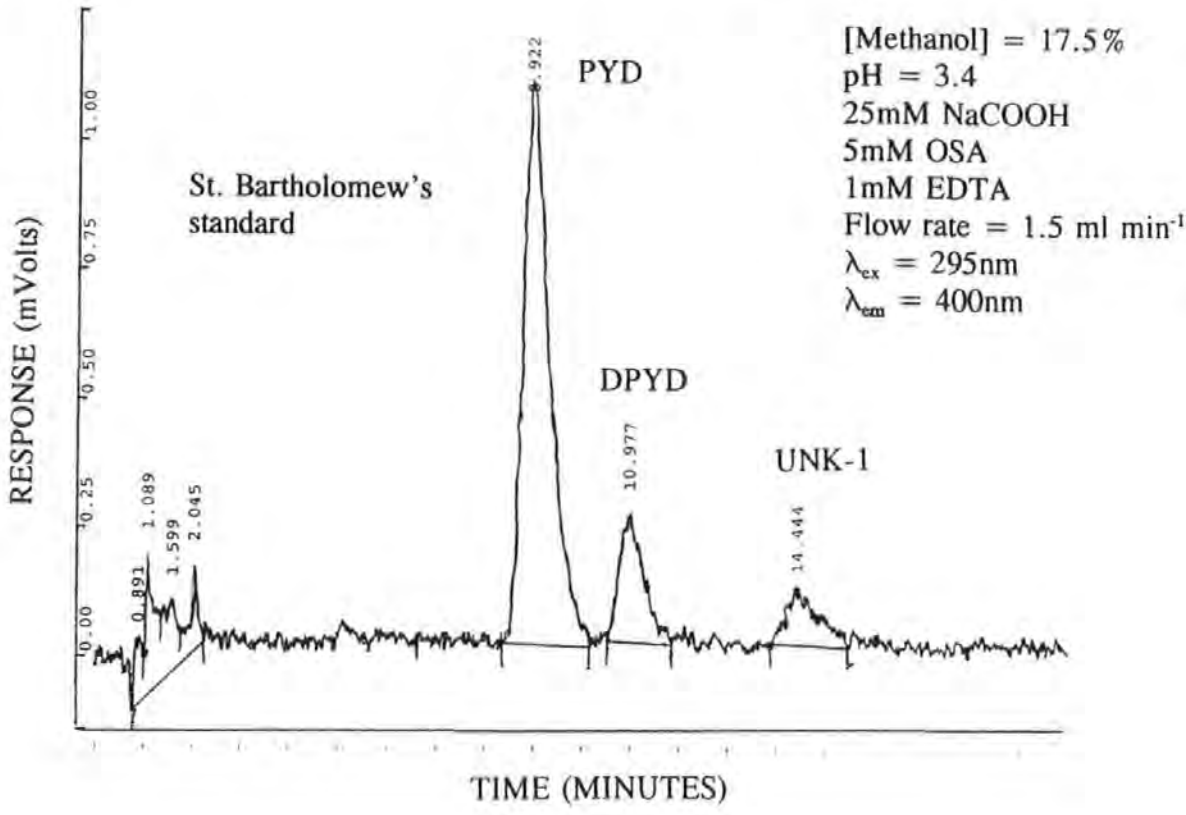


Figure 3.5: A typical chromatogram of Calibration solution 2, prepared from bovine pelvic bone, compared to the St. Bartholomew's crosslink standard.



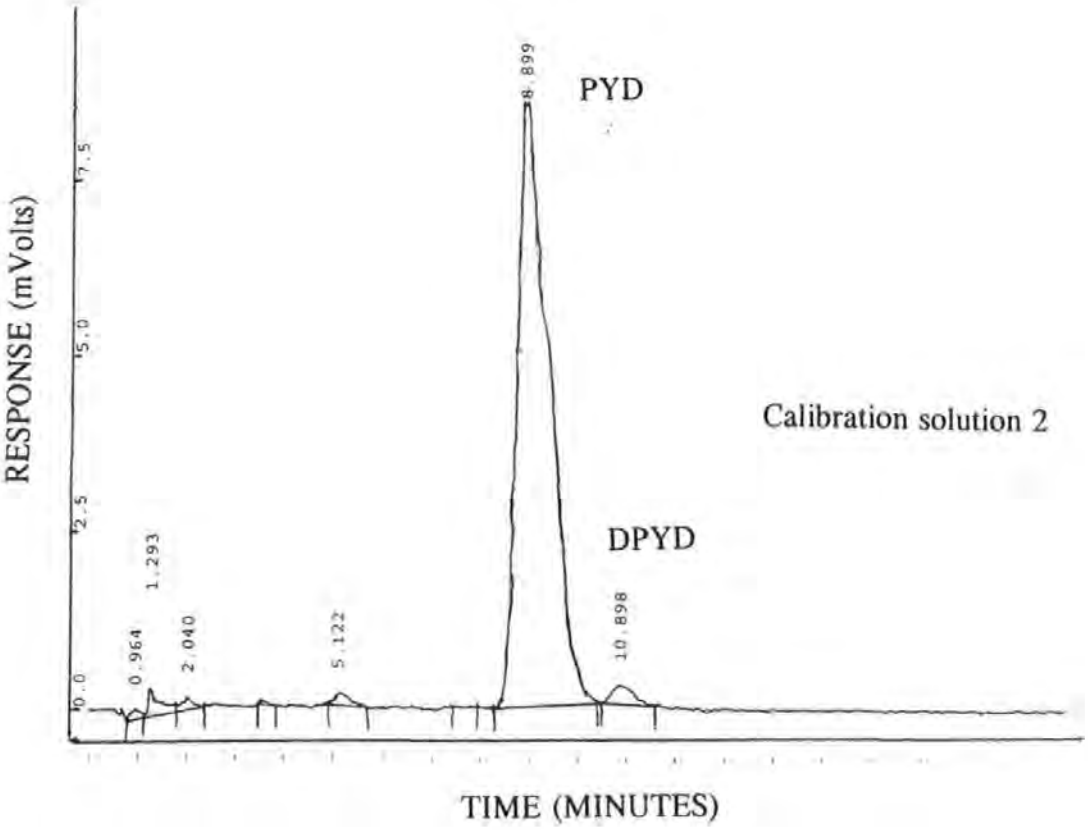


Figure 3.6: A typical chromatogram showing the crosslinks extracted from human femoral bone.

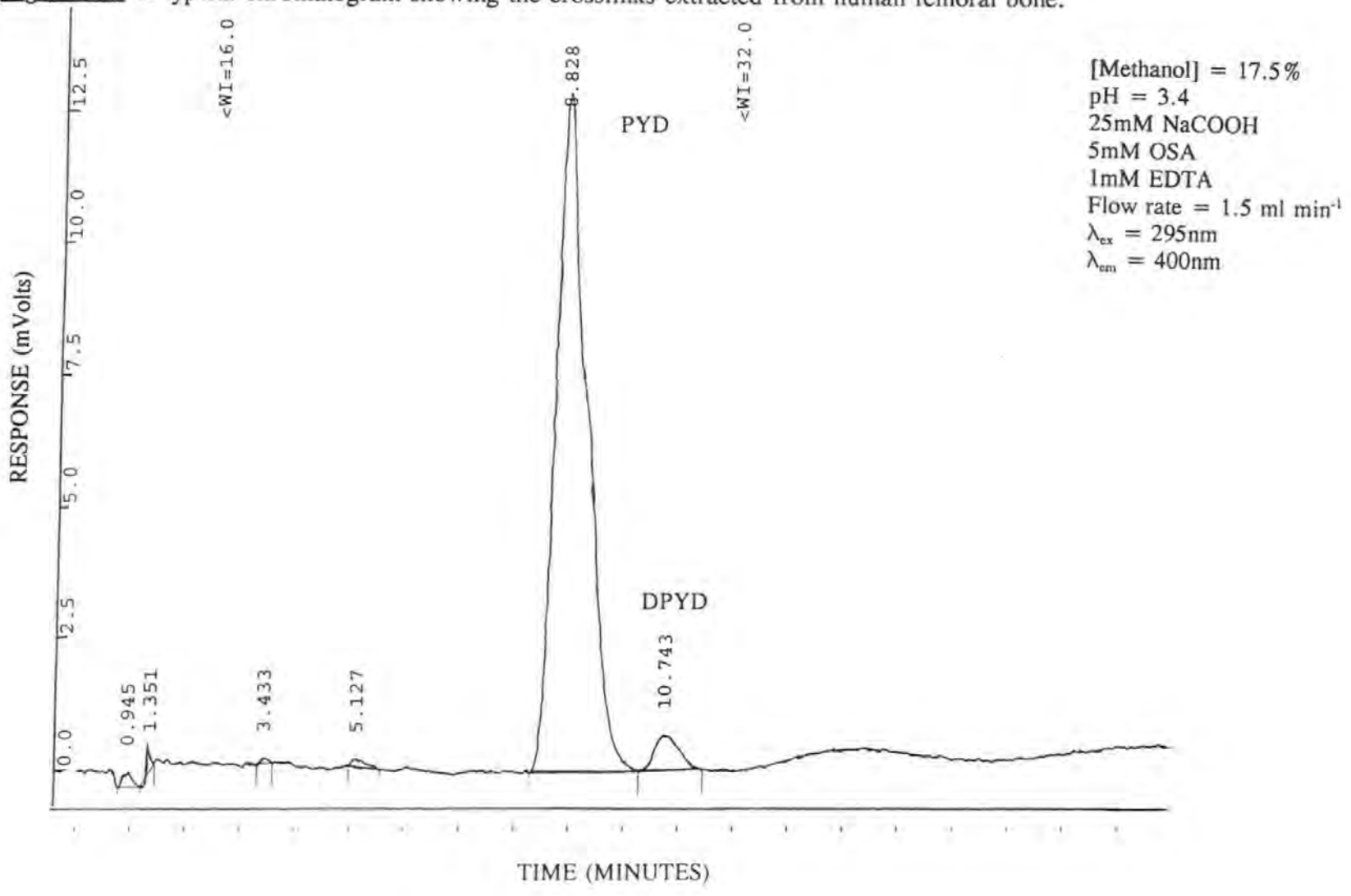


Table 3.2: Results for repeated measurements on the two calibration standards.

Sample	Standard 1 Retention time (minutes)	Standard 1 [PYD] (nM)[a]	Standard 2 Retention time (minutes) PYD	DPYD	Standard 2 [PYD] (nM)	[DPYD] (nM)
1	8.29	1067.88	8.90	10.90	8686.77	296.10
2	8.40	1060.03	8.78	10.76	8931.09	376.72
3	8.37	1031.25	8.75	10.71	9065.73	334.4
4	8.34	1006.63	8.75	10.84	9159.92	336.94
5	8.29	1053.59	8.76	10.72	9191.37	394.36
6	8.27	1000.68	8.76	10.73	9255.40	360.29
7	8.29	1029.73	8.71	10.67	9224.71	362.83
8	8.18	1030.52	8.70	10.66	9258.69	361.78
9	8.21	1030.28	8.70	10.64	9199.13	300.83
10	8.26	1041.29	8.72	10.67	9289.02	395.50
11	8.26	1048.81	8.76	10.71	8496.75	386.97
12	8.31	1037.44	8.74	10.68	8908.41	304.13
AVG	**8.29**	**1036.51**	**8.75**	**10.72**	**9055.58**	**350.90**
S.D.	**0.06**	**18.99**	**0.05**	**0.07**	**241.49**	**34.59**
(%RSD)	**(0.71)**	**(1.83)**	**(0.58)**	**(0.69)**	**(2.67)**	**(9.86)**

[a] DPYD values not given (below the limit of detection)

attained (Table 3.3), with % RSD's of 25% and 19% for PYD and DPYD respectively. The PYD:DPYD ratio also varied considerably. This clearly illustrated that the sample preparation procedure, rather than the chromatography, was responsible for the observed irreproducibility.

The linear response range using the Varian fluorescence detector for the crosslinks was evaluated using the "home made" bovine stock solution prepared as described in Section 3.2.2. A series of diluted samples were prepared and the peak area values for both PYD and DPYD were plotted against concentration. Figure 3.7 shows that PYD was linear up to 960nM and from Figure 3.8, DPYD was linear up to at least 700nM.

3.3.1.3: Evaluation of the resuspension solution.

The solution used to resuspend the freeze dried sample could also have a profound effect on the chromatography and\or its reproducibility, since it could interact and change the ion paring conditions of the column. Another series of extracts were therefore prepared as described in Section 3.2.3.

Different types of resuspension solutions were examined, as summarised in Table 3.4. A slight shoulder\split on the PYD peak, and the slight tailing on the DPYD peak were subtly changed by the resuspension solution. Hence the type of loading buffer used did affect the peak shape, particularly for the PYD crosslink. The best results were achieved when the samples were resuspended with 1mM octane sulphonic acid (OSA). Although the resuspension solution improved the peak shapes the % RSD values remained poor.

Table 3.3: Results for repeated measurements on real samples.

Sample	[PYD] (nM)	[DPYD] (nM)	PYD\ DPYD Ratio	PYD retention time (minutes)	DPYD retention time (minutes)
1	943.59	168.29	5.6:1	9.07	10.63
2	654.93	165.67	4:1	9.06	10.54
3	610.3	139.87	4.4:1	9.12	10.62
4	523.19	81.04	6.5:1	8.91	10.46
5	530.19	127.2	4.2:1	9.03	10.57
6	670.77	181.37	3.7:1	9.10	10.52
7	537.32	144.15	3.7:1	9.13	10.67
8	562.48	132.34	4.3:1	9.06	10.51
9	472.11	ND	-	9.08	-
10*	570.00	168.89	3.4:1	8.39	10.28
11*	505.16	174.86	2.9:1	8.73	10.08
12*	344.18	ND	-	8.19	-
13*	400.29	124.05	3.2:1	8.29	10.06
AVG $S.D_n$ (%RSD)	**571.12 141.17 (24.72)**	**146.16 28.24 (19.32)**			

* Samples analysed on a different day

ND = Not Detected

Figure 3.7: Fluorescence response for pyridinoline (PYD).

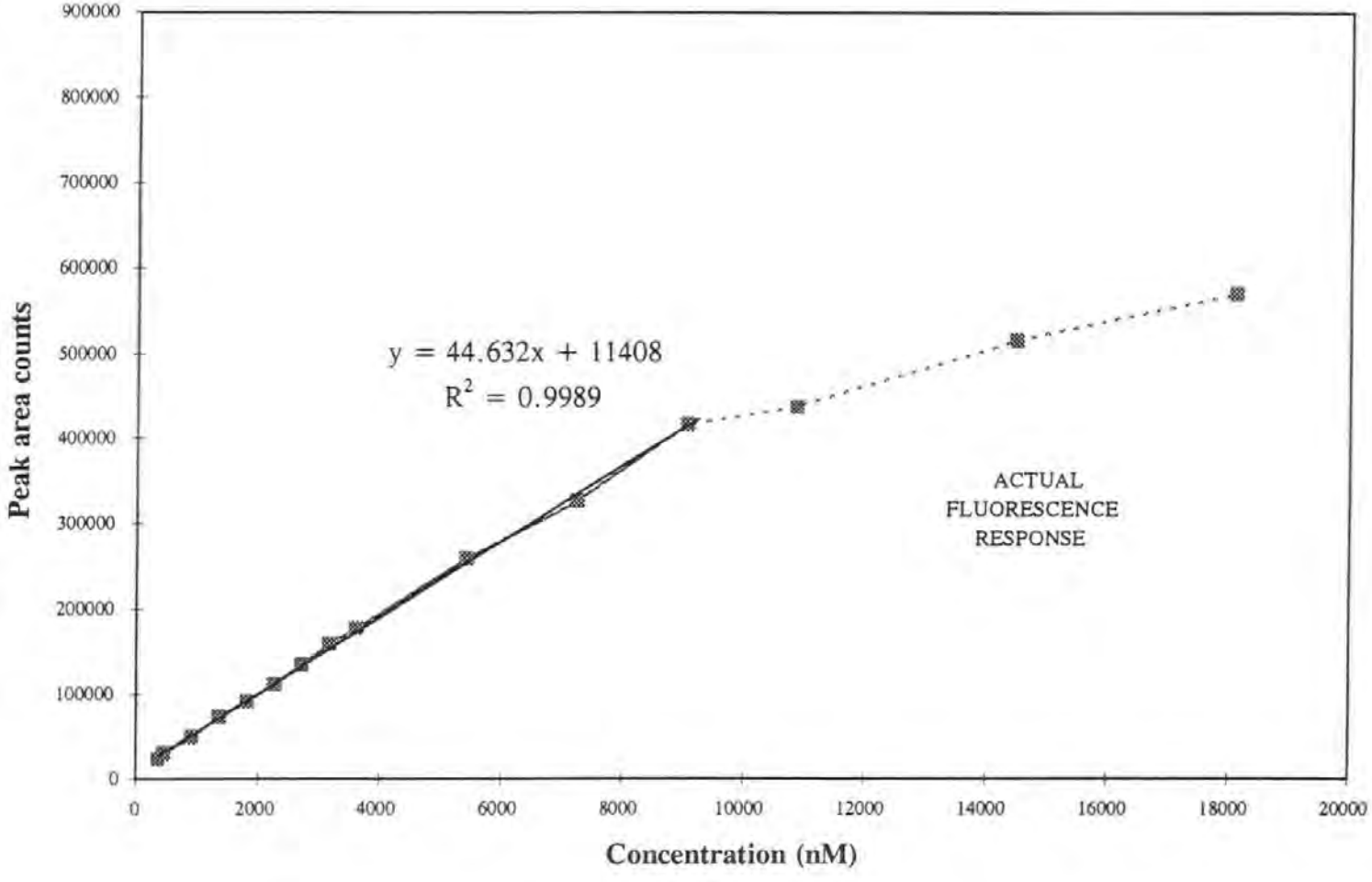


Figure 3.8: Fluorescence response for deoxypyridinoline (DPYD).

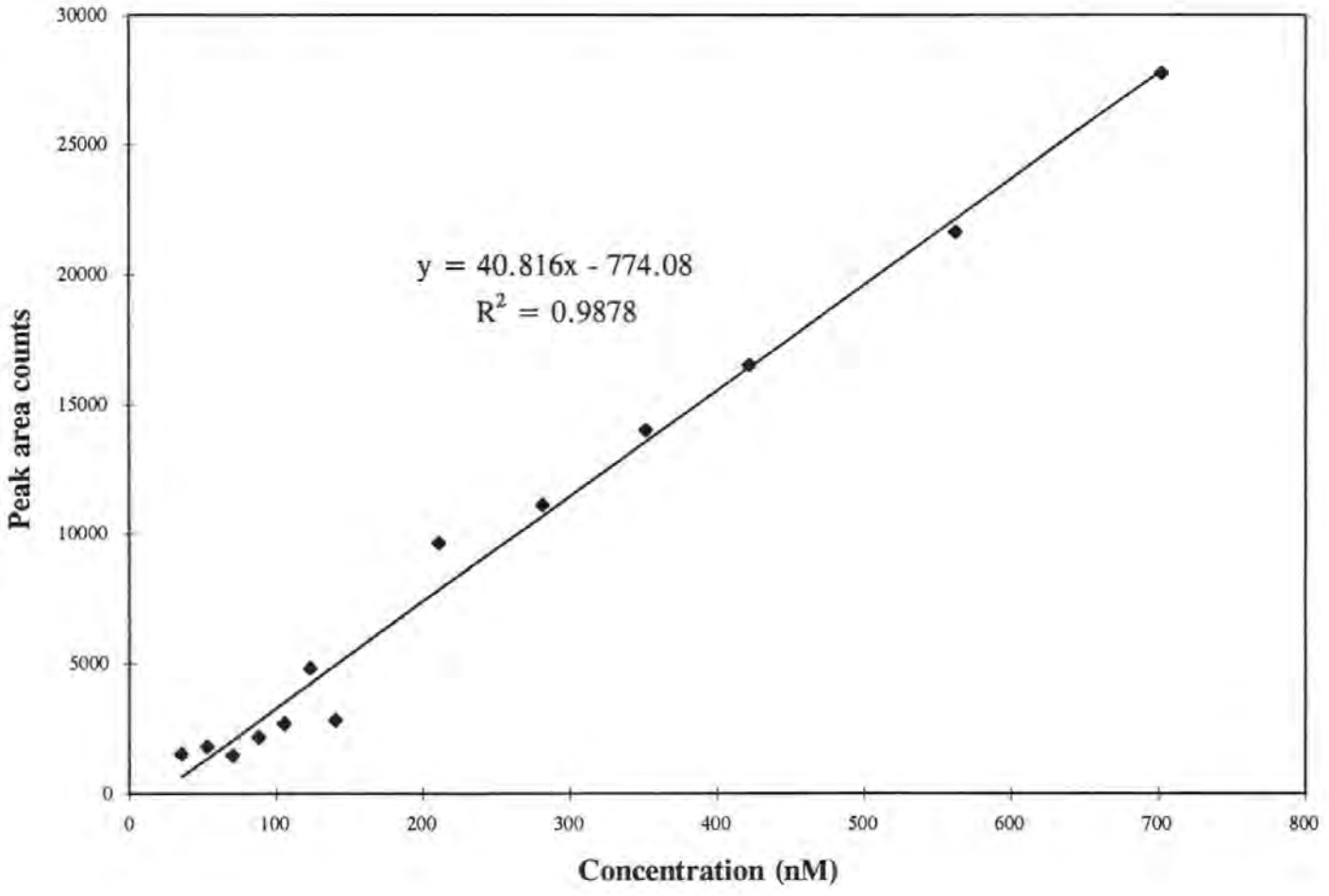


Table 3.4: Type of resuspension solutions evaluated.

Sample number	Resuspension Solution
1	0.05M HCl
2	1mM OSA
3	5mM OSA
4	5mM OSA*
5	50mM OSA
6	50mM OSA*
7	5mM sodium formate
8	5mM sodium formate*
9	25mM sodium formate
10	25mM sodium formate*
11	5mM sodium formate: 5mM OSA (50:50)
12	5mM sodium formate: 5mM OSA (50:50)*

* pH adjusted to 3.4 using 6M HCl.

3.3.2: An investigation into the sample preparation procedure.

Since neither the column condition, nor the chromatographic conditions were responsible for the poor peak shapes, further evaluation of the sample preparation procedure was conducted. Pooled urine samples were prepared, and the following parameters assessed to obtain the optimum conditions:-

A) Acid hydrolysis

B) Wash volume with butanolic eluent (6-30 mls).

C) Elution volume with MilliQ water (2-12 mls).

D) Cellulose column length (1-5 cm).

Table 3.5 shows the results for acid hydrolysed samples compared to non acid hydrolysed samples. As expected the acid hydrolysed samples (*i.e.* total crosslinks) gave higher values than the non acid hydrolysed samples (*i.e.* free fraction). However due to the lower concentrations, the analysis of the DPYD peak was more difficult and in many instances could not be quantified.

The literature states the free fraction constitutes approximately 40% of the total crosslink value (149). The experimental ratios for PYD and DPYD are given below:-

$$[\text{PYD}] = \frac{[\text{non-hydrolysed\free fraction}]}{[\text{hydrolysed\total fraction}]} = \frac{297.0}{554.9} \times 100 = \mathbf{53.5\%}$$

$$[\text{DPYD}] = \frac{[\text{non-hydrolysed\free fraction}]}{[\text{hydrolysed\total fraction}]} = \frac{121.2}{167.2} \times 100 = \mathbf{72.5\%}$$

Table 3.5: Summary of results attained for acid hydrolysed versus non acid hydrolysed samples.

	Acid hydrolysed	Non acid hydrolysed
PYD concentration (nM)		
average ± s.d	554.9 ± 161.0	297.0 ± 63.7
RSD (%)	29	21
no. of samples (n)	13	15
DPYD concentration (nM)		
average ± s.d	167.2 ± 43.6	121.2 ± 38.6
RSD (%)	26	32
no. of samples (n)	12	6
Ratio PYD:DPYD	3.3:1	2.5:1

The higher ratios particularly for DPYD, suggested that either the value for the non hydrolysed sample was too high or that not all the crosslinks were converted and\or extracted in the acid hydrolysed samples, thus giving a low value. Since the ratio of PYD:DPYD in the acid hydrolysed sample was in the normal range (3-4:1), whereas in the non acid hydrolysed sample it was low, this supported the view that the DPYD value in the non acid hydrolysed sample was too high. This could be attributed to the data handling by the chromatographic software, since the low concentrations involved made accurate quantification and peak identification difficult. Therefore on a practical basis, the analysis of the acid hydrolysed samples was easier and within the data handling capabilities of the software. However little difference in the RSD's were observed between the two types of samples, indicating that acid hydrolysis was an unlikely source of the quantitative irreproducibility observed.

To further support this conclusion, very little evaporation of the sample occurred during the acid hydrolysis stage (Table 3.6), indicating that none of the the HCl, present to convert the bound crosslink portion into free crosslinks was lost, hence complete conversion should take place. Also present in all the hydrolysates were fine black particles, which could interfere with the extraction of the crosslinks. Table 3.7 shows that under the normal preparation conditions (where the hydrolysate is shaken prior to an aliquot being taken), the correct PYD:DPYD ratio was attained, indicating the particulates do not appear to interfere with the crosslink extraction. However if the hydrolysate was centrifuged and the supernatant used, a lower ratio was attained. The PYD concentration was also lower, whilst the DPYD concentration was slightly higher. This suggested that some of the PYD becomes trapped or absorbed onto the fine

Table 3.6: Results showing evaporation rate during acid hydrolysis

Sample (1ml urine + 1ml HCl)	Weight before hydrolysis (g)	Weight after* hydrolysis (g)	% Loss
1	16.6039	16.5985	**0.03**
2	16.4705	16.4313	**0.07**
3	16.6778	16.6688	**0.05**
4	16.7994	16.7946	**0.03**
5	16.3277	16.3226	**0.03**
6	16.6144	16.6112	**0.02**

* Samples hydrolysed for 16 hours at 116°C

Table 3.7: Results for acid hydrolysates prepared under normal conditions (*i.e.* shaking) compared to centrifuging.

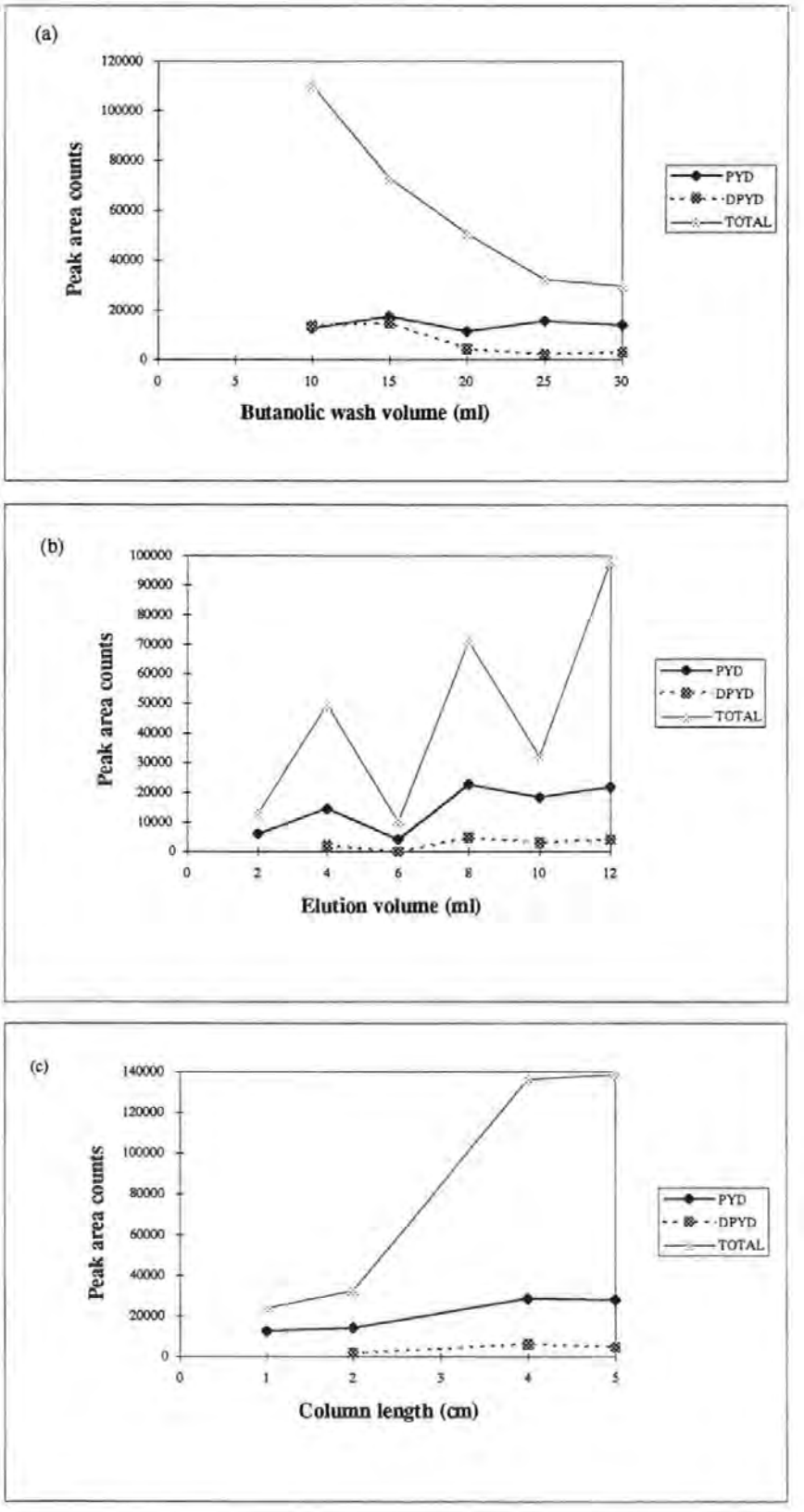
	Hydrolysates prepared after shaking	Hydrolysates prepared after centrifuging
PYD concentration (nM)		
average ± s.d	604.5 ± 158.0	497.1 ± 144.3
RSD (%)	26	29
no. of samples (n)	6	6
DPYD concentration (nM)		
average ± s.d	156.4 ± 37.8	178.1 ± 46.2
RSD (%)	24	26
no. of samples (n)	6	6
Ratio PYD:DPYD	3.9:1	2.8:1

particulates or that some is released or redissolved on shaking. However the RSD's for both types of sample were again high, further indicating that the acid hydrolysis stage was probably not the source of error.

Figure 3.9 summarises the results concerning the optimisation of the butanolic wash volume, elution volume and cellulose column length. Some trends can be seen which confirm the initial suspicions that the sample preparation stage is critical with respect to achieving reproducible quantitative results. The volume of butanolic wash solution (Figure 3.9a) should be 25mls or more in order to remove the bulk of the interfering fluorophores, since little change in the total peak areas was observed after this. The PYD peak areas did not change greatly, which indicated that the butanolic wash solution had a minimal effect on this crosslink. However the peak areas for the DPYD peak were reduced after washing with more than 15mls, suggesting that some of the DPYD was washed off the column along with the interfering fluorophores.

The elution volume (Figure 3.9b) should be greater than 8 mls of MilliQ water. This removed the maximum quantity of the crosslinks from the column, although the total peak area did vary. The column length (Figure 3.9c) should be 4-5 cm long in order to extract the maximum crosslink levels. The flow rate through the cellulose columns was the only parameter not controlled and since the amount of cellulose appeared to be important, it was possible that the flow rate would also play a critical role. This optimisation experiment was therefore repeated using polypropylene disposable columns, manufactured with a porous frit sealed in at the bottom (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, U.K.). It was hoped that the use of these columns would remove the

Figure 3.9: Optimisation of sample preparation procedure.



uncertainty associated with the flow rates through the columns and hence the time the crosslinks were in contact with the column for each sample. Unfortunately these columns made little difference to the % RSD values.

3.4: USE OF AN ALTERNATIVE CHROMATOGRAPHY METHOD.

When multiple samples were analysed during the method development work, the column conditions deteriorated quite quickly. Attention therefore focused on an alternative ion pair isocratic reverse phase HPLC method (444).

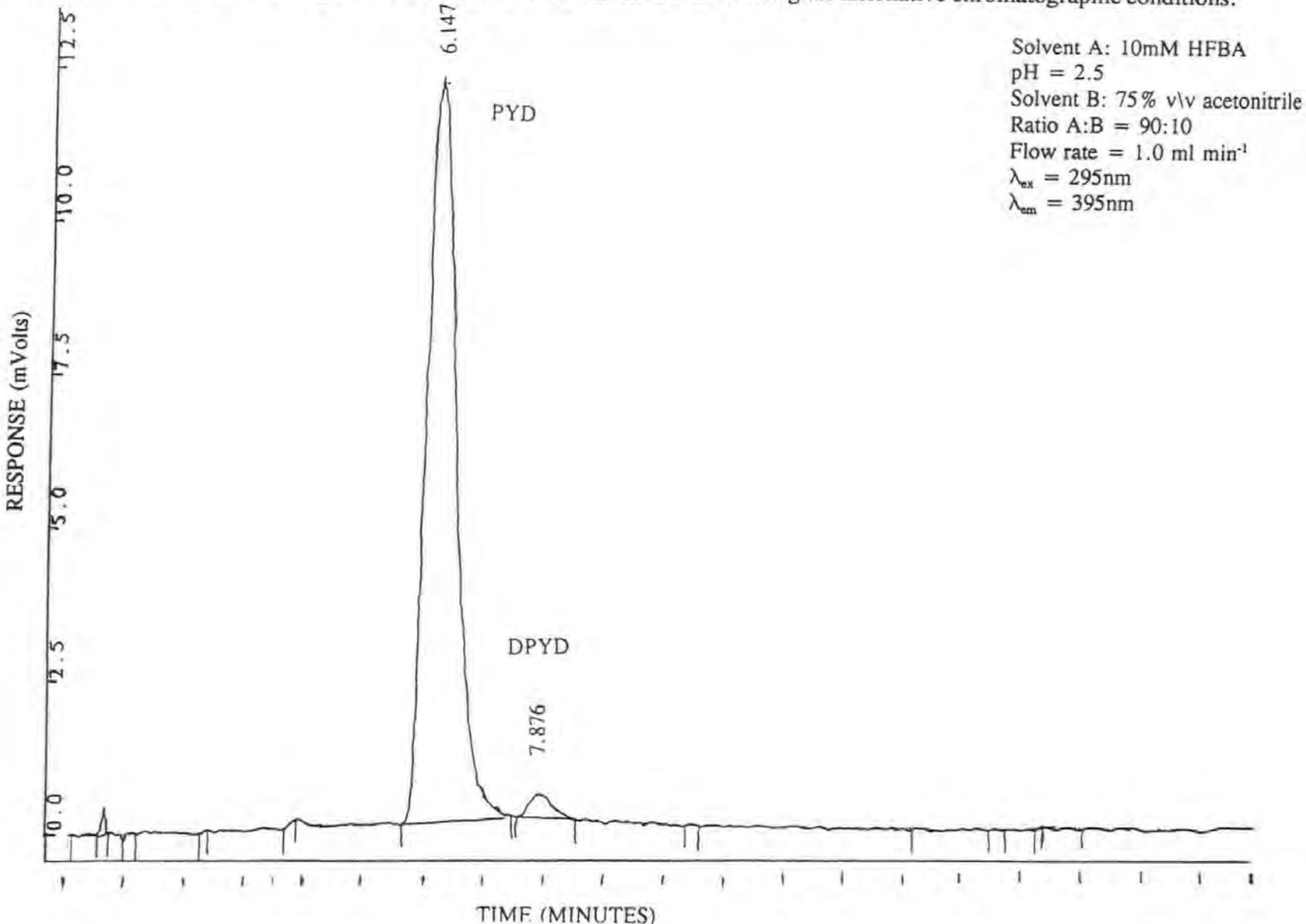
3.4.1: Experimental.

This alternative method's mobile phase consisted of solvent A (10mM HFBA, pH adjusted to 2.5 with 10M NaOH) and solvent B (75% v\v acetonitrile, 25% v\v solvent A). The final mixture of solvent A:solvent B was 90:10, using a flow rate of 1.0 ml min^{-1}. Samples were resuspended with 200 μl of a resuspension solution (1mM HFBA). The equipment and column used were as described in Section 2.2.3.

3.4.2: Results and discussion.

This alternative method also resulted in good resolution and peak shapes, within reasonable (less than 8 minutes) analysis time for both standards and samples (Figure 3.10). However better column stability and hence longer column life was possible when running a large number of samples. For these reasons this alternative method based on acetonitrile\HFBA was favoured over the methanol\OSA based method, and used in all the remaining experiments.

Figure 3.10: A typical chromatogram showing the St. Bartholomew's crosslink standard using the alternative chromatographic conditions.



Following on from the findings of the optimisation experiments described in Section 3.4.2, a series of spiked samples were prepared as summarised in Figure 3.11, to explore which stage of the sample preparation was responsible for the quantitative variability of the results. The % recovery results (Table 3.8) did not really clarify the nature of the problem in the sample preparation procedure. The results clearly showed each stage was susceptible to variable results and that no clear trend could be observed. The first stage gave % recovery results of 100% or greater? Stage two however only gave % recovery results of approximately 50%. It appeared the loss of crosslinks could occur during one or more of the preparation stages. These results underline the difficulties encountered with the HPLC method where the extraction procedure was not under control and thus giving good extraction efficiencies on some occasions whilst poor on others.

3.5: CONCLUSIONS.

Good separation of the PYD and DPYD crosslinks was possible using two different isocratic ion pair reverse phase HPLC methods. However the system based on acetonitrile and heptafluorobutyric acid, (as opposed to methanol and octanesulphonic acid), was preferred because it provided extra column stability and hence prolonged the column life. The HPLC technique provided excellent qualitative results, but the quantitative results were irreproducible. The sample preparation procedure appeared to be responsible for these variations but despite detailed examination and optimisation of the conditions, improvement to the quantitative precision was not achieved. Therefore the use of the HPLC methodology for routine assessment of the crosslinks was considered unsatisfactory. Either further improvements to this methodology or an alternative method, capable of the necessary accuracy and precision was therefore required.

Figure 3.11: Procedure used in recovery tests, where samples were spiked with calibrant 2 solution, at different stages of the preparation procedure, as indicated by the arrows.

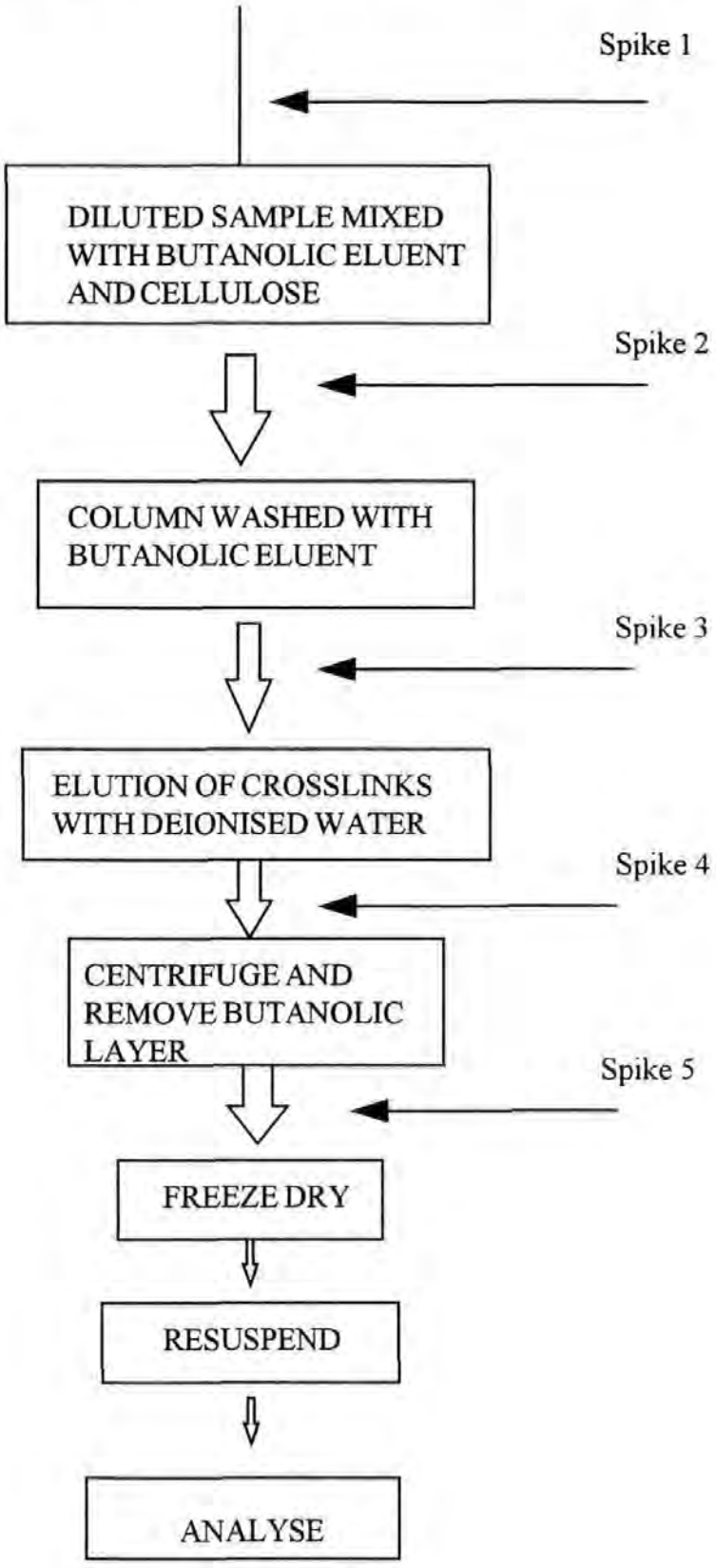


Table 3.8: % recovery results for the various stages of the sample preparation procedure.

Spike†		% Recovery PYD	% Recovery DPYD
1	(a)	90.4	105.3
		131.2 123.9*	- 169.7*
		97.8 125.2*	75.8 134.9*
		144.9 135.0*	141.4 156.8*
		146.5	120.9
2	(a)	35.1	46.3
	(b)	44.7	57.1
3	(a)	92.3	81.4
	(b)	87.9	94.5
4	(a)	88.0	99.2
	(b)	85.4	122.3
5	(a)	78.3	50.9
	(b)	82.9	102.3

† Refer to Figure 3.11 for details

* Same sample analysed 48 hours later

(a) spiked blank (water) samples

(b) spiked urine samples.

CHAPTER 4

ANALYSIS OF THE PYRIDINIUM CROSSLINKS BY ENZYME LINKED IMMUNOASORBANT ASSAY (ELISA).

CHAPTER 4: ANALYSIS OF THE PYRIDINIUM CROSSLINKS BY ENZYME LINKED IMMUNOASORBANT ASSAY (ELISA).

4.1: INTRODUCTION.

Alternative ways to HPLC to measure the pyridinium crosslinks, have been investigated in particular immunological methods. The first enzyme linked immunoassay (ELISA) method (432) demonstrated good sensitivity, but could not distinguish DPYD from PYD, and the desmosine crosslink of elastin also showed some cross-sensitivity (453).

It is now known that about 40% of urinary PYD and DPYD is present in the free form (149), and that measurements of the free forms correlate well with total PYD and DPYD in normal subjects and in those with levels elevated due to metabolic bone disease or menopause. Also the peptide bound forms of PYD and DPYD are mixtures of several molecular species (454). Based on this knowledge immunoassays have been developed using antibodies that preferentially recognise free PYD and DPYD, rather than the glycosolated and large peptide bound forms (433,434). Where only urinary free DPYD is measured, less than 1% cross-reaction with free PYD has been observed (434). These immunoassays require no sample preparation and suffers negligible sample interferences. In addition when compared to the HPLC technique (see Chapter 3) such techniques provide a rapid and simple method for evaluating the pyridinium crosslinks in urine (433,434,455). The only major disadvantage of this approach is that unlike HPLC it is unable to simultaneously distinguish between PYD and DPYD.

The ELISA method has demonstrated satisfactory correlation with the HPLC technique

(246) and has been used to monitor various metabolic diseases, such as vitamin D deficiency (246), Paget's disease (243), and osteoporosis (235). However these studies have shown that the ELISA method is not as sensitive as the HPLC technique, particularly where changes in calcium metabolism have occurred at menopause or during hormone replacement therapy (235). Whether this limitation will be balanced out by avoiding the inconvenience of the cumbersome, expensive and time consuming HPLC procedure is debatable. However, it is generally accepted that the ELISA technique offers the greatest potential for routine clinical use in a large number of metabolic bone diseases.

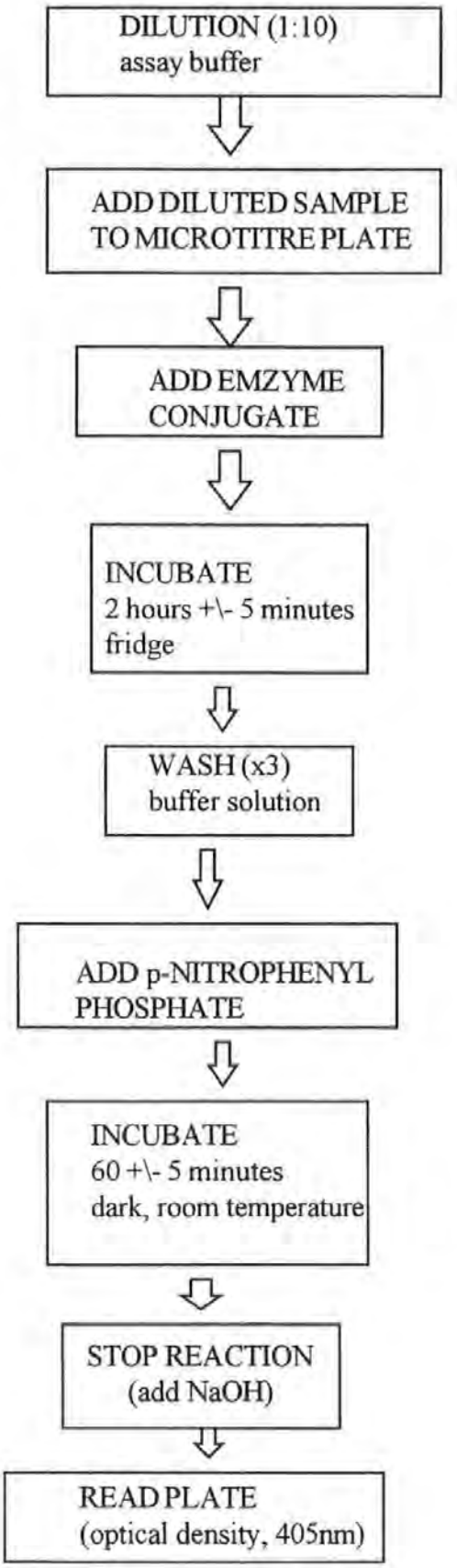
This chapter considers the potential of the ELISA method for the analysis of the urinary pyridinium crosslinks with the intention of utilising this approach (if successful) in the clinical trials. The ELISA kits manufactured and sold commercially by Metra Biosystems (Oxford, UK) were evaluated in order to ascertain their suitability for the analysis of a large number of samples, with both accurate and precise results, over a period of time.

4.2: EXPERIMENTAL.

The pyridinium crosslinks were measured using a commercially available ELISA kit (Metra Biosystems Ltd, Oxford, UK). Pyrilinks-D™ only measures the bone specific crosslink deoxypyridinoline, utilising a highly specific monoclonal antibody to determine free DPYD present in urine from the breakdown of bone collagen (434). The data analysis was performed using a Multiskan Mk II programme V1.5 (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK) and a curve fitting programme, Softmax V2.34 (Metra Biosystems Ltd, Oxford, UK).

For the deoxypyridinoline crosslink analyses, the procedures described in Section 2.3.2 and summarised in Figure 4.1 were followed. The samples, standards and controls were diluted 1 in 10 with a buffer solution (phosphate buffered saline [PBS] and Tween 20 {polyoxyethylene (20) sorbitan monolaurate}; 0.15M NaCl, 10M sodium phosphate, pH 7.0, and 0.05% Tween 20). These solutions were then added to individual wells coated with the DPYD antibody (rabbit or mouse anti-deoxypyridinoline monoclonal antibody) on a microtitre plate. Any DPYD present binds to the immobilised DPYD antibodies. An enzyme conjugate (DPYD-alkaline phosphatase) was added to the reaction mixture and left to incubate in the dark at between 2-8 °C for 120 $\pm$ 5 minutes. During this incubation period the DPYD in the sample competes with the conjugated DPYD-alkaline phosphatase for the antibody bound to the plate. The high immunological specificity of the DPYD antibody, combines with the catalytic ability of the alkaline phosphatase enzyme to bind with any DPYD molecules previously bound, creating an antibody-antigen-antibody sandwich. After washing away any unbound conjugate with a PBS-Tween solution, p-nitrophenyl phosphate was added to each well, resulting in a colour forming reaction to occur with the DPYD during an incubation period of 60 $\pm$ 5 minutes at room temperature in the dark. The colour development was stopped by the addition of sodium hydroxide (1N) and the optical density was measured at 405nm using a microplate reader (Titertek Multiskan Plus Mk II, Labsystems Life Science International (UK) Ltd, Basingstoke, Hampshire, UK). The amount of substrate converted is proportional to the concentration of bound DPYD.

Figure 4.1: Summary of the ELISA procedure to measure the DPYD crosslink.



4.3: RESULTS.

Table 4.1 shows that excellent % recovery's for DPYD (range: 96.6% to 106.5%) were attained at different concentration levels. Both of the control standards also gave values within the accepted reference ranges, *i.e.* 14.3 $\pm$ 0.9 nM (range: 12.4 - 17.7 nM), and 91.1 $\pm$ 5.2 nM (range: 82.2 - 117.3 nM), and with RSD's of 6.1% and 5.7% respectively (Table 4.2).

4.4: CONCLUSIONS.

The ELISA technique utilising the commercial kits from Metra Biosystems provided both an accurate and precise way of measuring the deoxypyridinoline crosslink in urine samples. The technique offered several other advantages when compared to the HPLC method in that it was quick and simple to use, required very little sample preparation (*i.e.* just dilution as opposed to extraction), was more robust, less prone to interferences and could easily be introduced into clinical laboratorys on a routine basis. For these reasons the ELISA method was adopted for the clinical trials.

Table 4.1: Results obtained from % recovery trials for deoxypyridinoline (DPYD).

Test	[DPYD] (nM)	Added [DPYD] (nM)	Observed [DPYD] (nM)	Recovery (%)
1	71.2	12.6	80.9	96.6
2	71.2	89.0	161.9	101.1
3	37.5	12.6	53.35	106.5
4	37.5	89.0	130.6	103.2

Table 4.2: Results obtained for deoxypyridinoline (DPYD) control standards.

Control Sample	Reference Range (nM)	Average [DPYD] ± s.d (n=6)	RSD (%)
Low	12.4 - 17.7	14.3 ± 0.9	6.1
High	82.2 - 117.3	91.1 ± 5.2	5.7

CHAPTER 5

A PRELIMINARY INVESTIGATION INTO THE BLOOD LEAD LEVELS IN PATIENTS DIAGNOSED WITH BONE METASTASES.

CHAPTER 5: A PRELIMINARY INVESTIGATION INTO THE BLOOD LEAD LEVELS IN PATIENTS DIAGNOSED WITH BONE METASTASES.

5.1: INTRODUCTION.

5.1.1: Sources of lead.

Lead is widely distributed in the earth's crust, although lead does not exist in its elemental state in nature. Natural lead consists of four stable isotopes: Pb^{204} (1.48%), Pb^{206} (23.6%), Pb^{207} (22.6%) and Pb^{208} (52.3%). The principle lead ores are galena, cerrusite and anglesite containing lead sulphide, lead carbonate and lead sulphate respectively. Lead is mined from mixed lead and zinc ores and has been used extensively for many centuries (456,457).

Mans exposure to lead from natural sources is minimal compared to the exposure from anthropogenic sources of lead (458). The preparation and\or storage of food, especially acidic foods and drinks in containers such as glazed earthenware, pewterware, leaded decanters, and other packaging materials can result in contamination of the food with variable levels of lead (456,457).

Paper, newsprint and highly coloured magazines contain lead pigments, as do paints, hair dyes and eyepaints, all these can be potentially harmful not only to the user but to young children in the house (456,457). Airborne dust and soils are other sources of lead exposure, with higher lead levels found in inner cities and around highways, compared to rural areas. These levels have been ascribed to industrial pollution from, for example,

lead smelters, battery plants, scrap production, glassworks etc, and from the exhaust fumes from leaded petrol (456-463). Children are particularly susceptible to lead poisoning and the relationship between pica (*i.e.* the habit of eating non-food stuffs) and lead poisoning has been recognised for many years (456,464).

However the principle source of exposure to the general population and the main non-industrial source of lead is from the diet, *i.e.* food, drinking water and other beverages (458,465). Exposure to lead from drinking water is on average very small, unless the water treatment and distribution system utilises leaded pipes, particularly in areas with acidic or "soft" water. Alcoholic drinks, particularly in combination with smoking can be a significant source of lead (466,467). The amount of lead intake in man from food depends on four major inter-related factors: the concentration of lead in the food, the amount of food consumed, the species or the chemical form in which the lead is present in the food and the degree of absorption of the lead into the body (456-458,461).

5.1.2: Physiology of lead.

Lead is a general protoplasmic poison that is cumulative and slow acting. Lead inhibits nearly all the enzymatic steps involved in the haem synthesis and inhibits the uptake of iron from transferrin (456). Inorganic lead is an effective substituent for calcium (Ca) and influences calcium dependent processes (461).

Lead may be absorbed into the body by ingestion, inhalation and through the skin, although the degree and rate of absorption is dependant on the chemical form, for example, organic lead, such as tetraethyl lead is absorbed more rapidly by body tissues

compared to inorganic lead (461). Major routes of lead absorption include the respiratory and gastrointestinal tract. About 5-10% of lead ingested is absorbed into the body, the remainder is excreted in the faeces. Children generally exhibit higher absorption rates (456-458,461).

5.1.3: Distribution of lead in the body.

Following absorption into the blood stream, the bulk of the lead is transferred to the erythrocytes, primarily bound to the haemoglobin (468,469). The plasma concentrations are significantly related to whole blood concentration (470), and accounts for about 6% of the blood lead content (Figure 5.1). Lead disappears from the blood into the tissues, with first order kinetics, diffusing from the red cells to plasma then to extracelluar spaces, and then into the intracellular spaces or tissue cells. The liver and kidney, among soft tissues, contain the highest concentrations. Lead then diffuses from the soft tissues, such that part is stored in the bone and teeth, and part is excreted. Several other factors influence the lead body burden, the most important are:- sex and age, lifestyle, and geographic region for living and occupation (461). Absorbed lead is excreted primarily in the urine (76%), gastrointestinal secretions (16%), hair, sweat, nails and others accounting for the remaining 8% (461). Lead is excreted in human milk (471) and organolead is rapidly excreted in urine, partly metabolised to inorganic lead (472,473).

5.1.3.1: Lead in bone.

Lead approximates other bone seeking elements such as calcium, strontium, barium and fluoride, although the rates are different for each element (474,475). In the stable state more than 90% of the total body burden of lead is stored in the skeleton (476,477). The

Figure 5.1: Lead content in various fractions of blood (461).

<table>
<tr><td rowspan="5">WHOLE BLOOD

Pb = 100%</td><td rowspan="3">PLASMA

Pb = 6%</td><td>PROTEIN (4.5%)</td><td>ALBUMIN (4%)</td></tr>
<tr><td>LIPIDS (1.3%)</td><td rowspan="2">GLOBULINS (0.5%)</td></tr>
<tr><td>SOLUBLE (0.2%)</td></tr>
<tr><td rowspan="2">ERYTHROCYTES

Pb = 94%</td><td rowspan="2">MEMBRANES (14%)</td><td>CARBOXYL (9.5%)</td></tr>
<tr><td>SULPHYDROXYL (2.5%)</td></tr>
<tr><td></td><td>HAEMOGLOBIN (80%)</td><td>PHOSPHOLIPIDS (2%)</td></tr>
</table>

lead stored in the bone is believed to assume a position within the bone crystal either by displacing other cations or by occupying lattice intersities (474). However the possibility that lead is also bound to organic compounds or deposited as a discrete crystal of an insoluble compound cannot be excluded. At very low concentrations lead is an effective nucleating agent for inducing calcium phosphate crystal formation (478), which may be important in trapping lead at the surface of bone crystal.

Lead in bone is unevenly distributed between cortical and trabecula bone hence will contribute different lead concentrations to the total skeletal burden (479,480). An indication of the different bone lead concentrations between cortical and trabecula bone, as well as differences found between bone samples of the same type but collected from different anatomical sites is given in Table 5.1. Trabecula bone represents a lead pool with a faster turnover, whereas cortical bone represents a pool with a slower turnover\kinetics (480,481), hence why the lead levels in cortical bone are higher than in trabecula bone (477,480,481), accounting for more than 70% of the total body burden (476,477).

The lead levels in bone of exposed individuals is greater than in non exposed individuals in the same age group (476,477). Table 5.2 gives an example of the effects exposure can have on the bone lead concentrations. Adult males have higher lead levels than females (476,477,481). It has been found that the lead concentration in cortical bone is dependent on the intensity and duration of exposure, whereas the lead concentration in trabecula bone is dependent on intensity rather than duration of exposure (483).

Table 5.1: Comparison of the mean bone lead concentrations (μg Pb\g bone ash) taken from five different anatomical sites, from different age groups (479).

Age group (years)	Age	Tibia	Ilium	Rib	Vertebra	Skull
Senior adults (>75)	86.3±1.0 (31)*	29.0±3.4 (28)	17.0±2.6 (29)	20.5± 2.4 (31)	18.8±2.6 (30)	26.1± 3.2 (28)
Mature adults (51-75)	63.9±1.1 (42)	24.2±2.3 (38)	19.2±2.4 (40)	22.3± 2.6 (40)	22.4±2.6 (41)	22.8± 2.9 (29)
Mid adults (36-50)	42.3±1.3 (15)	16.6±4.1 (14)	9.9±1.6 (15)	9.7±1.7 (15)	11.9±2.1 (15)	15.2± 3.3 (15)
Young adults (21-35)	24.6±1.0 (18)	5.9±1.2 (18)	5.3±1.6 (16)	5.0±1.2 (18)	6.3±1.3 (17)	4.9± 1.1 (17)
Adolescents (14-20)	17.6±0.5 (13)	2.3±1.0 (13)	2.3±0.9 (13)	2.9±1.4 (12)	3.8±1.4 (12)	3.2± 1.7 (12)
Infants (0-2)	0.3±0.1 (12)	0.3±0.2 (11)	0.0±0.0 (11)	0.7±0.4 (12)	0.6±0.6 (12)	0.6± 0.4 (12)

N.B. Sample population contains no subjects between the ages of 3 and 13 years.

* Numbers in parentheses represent the total number of samples contributing to the mean value.

Table 5.2: Results from vertebral bone biopsies, showing the lead concentration differences between exposed and non-exposed individuals (481).

	Exposed (n= 27)	Retired (n=9)	Controls (n=14)
Mean age (range)	46 (26-65)	67 (61-71)	62 (34-88)
Mean exposure time (range)	11 (0.6-39)	29 (6-46)	-
Mean lead concentration {vertebral bone} (μg\g wet weight)	29 (2-155)	19 (5-76)	1.3 (1-4)

N.B. All participants were male.

The lead content of childrens bone is lower compared to adults, accounting for approximately 75% of the total body burden (477), which can be attributed to the fact that children have had lower exposure levels, and that they do not possess the same capacity to retain lead in the bone (471). Bone turnover rates vary with age and health (475), with the bone lead content generally increasing with age at a rate dependent on the skeletal site (see Table 5.1) and extent of lead exposure (475,479,482,484). This is in contrast to the lead levels in soft tissues which tend not to change with age (476,479,485).

Skeletal lead is fairly inert and is in equilibrium with the blood where the skeletal lead leaves the skeleton very slowly, accompanying calcium in its osteoclastic transfer from the bone to the blood and the osteoblastic transfer from blood to bone (480). However a number of different physiological, pathological and degenerative conditions, can result in a release of lead stored in the bone into the blood (486,487). This mobilisation of skeletal lead can therefore act as a major endogenous source of lead exposure and may result in symptoms of acute lead intoxification (486,487). The degree of mobilisation is dependent on the lead content of the bone, which is in turn dependent on the lead exposure rate which will influence the location and concentration of lead in different sites, and hence may influence its availability for mobilisation (475). For example in one study the leukaemic infiltration of bone marrow was believed to have caused mobilisation of lead stored in the skeleton (488), and further studies have shown that even without undue exposure, osteolytic processes may result in high blood lead levels (489). Other conditions known to cause such mobilisation include renal disease (490), immobilisation (491), pregnancy (492,493), lactation (494), postmenopausal osteoporosis (495) and

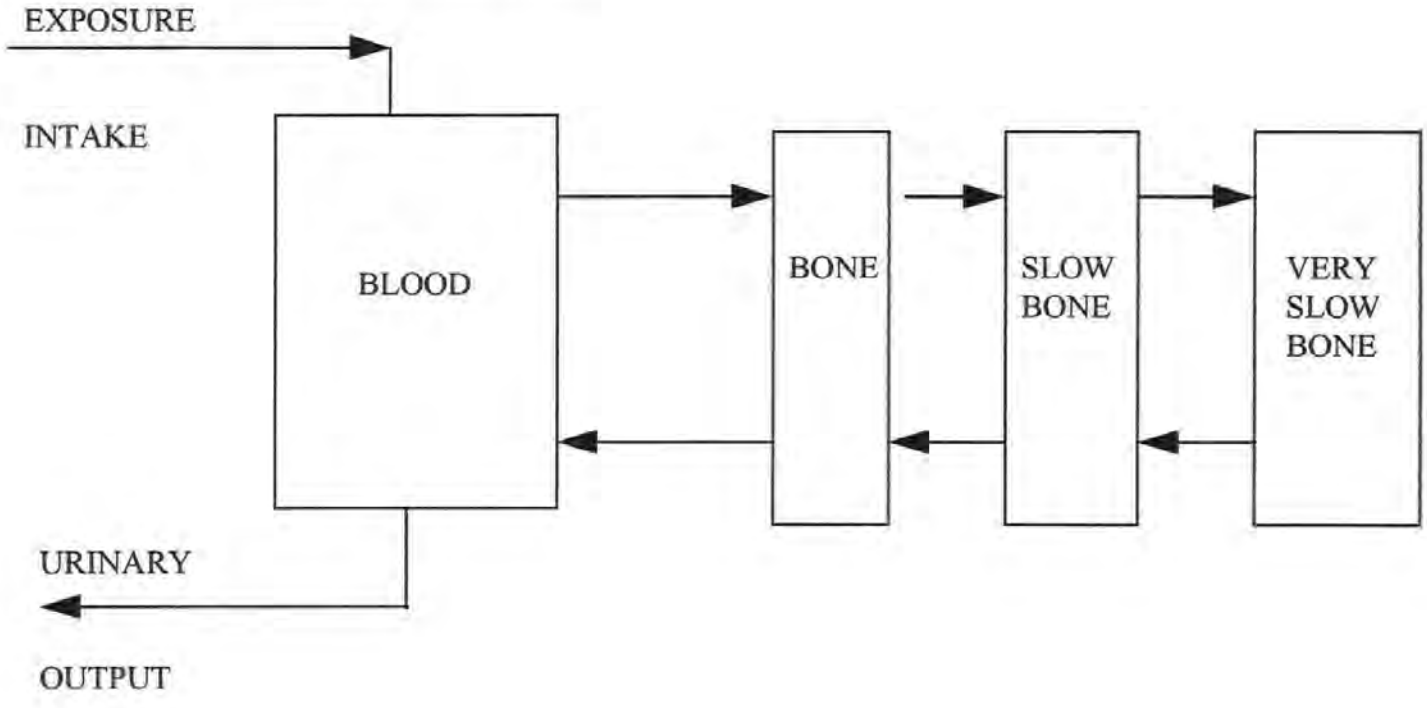
chemotherapy treatment with cis-platin (496-499).

5.1.3.2: Blood lead levels.

The measure of bone lead reflects the cumulative exposure to lead, due to its long residence time [30-70 years] (500). In contrast the lead residence time in blood is only a few weeks, hence blood lead levels can reflect recent accumulation\exposure (refer to Table 5.3), mainly to inorganic lead (484,487,501), although a recent study has shown that organolead compounds may also influence the blood lead content (502). However interactions between the skeleton, blood and soft tissues means that even if exposure is removed, the skeleton continues to release lead into the blood (Figure 5.2) and thus contribute to the lead concentration found in the blood and soft tissues. Therefore chelateable lead in general is not a good indicator of total body burden, since it mainly reflects the blood and soft tissue lead pool, and a fraction of the trabecula bone lead pool, which has a relatively rapid turnover (503,504). The concentration of lead in blood is widely used to assess environmental lead exposure (505,506), and as with the bone lead concentrations, the levels of blood lead are influenced by lifestyles such as smoking and drinking habits, consumption of dairy products etc, and environmental factors such as community size, density of traffic, nearness to smelters etc (466,467,507,508).

Over the years a decrease in the measurable amounts of lead in blood has been observed. This is due to a natural fall in lead levels found in the diet, the introduction of "unleaded" petrol (458) and improvements in the analytical methodology and instrumentation used.

Figure 5.2: Multiple bone pool kinetic model (475).



Not all the lead in bone is equally exchangeable with blood. Pools maybe envisaged with varying degrees of accessibility. The lead at the surface of the bone is readily exchangeable with blood, but some lead becomes buried and more unavailable for exchange (denoted as slow bone and very slow bone). With the passage of years and the turnover and remodelling of bone by osteon activity, this buried lead may become available for exchange.

Table 5.3: Summary of chelatable lead and the lead levels in blood and bone in active and retired lead workers and in occupationally non exposed individuals (503).

Parameter	Active lead workers N	Median	Range	Retired lead workers N	Median	Range	Non-exposed subjects N	Range
Blood lead (μmol l^{-1})	21	2.2	0.77-3.96	14	1.49	0.72-1.91	2	0.19-0.22
Bone lead:- Vertebra [trabecula] (μg g^{-1} Ca)	11	0.15	0.03-0.56	10	0.34	0.06-1.17	2	0.01-0.04
Finger [compact] (μg g^{-1} Ca)	16	32	<20-79	14	72	<20-135	2	<20
Urinary lead (μmol/mmol creatinine)	20	0.023	0.002-0.156	14	0.014	0.004-0.048	2	0.19-0.22
Chelatable lead 6 h after PCA (μmol/mmol creatinine)	21	0.085	0.003-1.225	14	0.049	0.012-0.116	2	0.004
(μmol)	21	0.37	0.06-5.17	14	0.17	0.08-0.58	2	0.01-0.02
Chelatable lead 24 h after PCA (μmol/mmol creatinine)	21	0.042	0.002-0.552	14	0.029	0.007-0.062	2	0.002
(μmol)	19	0.61	0.14-6.54	14	0.32	0.10-1.02	2	0.04

PCA Penicillamine

5.1.4: Lead toxicity.

The blood lead concentrations, regarded as a marker of the permissible occupational exposure levels to lead have deceased over the past two decades due to the growing awareness and concern that toxic biochemical and functional effects were occurring at lower levels of exposure than those that produce overt clinical and pathological signs and symptoms (509). A recent study has stated that the use of $< 100\ \mu g\ l^{-1}$ as the normal reference limit for blood in non occupationally exposed adults appears appropriate for today's population (510). However with respect to controlling lead poisoning, particularly in children, it has now been suggested that there may be no level of blood lead that does not produce a toxic effect, particularly in the developing central nervous system. This also applies to the fetus *in vivo* and in women of child bearing age. As a consequence research has been concerned with all aspects of lead toxicity such as neuropsychological effects, cardiovascular disease, bone, reproductive effects, nephrotoxicity and carcinogenesis (509).

5.1.5: Blood lead analysis.

Lead is probably one of the most widely determined elements in biological samples. Several methods have been developed, with the Delves Cup (DC) AAS method being one of the most routinely used (511). However the need for more sensitive methods to measure lower levels of blood lead has resulted in electrothermal atomic absorption spectrometry (ETAAS) becoming the main analytical technique used. Several methods for the determination of lead in blood by ETAAS have been published over the past decade (263,278,279,512,518-521), as summarised in Table 5.4. A number of analytical problems do exist but these can be eliminated or minimised by appropriate sample

Table 5.4: Summary of ETAAS methods for measuring lead in blood.

Procedure	L.O.D	Linear Range	% RSD	Comments	Refs.
1 + 19 dilution with 1% v\v ammonia solution, 0.003M $(NH_4)_2H_2EDTA$, 0.29M $NH_4H_2PO_4$ Cal: spiked blood Vol: 10μl	0.08 μmol l^{-1}	<4.8 μmol l^{-1}	6.9% (within) 7.3% (between) at 0.5 μmol l^{-1}	Zeeman background correction, L'vov platform	513
1 + 9 dilution with 0.5% v\v Triton X-100, 0.2% v\v conc. HNO_3, 0.2% m\v $(NH_4)_2HPO_4$ Cal: spiked blood Vol: 20μl	0.07 μmol l^{-1}	< 4 μmol l^{-1}	2 - 5% at 0.24 - 2.4 μmol l^{-1}	Deuterium background correction, L'vov platform	514
1 + 4 dilution for [Pb] < 300 μmol l^{-1}, and 1 + 9 dilution for [Pb] > 300 μmol l^{-1}, with 0.005M $(NH_4)_2HPO_4$, 0.2% Triton X-100, 0.014M HNO_3 Cal: Spiked blood Vol: 20μl	36 pg Pb	0 - 60 μg l^{-1}	4.6% (within) 9% (between)	Deuterium background correction, L'vov platform	515

Table 5.4: Continued.

Procedure	L.O.D	Linear Range	% RSD	Comments	Refs.
1 + 9 dilution with 0.1% $NH_4H_2PO_4$ and 0.05% $Mg(NO_3)_2$ Vol: 10μl	0.7 $\mu g\ l^{-1}$			Deuterium background correction with L'vov platform	516
1 + 9 dilution with 0.1% Triton X-100 Vol: 20μl				Zeeman background correction with L'vov platform	517
1 + 9 dilution with 0.01% v\v Triton X-100, inject 10μl of diluted sample plus 10μl of matrix modifier [0.6% m\v $NH_4H_2PO_4$ and 0.15% m\v $Mg(NO_3)_2$ in 0.01M HNO_3] Cal: Aqueous Vol: 10μl	1.2 $\mu g\ l^{-1}$ 0.7 $\mu g\ l^{-1}$ (with L'vov platform)		1 - 4% (within) 1 - 6% (between)	Deuterium background correction with pyrolytic graphite coated tubes and L'vov platform	518

Table 5.4: Continued.

Procedure	L.O.D	Linear Range	% RSD	Comments	Refs.
1 + 9 dilution with 0.05% m\v Triton X-100 Cal: Aqueous Vol: 5-10μl	10 μg dl^{-1}		5% (5μl) > 10% (10μl)	Deuterium background correction, with pyrolytic graphite coated tubes and L'vov platforms. Better sensitivity observed without the addition of $(NH_4)_2HPO_4$ modifier when using platform atomisation.	519
1 + 9 dilution with 0.5% v\v Triton X-100, 0.2% m\v $NH_4H_2PO_4$, 0.2% HNO_3 Cal: Aqueous Vol: 10μl	0.5 μg dl^{-1}	< 60 μg dl^{-1}	1 - 3%	Longitudinal Zeeman background correction with transversely heated graphite atomizer (THGA) and L'vov platform	520
1 + 9 dilution with 0.1% Triton X-100, 10μl injected followed by 10μl matrix modifier [0.5 mg l^{-1} Pd and 2% m\v citric acid] Cal: Aqueous Vol: 10μl	0.1 μg l^{-1}	< 50 μg l^{-1}	2.2%	Deuterium backround correction	521

preparation, for example sample dilution, addition of matrix modifiers, deprotonisation, background corrections and calibration by matrix matched standards (512).

Typically Triton X-100 (a surfactant) is added to whole blood to eliminate dispensing problems by reducing the viscosity of the sample, improve contact between sample and furnace wall, and homogenise the sample by lysis of the erythrocytes (517,519). Other matrix modifiers such as $NH_4H_2PO_4$ or $(NH_4)_2HPO_4$ may also be added, allowing higher ashing temperatures and ensuring the complete removal of any carbonaceous material that may otherwise build up, reducing sensitivity and precision. It is believed the NaCl interference is removed by forming volatile NH_4Cl at high temperatures, while the phosphate ions react with Pb (II) to form the relatively thermally stable $Pb_3(PO_4)_2$ (278). Alternative modifiers include $Mg(NO_3)_2$, which probably acts by embedding lead in a matrix of MgO, thereby delaying volatilisation (516,518). A palladium modifier has been used to control the volatilisation loss of lead in conjunction with the carbon reducing effect achieved by the addition of citric acid (521,522). Other AAS methods have been developed, such as flow injection hydride generation AAS [FI-HG-AAS] (523) and a second generation filter paper based Delves Cup flame AAS (FPDC) procedure for blood lead screening purposes in children (524).

Alternative methods to AAS techniques include electrochemical methods such as voltammetry (461) and proton induced X-ray emission spectrometry [PIXE] (525). However more recently both inductively coupled plasma-atomic emission spectrometry (ICP-AES) and particularly inductively coupled plasma-mass spectrometry (ICP-MS) have been used, owing to their multi-element capacity and high sensitivity. Inductively coupled

plasma mass spectrometry has been successfully used to measure total lead levels in blood samples (526) as well as the lead isotope ratios, in order to identify potential sources of environmental pollution and lead poisoning in children (526-528). Isotope dilution ICP-MS (ID-ICP-MS) gives particularly accurate and precise measurements (417,529,530), and size exclusion chromatography ICP-MS has been used to determine lead species in blood components (531). More recently a capacitively coupled microwave plasma atomic emission spectrometry (CCMP-AES) method has been developed, which offers similar sensitivity and accuracy as both the GFAAS and ICP-MS methods, but has the advantage that no sample dilution or pretreatment is required, and thus has potential as a more appropriate screening method (532,533).

5.2: AIMS OF THIS STUDY.

Since bone metastases results in extensive bone resorption the release of stored skeletal lead into the blood may potentially be used as a marker of bone breakdown. The aim of this preliminary investigation was to develop a simple, accurate and precise method to measure lead levels in whole blood samples, to assess the practicality of measuring the blood samples taken from patients diagnosed with bone metastases, and to observe whether any changes in the blood lead concentrations could be measured over time and with treatment.

5.3: EXPERIMENTAL.

5.3.1: Reagents and chemicals.

All the water used to rinse laboratory ware and for the preparation of solutions, standards and samples was deionised using either a MilliQ purification system (Millipore, Bedford, MA, USA) or an Elgastat Maxima purification system (Elga Ltd, High Wycombe, Bucks., UK) at a resistivity of 18 MΩ.

Aristar nitric acid and Spectrosol lead nitrate standard solution (1000 mg l^{-1}) were obtained from Merck (Poole, Dorset, UK). Thallium nitrate standard reference material, NBS SRM 3158, (10,000 mg l^{-1}) was obtained from the National Institute of Standards and Technology (Washington DC, USA) and Triton X-100 was obtained from Aldrich (Gillingham, Dorset, UK).

Lead and cadmium in whole blood certified reference material BCR-194 was obtained from the Community Bureau of Reference (Brussels, Belgium). Lead in whole blood quality control samples were kindly donated by Dr. Andrew Taylor (Robens Institute, University of Surrey, Guildford, UK) and pooled blood samples, used as internal quality control samples, were obtained from Freedom Fields Hospital (Plymouth, Devon, UK).

5.3.2: Procedures.

Blood samples from patients diagnosed with bone metastases and receiving treatment at the Department of Oncology, Freedom Fields Hospital, Plymouth, were collected by venepuncture into Vacutainer tubes, B-D 6484 (Beckton-Dickinson, East Rutherford, NJ,

USA), containing lithium heparin anticoagulant, and stored at -20°C. Control samples were collected in the same manner from healthy volunteers (*i.e.* nursing staff) in the Department of Oncology, at Freedom Fields Hospital, Plymouth.

After defrosting, the blood samples were shaken thoroughly and 1ml of blood was pipetted into 15ml trace metal free plastic tubes (Lasbsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK) and 9mls of diluent was added using an automatic dispenser (Optifix, Merck, Poole, Dorset, UK). The diluent consisted of 0.1% v\v Triton X-100, used to promote cell lysis and to improve nebuliser efficiency and sample transport; 0.1% v\v Aristar nitric acid, to provide a stable pH environment; and 100 $\mu g\ l^{-1}$ thallium internal standard, to correct for any instrumental drift. The samples were shaken and then analysed by inductively coupled plasma-mass spectrometry (ICP-MS) as discussed in Section 2.1.8.

All laboratory ware was soaked at least overnight in 10% v\v nitric acid, rinsed thoroughly with MilliQ or Elga water and left to drain. A 10mg l^{-1} lead stock solution (in 5% v\v Aristar nitric acid) was prepared from the Spectrosol lead standard (1000 mg l^{-1}). This stock solution was used to freshly prepare a series of calibration standards by pipetting 0, 10, 50, 100, 250 and 500 μl into 100ml plastic volumetric flasks and made up to the mark with the same diluent to give 0 (blank), 1, 5, 10, 25 and 50 $\mu g\ l^{-1}$ lead calibration standards.

5.4: RESULTS AND DISCUSSION.

5.4.1: Analytical figures of merit.

The method developed gave excellent linear calibration over the sample concentration range (1-50 $\mu g\ l^{-1}$), with a limit of detection of 0.4 $\mu g\ l^{-1}$ (average + 3 x S.D, n=20). The accuracy and precision of the method was evaluated initially against the quality control samples from the Robens Institute, University of Surrey. Table 5.5 summarises the results attained. In general the initial results were in close agreement with the indicated values and within the accepted tolerance limits, with the exception of samples 1 and 7. The concentration of sample 1 was within the limit of determination (10 x LOD), which may account for the experimental value falling outside the accepted tolerance limits. With respect to sample 7, an experimental error or some contamination may be possible. However these initial results generally provided a satisfactory indication that the methodology used could provide an accurate and precise measure of lead levels in whole blood samples. Confirmation was achieved by the analysis of a certified reference material BCR 194. Table 5.6 shows that the experimental value (128.24 $\pm$ 1.34 $\mu g\ l^{-1)}$ agreed closely with the certified value (126 $\pm$ 4 μg l-1), with an excellent RSD value of 1% (n=6).

During the preliminary trials, some form of quality control procedure was essential to ensure the method remained under control. The pooled blood samples were standardised against the certified reference material BCR 194, assigned values and used as internal quality control samples (IQC's). The standardisation of the IQC's was conducted on five separate days, and Table 5.7 summarises the results (IQC 1 assigned value = 152 $\mu g\ l^{-1}$

Table 5.5: Summary of results for the quality control samples (supplied by the Robens Institute, University of Surrey).

Sample	Actual lead concentration ($\mu g\ dl^{-1}$)	Measured lead concentration ($\mu g\ dl^{-1}$), n=2	Difference (%)
1	2.53	2.33 ± 0.11	-7.9
2	3.85	3.83 ± 0.04	-0.5
3	4.14	4.03 ± 0.14	-2.7
4	5.5	5.25 ± 0.01	-4.5
5	7.14	7.08 ± 0.07	-0.8
6	12.15	12.29 ± 0.18	1.2
7	22.98	27.37 ± 0.04	19.1
8	34.93	36.06 ± 0.71	3.2
9	46.91	49.02 ± 1.07	4.5

Accepted tolerance levels:-

Samples 1 - 5 = ±5%

Samples 6 - 9 = ±15%

Table 5.6: Summary of results for certified reference material BCR 194 lead and cadmium in whole blood.

Run number	Measured lead concentration ($\mu g\ l^{-1}$)
1	128.35 ± 2.8
2	127.95 ± 3.2
3	128.85 ± 2.5
4	128.18 ± 3.0
5	125.80 ± 2.0
6	130.31 ± 0.7
Overall average	**128.24 ± 1.34**
Certified value	**126 ± 4**
RSD	**1%**

Table 5.7: Results summary for the standardisation of the pooled blood samples (IQC's) against the certified reference material BCR 194.

Sample	IQC 1 measured [Pb] ($\mu g\ l^{-1}$)	IQC 1 average [Pb] ($\mu g\ l^{-1}$) (%RSD)	IQC 2 measured [Pb] ($\mu g\ l^{-1}$)	IQC 2 average [Pb] ($\mu g\ l^{-1}$) (%RSD)
Day 1	149.99 ± 1.6 154.21 ± 2.4 150.97 ± 3.9	151.7 ± 2.2 (1.5%)	70.88 ± 1.3 72.75 ± 1.3	71.82 ± 1.3 (1.85%)
Day 2	151.51 ± 2.6 154.04 ± 4.1	152.8 ± 1.8 (1.2%)	69.58 ± 0.6 73.12 ± 1.6	71.35 ± 2.5 (3.5%)
Day 3	156.51 ± 3.3 150.9 ± 1.4	153.7 ± 4.0 (2.6%)	72.29 ± 0.7 I.F	72.29 ± 0.7 (0.9%)
Day4	150.84 ± 1.7 148.33 ± 3.4	149.6 ± 1.8 (1.2%)	71.08 ± 0.6 72.07 ± 1.66	71.58 ± 0.7 (1.0%)
Day 5	150.73 ± 0.9 153.17 ± 2.7	152.0 ± 1.7 (1.1%)	73.65 ± 1.4 I.F	73.65 ± 1.4 (1.8%)
Overall average (RSD)	151.93 ± 2.2 (1.5%)	151.96 ± 1.5 (1.0%)	71.93 ± 1.3 (1.7%)	72.14 ± 0.9 (1.3%)
Assigned value	**152.0 $\mu g\ l^{-1}$**		**72.0 $\mu g\ l^{-1}$**	

I.F = instrument failure

and IQC 2 assigned value = 72 $\mu g\ l^{-1}$). Table 5.7 also shows that both the inter and intra assay RSD's were less than 2% in most cases.

5.4.2: Preliminary trial.

During the course of the preliminary trials, the methodology was checked by analysing the IQC's after every five - ten patient samples. Tables 5.8 and 5.9 gives the results for IQC 1 and IQC 2 respectively. The results clearly show that the methodology remains under control throughout the trials, giving inter and intra assay RSD's of 5% or less.

A total of 44 patients (24 male, 20 female) diagnosed with bone metastases and receiving treatment were studied over a period of several months. Blood samples were taken each time the patient attended the outpatient's clinic, although this meant that the samples were collected at random time intervals. This factor along with the trial drop out rate resulted in only 18 out of the 44 patients studied having three or more samples collected within the trial period. Overall these results could be categorised into those patients who: 1) showed a downward trend in blood lead levels over time, 2) showed an upward trend in blood lead levels with time, and 3) showed no clear trend with respect to the blood lead levels over time. None of the results were eliminated from the study, unless the IQC results were out of specification for that batch. Although there was a fair amount of variation, both between and within individuals, all the results were retained, since there was no evidence to support the case that a value was an outlier, caused by contamination or some other experimental error, or whether a result was as a consequence of some biological\clinical factor, not studied during the trials.

Table 5.8: Summary of the results for IQC 1 during the preliminary trials.

Trial number	Average lead concentration ($\mu g\ l^{-1}$)	Number of samples (n)	RSD (%)
1	152.51 ± 4.04	10	2.6
2	154.97 ± 4.42	12	2.9
3	150.63 ± 6.28	5	4.2
4	148.93 ± 2.04	3	1.4
5	165.79 ± 4.26	3	2.6
6	145.52 ± 4.91	9	3.4
Overall average	153.06 ± 4.91		
% RSD	4.2		

Table 5.9: Summary of the results for IQC 2 during the preliminary trials.

Trial number	Average lead concentration ($\mu g\ l^{-1}$)	Number of samples (n)	RSD (%)
1	70.23 ± 3.34	5	4.8
2	75.78 ± 1.62	3	2.1
3	70.33 ± 2.78	8	4.0
4	68.82 ± 3.65	3	5.3
5	78.39 ± 1.18	8	1.5
6	68.94 ± 3.13	6	4.5
Overall average	72.08 ± 3.66		
% RSD	5.1		

Table 5.10 and Figures 5.3 and 5.4 depict the results for patient category 1 (*i.e.* those patients who showed a decrease in blood lead levels over time), for male and female patients respectively. All the male patients had higher initial blood lead levels compared to the females which is not unexpected since males are more likely to have had a higher occupational lead exposure. The lead levels appear not to be influenced by other parameters such as age and bone scan rating.

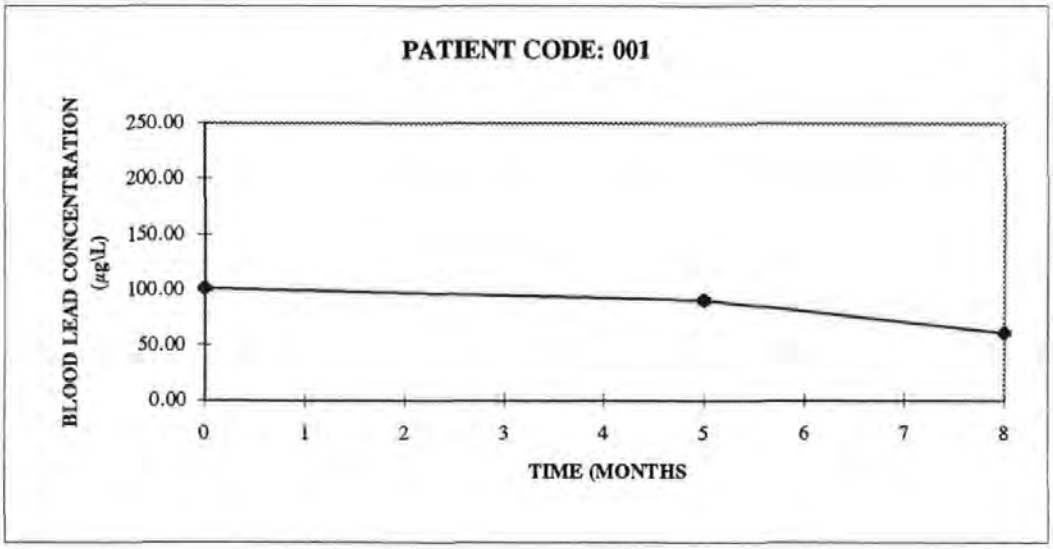
The results for patient category 2, *i.e.* those who showed an increase in blood lead levels over time, are depicted in Table 5.11 and Figure 5.5. These two patients, one male and one female, are of the same age, and showed different initial blood lead levels. The male patient had a higher bone scan rating, indicating a greater extent of skeletal metastases, but had a lower initial blood lead concentration in comparison to the female patient. This result appears to be unaffected by sex and bone scan rating.

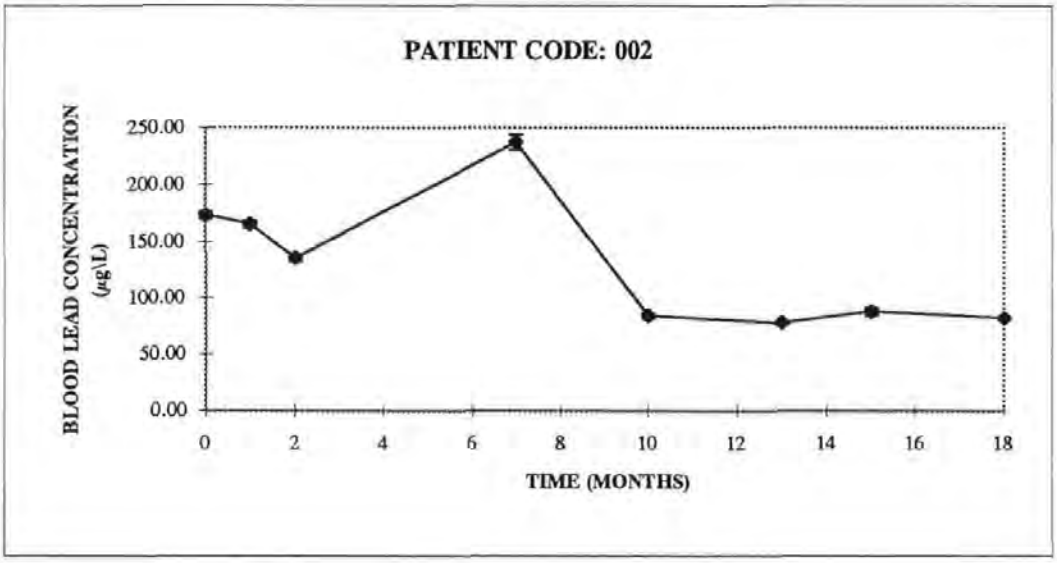
Table 5.12 summarises the results for patient category 3 (*i.e.* those who showed no clear trends in blood lead levels over time), which has been sub-divided, according to the initial blood lead concentration:- A = >80 $\mu g\ l^{-1}$ (Figure 5.6), B = 40 - 80 $\mu g\ l^{-1}$ (Figure 5.7), and C = <40 $\mu g\ l^{-1}$ (Figure 5.8). Again these results indicate there is no relation with age or bone scan rating. Only female patients made up sub-division C.

Table 5.10: Patients who showed a downward trend in blood lead concentration over time.

Patient Code	Sex	Age at start of trial	Bone Scan Rating	Primary Cancer	Treatment	Comments
001	Male	75	2	Prostate	Casodex Zoladex Estracyt	Refer to Figure 5.3: High initial blood [Pb] compared to controls (10-80 $\mu g\ l^{-1}$).
002	Male	73	2	Prostate	Casodex Zoladex	Refer to Figure 5.3: High initial blood [Pb] compared to controls (10-80 $\mu g\ l^{-1}$). Possible error at 7 months.
003	Male	65	3	Prostate	Casodex Zoladex Estracyt	Refer to Figure 5.3: High initial blood [Pb] compared to controls (10-80 $\mu g\ l^{-1}$)
004	Female	80	2	Breast	Pamidronate Radiotherapy	Refer to Figure 5.4: Blood lead levels lower than for male couterparts. Possible error at 14 months.
005	Female	52	1	Breast	Chemotherapy Radiotherapy	Refer to Figure 5.4: Initial blood [Pb] on the upper level of control values. Again values are lower than for the male counterparts.

Figure 5.3: Preliminary trial results - male patients who showed a downward trend in blood lead concentration with time.





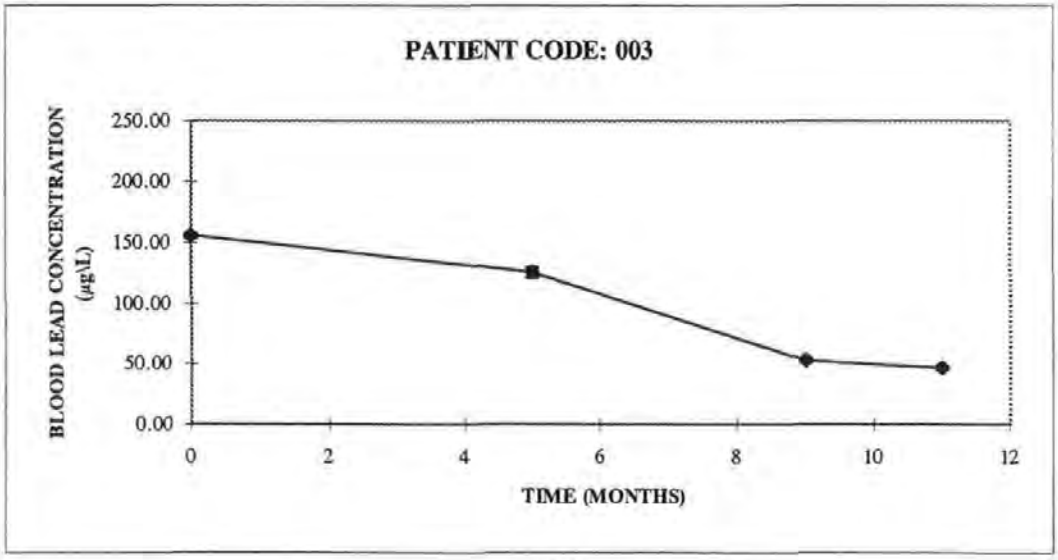
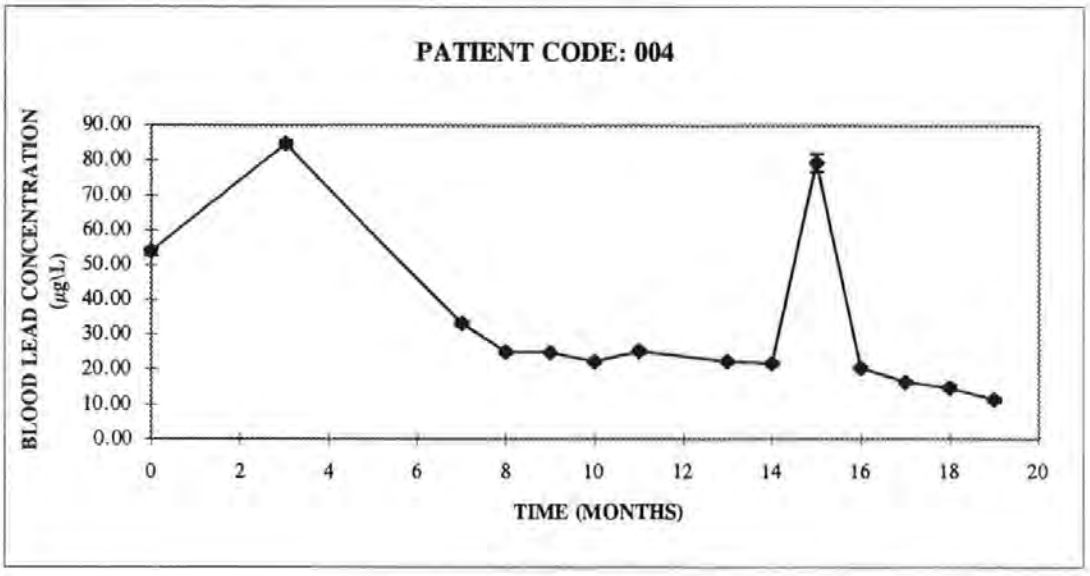


Figure 5.4: Preliminary trial results - female patients who showed a downward trend in blood lead concentration over time.



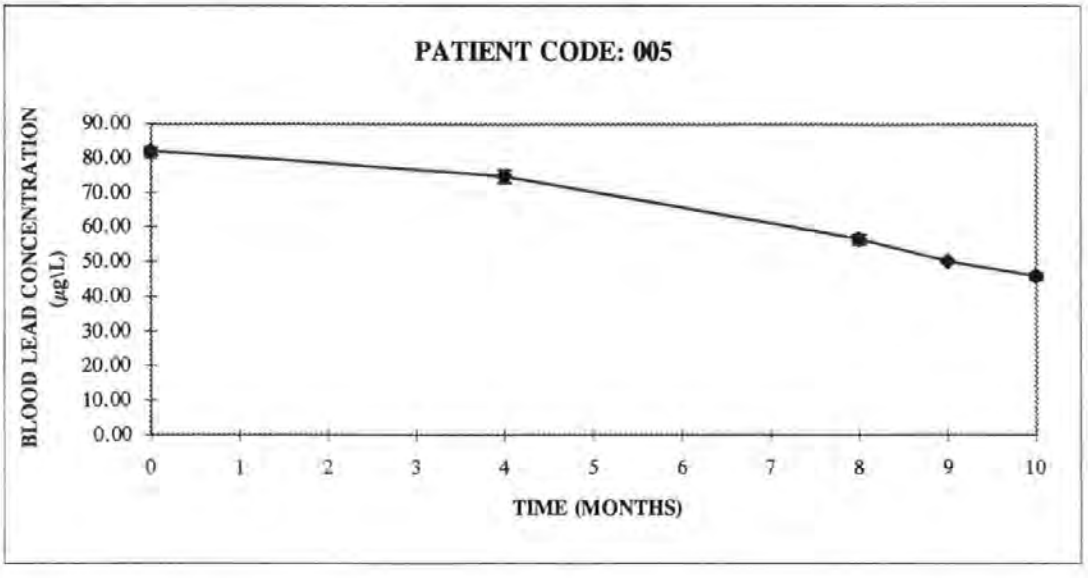
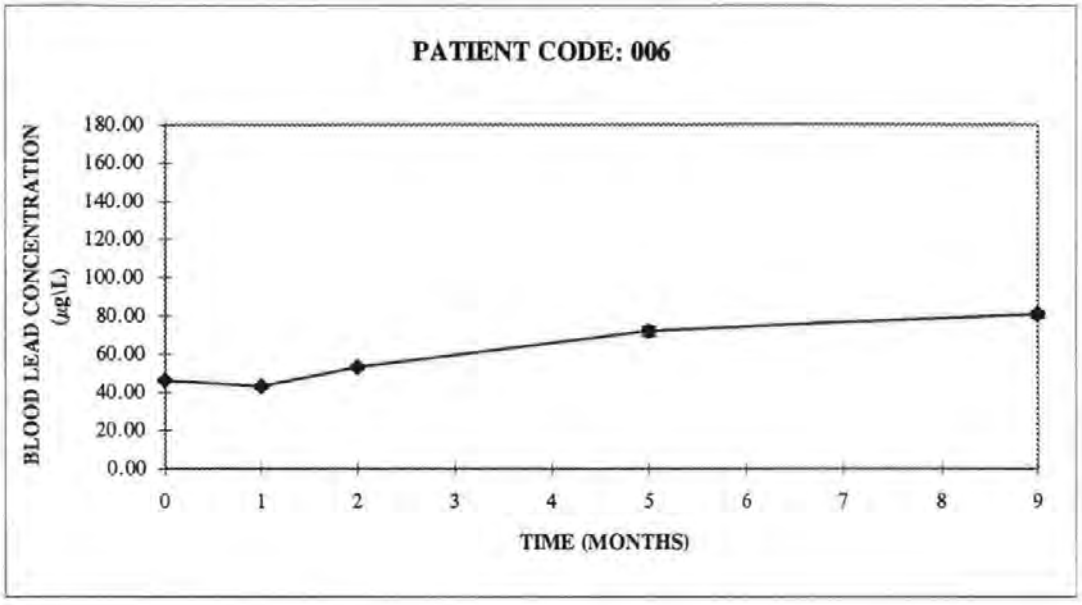


Table 5.11: Patients who showed an upward trend in blood lead concentration over time.

Patient Code	Sex	Age at start of trial	Bone Scan Rating	Primary Cancer	Treatment	Comments
006	Male	83	3	Prostate	Zoladex	Refer to Figure 5.5: High bone scan rating, indicating extensive bone metastases, but a lower blood [Pb] compared to age matched female counterpart.
007	Female	83	1	Breast	Arimadex B	Refer to Figure 5.5: High blood [Pb] compared to controls and age matched male counterpart, despite having a lower bone scan rating.

Figure 5.5: Preliminary trial results - patients who showed an upward trend in blood lead concentration over time.



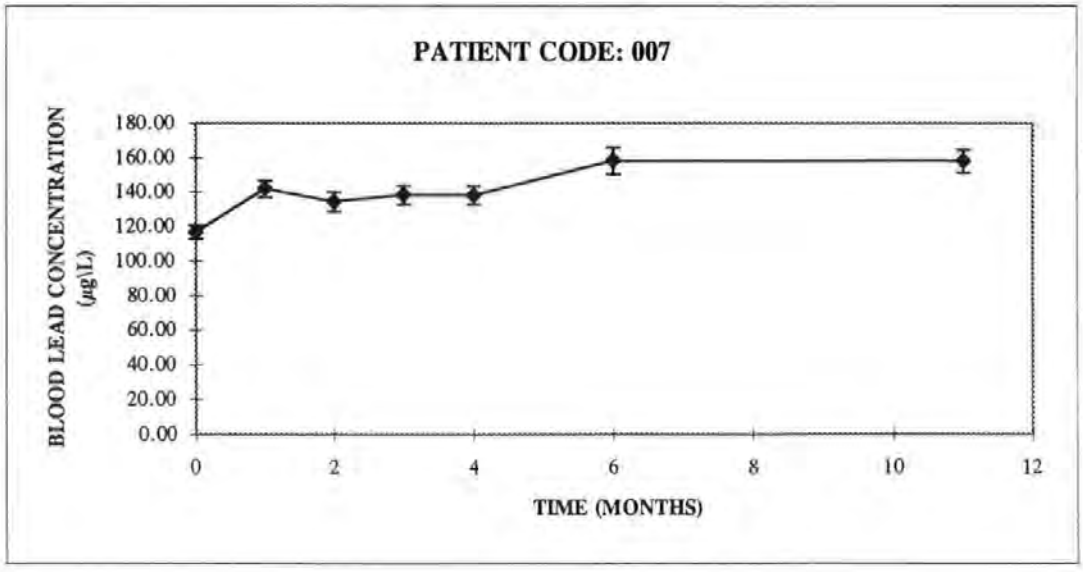


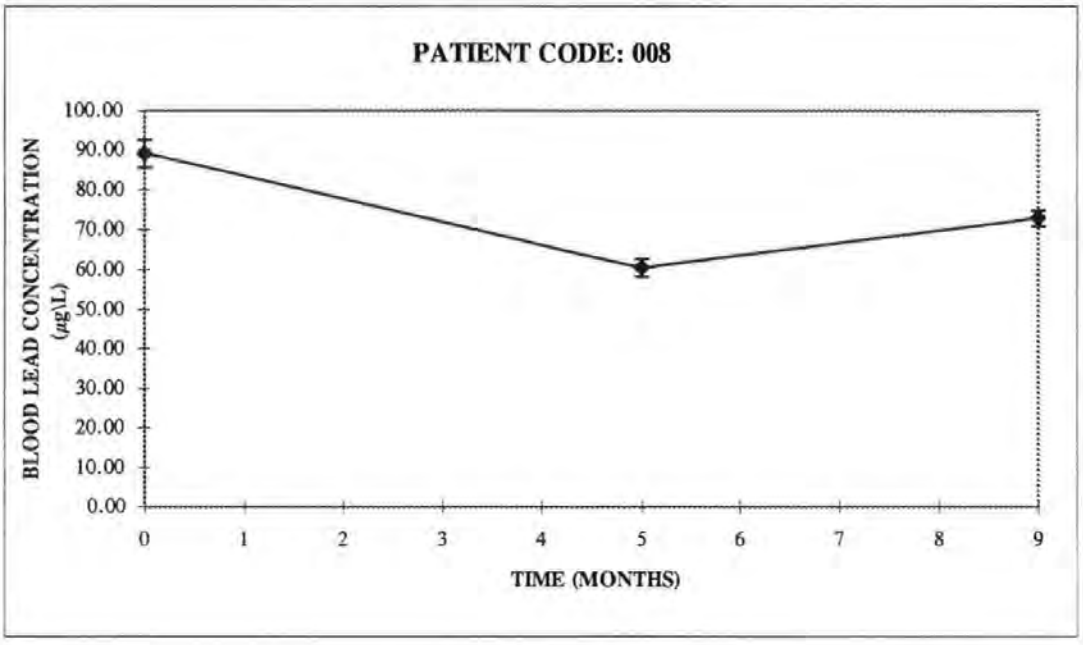
Table 5.12: Patients who showed no clear trends in blood lead concentration over time.

Patient Code	Sex	Age at start of trial	Bone Scan Rating	Primary Cancer	Treatment	Comments
008	Male	64	1	Prostate	Zoladex	Refer to Figure 5.6: Initial blood [Pb] greater than 80 $\mu g\ l^{-1}$
009	Female	70	1	Breast	Pamidronate	Refer to Figure 5.6: Initial blood [Pb] greater than 80 $\mu g\ l^{-1}$
010	Male	63	1	Prostate	Zoladex	Refer to Figure 5.7: Initial blood [Pb] between 40 - 80 $\mu g\ l^{-1}$
011	Female	69	2	Breast	Pamidronate	Refer to Figure 5.7: Initial blood [Pb] between 40 - 80 $\mu g\ l^{-1}$
012	Female	65	2	Breast	Pamidronate Pharmarubicin Mitomycin	Refer to Figure 5.7: Initial blood [Pb] between 40 - 80 $\mu g\ l^{-1}$
013	Female	60	2	Breast	Pamidronate	Refer to Figure 5.7: Initial blood [Pb] between 40 - 80 $\mu g\ l^{-1}$
014	Female	52	1	Breast	Pamidronate Pharmarubicin	Refer to Figure 5.7: Initial blood [Pb] between 40 - 80 $\mu g\ l^{-1}$

Table 5.12: Continued.

Patient Code	Sex	Age at start of trial	Bone Scan Rating	Primary Cancer	Treatment	Comments
015	Female	66	2	Breast	Pamidronate	Refer to Figure 5.8: Initial blood [Pb] below 40 $\mu g\ l^{-1}$
016	Female	65	2	Breast	Pamidronate Clodronate	Refer to Figure 5.8: Initial blood [Pb] below 40 $\mu g\ l^{-1}$
017	Female	64	2	Breast	Pamidronate Pharmarubicin Clodronate	Refer to Figure 5.8: Initial blood [Pb] below 40 $\mu g\ l^{-1}$
018	Female	58	2	Breast	Arimadex Pamidronate	Refer to Figure 5.8: Initial blood [Pb] below 40 $\mu g\ l^{-1}$

Figure 5.6: Preliminary trial results - patients who showed no clear trends in blood lead concentration over time, catogory A (initial blood [Pb] > 80 $\mu g\ l^{-1}$).



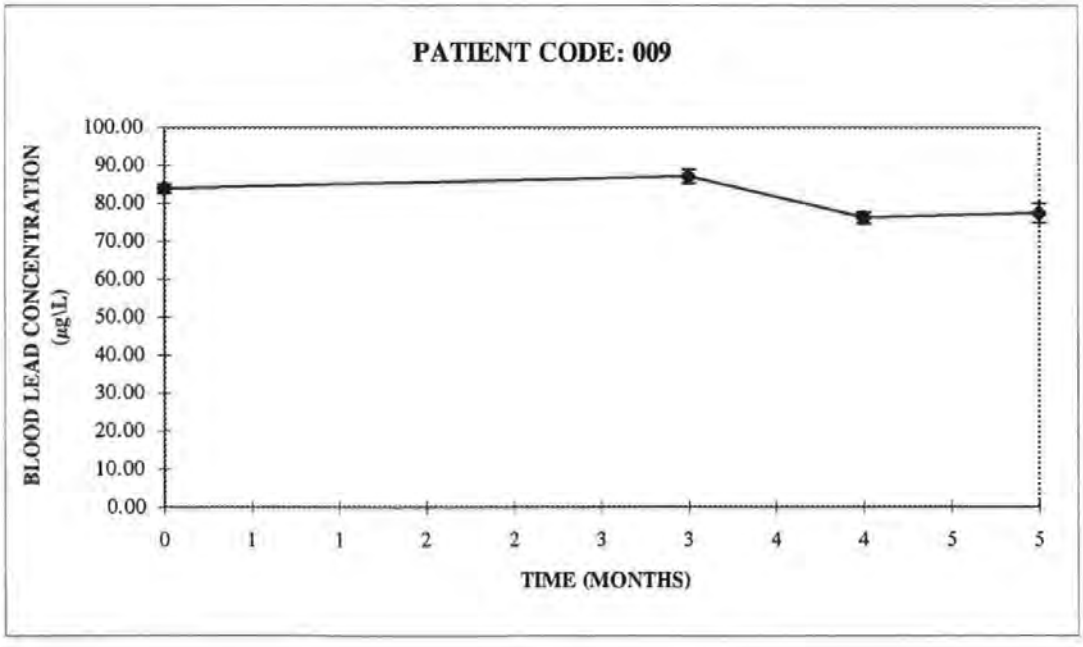
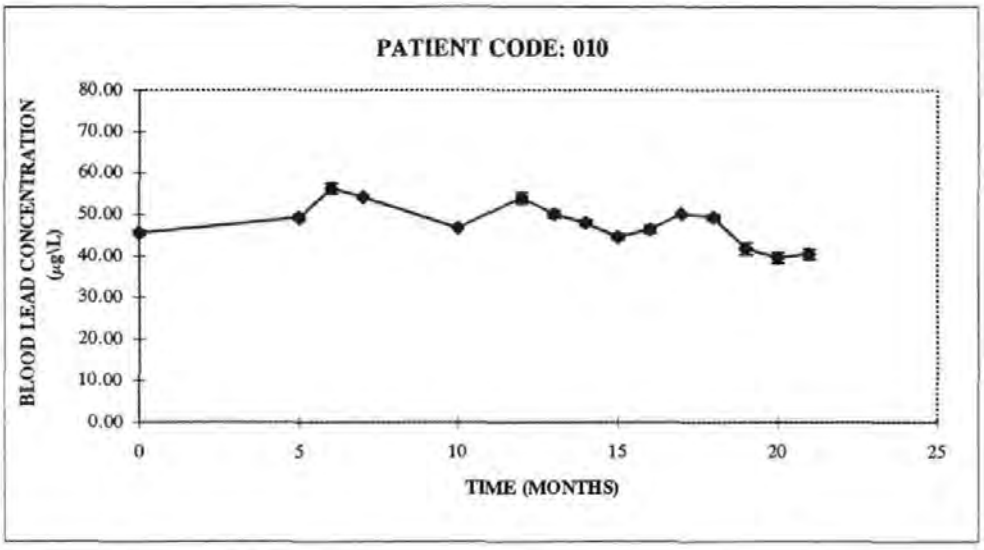
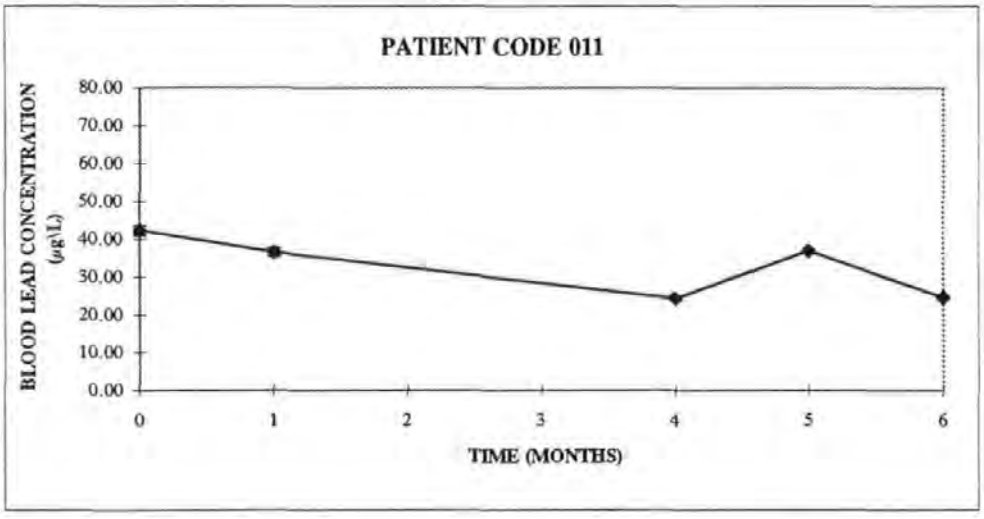


Figure 5.7: Preliminary trial results - patients who showed no clear trends in blood lead concentration over time, catogory B (initial blood [Pb] 40 - 80 $\mu g\ l^{-1}$).





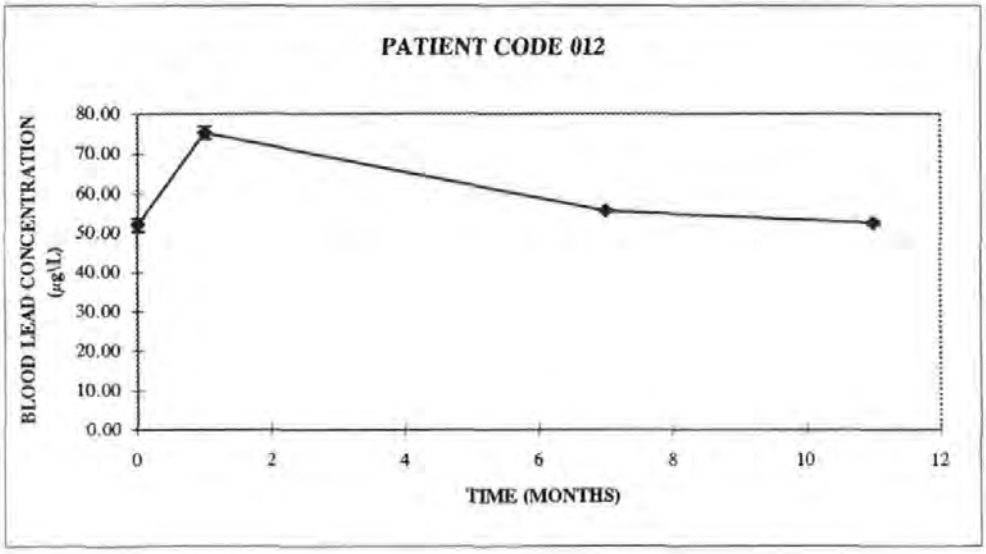
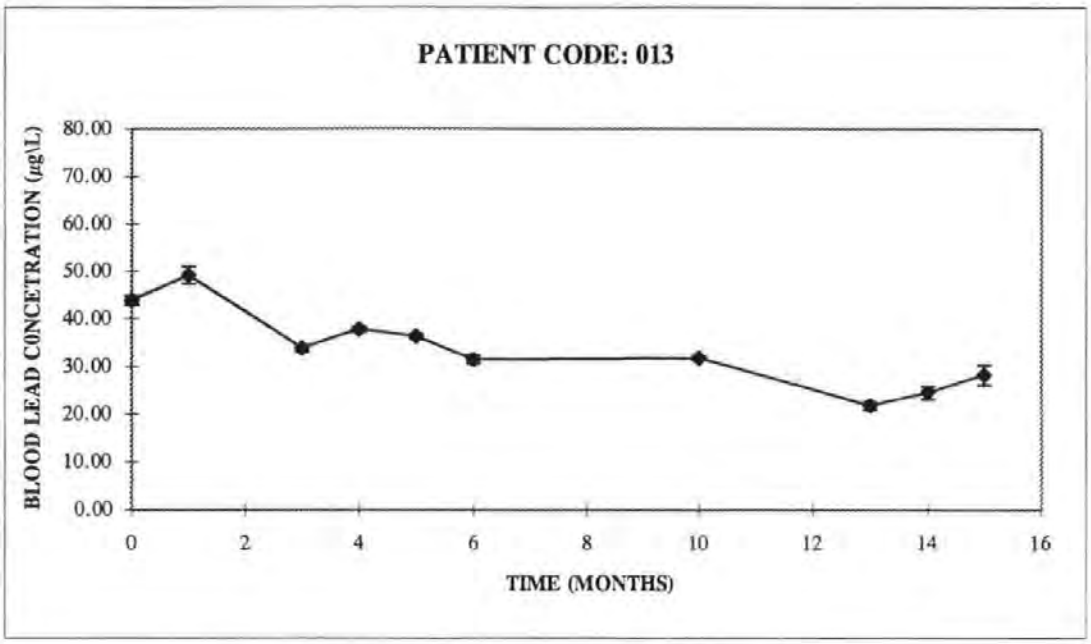


Figure 5.7: Continued.



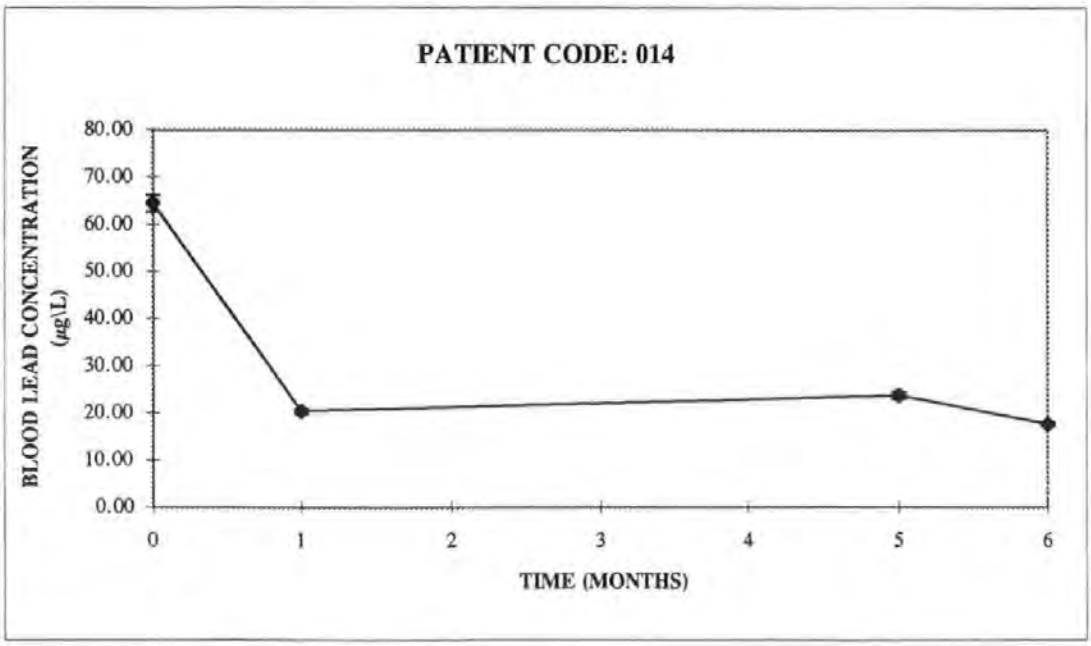
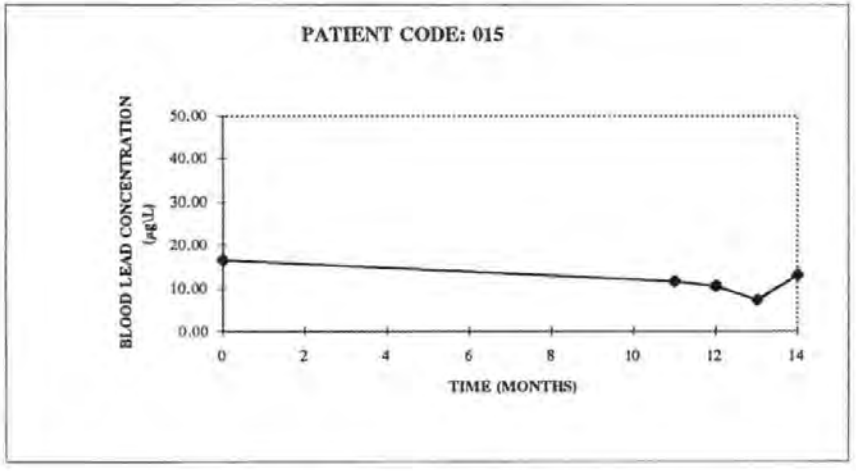
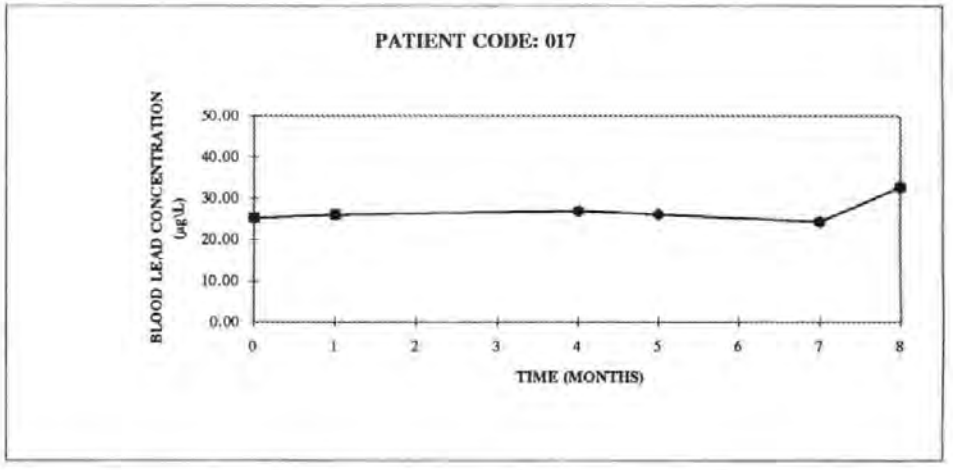
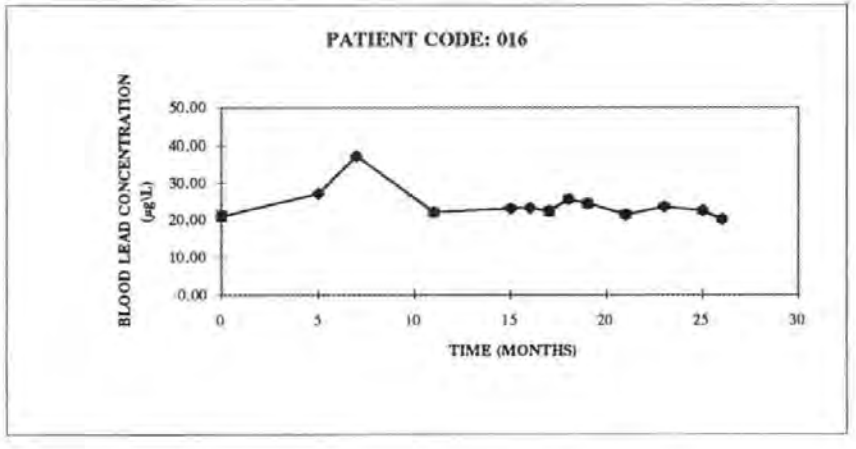
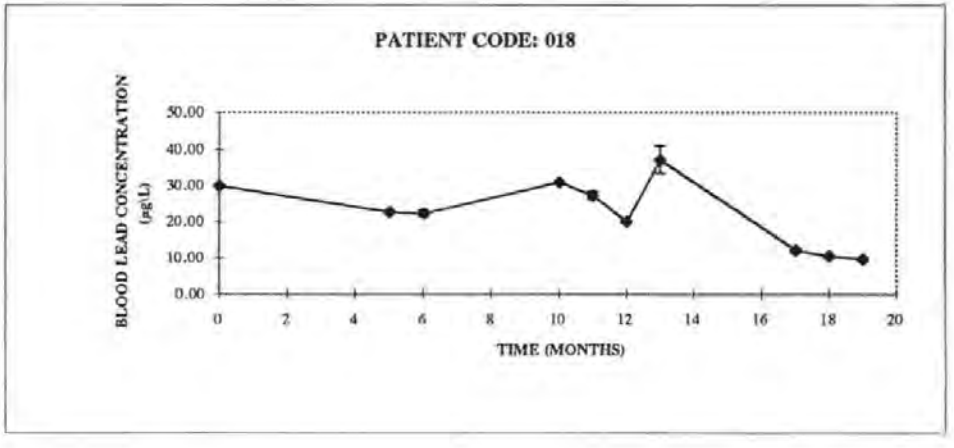


Figure 5.8: Preliminary trial results - patients who showed no clear trends in blood lead concentration over time, catogory C (initial blood [Pb] $< 40\ \mu g^{-1}$).









5.5: CONCLUSIONS.

An accurate and precise method for the determination of lead in whole blood by inductively coupled plasma-mass spectrometry has been developed. The method is simple and has been shown to remain under full control over time. The results from the preliminary trials were in general encouraging and demonstrated the ability of the method to measure and monitor changes in blood lead levels. It also demonstrated the individual variability and uncertainties encountered with respect to measuring blood lead levels. There are many factors which could be responsible for the blood lead levels observed which must be taken into consideration if correct and accurate conclusions are to be drawn.

These results indicate a fuller clinical trial is warranted, where parameters such as age, sex, primary diagnosis, treatment, bone scan rating etc should be assessed by full statistical analysis. Samples need to be collected in a more systematic and regular fashion, *i.e.* once a month, and more information concerning the patient's history and lifestyle should be collated, *i.e.* details concerning their nutritional habits, drinking and smoking habits, present and previous occupation(s), and place(s) of residence. All these factors could have a significant bearing on the blood lead levels measured.

CHAPTER 6

OTHER POTENTIAL TRACE METAL MARKERS FOR BONE METASTASES.

CHAPTER 6: OTHER POTENTIAL TRACE METAL MARKERS FOR BONE METASTASES.

6.1: INTRODUCTION.

The preliminary trial results (Chapter 5) showed that further investigations into the use of trace metals as biochemical markers to measure bone breakdown, via a fuller clinical trial was needed. It is known that bone contains many trace metals (309), and in order for this study to be more robust and potentially clinically useful, a number of elements, including lead should be studied in both blood and urine samples. The metabolic pathways for elements differ from one another, hence the analysis of urine samples taken at the same time would be useful in order to assess whether any trends and\or relationships between the blood and urine levels exist. Urine samples can be easier and more convenient to collect and are generally easier to analyse, due to their less viscous nature. However it is also recognised that the analysis of urine samples does introduce additional problems, such as greater concentration variations due to renal efficiency and dilution effects.

This chapter discusses studies undertaken to evaluate the selection of other elements to be incorporated along with lead in the clinical trials. Initially trace metal profiles were obtained by semi-quantitative analyses, with more accurate data collected in subsequent fully quantitative assessments, yielding a pool of potential elements. Whilst it was known that the developed method (Chapter 5) gave accurate and precise measurements for the blood lead concentrations, it was not clear whether or not the same methodology was suitable for the measurement of the other elements selected. Consequently the analytical

methodology was reviewed, and the collection, storage and analysis procedures employed investigated to ensure accurate and precise measurement of the selected elements was possible. Based on this data, the final selection of which elements to include in the subsequent clinical trials was made.

6.2: REAGENTS AND CHEMICALS.

All the water used to rinse laboratory ware and for the preparation of solutions, standards and samples was deionised using either a MilliQ purification system (Millipore, Bedford, MA, USA) or an Elgastat Maxima purification system (Elga Ltd, High Wycombe, Bucks., UK) at a resistivity of 18 MΩ. All laboratory ware was soaked in 10% v\v nitric acid and rinsed several times with Elga or MilliQ water.

Aristar nitric acid was obtained from Merck (Poole, Dorset, UK), and the Triton X-100 was obtained from Aldrich (Gillingham, Dorset, UK). Thallium nitrate standard reference material, NBS SRM 3158, (10,000 mg l^{-1}) was obtained from the National Institute of Standards and Technology (Washington DC, USA) and Spectrosol standard solutions or equivalent (1000 mg l^{-1}) for the various elements studied (see Section 6.3) were obtained from Merck and Aldrich respectively. All the standards were prepared, at appropriate dilutions from these solutions.

Seronorm whole blood and urine reference materials (Nycomed (UK), Birmingham, UK), certified reference materials: BCR 195 whole blood (Community Bureau of Reference, Brussels, Belgium) and NIST freeze dried urine 2670 (National Institute of Standards and Technology, Laboratory of the Government Chemist, Teddington, London), were used

to verify the analytical methodology. Pooled blood samples, used as internal quality control samples (refer to Section 5.4.1), were provided by Freedom Fields Hospital (Plymouth, Devon, UK).

6.3: SELECTION OF OTHER TRACE METALS.

6.3.1: Experimental.

In order to ascertain which other elements should be studied, those samples collected from the patients recruited for the preliminary trial, which had initial blood lead levels greater than 100 $\mu g\ l^{-1}$ were analysed and compared to the blood metal levels of the control samples from healthy volunteers (*i.e.* nursing staff). Initial investigations using ICP-MS in the semi-quantitative mode provided trace metal profiles, and indicative values for suitable elements which could then be selected for fully quantitative assessment.

To a clean plastic 100 ml volumetric flask, 0.1ml of a pre-prepared solution (containing 10 mg l^{-1} Be, Mg, Co, In, Pb, U) was pipetted and made up to the mark with diluent to give a 100 $\mu g\ l^{-1}$ standard solution for use in the semi quantitative study. A separate plastic flask was filled with diluent only, to serve as the blank. The blood samples were prepared as described in Section 5.3.2, where 1ml of blood was pipetted into 15 ml trace metal free plastic tubes and 9mls of diluent was added using the automatic dispenser. The diluent consisted of 0.1% v\v Triton X-100, 0.1% v\v Aristar nitric acid and 100 $\mu g\ l^{-1}$ thallium internal standard.

The elements selected following semi-quantitative analysis of the patient and control

blood samples, were subsequently analysed by ICP-MS in the fully quantitative mode. The procedures used were as described in Section 5.3.2.

6.3.2: Results and discussion.

All the elements with known spectral interferences [refer to Table 2.1] were not considered further, neither were elements whose concentrations were known to be above trace levels, such as Na, K, Mg, Ca, Fe (refer to Table 1.10), since the release of these elements into the blood would in comparison be very small and therefore negligible. Although the semi-quantitative experiments conducted during the preliminary trials, gave variable results for many elements, the trace metal profiles were carefully evaluated. The remaining elements which showed levels above 1 $\mu g\ l^{-1}$ and reasonable (>25%) percentage differences between the patient and control samples, were considered to offer the most potential. This data coupled with information from the literature (309) resulted in the following elements being selected for fully quantitative analysis:- Al, Ba, Cd, Ce, Cs, Pb, Rb, Sb, Sr and Zr, along with some rare earth elements (e.g La, Sm, Tb, Th, Tm, Y).

Following the fully quantitative analyses, elements were excluded if the concentrations were both too low (*i.e.* < 1 $\mu g\ l^{-1}$) or too high (*i.e.* >1000 $\mu g\ l^{-1}$), such as Rb. The remaining elements which showed consistently measurable levels (generally between 1-100 $\mu g\ l^{-1}$) were Al, Ba, Cd, Ce, Pb, Sr and Zr. From the literature, all these elements, with the exception of Ce and Zr, are known to be present in bone (309). Following the selection of these elements, further evaluation of the analytical methodology was required to ensure both accurate and precise measurement of the selected elements was possible.

6.4.: DETERMINATION AND CONTROL OF BLANK VALUES.

The blood collection tubes and the urine collection containers were assessed as to their suitability for all the selected elements (Al, Ba, Cd, Ce, Pb, Sr and Zr). An investigation was carried out in order to determine whether or not any contamination from the collection vessels occurred.

6.4.1: Experimental.

In order to assess any leaching\contamination from the sample collection vessels a "worse case scenario" was employed where the containers were studied using a test solution consisting of 2% v\v Aristar nitric acid and internal standards. Although the acidity of this solution was much greater than the actual samples, under these conditions any potential contamination sources could be identified.

Two types of blood collection tubes were evaluated: evacuated glass Vacutainer tubes coated with lithium heparin anticoagulant (B-D 6484, Beckton-Dickinson, East Rutherford, NJ, USA) and a plastic non-evacuated tube coated with lithium heparin anticoagulant (Teklab, [Medical Laboratories] Ltd., Sacriston, Durham, UK). Both these tubes were compared against the plastic trace metal free tubes (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK). Each tube was filled with 10 mls of the test solution (2% v\v Aristar nitric acid and 100 $\mu g\ l^{-1}$ thallium internal standard), stoppered, shaken thoroughly and left for a period of a)1 hour and b)1 week.

With respect to the urine collection procedure, 100mls of the test solution was poured into a 250ml plastic beaker, a portion was then decanted into a 30ml plastic amber screw

cap bottle (Merck, Poole, Dorset, UK), from which 10ml aliquots were pipetted into the plastic trace metal free tubes.

A 10mg l^{-1} stock solution containing Al, Ba, Cd, Ce, Pb, Sr and Zr in 5% v\v Aristar nitric acid was prepared from the respective Spectrosol or equivalent standards (1000 mg l^{-1}). This stock solution was used to freshly prepare a series of calibration standards by pipetting 0, 10, 50, 100, 250 and 500 μl into 100ml plastic volumetric flasks and making up to the mark with the diluent (2% v\v Aristar nitric acid and 100 μg l^{-1} thallium internal standard), to give 0, 1, 5, 10, 25 and 50 μg l^{-1} calibration standards.

6.4.2: Results and discussion.

Table 6.1 summarises the results obtained for the test solutions left for one week. The concentrations of all the selected elements in the plastic trace metal free tubes (Labsystems) were virtually the same as the blank concentrations *i.e.* <1 μg l^{-1}, except for aluminium. The high aluminium levels could be attributed to its ubiquitous nature (534,535) and the environmental condition in the laboratory. These results clearly showed that the trace metal free plastic tubes from Labsystems did not suffer from any contamination problem for the selected elements, and hence were suitable for trace metal analysis purposes.

A comparison of the plastic blood collection tubes containing lithium heparin anticoagulant (Teklab), with the trace metal free plastic tubes, showed an increase in the cadmium and barium levels and a slight increase in the strontium levels. The glass Vacutainer tubes, also showed increased levels of Ba and Sr compared to the trace metal

Table 6.1: Summary of blank study on blood collection tubes filled with test solution (2% v\v Aristar nitric acid and 100μg l^{-1} Tl internal standard) and left for 1 week.

Element	Blank concentration (μg l^{-1}) n = 3	Concentration in trace metal free plastic tubes, no Li Heparin (μg l^{-1}) (Labsystems) n = 5	Concentration in plastic tubes with Li Heparin (μg l^{-1}) (Teklab) n = 3	Concentration in glass Vacutainer tubes, with Li Heparin (μg l^{-1}) (Beckton-Dickinson) n = 10
Al^{27}	7.64 ± 0.21	9.97 ± 0.27	8.41 ± 0.37	61.42 ± 3.36
Sr^{88}	0.20 ± 0.04	0.18 ± 0.01	0.48 ± 0.02	1.63 ± 0.08
Zr^{90}	0.69 ± 0.06	0.58 ± 0.06	0.55 ± 0.06	8.02 ± 0.83
Cd^{111}	0.38 ± 0.05	0.35 ± 0.01	7.03 ± 0.88	0.36 ± 0.05
Ba^{138}	0.18 ± 0.06	0.15 ± 0.01	4.51 ± 0.44	70.38 ± 5.54
Ce^{140}	0.14 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	16.54 ± 0.91
Pb^{208}	0.21 ± 0.05	0.22 ± 0.07	0.24 ± 0.02	0.47 ± 0.08

free plastic tubes. These increased levels were greater than those seen for the Teklab plastic blood collection tubes, particularly for barium. The glass Vacutainer blood collection tubes also showed increased levels of aluminium, cerium and zirconium, but no increase in the cadmium levels.

These results suggest that both the lithium heparin and glass were responsible for significant increases in the barium levels and only a slight increase in the strontium levels, whereas the glass was also responsible for significant increases in the aluminium, cerium and zirconium levels. The increased levels of cadmium observed in the Teklab tubes could be attributed to the orange stoppers used. This observation was supported by comments in the company literature, and the replacement of these stoppers with white stoppers should overcome this problem.

The glass Vacutainer tubes clearly gave the worse results especially for aluminium, barium, cerium and zirconium and to a lesser extent strontium. Table 6.2 summarises the results obtained for tubes in the one hour soak test. The results again show an increase in the aluminium, barium, cerium and zirconium levels and a slight increase in the strontium levels. The increases are similar to those for tubes soaked for one week, indicating that significant increases in contamination occur after only a short period of time.

It is clear from these results that the collection of the blood samples using the glass Vacutainer tubes resulted in significant increases in the Al, Ba, Ce and Zr levels. The collection of blood samples therefore would be best in the Teklab plastic tubes with

Table 6.2: Summary of blank study on blood collection tubes filled with test solution (2% v\v Aristar nitric acid and 100μg l^{-1} Tl internal standard) and left for 1 hour.

Element	Blank conc. (μg l^{-1}) n = 3	Conc. in trace metal free plastic tubes, no Li Heparin (μg l^{-1}) (Labsystems) n = 5	Conc. in glass Vacutainer tubes, with Li Heparin (μg l^{-1}) (Beckton-Dickinson) n = 5
Al^{27}	10.07 ± 0.16	23.98 ± 2.65	65.79 ± 6.11
Sr^{88}	0.24 ± 0.01	0.18 ± 0.10	0.80 ± 0.10
Zr^{90}	0.32 ± 0.01	0.31 ± 0.01	2.80 ± 0.24
Cd^{111}	0.24 ± 0.01	0.22 ± 0.01	0.23 ± 0.01
Ba^{138}	0.44 ± 0.01	0.50 ± 0.04	43.34 ± 2.75
Ce^{140}	0.29 ± 0.01	0.30 ± 0.01	13.71 ± 0.85
Pb^{208}	0.11 ± 0.01	0.04 ± 0.01	0.18 ± 0.05

lithium heparin anticoagulant. However since these tubes are non-evacuated, a syringe and needle would be necessary in order to collect the blood samples, and this process may act as another source of contamination. Table 6.3 shows the results obtained in an experiment to evaluate this possibility. As can be seen the use of plastic syringes (Termo Europe NV, Leuven, Belgium) and needles (Beckton-Dickinson, Dublin, Ireland) did not result in contamination of the selected elements. Unfortunately due to the quantity of blood that must be taken for both the clinical trial and for the patients' normal routine medical tests, and the frailty of the patients, the use of a syringe and needle may cause the veins to collapse. The use of the evacuated Vacutainer tubes of course, do not suffer from this problem.

Due to this limitation, the next best available option was to collect the blood samples using the Vacutainer tubes, and then immediately decant the contents into the Teklab plastic tubes. Unfortunately significant contamination still occurred (Table 6.4). The samples were collected in the glass Vacutainer tubes, stoppered, shaken, and then decanted immediately into the plastic Teklab tubes (with white stoppers), and compared to values for solutions collected directly into the Teklab tubes. Significant increases in the Al, Ba and Ce levels was evident, as well as a slight increase in the strontium levels. The cadmium levels this time remained constant at a similar level to the blanks, confirming the original suspicion that the orange stoppers were the source of contamination.

A compromise between the clinical and analytical factors therefore had to be made. Many of the samples had already been collected and further blood collection was only possible

Table 6.3: Summary of blank study on test solution (2% v\v Aristar nitric acid, 100 $\mu g\ l^{-1}$ Tl internal standard), collected using plastic syringes and metal needles, then injected into plastic trace metal free tubes (Labsystems), {n=3}.

Element	[Blank] ($\mu g\ l^{-1}$)	[Control 1] ($\mu g\ l^{-1}$)	[Control 2] ($\mu g\ l^{-1}$)	[Sample] ($\mu g\ l^{-1}$)
Al^{27}	9.04 ± 0.25	6.16 ± 0.27	6.13 ± 0.09	6.74 ± 0.09
Sr^{88}	0.78 ± 0.01	0.44 ± 0.02	0.43 ± 0.01	0.44 ± 0.01
Zr^{90}	2.73 ± 0.14	2.11 ± 0.27	1.85 ± 0.02	1.82 ± 0.02
Cd^{111}	0.35 ± 0.05	0.29 ± 0.02	0.29 ± 0.04	0.30 ± 0.02
Ba^{138}	0.26 ± 0.02	0.15 ± 0.06	0.10 ± 0.01	0.12 ± 0.03
Ce^{140}	0.08 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Pb^{208}	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

Control 1: Trace metal free plastic tubes (Labsystems) filled with the test solution directly from the plastic volumetric flask (blank).

Control 2: The amber plastic bottles used in the collection of urine samples were filled with the test solution, and then 10 ml aliquots were pipetted into the trace metal free plastic tubes (Labsystems).

Sample: A plastic syringe and metal needle were used to draw up 10 mls of the test solution from the amber plastic bottles. The contents were then injected into the trace metal free tubes (Labsystems).

Table 6.4: Summary of the blank study for test solution collected initially in the glass Vacutainer tubes, and then immediately decanted into the plastic tubes (Teklab).

Element	Concentration in trace metal free plastic tubes, no Li Heparin ($\mu g\ l^{-1}$) (Labsystems) n = 3	Concentration in plastic tubes with Li Heparin ($\mu g\ l^{-1}$) (Teklab) n = 3	Concentration after transfer form glass Vacutainer tubes, to plastic Teklab tubes ($\mu g\ l^{-1}$) n = 3
Al^{27}	4.85 ± 0.58	3.36 ± 0.19	23.11 ± 3.42
Sr^{88}	0.31 ± 0.01	0.48 ± 0.02	1.16 ± 0.11
Zr^{90}	3.07 ± 0.50	2.64 ± 0.11	3.10 ± 0.08
Cd^{111}	0.05 ± 0.01	0.05 ± 0.03	0.04 ± 0.02
Ba^{138}	0.09 ± 0.03	2.34 ± 0.11	26.02 ± 3.07
Ce^{140}	0.04 ± 0.01	0.05 ± 0.02	7.81 ± 1.15
Pb^{208}	0.11 ± 0.01	0.15 ± 0.03	0.25 ± 0.08

using the glass Vacutainer tubes. Consequently aluminium, barium, cerium and zirconium were dropped from the clinical trials. Aluminium and zirconium were also prone to high and variable blank levels, and since the clean up of the water supply appeared to make little difference, this contamination was attributed to the laboratory environment and ICP-MS instrument respectively. The collection of blood samples with the glass Vacutainers resulted in no contamination in the levels of cadmium and lead and only a very slight increase in the strontium levels.

The blank studies conducted on the urine collection containers, summarised in Table 6.5, showed that no contamination occurred. Thus the integrity of the urine samples was not compromised during the collection of the samples by the given methodology.

6.5: AN INVESTIGATION INTO THE STORAGE AND PRETREATMENT OF URINE SAMPLES.

Biological samples are prone to extraneous contamination, while urine, which is supersaturated at room temperature, has the further problem of precipitation following collection. The exact nature of the precipitate is unclear, although it is probably a calcium-base precipitate, such as calcium phosphate (536). This precipitation is intensified during sample storage at low temperatures, and any analyte within this precipitate will not be fully detected, since ICP-MS requires the sample to be in an homogenous form. The precipitate formed is not easily redissolved by dilution and can therefore significantly reduce the concentration of certain cations in solution. Therefore it is necessary to ascertain whether or not such precipitation and any pretreatment of the urine samples affected the measurement of the cadmium, lead and strontium levels.

Table 6.5: Summary of the blank studies on the urine collection procedure.

Element	Blank concentration ($\mu g\ l^{-1}$)	Concentration in trace metal free tubes following urine collection procedure ($\mu g\ l^{-1}$)
Al^{27}	3.52 ± 0.05	3.46 ± 0.18
Sr^{88}	0.58 ± 0.05	0.30 ± 0.01
Zr^{90}	3.70 ± 0.53	2.50 ± 0.05
Cd^{111}	0.09 ± 0.02	0.03 ± 0.01
Ba^{138}	0.18 ± 0.02	0.12 ± 0.03
Ce^{140}	0.07 ± 0.02	0.04 ± 0.01
Pb^{208}	0.23 ± 0.03	0.1 ± 0.01

6.5.1: Experimental.

A recent study (536) suggested that if the urine sample was diluted with nitric acid, warmed to 40°C and then re-equilibrated to room temperature, all the elements would be maintained in a soluble form as required for accurate analysis. Based on this study the following experiments were conducted. The urine samples were collected in 250 ml plastic beakers, and then immediately decanted into 30 ml amber plastic screw cap bottles (Merck, Poole, Dorset, U.K). From these, 10 ml aliquots were pipetted into 15ml trace metal free plastic tubes (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK). These tubes were stored at room temperature, 4°C and -20°C. At each temperature, one tube was stored with 0.1% v\v Aristar nitric acid added, and one tube stored with no acid added. All the samples were left overnight and then re-equilibrated to room temperature and the amount of precipitate assessed. The samples were analysed following a simple dilution method (404), where the samples were shaken, 1ml of urine pipetted into 15ml trace metal free tubes and 9mls of diluent added using an automatic dispenser (Optifix, Merck, Poole, Dorset, UK). The diluent consisted of 2% v\v Aristar nitric acid and 100 $\mu g\ l^{-1}$ thallium internal standard. Each sample was analysed by ICP-MS and a comparison made between samples heated to 40°C and those without heating to see if the metal concentrations differed. Calibration solutions were prepared as described in Section 6.4.1.

6.5.2: Results and discussion.

The amount of precipitate formed at -20°C was found to be greater than that formed at 4°C, which in turn was greater than that compared to the samples stored at room temperature. In all cases the addition of acid appeared to increase the relative amounts

of precipitate. However the measured metal concentrations appeared to be unaffected by the addition of acid (Table 6.6). The results also showed that very little difference was observed between samples heated to 40°C and left to re-equilibrate prior to analysis, compared to those samples analysed directly. Therefore, with respect to the analysis of strontium, cadmium and lead, the addition of 0.1% v\v nitric acid did not cause major problems, and maintained a stable pH environment for the cations. In addition the urine samples could be analysed after simple dilution, without prior heating.

6.6: INTERFERENCE STUDY.

Biological samples are known to be prone to a number of non-spectral interferences (refer to Section 2.1.7). Initially a series of experiments were conducted to evaluate whether or not such interferences were present, by comparing the slopes of calibration graphs obtained using both aqueous standards and matrix matched standards. This was followed by a study of the effects of increasing the concentration of the main potential interfering ions with respect to the ratio of the analyte and internal standard signal, to ascertain whether or not internal standardisation satisfactorily corrected for any enhancement or suppression of the analyte signal.

6.6.1: Experimental.

Aqueous calibration solutions were prepared by pipetting 0, 10, 50, 100, 250 and 500 μl of a 10mg l^{-1} stock solution (Cd, Pb and Sr) into 100ml plastic volumetric flasks and made up to the mark with diluent to give 0, 1, 5, 10, 25 and 50 μg l^{-1} calibration standards. The diluent consisted of 2% v\v Aristar nitric acid containing 100 μg l^{-1} thallium internal standard for the urine analyses, and a solution of 0.1% v\v Triton X-

Table 6.6: Summary of the investigation into the storage and pretreatment of urine samples.

Element	Storage condition	Addition of 0.1% v\v Aristar nitric acid	Concentration with no heating to 40°C prior to analysis ($\mu g\ l^{-1}$)	Concentration with heating to 40°C prior to analysis ($\mu g\ l^{-1}$)
Sr^{88}	Room Temp.	Yes	14.62 ± 0.29	15.86 ± 0.15
	Room Temp.	No	12.66 ± 0.41	16.17 ± 0.67
	4°C	Yes	15.18 ± 0.31	15.45 ± 0.19
	4°C	No	14.73 ± 0.19	14.95 ± 0.77
	-20°C	Yes	15.36 ± 0.46	14.82 ± 0.20
	-20°C	No	15.44 ± 0.62	14.45 ± 0.19
Cd^{111}	Room Temp.	Yes	0.36 ± 0.04	0.55 ± 0.07
	Room Temp.	No	0.31 ± 0.05	0.54 ± 0.05
	4°C	Yes	0.39 ± 0.07	0.47 ± 0.07
	4°C	No	0.44 ± 0.01	0.53 ± 0.18
	-20°C	Yes	0.57 ± 0.07	0.42 ± 0.04
	-20°C	No	0.51 ± 0.11	0.46 ± 0.02
Pb^{208}	Room Temp.	Yes	0.32 ± 0.05	0.20 ± 0.01
	Room Temp.	No	0.41 ± 0.03	0.31 ± 0.08
	4°C	Yes	0.30 ± 0.04	0.25 ± 0.07
	4°C	No	0.75 ± 0.07	0.24 ± 0.04
	-20°C	Yes	0.18 ± 0.02	0.32 ± 0.05
	-20°C	No	0.17 ± 0.01	0.23 ± 0.05

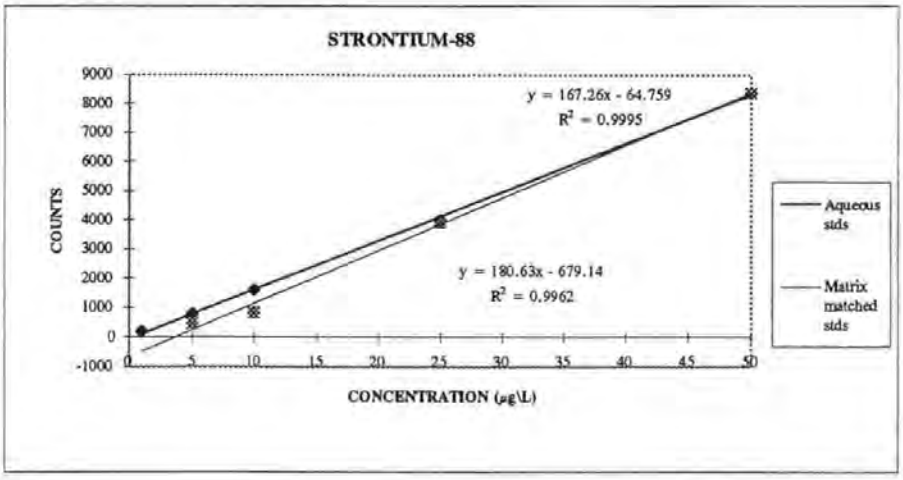
100, containing 0.1% v\v Aristar nitric acid and 100 $\mu g\ l^{-1}$ thallium internal standard for the blood analyses. The matrix matched standards were prepared in a similar fashion using a 1 in 10 dilution of blood or urine. The appropriate amount of the 10mg l^{-1} stock solution was pipetted into 30 ml plastic trace metal free tubes (Merck, Poole, Dorset, UK), along with 2 mls of blood or urine and 18 mls of the respective diluent as described above, to give a series of calibration solutions (0 - 50 $\mu g\ l^{-1}$).

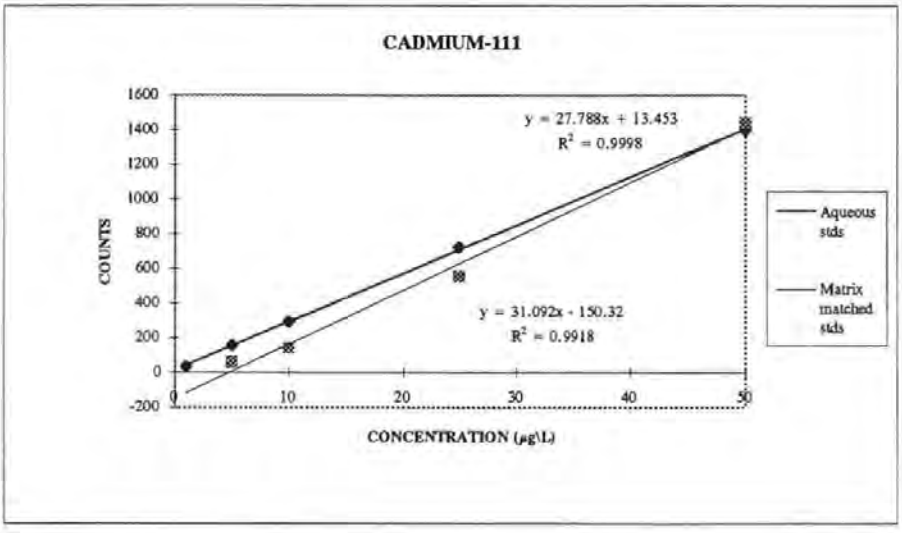
For the second interference study, a series of solutions were prepared in plastic flasks containing between 0 - 10 g l^{-1} of KNO_3, $NaNO_3$, $Mg(NO_3)_2$, $Ca(CO_3)_2$, HCl, H_2SO_4, and H_3PO_4, in 2% v\v Aristar nitric acid. Each flask also contained 10 $\mu g\ l^{-1}$ Cd, Pb and Sr (analyte ions) and 100 $\mu g\ l^{-1}$ of Cs, Y, In and Tl (internal standards). ICP-MS in the fully quantitative mode was used to collect the raw data (*i.e.* counts), to study the effects on the ratio of the analyte to internal standard signal ratio, as the concentration of the potential interfering ions (*i.e.* K^+, Na^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-} and PO_4^{3-}) increased.

6.6.2: Results and discussion.

Figures 6.1 and 6.2 show the calibration graphs for aqueous versus matrix matched standards for blood and urine samples respectively for each of the three analytes - strontium, cadmium and lead. These graphs are linear within the sample concentration range tested. The slopes for Cd and Sr in blood were similar, with only a slight suppression of signal at the lower concentration ranges compared to that of the aqueous standards. Lead in blood showed greater variation but the sensitivities of the two types of calibration standards were still close to one another, indicating only minor matrix effects. The slopes of the graphs for the urine samples clearly showed greater matrix

Figure 6.1: Calibration graphs comparing aqueous standards with matrix matched standards for blood analyses.





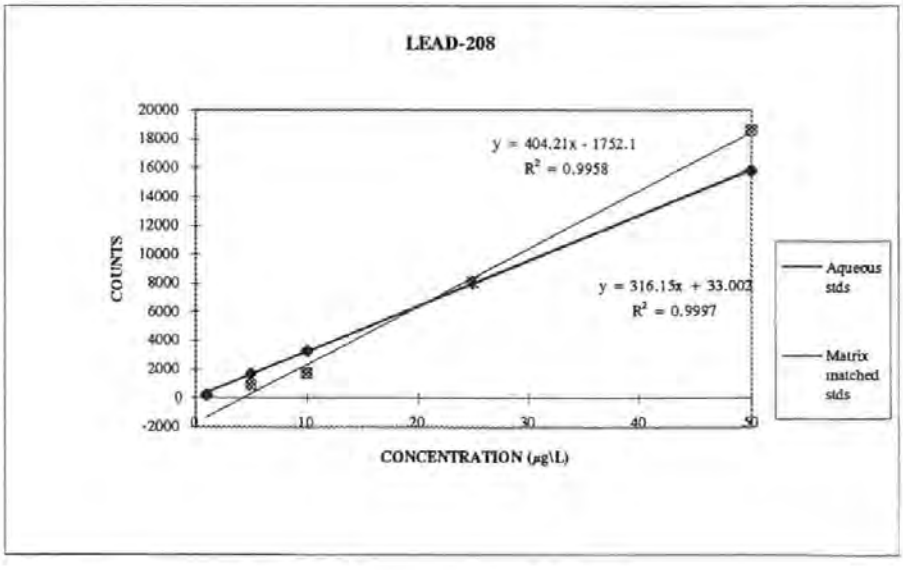
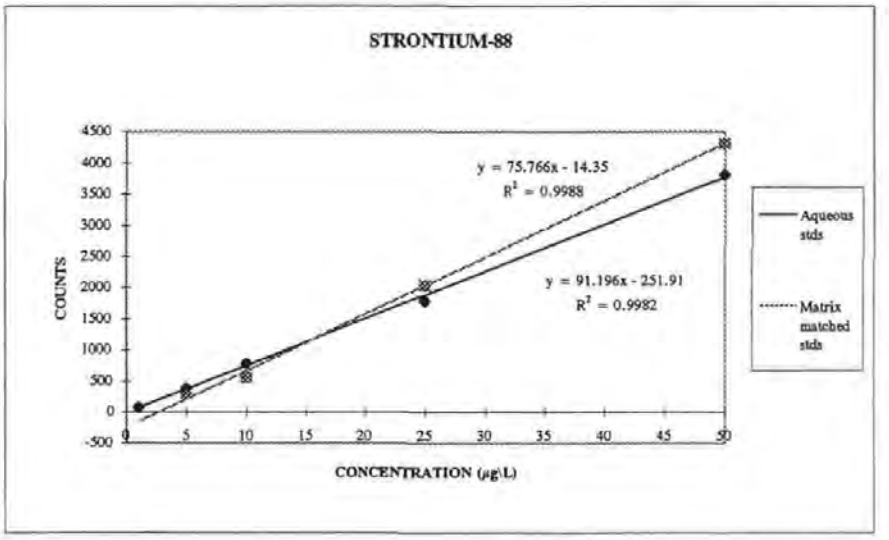
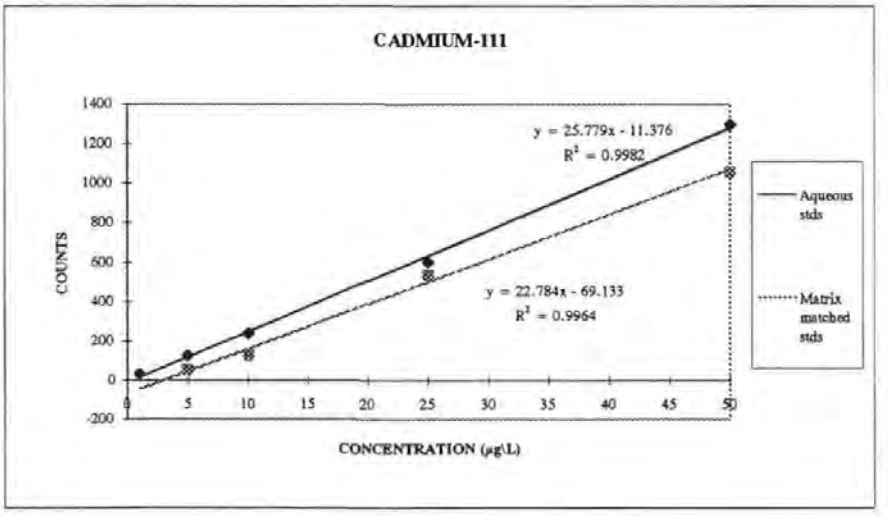
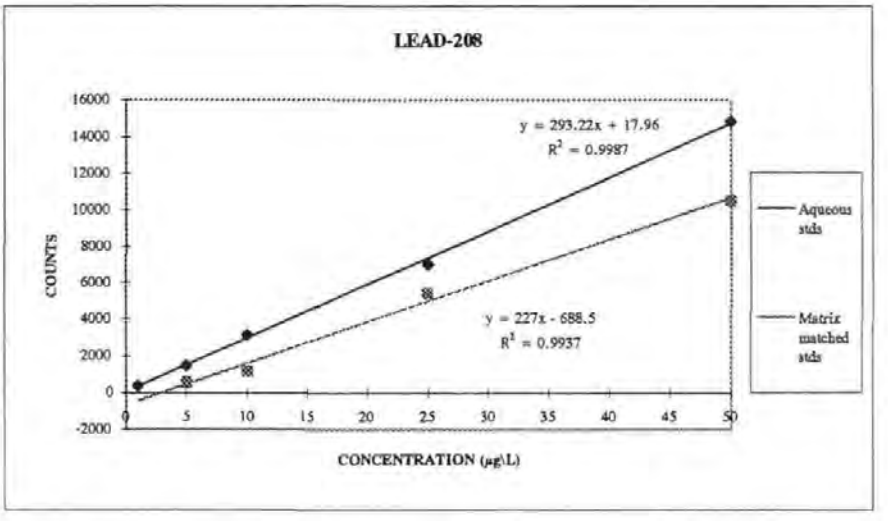


Figure 6.2: Calibration graphs comparing aqueous standards with matrix matched standards for urine analyses.







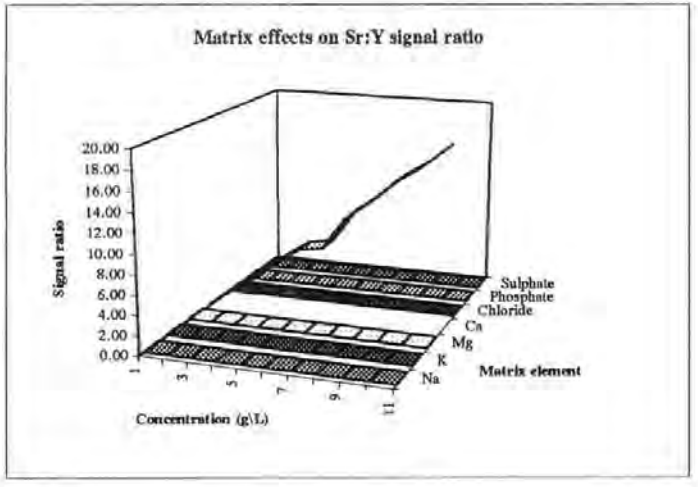
effects compared to the blood samples, particularly at the higher concentration range. These effects became more pronounced as the analyte mass increased. These results indicated that the use of aqueous standards would probably suffice for the blood analyses, however their use in the urine analyses may be problematic.

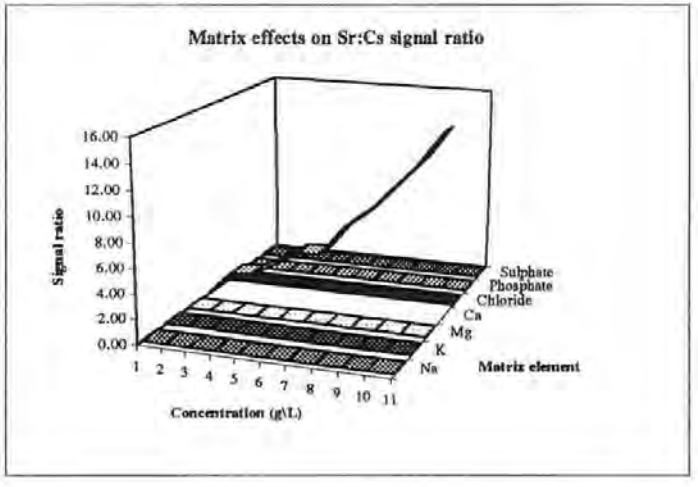
Figure 6.3 depicts the effects of individual potentially interfering ions on the ratio between strontium and the various internal standards. These graphs clearly show that all of the internal standards evaluated, satisfactorily correct for any changes in the analytical signal, except in the presence of calcium. However using a 1:10 dilution, the concentration level of Ca in the sample would be approximately 1 gl^{-1} and therefore, based on these results would only have minimal consequences on the strontium: internal standard ratio.

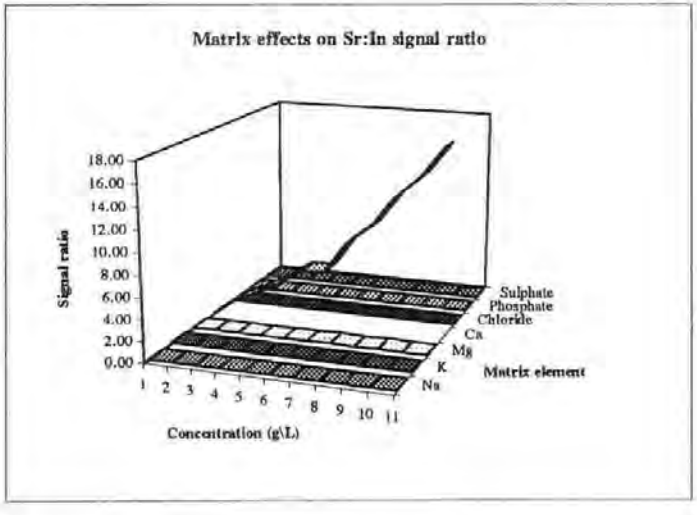
Figure 6.4 shows that all the internal standards evaluated satisfactorily corrected for any analytical signal changes for cadmium. However, as expected the internal standards, In and Cs, with masses closest to the analyte mass, demonstrated marginally more stable ratios compared to Y and Tl. Similarly Figure 6.5 clearly shows that thallium was the best internal standard for the lead measurements.

It was noted that throughout these experiments the stability of the In internal standard in the blood diluent solution was responsible for inconsistent results, with the indium counts varying throughout a run, whereas the other internal standards gave more consistent readings. This observation was probably due to a pH effect, with the In requiring a slightly more acidic environment. However the addition of more acid would result in

Figure 6.3: Graphical presentation of the matrix effects of potential interfering ions on the Sr:Internal standard ratio.







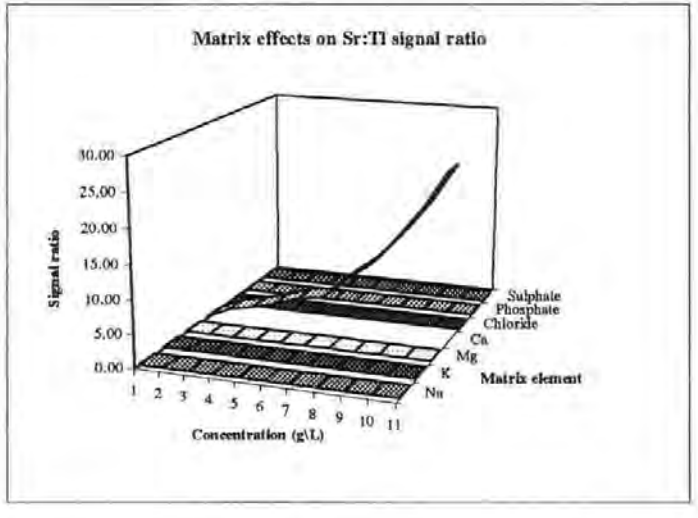


Figure 6.4: Graphical presentation of the matrix effects of potential interfering ions on the Cd:Internal standard ratio.

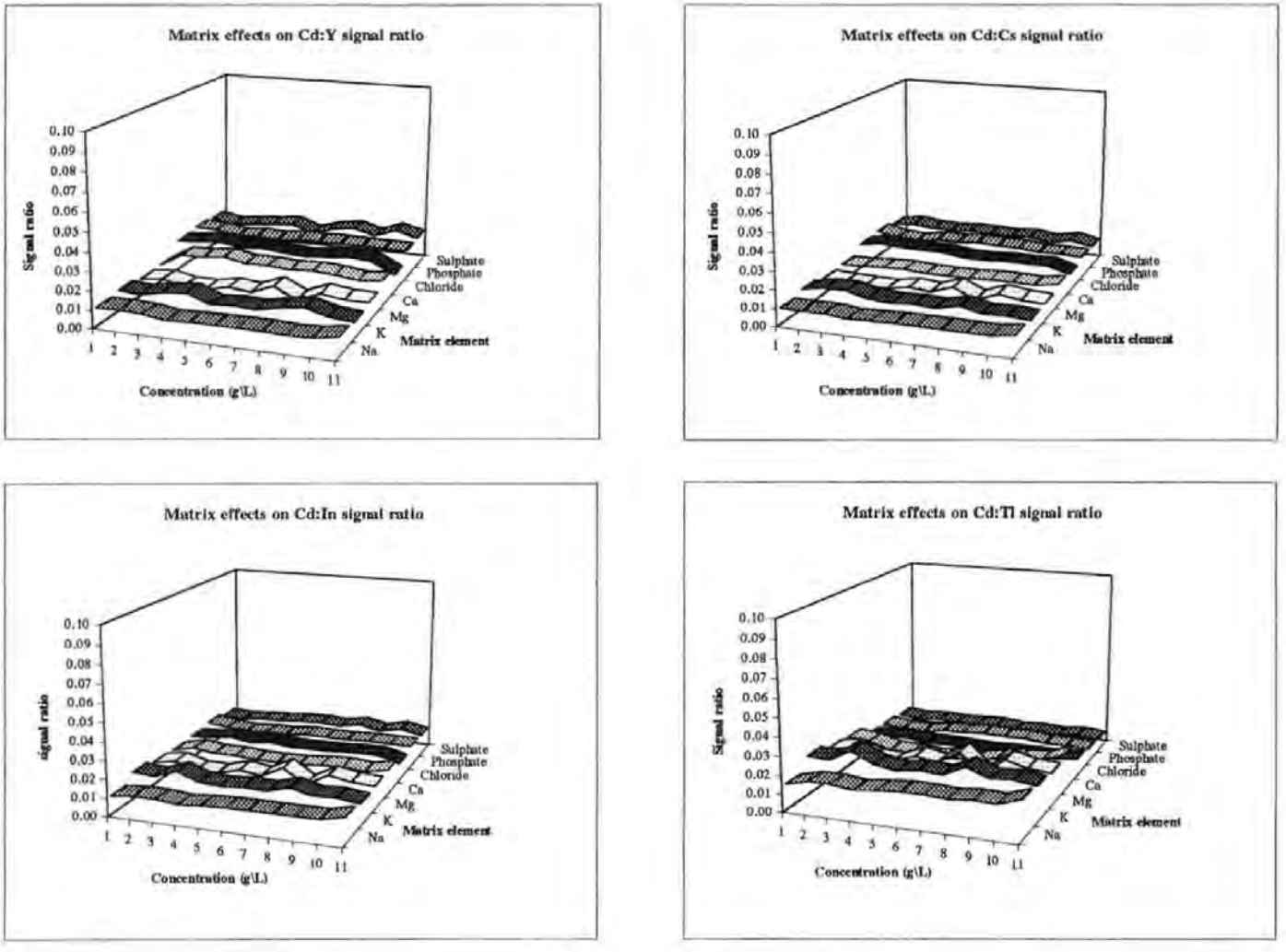
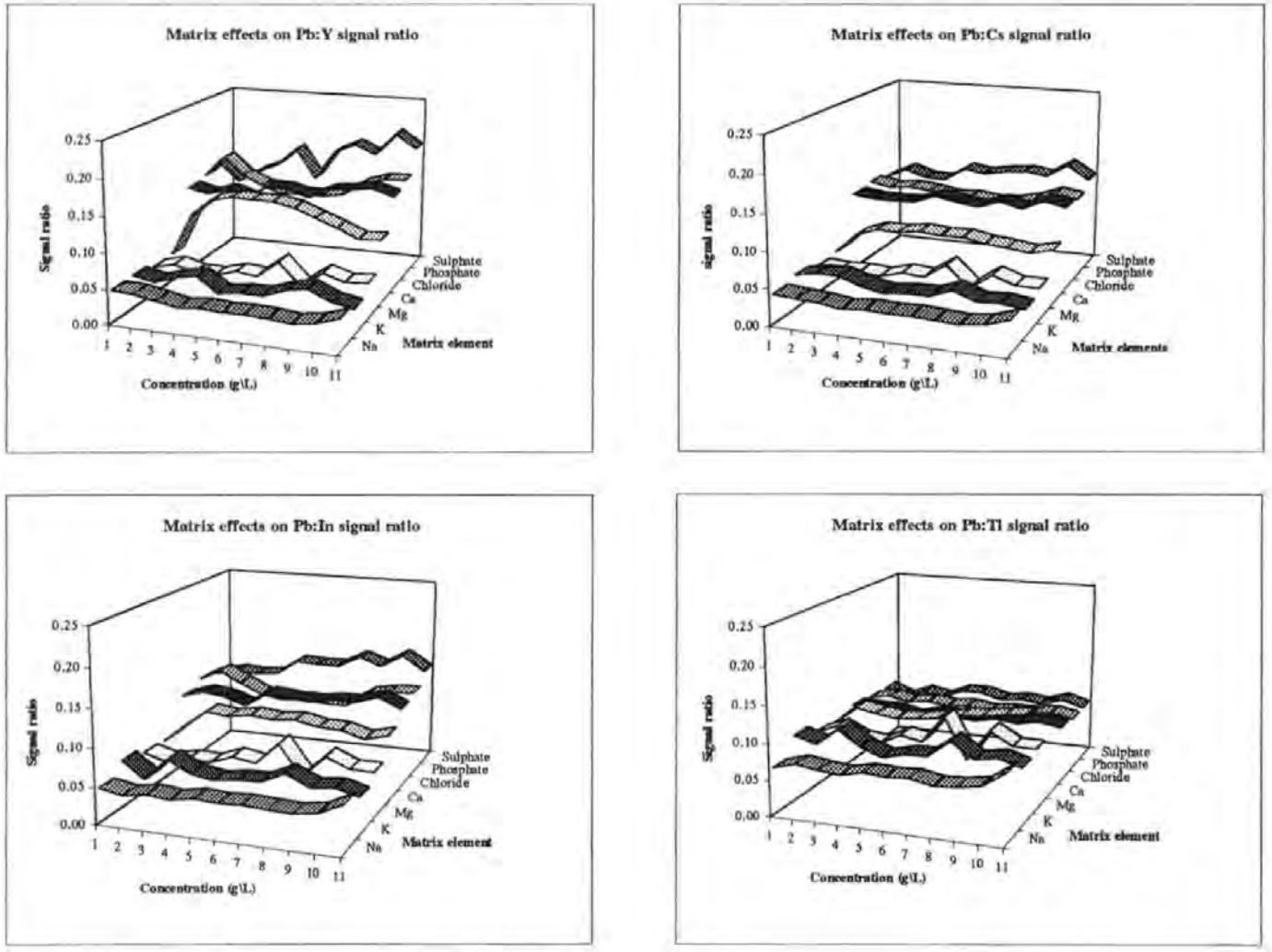


Figure 6.5: Graphical presentation of the matrix effects of potential interfering ions on the Pb:Internal standard ratio.



more rapid deprotonisation of the blood samples, which would in turn lead to clogging of the nebuliser. Consequently Cs was used as the internal standard instead of indium in the blood analyses.

6.7: ANALYTICAL FIGURES OF MERIT.

The experiments into the collection and storage of the samples, the methods and procedures employed, plus the equipment used, demonstrated that no major contamination of Sr, Cd and Pb occurred. Verification of the accuracy and precision of the analytical methodology for these analytes was achieved by use of spiking experiments and use of reference\certified reference materials.

6.7.1: Experimental.

The blood samples (pooled blood samples (IQC's), or Seronorm samples) were diluted 1 in 10 with a diluent consisting of 0.1% v\v Triton X-100, 0.1 %v\v Aristar nitric acid and 100 $\mu g\ l^{-1}$ internal standard (Y, Cs and Tl). The urine samples (Seronorm or pooled sample) were diluted 1 in 10 with a diluent consisting of 2% v\v Aristar nitric acid containing 100 $\mu g\ l^{-1}$ internal standard (Y, In and Tl).

Calibration was achieved using standards (0-50 $\mu g\ l^{-1}$) made up to the mark with the appropriate diluent in plastic volumetric flasks. The % recovery tests were conducted by spiking the samples at three different concentration levels (10, 20 and 30 $\mu g\ l^{-1}$), with a standard stock solution (10 mg l^{-1} Sr, Cd and Pb). Seronorm whole blood and urine reference materials were initially used to assess the accuracy and precision of the methodology for all three elements. Further verification for both lead and cadmium was

achieved by analysing certified reference materials: BCR 195, lead and cadmium in whole blood, and NIST SRM 2670 freeze dried urine. All the reference\certified reference materials were supplied in a freeze dried form, which were stored at 4°C, (as directed), and reconstituted as instructed using MilliQ or Elga water.

6.7.2: Results and discussion.

The limits of detection (3σ) for all three selected elements in the respective diluents used for both blood and urine analyses were less than 1 $\mu g\ l^{-1}$ (Table 6.7). In general the percentage recovery results for spiked pooled blood samples for the selected elements was very good (Table 6.8), although the % RSD's were slightly high for both Sr and Cd. Table 6.9 shows the results attained for Seronorm whole blood reference material. Although a reference value is not available for Sr the results indicate reasonable reproducibility at all three concentration levels. High cadmium values for level 1 (low) could be attributed to the reference value being within the determinable limit. The Cd values for level 2 were within range and only slightly high at level 3. The lead levels were well within specification for level 1 but consistently high at both levels 2 and 3. This could be attributed to the enhancement of signal due to the blood matrix at these higher concentration as depicted in Figure 6.2, and in the case of level 3 compounded by the fact that this value was outside the tested calibration range. However the analysis of certified reference materials BCR 194 (Chapter 5) and BCR 195 showed that the results for lead at both levels were within the certified range, although the cadmium value was again slightly high, but still within range (Table 6.10).

The urine samples gave excellent percentage recovery results for all the elements with

Table 6.7: Limit of detection (LOD) results for the three selected elements (n=11)

Element	LOD (3σ) for Blood Analysis [a] (μg l^{-1})	LOD (3σ) for Urine Analysis[b] (μg l^{-1})
Sr^{88}	0.15	0.27
Cd^{111}	0.24	0.36
Pb^{208}	0.06	0.06

[a] Repeated measurements on blank solution consisting of 0.1% v\v Triton X-100, 0.1% v\v Aristar nitric acid and 100 μg l^{-1} Y, Cs and Tl internal standards.

[b] Repeated measurements on blank solution consisting of 2% v\v Aristar nitric acid and 100 μg l^{-1} Y, In and Tl internal standards.

Table 6.8: Percentage recovery results for pooled blood samples (n=9)

Element	10 μg l^{-1} spike average (%RSD)	20 μg l^{-1} spike average (%RSD)	30 μg l^{-1} spike average (%RSD)
Sr^{88}	109.7 (11.8)	113.8 (9.3)	111.2 (10.1)
Cd^{111}	98.2 (11.6)	99.2 (12.2)	102.2 (11.9)
Pb^{208}	106.0 (6.6)	107.8 (5.9)	109.7 (4.6)

Table 6.9: Results for Seronorm whole blood reference material (n=10)

Seronorm sample		Reference Range ($\mu g\ l^{-1}$)	Experimental Result ($\mu g\ l^{-1}$) Average ± s.d
Level 1	Sr^{88}	-	70.6 ± 2.5
	Cd^{111}	0.8 - 1.0	2.33 ± 1.2
	Pb^{208}	31 - 41	32.9 ± 0.7
Level 2	Sr^{88}	-	17.0 ± 3.5
	Cd^{111}	5.9 - 6.8	6.7 ± 0.9
	Pb^{208}	361 - 396	416.3 ± 6.0
Level 3	Sr^{88}	-	75.0 ± 4.8
	Cd^{111}	11.2 - 12.5	12.9 ± 0.9
	Pb^{208}	626 - 702*	707.5 ± 7.4

* Outside calibration range

Table 6.10: Results for BCR certified reference material for blood (n=6)

CRM		Certified Value ($\mu g\ l^{-1}$)	Experimental Result ($\mu g\ l^{-1}$) Average ± s.d
BCR 194	Pb	126 ± 4	128.2 ± 1.3
BCR 195	Cd	5.37 ± 0.24	6.92 ± 1.60
	Pb	416 ± 9	422.8 ± 2.8

The urine samples gave excellent percentage recovery results for all the elements with good RSD's (Table 6.11). Similarly no reference value is available for strontium in Seronorm urine reference material, however repeated measurements gave reproducible results. The cadmium level was again slightly high, whilst lead agreed very closely with the reference value (Table 6.12). The analysis of a certified reference material NIST 2670, (Table 6.13) gave values in the correct area at the low level where only a guideline value was indicated. However both cadmium and lead were in good agreement with the certified value at the elevated level.

6.8: SUMMARY.

The semi-quantitative and fully quantitative modes of ICP-MS analysis provided the trace metal profiles from which a number of potential elements to measure alongside lead in the clinical trials were identified. Most of the selected elements agreed closely with the literature with respect to the type of elements normally found within the skeletal system. Following detailed investigations into the collection, storage and analytical procedures, along with the clinical practicalities, only strontium, cadmium and lead were selected to be measured in the subsequent extended clinical trials in both blood and urine samples.

Table 6.11: Percentage recovery results for pooled urine samples (n=12)

Element	10 $\mu g\ l^{-1}$ spike average (%RSD)	20 $\mu g\ l^{-1}$ spike average (%RSD)	30 $\mu g\ l^{-1}$ spike average (%RSD)
Sr^{88}	96.4 (13.3)	103.7 (3.8)	108.2 (5.8)
Cd^{111}	98.6 (6.7)	103.5 (4.2)	106.7 (7.1)
Pb^{208}	99.2 (5.2)	104.0 (2.2)	103.6 (7.4)

Table 6.12: Results for Seronorm urine reference material (n=10)

Element	Reference Value ($\mu g\ l^{-1}$)	Experimental Result ($\mu g\ l^{-1}$) Average $\pm$ s.d
Sr^{88}	-	105.5 $\pm$ 3.7
Cd^{111}	5.0	6.2 $\pm$ 1.0
Pb^{208}	100.0	98.2 $\pm$ 4.1

Table 6.13: Results for NIST 2670 urine certified reference material (n=6)

CRM: NIST 2670		Certified Value ($\mu g\ l^{-1}$)	Experimental Result ($\mu g\ l^{1}$) Average $\pm$ s.d
Normal level	Cd	(0.4)	2.29 $\pm$ 0.4
	Pb	(10.0)	6.2 $\pm$ 0.5
Elevated level	Cd	88 $\pm$ 3.0	88.4 $\pm$ 3.8
	Pb	109 $\pm$ 4.0	109.7 $\pm$ 1.3

Values given in parentheses are non-certified and are for information only

CHAPTER 7

A COMPARISON OF THE BLOOD AND URINARY LEVELS OF CADMIUM, LEAD AND STRONTIUM, WITH URINARY DEOXYPYRIDINOLINE CROSSLINK LEVELS, AS A MEASURE OF BONE RESORPTION IN PATIENTS WITH SKELETAL METASTASES (EXTENDED CLINICAL TRIAL).

CHAPTER 7: A COMPARISON OF THE BLOOD AND URINARY LEVELS OF CADMIUM, LEAD AND STRONTIUM, WITH URINARY DEOXYPYRIDINOLINE CROSSLINK LEVELS, AS A MEASURE OF BONE RESORPTION IN PATIENTS WITH SKELETAL METASTASES (EXTENDED CLINICAL TRIAL).

7.1: INTRODUCTION AND AIMS.

The preliminary trial results presented in Chapter 5, where the blood lead levels were measured in patients with skeletal metastases and receiving treatment, indicated that the measurement of lead showed some potential as a bone resorption marker and warranted further and more detailed investigation. In Chapter 6, several other potential trace metals were also identified and assessed. However after assessing both analytical and clinical practicalities, only cadmium and strontium were selected alongside lead to be measured in the extended clinical trials.

The purpose of these extended clinical trials was to measure the urinary deoxypyridinoline (DPYD) crosslink levels, using the ELISA technique (serving as the reference marker), and to compare these levels with the selected trace metal levels (Cd, Pb, and Sr), measured by ICP-MS, in blood and urine samples taken at the same time. The trials were essentially a feasibility study, where examination of the diagnostic potential, coupled with the ability to monitor the effectiveness of treatment would be made, along with associated studies of other influencing factors on the results such as, age, sex, treatment, primary cancer and bed rest.

7.1.1: Biological significance of lead in the human body.

Many of the key areas relating to the biological significance of lead in the human body have already been discussed in Chapter 5. The majority of measurements have centred on lead levels in whole blood, since lead is primarily found in the erythrocytes, bound to haemoglobin (468,469). The lead concentration of plasma and serum are generally in the region of 0.1 - 0.5 $\mu g\ l^{-1}$ (306) and often considered too low to measure. The presence of lead in urine although lower than for blood, (usually $< 20\ \mu g\ l^{-1}$ (263,306)), can be regarded as an indirect index of the renal and total body burden of this metal. Hence the determination of lead in urine may be useful for monitoring occupational and environmental exposure to this element. However due to the low level of lead in urine, only the most sensitive analytical techniques may be used successfully, such as ETAAS (263,537,538) and ICP-MS (384).

7.1.2: Biological significance of cadmium in the human body.

7.1.2.1: Sources of cadmium.

Cadmium occurs naturally in soils and rocks (539,540). Anthropogenic sources of cadmium include smelter emissions, steel production, non-ferrous metal production, refining, cement manufacture, pigment manufacture, electroplating, battery manufacture and plastic stabiliser manufacture. Other sources of cadmium include combustion: oil combustion, waste incineration, coal combustion, along with applications of phosphate fertilisers and sewage sludge to land (539-543).

Such contamination of the environment, results in cadmium becoming incorporated into

the food chain and thus potentially causing serious health problems (542). The main routes of human exposure to cadmium include the working environment, for example by the inhalation of fumes and dust, and occasionally by oral intake (542), and more generally from food, air and water (539-541,543). The concentration of cadmium found in food is low, although elevated levels can be found in some foods, such as kidney, shellfish, cereals and leafy vegetables (539,540). The cadmium levels in drinking water are generally low and the contributions from storage tanks and plumbing is generally regarded as negligible (539,540).

There are a number of factors which affect the susceptibility to cadmium toxicity in man, although the relationship between toxicity and intake is complex. Such factors include age, sex, tobacco, and alcohol consumption. Smokers have a higher cadmium intake compared to non-smokers (467, 544-547). The blood cadmium levels increase from age 20 to 60 years, and then decreases again (546). The cadmium levels of the liver, pancreas and kidney increase with age, although the average concentration found in the kidney tends to decrease after about 50 years of age (544). The urinary cadmium levels also tend to increase with age (548), and are generally higher in females compared to males (548). However the cadmium levels in blood tend to be higher in males than females (467,546).

In contrast to lead, the consumption of alcohol has been associated with a decrease, as opposed to an increase, in the blood cadmium levels (467). However a study of different alcoholic beverages has shown a wide range of cadmium concentrations (Table 7.1), such that their consumption could contribute a large fraction of the cadmium intake and therefore this factor should be taken into consideration and control may be advisable in

Table 7.1: Cadmium levels measured in various alcoholic beverages (549).

Sample	Cadmium concentration ($\mu g\ l^{-1}$)	
Brandy	5.31	(n.d - 11.52)
Red wine	3.34	(0.19 - 15.05)
White wine	3.44	(0.10 - 15.38)
Rose wine	3.26	(2.00 - 4.50)
Whisky	3.20	(0.15 - 10.21)
Gava	1.11	(0.70 - 1.35)
Gin	0.64	(0.08 - 1.12)
Olossa	0.52	(0.34 - 0.70)
Sherry	0.36	(0.30 - 0.45)
Rum	0.36	(n.d - 0.70)
Cider	0.34	(0.21 - 0.66)
Beer	0.21	(n.d - 0.80)
Liquor	0.13	(n.d - 0.10)
Anisette	0.04	(n.d - 0.23)

Mean values with range in parentheses

n.d = not detected

any study (549).

7.1.2.2: Metabolism and body burden of cadmium.

The absorption of cadmium compounds varies considerably depending on its chemical form and particle size. Oral absorption of inhaled cadmium in air is 10-50%, while gastrointestinal absorption accounts for about 5% (539,540). Absorption of cadmium is higher for females than males, due to differences in iron stores (540). Transport of cadmium in the gastrointestinal tract is also influenced by compounds, such as proteins and amino acids (539,540).

After inhalation and gastrointestinal absorption, the cadmium accumulates in the body organs, particularly the liver, kidney cortex, kidney medulla, urine, bladder, lungs and glottis (539). Accumulation within the liver and kidney constitutes 50% of the total body burden of cadmium (550). There is a limit to the renal storage of cadmium, above which renal damage prevents further metal accumulation, and results in a decrease in the cadmium kidney concentration. However the body burden of the liver appears to be independent of the functional state of the kidney. Changes in the environmental cadmium levels have been reflected in a change in the body burden of cadmium in the general population (305,539).

7.1.2.3: Blood cadmium levels.

Greater than 90% of the cadmium in blood is bound to the erythrocytes (551), and it is believed that the binding in the erythrocytes may be partly due to the haemoglobin, but also to higher as well as lower molecular mass proteins (metallothienein) (540). The

blood cadmium levels represent the recent exposure levels and in the general population is usually $\leq 4\ \mu g\ l^{-1}$ (552). Smokers have higher blood cadmium levels compared to non smokers, similarly workers slightly exposed to cadmium tend to show higher levels, with the blood cadmium levels correlating with the intensity but not the duration of cadmium exposure.

7.1.2.4: Urine cadmium levels.

At low exposure, urine cadmium reflects the total accumulation of cadmium in the body. In the presence of short term occupational exposure the urinary cadmium levels fluctuate depending on the cadmium exposure intensity concerned. However the urinary cadmium levels generally correlate with the blood cadmium levels (553). In the more common long term exposure scenario the urinary cadmium levels reflect and correlate more closely with the total body burden before severe renal damage has occurred (554). For adults, not occupationally exposed and under 65 years of age, the urine cadmium levels are usually $\leq 2\ \mu g\ g^{-1}$ creatinine (555).

If exposure to cadmium has been excessive, the Cd binding sites become progressively saturated and despite continued exposure, the cadmium concentration in the kidney tends to plateau. At this point, the cadmium that is still absorbed cannot be further retained in the kidney and is rapidly excreted into the urine. Although this excretion is usually low (*i.e.* 0.001% - 0.01% of the total body burden of Cd in 24 hours), it increases as renal damage increases. Therefore the excretion of urinary cadmium and hence the urinary cadmium levels is influenced both by the body burden of cadmium as well as the degree of renal impairment (541).

7.1.2.5: Measurement of cadmium in blood and urine samples.

The determination of cadmium in blood and urine has suffered from several problems, mainly due to the low concentrations involved, coupled with matrix interference effects and contamination problems. Electrothermal atomic absorption spectrometry (ETAAS) has become the most popular and widely used technique to date and a number of procedures have been employed to overcome the above problems (263). Some of the more recent methods are summarised in Table 7.2. The methods have included preconcentration of the Cd by solvent extraction (557,560) and electrodeposition (566) as a means of reducing the interferences from the matrix, whilst improving the sensitivity. However such methods are prone to losses and contamination. Other applications have employed direct injection of the sample into the furnace in ETAAS followed by the addition of matrix modifiers to overcome the interference effects. A wide variety of modifiers, or combinations of, have been tried, such as $(NH_4)_2HPO_4$, $(NH_4)H_2PO_4$, $Pd(NO_3)_2$, NH_4NO_3, nitric acid and Triton X-100 (555-559,562,563,565,567-569). Although these modifiers are known to provide separation of the Cd analytical signal from the matrix interferences by allowing higher charring temperatures, they can also act as a source of contamination at the low levels of Cd normally found in blood and urine. Other workers have tried novel furnace atomisers that provide more precise temperature control, such that the analytical signal can be differentiated from the background signal (563,570), and continuum source ETAAS (564).

Other methods that have been evaluated include electroanalytical techniques (539,540,543), ICP-AES (571), isotope dilution gas chromatography mass spectrometry

Table 7.2: Summary of ETAAS methods for measuring cadmium in blood and urine.

Sample type	Procedure	L.O.D	Linear Range	% RSD	Comments	Ref.
Blood	1 + 9 dilution with 0.05 % m\v Triton X-100 with 1% nitric acid added separately. Cal: Aqueous (1% v\v nitric acid with 0.2% m\v $(NH_4)_2HPO_4$ and 1 mg\ml $Mg(NO_3)_2$ solution). Vol: 5μl	1.3pg	3 ng ml^{-1}		Deuterium background correction with L'vov platform. Stabilised temperature platform furnace conditions (STPF). Addition of the stabiliser mixture to the aqueous calibration solution results in thermal behaviour resembling that of Cd in the blood matrix.	555
Blood Serum Urine	1 + 4 dilution with Triton X-100 (blood), and 1 + 2.5, and 1 + 2 dilution with 0.2% nitric acid (serum and urine respectively). Palladium nitrate and ammonium nitrate used as the matrix modifier. Cal: Matrix free reference solutions	0.4 $\mu g\ l^{-1}$ 0.14 $\mu g\ l^{-1}$ 0.10 $\mu g\ l^{-1}$		3.9% 33.3% 17.5%	Zeeman background correction. Values for reference materials within their respective certified ranges.	556

Table 7.2: Continued.

Sample type	Procedure	L.O.D	Linear Range	% RSD	Comments	Ref.
Blood Urine	1 + 3 dilution with 1 M nitric acid (blood), 1 + 3 or 1 + 5 dilution with ultrapure water (urine - direct determination). Urine samples also prepared by solvent extraction with aqueous 2% sodium diethyl dithiocarbamate (NaDDC) solution, followed by methyl isobutyl ketone (MIBK). Cal: Matrix matched Vol: 10 - 25μl (blood) 10μl (urine - direct) 10 - 50μl (urine - solvent extraction)	$\leq$2 μg l^{-1} (blood) $\leq$0.2 μg l^{-1} (urine, direct) $\leq$0.1 μg l^{-1} (urine, solvent extraction)		Blood: 30% at 0.4μg l^{-1} 3.8% at 9.3μg l^{-1} Urine: 26% at 0.6μg l^{-1} 7.1% at 27μg l^{-1}	Stabilised temperature platform furnace (STPF) with Zeeman background correction.	557
Blood Urine	1 + 4 dilution with 0.1% Triton X-100 (blood) or 0.2% nitric acid (urine), added to graphite tube platform where the "*in-situ*" dried and decontaminated modifier already added. Cal: Matrix matched and Aqueous Vol: 10μl	0.22 μg l^{-1}	<400pg	<3%	Zeeman background correction and stabilised temperature platform furnace conditions (STPF). Palladium based modifiers and "*in-situ*" decontamination studied. Comparison of modifiers:- Pd, Pd with ammonium nitrate and Pd with magnesium nitrate .	558

Table 7.2: Continued.

Sample type	Procedure	L.O.D	Linear Range	% RSD	Comments	Ref.
Urine	1 + 4 dilution with ultrapure water. Matrix modifier consisted of 200 μg $(NH_4)_2HPO_4$ in 30ml l^{-1} HNO_3. Cal: Aqueous standards. Vol: 10μl.	0.04μg l^{-1}		5%	Stabilised temperature platform furnace (STPF) with Zeeman background correction.	559
Urine	Solvent extraction sample preparation used: The sample is dried, ashed in the presence of nitric acid, the residue is dissolved in hydrochloric acid and the Cd is extracted as its tetrahexylammonium iodide into methyl isobutyl ketone. The organic phase is then analysed by ETAAS. Cal: Matrix matched Vol: 20μl	1 ng g^{-1}	$\leq$4.2 ng g^{-1}	10%	Deuterium background correction.	560
Urine	1 + 1 dilution with deionised water. No matrix modifiers used. Cal: Standard additions Vol: 20μl	0.06 μg l^{-1}	up to 12 μg l^{-1}		L'vov platform and selective atomisation.	561

Table 7.2: Continued.

Sample type	Procedure	L.O.D	Linear Range	% RSD	Comments	Ref.
Urine	a) 1 + 4 dilution with 6% v\v nitric acid. or b) 1 +1 dilution with 1% v\v nitric acid. Cal: Standard additions Vol: a) 10μl b)20μl	a) 0.07 μg l^{-1} b) 0.13 μg l^{-1}	up to 8 μg l^{-1}	a) < 5% (intra), 15-20% (inter) b) <2% (intra), 10% (inter)	a) 4 stage furnace programme using L'vov platform in an uncoated graphite tube. Selective volatilisation separates Cd from background absorbance. b) Analyses off the wall of an uncoated tube with a short 3 stage programme using no ashing stage.	562
Urine	1 + 1 dilution with 1.5% nitric acid. Cal: Standard additions Vol: 30μl	0.05 μg l^{-1}		< 10%	Zeeman background correction.	563
Urine	1 + 3 dilution with ultrapure water. No chemical modifier used or sample pretreatment. Cal: Aqueous Vol: 10μl	0.3 μg l^{-1}	3-40 μg l^{-1}	2% at 3.5 μg l^{-1}	Deuterium background correction. Probe atomisation.	564

Table 7.2: Continued.

Sample type	Procedure	L.O.D	Linear Range	% RSD	Comments	Ref.
Urine	1 + 1 dilution with nitric acid, digest at 60°C in water bath for 1 hour in a sealed plastic vial. No matrix modifiers used Cal: Aqueous	0.03 ng ml^{-1}	0.03-3 ng ml^{-1}	3.5%	Continuum source AAS with a diode array detector used with a conventional graphite furnace atomiser.	565
Urine	Digest 20 ml of urine with either conc. perchloric acid and nitric acid or just nitric acid. Dissolve and dilute residue in 0.1M sulphuric acid (100ml). Add 20 mls of solution into the electrolysis cell. Insert electrodes and after 2 minutes preconcentration time, remove electrodes and carefully remove the residue from the tungsten wire into the graphite tube. Cal: Aqueous	0.01 ng ml^{-1}	$\leq$ 0.55 ng ml^{-1}	3.35%	Preconcentration of Cd by electrodeposition onto a tungsten wire .	566

(572) and isotope dilution ICP-MS (529,573).

7.1.3: Cadmium and cancer.

The potential carcinogenic effects of cadmium might be affected by several factors such as smoking, hormones and the presence of other metals, such as selenium and zinc (574). A recent study could neither prove or disprove the role of cadmium in breast cancer initiation, promotion or progression (574). However in another study cadmium induced prostate tumours in rats appeared to be associated with metallothienein deficiency (575). It is known that upon entering the body system *via* absorption through the lungs and intestine into the blood stream, cadmium first accumulates in the liver where it induces the synthesis of metallothienein, to which the cadmium binds.

Therefore since the patients recruited for this clinical trial were suffering from primary cancers of the breast and prostate, the suggestion that cadmium may be linked with these cancers, or may affect the pathology of such cancers should be taken into consideration, since it could influence the findings of this study. Doubt may well be cast over the suitability for using cadmium as a biochemical marker of bone resorption in this oncology study.

7.1.4: Biological significance of strontium in the human body.

7.1.4.1: Sources of strontium.

Strontium occurs naturally in the sea, rocks and land (576) and possesses four stable isotopes: Sr^{84} (0.55%), Sr^{86} (9.75%), Sr^{87} (6.96%) and Sr^{88} (82.74%), and

several radioisotopes. Strontium resembles calcium and barium and has properties intermediate between the two elements (576). Strontium is used as a scavenger to remove traces of gases from vacuum tubes and as a colouring agent in fireworks. Strontium compounds are also used in ceramics, plastics, greases, permanent magnets and iron castings (576). In addition to these chemical applications Sr plays an important role in medicine, where stable and radioactive Sr isotopes are used as markers for calcium metabolism (577) and Sr^{89} is used as a palliative agent in the treatment of bone metastases (578-580).

Strontium is an element naturally occurring in foods and beverages. Components that contribute to a major portion of the diet (meat, poultry, fruit and vegetables) contain lowest amounts of strontium (0.3-5.1 $\mu g\ ml^{-1}$). Cereals, grains and sea foods contain up to 25 $\mu g\ ml^{-1}$. Amounts over 100 $\mu g\ ml^{-1}$ have been found in brazil nuts, cinnamon, and some kinds of fish flour made with white fish including bones (576). Daily intake therefore varies depending on the type of diet. Lower Sr intake is associated with diets in societies where Sr and Ca primarily originate in dairy products. Whereas higher amounts are found in foods in societies where cereal or other grain products constitute 55-65% of the Ca intake and the remainder is derived from milk and dairy products (576).

7.1.4.2: Metabolism and body burden of strontium.

The gastrointestinal tract represents the main route of entry into the body, but the fraction absorbed by the intestine is relatively low (30%), in comparison to the fraction absorbed *via* the lungs (85%). The skin absorbs 0.26% in an undamaged state, compared to 57.4%

through damaged skin (576).

It is known that strontium can replace calcium in many biological processes, but the intestinal absorption of Sr is lower compared to calcium. The fraction absorbed from dietary intake is calculated to be approximately 20%. Intestinal Sr absorption is negatively affected by age, chelating agents (e.g. sodium alginates), food in general and high dietary contents of Ca and\or phosphates (576).

The distribution of Sr is similar to Ca, *i.e.* 99% of the body burden is in bone (576). It is believed that the incorporation of Sr in bone is by ion exchange with calcium (474,581,582). The preference of hydroxyapatite crystals to bind with calcium rather than strontium seems mainly due to the larger size of the Sr ion, which produces a mild distortion of the crystal lattice and this probably results in a weaker binding of Sr to the components of the hydroxyapatite crystal (583). Within long bones Sr is preferentially deposited in the shafts (584).

The Sr concentration in bone varies, possible due to the fact that the content is positively affected by dietary intake or aging (585) and negatively affected by dietary intake of calcium and phosphate. The various techniques used to analyse the bone samples also adds to the diversity of values measured (576).

The second important compartment for Sr is blood. Analogous to bone, the blood-strontium concentration is influenced by the dietary intake of Sr, Ca and phosphate (586), plus the values measured tend to vary with analytical technique used. Strontium in blood

is bound by serum proteins and transferred to the interstitial fluid to the same extent as Ca but intra cellular penetration appears to be more limited. However Sr has the unique capacity of being able to replace Ca even at sites highly specific for Ca. The normal levels of Sr found in the blood range between 16 - 43 $\mu g\ l^{-1}$ (587). Sr is eliminated from the body in the urine, and to a lesser extent in the faeces, milk and sweat.

7.1.4.3: Analysis of strontium in blood and urine.

A wide range of analytical techniques have been used for the measurement of strontium in biological fluids (576), with some of the more recent examples summarised in Table 7.3. However many of these techniques are not available in clinical laboratories, hence procedures such as FAAS and ETAAS have found more widespread applications. The behaviour of strontium in FAAS is intermediate of calcium and barium, *i.e.* it can be determined in both nitrous oxide\acetylene and air\acetylene flames, although the latter is prone to a larger number of chemical interferences. This can be overcome by the use of lanthanum chloride (595). The nitrous oxide\acetylene flame reduces the chemical interferences but gives rise to ionisation interference, although this can be avoided by the addition of an excess of an easily ionising element such as rubidium (596) or potassium (597).

Electrothermal AAS (ETAAS) is the preferred technique, mainly due to its greater sensitivity as well as its greater suitability for analysing smaller sample volumes. A variety of furnace tubes have been studied, such as graphite tubes (591) and pyrolytic coated tubes with or without a platform (587,591,598).

Table 7.3: Analytical methods used to determine strontium in biological fluids.

Technique	Sample type	L.O.D	Linear Range	% RSD	Comments	Ref.
FAAS	Blood	0.56 μg 100ml^{-1}	20 μg 100ml^{-1}	$<8\%$	A selective extraction into isobutyl methyl ketone used, followed by determination of Sr in the organic phase using an air\acetylene flame.	588
FAES	Serum Urine Saliva CSF	3 ng ml^{-1}	100 ng ml^{-1}	$<5\%$	A rapid, direct method for the determination of normal levels in serum without separation or preconcetration. A nitrous oxide-acetylene flame used	589,590
ETAAS	Blood	4.6 μg l^{-1}	≤ 260 μg l^{-1}	$<10\%$	Zeeman background correction used. Blood (50μl) diluted 20 fold with 950μl of Triton X-100. A 10μl aliquot introduced to the pyrolytically coated graphite tubes. Calibration was achieved using either aqueous standards (in 1% v\v HNO_3) or spiked diluted blood (standard additions).	587

Table 7.3: Continued.

Technique	Sample type	L.O.D	Linear Range	% RSD	Comments	Ref.
ETAAS	Plasma Urine	2 $\mu g\ l^{-1}$ 3 $\mu g\ l^{-1}$	$\leq 250\ \mu g\ l^{-1}$	$<10\%$	The plasma and urine samples were diluted with dilute nitric acid 20 fold and 50 fold respectively. Samples (20 μl aliquot) were analysed using pyrolytically coated graphite tubes, by optimised temperature programming in the automatic background correction mode (tungsten, halogen source). Matrix matched calibration standards were used. Good accuracy and precision was achieved.	591
ETAAS	Plasma	0.02 $\mu g\ l^{-1}$	$\leq 5\ \mu g\ l^{-1}$	$<3\%$	Zeeman background correction and pyrolytically coated graphite tubes were used. Samples were diluted 451 fold by adding 50μl of sample to 2ml of 0.2% HCl (dilution 1), followed by addition of 100μl of dilution 1 to 1ml 0.2% HCl. An optimised temperature programme and matrix matched calibration standards used.	592

Table 7.3: Continued.

Technique	Sample type	L.O.D	Linear Range	% RSD	Comments	Ref.
ICP-AES	Blood	0.3 $\mu g\ l^{-1}$	$\leq 25\ \mu g\ l^{-1}$	<5%	Blood samples are digested in a mixture of nitric and perchloric acid in combination with a microwave oven. Aqueous calibration solutions were used.	593
ICP-MS	Serum				Serum was diluted 10 fold with deionised water (MilliQ). A study into internal standardisation and interference effects revealed that accurate measurement could be achieved providing a suitable internal standard *i.e.* Y was used.	384
ICP-MS	Serum	0.05 $\mu g\ l^{-1}$		<3%	Serum was diluted 10 or 5 fold with 0.14 M nitric acid. Indium was used as the internal standard (100 $\mu g\ l^{-1}$) along with aqueous calibration solutions.	594
NAA	Serum	0.02-0.05$\mu g\ l^{-1}$		<10%	The Sr^{87m} produced was radiochemically separated by extraction with oxine in chloroform	594

FAAS Flame atomic absorption spectrometry
ETAAS Electrothermal atomic absorption spectrometry
ICP-MS Inductively coupled plasma-mass spectrometry
CSF Cerebral spinal fluid
FAES Flame atomic emission spectrometry
ICP-AES Inductively coupled plasma-atomic emission spectrometry
NAA Neutron activation analysis

7.1.5: Correction factors to adjust urine analyte concentrations.

It is usually not practical to collect urine samples over long and fixed periods of time. Therefore, it is now common place to do spot tests and adjust the analytical values obtained according to reference parameters, such as creatinine, specific gravity and osmolarity. All of these parameters are used to eliminate the influence of dilution, although creatinine is also used to obtain values comparable to that observed with a 24 hour sample. Since the quantity of creatinine excreted in 24 hours is generally accepted to be consistent and little influenced by diuresis, correction with creatinine has become a common and accepted practice.

However doubts have now been expressed as to creatinine's suitability as an adjustment parameter, since contrary to expectations the level of creatinine has showed marked inter and intra individual variations (548,599). Some studies have shown that analytes should be corrected using specific gravity and osmolarity, rather than with creatinine (600), whilst other studies suggest that adjustment is not always necessary (544,601). Each analyte should therefore be assessed individually with respect to the merits of adjusting values with one or more reference parameters.

7.2: EXPERIMENTAL.

7.2.1: Reagent and chemicals.

All of the water used to wash laboratory ware and for the preparation of solutions, standards and samples was deionised using either a MilliQ purification system (Millipore, Bedford, MA, USA) or an Elgastat Maxima purification system (Elga Ltd, High

Wycombe, Bucks., UK) at a resistivity of 18 MΩ. All laboratory ware was soaked in 10% v\v nitric acid and rinsed several times with Elga or MilliQ water.

Aristar nitric acid was obtained from Merck (Poole, Dorset, UK), and the Triton X-100 was obtained from Aldrich (Gillingham, Dorset, UK). Thallium nitrate standard reference material, NBS SRM 3158, (10,000 mg l^{-1}) was obtained from the National Institute of Standards and Technology (Washington DC, USA) and cadmium, cesium, indium, lead, strontium and yttrium Spectrosol standard solutions or equivalent (1000 mg l^{-1}) were obtained from Merck and Aldrich respectively. All of the calibration standards were prepared, at appropriate dilutions from these standard solutions.

Seronorm whole blood and urine reference materials (Nycomed (UK), Birmingham, UK), and NIST freeze dried urine 2670 (National Institute of Standards and Technology, Laboratory of the Government Chemist, Teddington, London), were used as quality control measures.

7.2.2: Patient selection.

During the extended clinical trials it was proposed to collect blood and urine samples concurrently, once a month. The following selection criteria (see Section 1.8) were chosen:-

i) Patients with primary cancers of the prostate or breast, diagnosed with bone metastases and receiving treatment (Group 1).

ii) Patients with primary cancers of the prostate or breast, diagnosed with bone metastases but who had not commenced on treatment (Group 2).

iii) Patients diagnosed with primary cancers of the prostate or breast but with no known bone metastases (Group 3).

iv) Age and sex matched patients with no known primary or secondary cancers, *e.g.* spouses (Group 4).

7.2.3: Sample collection.

Urine sampling kits were given to each patient involved in the clinical trials. These kits, sealed in a plastic bag, consisted of a 250ml plastic beaker, and 30ml amber screw cap plastic bottle. To minimise any possible contamination the beaker and bottle were soaked for at least 24 hours in 5% v\v Decon-90, rinsed thoroughly with distilled water, soaked for a further 24 hours in 10% v\v Aristar nitric acid, rinsed thoroughly with MilliQ or Elga deionised water, dried in an oven, and then immediately sealed in the plastic bags. The sampling kits were taken home by the patients, who were instructed to keep the kits in a clean, dry place and only to use them if the seal had not been broken.

On the morning of the patient's clinic, they were instructed to collect their first void urine sample, providing the integrity of the sampling kits had not been compromised, and to store the bottle in a clean, dry, cool place out of direct sunlight. Upon arrival at the clinic the urine sample was collected by the nursing staff who issued a new sterilised kit ready for the next visit. The urine sample was shaken and two 10 ml aliquots were removed. One aliquot was placed into a trace metal free plastic tube with 0.1% v\v Aristar nitric acid added (for the trace metal analysis by ICP-MS), whilst the other aliquot was placed in a trace metal free plastic tube with no acid added (for the creatinine tests and deoxypyridinoline crosslink analysis by ELISA). Both the tubes were stored at -20°C.

The blood samples were taken on the same day, whilst the patients attended the clinic as

part of their routine treatment. The blood samples were collected by venepuncture into Vacutainer tubes, B-D 6484 (Beckton-Dickinson, East Rutherford, NJ, USA), containing lithium heparin anticoagulant, and stored at -20°C.

7.2.4: Procedures.

The procedures employed have been discussed in detail in Chapters 5 and 6. In summary, after defrosting, the blood samples were shaken thoroughly and 1ml of blood was pipetted into 15ml trace metal free plastic tubes (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK) and 9mls of diluent was added using an automatic dispenser (Optifix, Merck, Poole, Dorset, UK). The diluent consisted of 0.1% v\v Triton X-100 (to promote cell lysis, to improve nebuliser efficiency and sample transport), 0.1% v\v Aristar nitric acid (to provide a stable pH environment), and 100 $\mu g\ l^{-1}$ yttrium, cesium and thallium internal standards (to correct for any instrumental drift). The samples were shaken and then analysed by ICP-MS as discussed in Section 2.1.8.

The urine samples were defrosted and shaken thoroughly. For the trace metal analysis, 1 ml of urine was pipetted from the tubes (with added acid) into a clean trace metal free plastic tube (Labsystems, Life Science International (UK) Ltd, Basingstoke, Hampshire, UK), and 4 mls of diluent was added using the automatic dispenser. The diluent consisted of 2% v\v Aristar nitric acid, and contained 100 $\mu g\ l^{-1}$ yttrium, indium and thallium as internal standards to correct for any instrumental drift. For the deoxypyridinoline crosslink analysis the procedure used was exactly as described in Chapter 4.

Creatinine measurements were made on each urine sample (analyses performed by the

Clinical Biochemistry Laboratory, at Derriford Hospital, Plymouth, Devon, UK). The ELISA technique for measuring the DPYD crosslinks was based on spot tests followed by correction with creatinine values. For this reason creatinine was selected as the reference adjustment factor and the trace metal concentrations measured in the clinical trial are presented below in both a corrected and non corrected format.

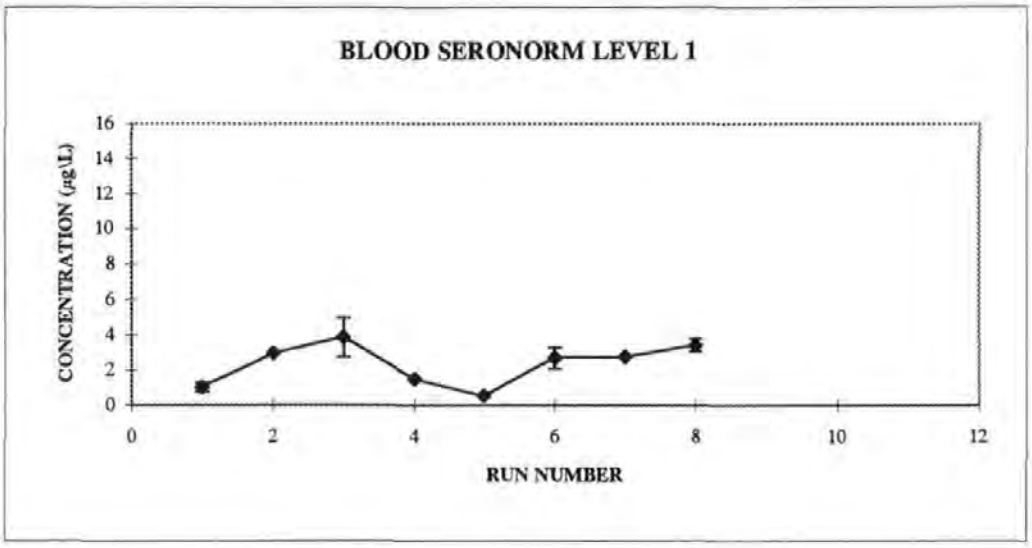
7.3: RESULTS AND DISCUSSION.

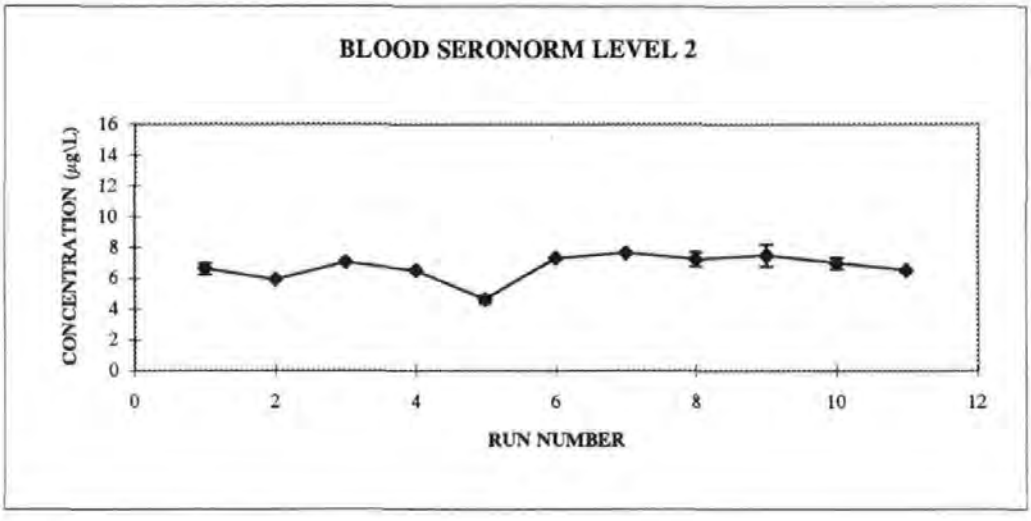
7.3.1: Quality control measures.

Throughout the clinical trial standards were measured at regular intervals in order to ensure the methodology remained under control for all the analytes of interest. Figures 7.1, 7.2 and 7.3 show charts plotting the analytical performance of Seronorm blood standards for cadmium, lead and strontium respectively. Figure 7.1 shows that the experimental cadmium results for level 1 were more variable and generally higher than the certified range (0.8 - 1.0 $\mu g\ l^{-1}$) although this was not unexpected since this was near the limit of detection for the method. The results for level 2 were more stable and generally within the certified analytical range (5.9 - 6.8 $\mu g\ l^{-1}$) whilst the experimental results for level 3 tended to be slightly higher than the certified analytical range (11.2 - 12.5 $\mu g\ l^{-1}$), although the results were again reasonably consistent over time.

The experimental results for lead, as depicted in Figure 7.2, show that consistent results within the certified analytical range (31 - 41 $\mu g\ l^{-1}$) was attained for level 1, which correlates with the normal blood lead levels expected and measured. The results for both levels 2 and 3 were also consistent but higher than the upper certified range values (361

Figure 7.1: Chart plotting the analytical performance for cadmium in Seronorm blood reference material.





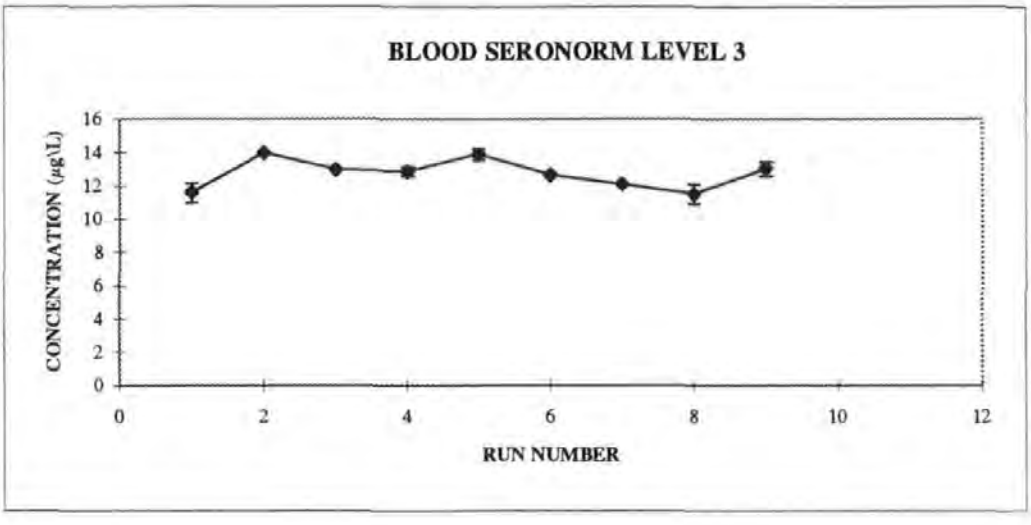


Figure 7.2: Chart plotting the analytical performance for lead in Seronorm blood reference material.

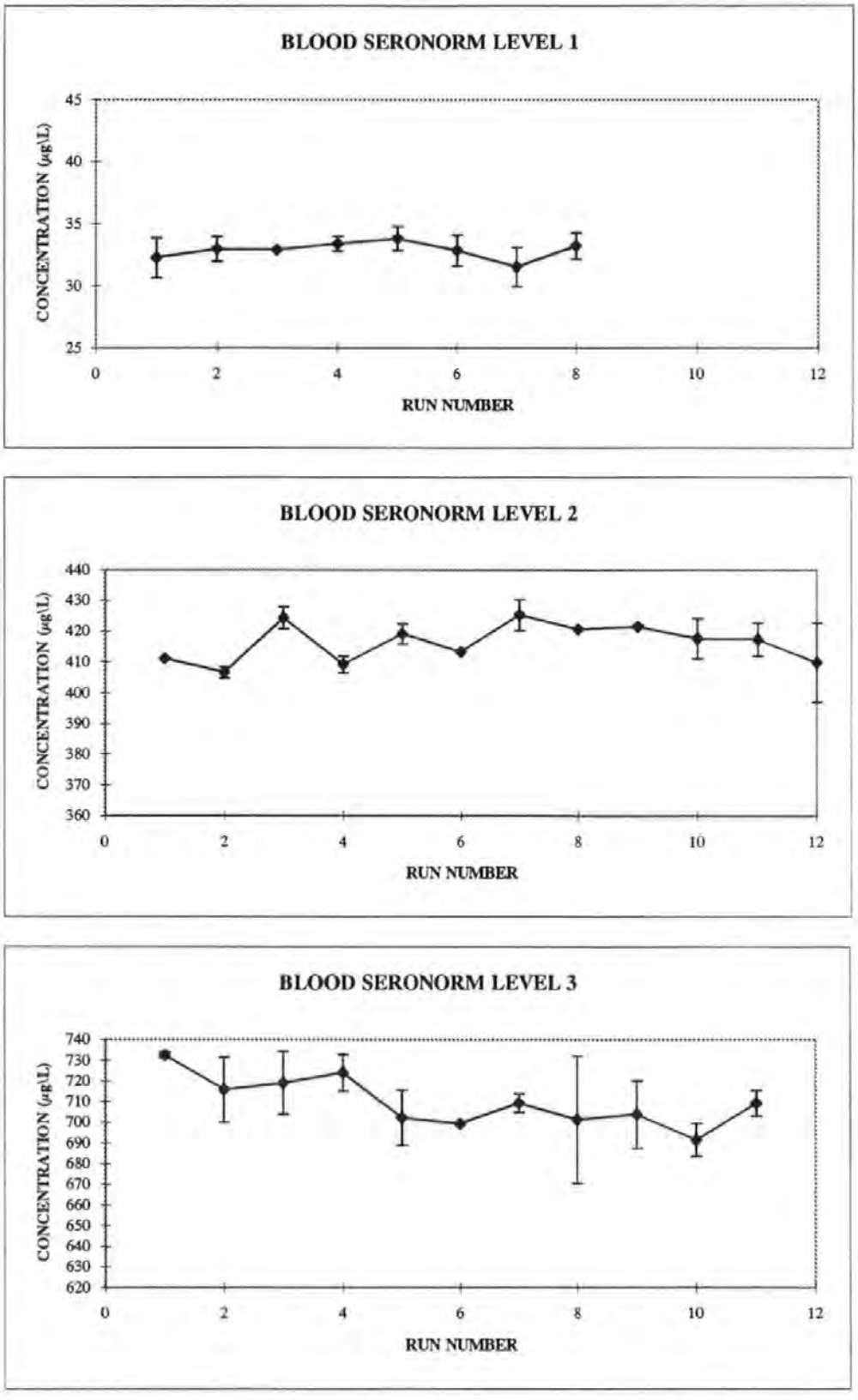
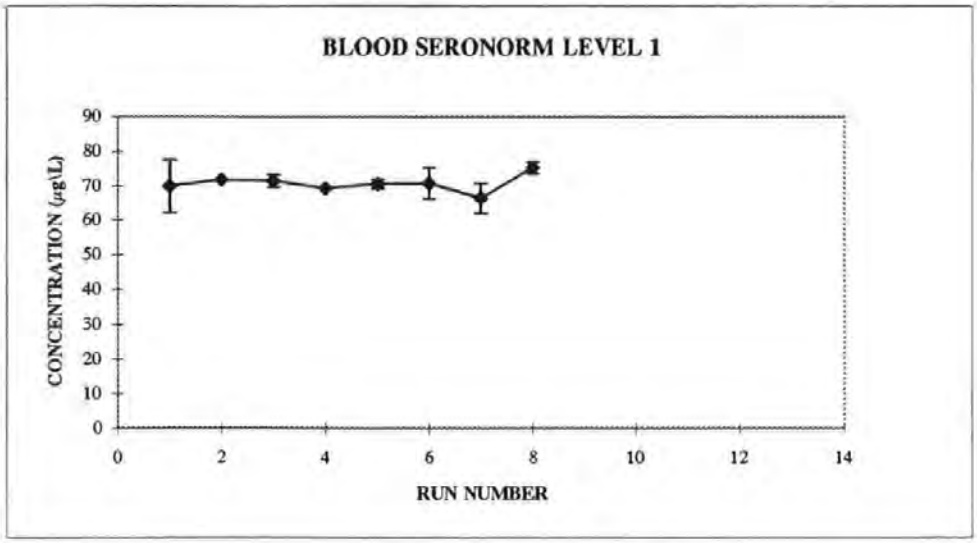
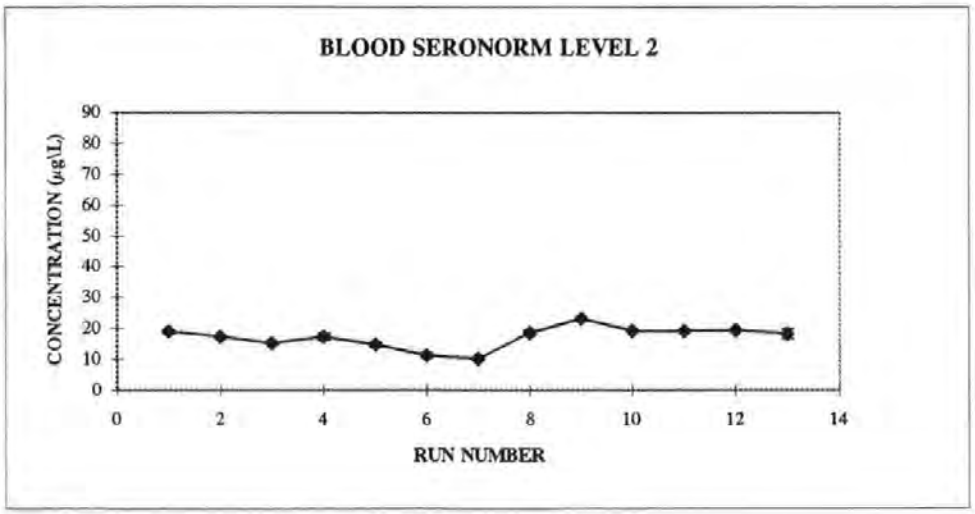
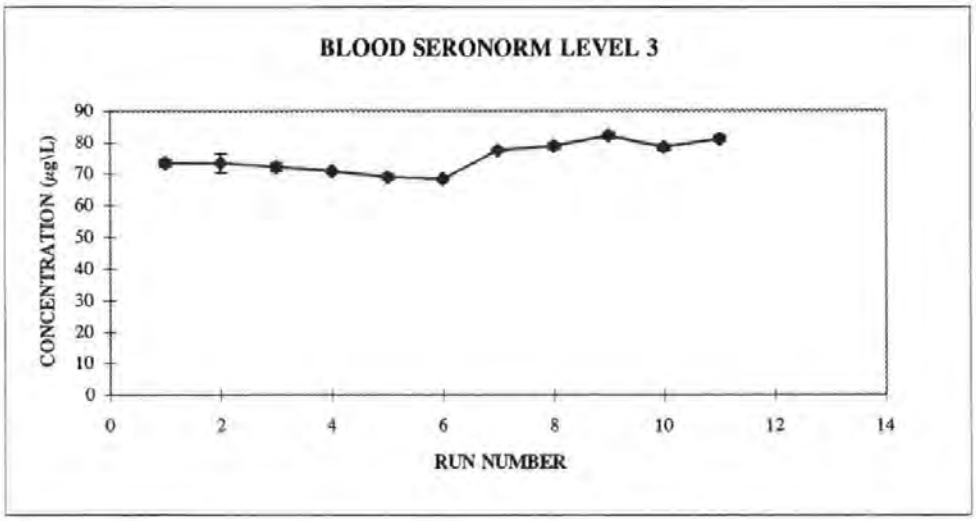


Figure 7.3: Chart plotting the analytical performance for strontium in Seronorm blood reference material.





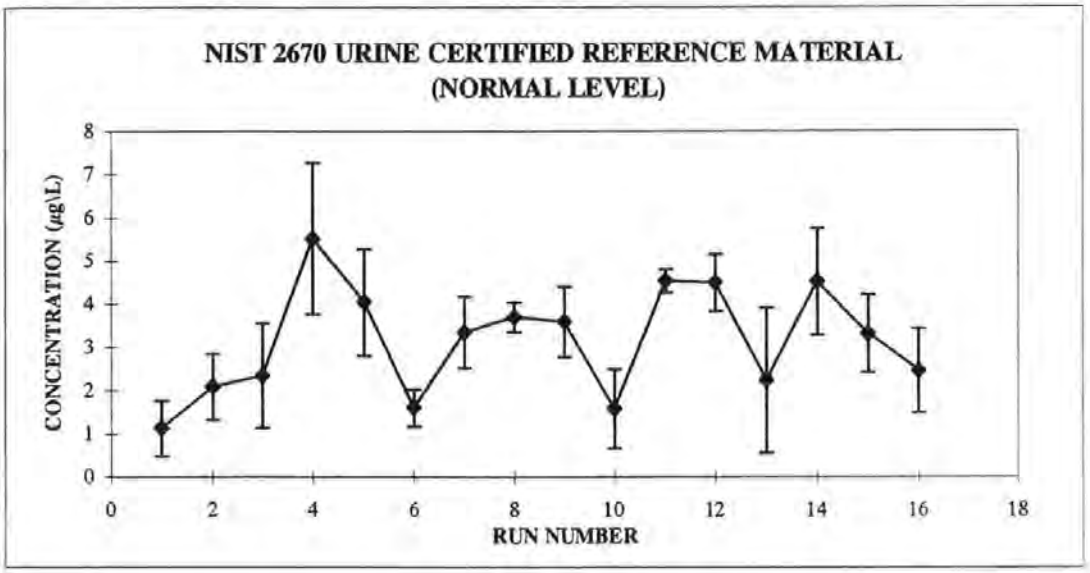


- 396 $\mu g\ l^{-1}$ and 626 - 702 $\mu g\ l^{-1}$ respectively). Since these high levels of blood lead were not determined during the course of the trial the results were considered less relevant compared to results for level 1.

No certified values was given for strontium in the Seronorm samples and Figure 7.3 shows that the results obtained were quite variable. Although level 1 gave the most consistent results it also appeared most prone to large errors for each measurement, compared to levels 2 and 3. All three levels showed a similar pattern over time, in that the concentrations measured tended to decrease over runs 1-6 (day 1), followed by an increase on day 2 (up to run number 9), and then decreased and plateaued on day 3. The results showed that although the blood strontium measurements were subject to some variation, the values did remain within a sufficiently narrow analytical range.

Figures 7.4, 7.5 and 7.6 show charts plotting the analytical performance of NIST CRM 2670 urine for cadmium, lead and strontium respectively. Guideline values were given for the normal level standard, whilst the values for the elevated level standard were certified. In Figure 7.4, the cadmium levels for the normal level standard were more variable, which is not unusual at low concentrations. Consistent results were attained at the elevated level, although slightly below the certified reference range ($88 \pm 3\ \mu g\ l^{-1}$). In Figure 7.5, more consistent levels were shown for lead at both levels. The values were just below the guideline value (*i.e.* $10\ \mu g\ l^{-1}$) in the normal level standard, and were within the certified range for the elevated level standard ($109 \pm 4\ \mu g\ l^{-1}$) throughout the trial period, despite the tendency towards a downward drift. As with the Seronorm samples, no reference value was given for strontium in the NIST CRM urine sample but

Figure 7.4: Chart plotting the analytical performance for cadmium in NIST CRM 2670 urine.



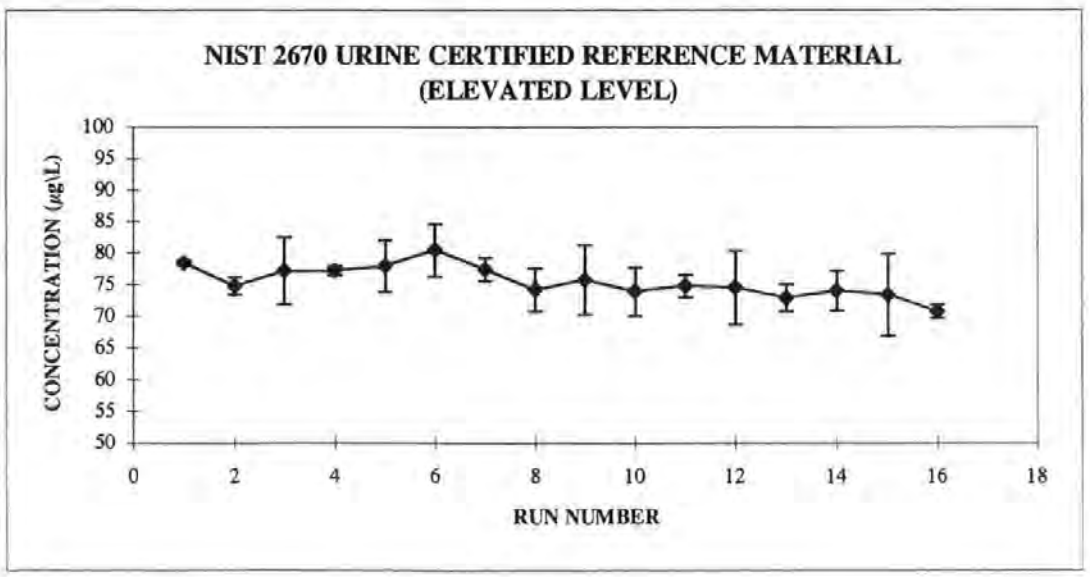
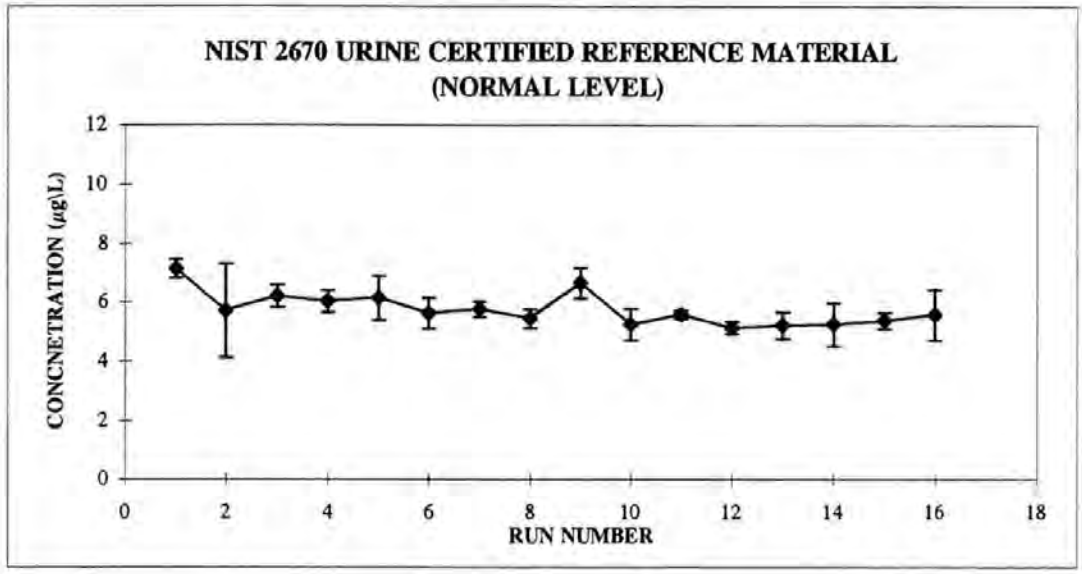


Figure 7.5: Chart plotting the analytical performance for lead in NIST CRM 2670 urine.



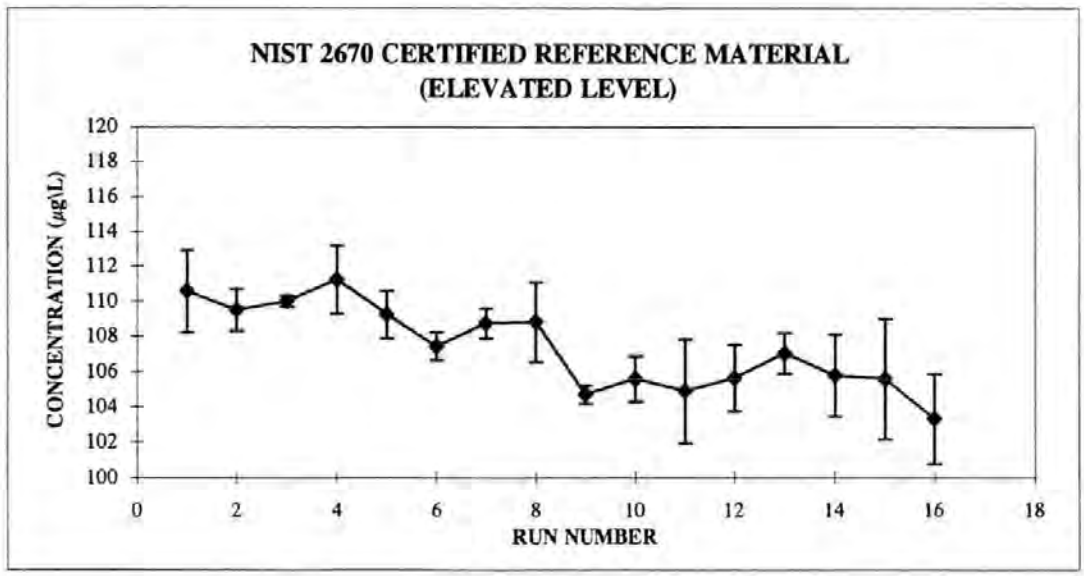
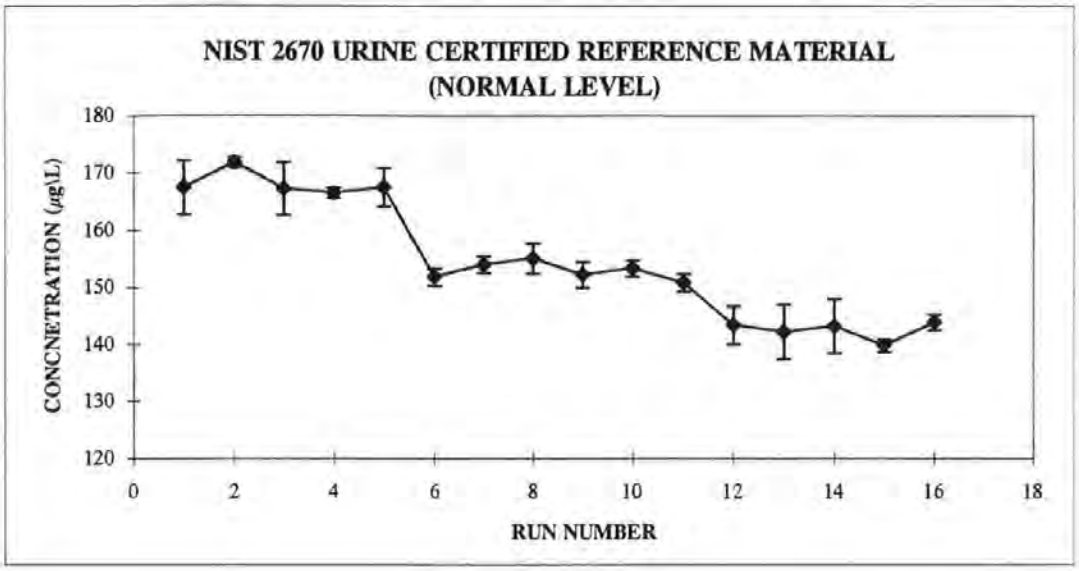


Figure 7.6: Chart plotting the analytical performance for strontium in NIST CRM 2670 urine.



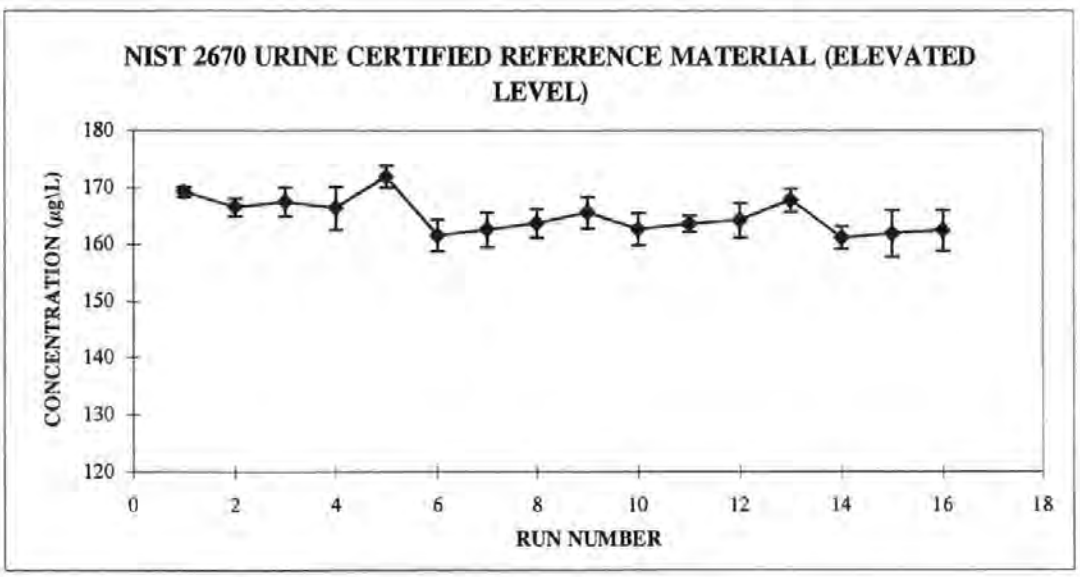


Figure 7.6 shows that at the elevated level the values were reasonably consistent over time. However the values for the normal level inexplicably suffered from a downward drift (about 17% over 16 runs).

For the DPYD measurements, high and low controls were analysed on each plate. The results were in excellent agreement and within the reference ranges, as discussed in Chapter 4.

Overall although some of the experimental values fell outside the certified ranges for all the elements involved, the results were generally considered to be satisfactory, and in all cases close to the analytical concentration areas indicated. Therefore for the purposes of this clinical trial, since the results were only to be used in-house, and therefore subjected to the same procedures, instruments and conditions etc, and coupled with the fact that the best results were often attained for the normal concentration ranges for each of the elements (particularly in the blood analyses), the analytical methodology was considered to remain under control.

7.3.2: Extended clinical trial.

Ideally between 25 - 50 patients were required for each subject group (refer to Section 7.2.2) with a full medical and personal history. These patients would be studied for at least 6 months with one blood and urine sample taken concurrently once a month. Unfortunately major problems with respect to recruiting suitable and willing candidates, along with a high "drop out rate", resulted in three or more samples being collected from only 27 patients, of which 6 participated in the preliminary lead trial, discussed in

Chapter 5. The fact that only a small number of patients were recruited for groups 1, 3 and 4, and no patients at all for group 2, (as summarised in Table 7.4), meant that comparisons between analyte levels for each group was difficult. Also information concerning the patient's lifestyles and habits, such as smoking, alcohol consumption, diet, occupational and geographical history were not available. These factors can have a very influential effect on the levels of the trace metals measured. The information available concerning the patients recruited for this study is summarised in Table 7.5. The lack of numbers and subsequent lack of personal details meant that any major, significant or conclusive comments on differences between the groups would be difficult to make.

7.3.2.1: Comparison of initial levels of Cd, Pb, Sr and DPYD.

The relationship between the trace metal levels and age and sex would also be dependant to some degree on the above mentioned factors. The length of time the patient had received treatment, combined with the success of such treatment would also be important. This would influence the levels observed for patients in group 1, and possibly in group 3. Consequently the data on the initial levels of Cd, Pb and Sr measured in blood and urine samples was quite variable as expected. No clear trends or differences could be distinguish between the sexes, and no change was observed with age. The level of trace metals measured also did not appear to relate to the degree of metastic disease (*i.e.* bone scan rating).

The initial blood cadmium levels, (Figure 7.7), for each patient within each group, were all fairly constant within the range from 1 - 6 $\mu g\ l^{-1}$. Only a few results were at a slightly higher level, the majority of these being in the group 1 category (*i.e.* cancer with bone

Table 7.4: Patient numbers recruited for the extended clinical trial.

	Group 1	Group 2	Group 3	Group 4
Males	8	0	6	2
Females	7	0	2	2
Total	15	0	8	4

Table 7.5: Summary of patients recruited for the full clinical trials.

Patient code	Group	Sex	Age at start of trial	Bone scan rating	Diagnosis	Treament
004*	1	Female	83	2	Breast	Pamidronate Radiotherapy
008*	1	Male	66	1	Prostate	Zoladex
010*	1	Male	73	1	Prostate	Zoladex
015*	1	Female	69	2	Breast	Pamidronate
016*	1	Female	69	2	Breast	Pamidronate Clodronate
018*	1	Female	61	2	Breast	Anmadex Pamidronate
019	1	Male	64	3	Prostate	Zoladex
020	1	Male	65	3	Prostate	Casodex Zoladex
021	1	Male	70	1	Prostate	Triptorelin
022	1	Male	70	2	Prostate	Triptorelin
023	1	Male	76	3	Prostate Kidney	Zoladex
024	1	Male	81	3	Prostate	Triptorelin

Table 7.5: Continued.

Patient code	Group	Sex	Age at start of trial	Bone scan rating	Diagnosis	Treament
025	1	Female	49	2	Breast	Radiotherapy Zoladex BM21
026	1	Female	52	1	Breast	Surgery Bisphosphonate
027	1	Female	66	1	Breast	Pamidronate
028	3	Male	66	0	Prostate	Casodex
029	3	Male	69	0	Prostate	N.D
030	3	Male	70	0	Prostate	N.D
031	3	Male	71	0	Prostate	N.D
032	3	Male	74	0	Prostate	N.D
033	3	Male	76	0	Prostate	Triptorelin
034	3	Female	44	0	Breast	Surgery
035	3	Female	62	0	Breast	BM21

Table 7.5: Continued.

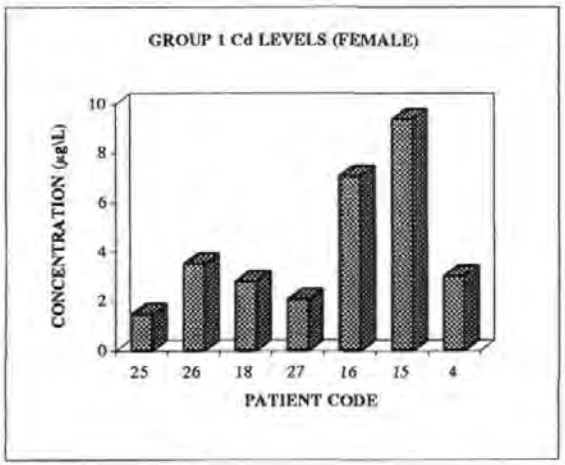
Patient code	Group	Sex	Age at start of trial	Bone scan rating	Diagnosis	Treament
036	4	Male	N.D (61)#	0	Control	None
037	4	Male	N.D (62)#	0	Control	None
038	4	Female	N.D (70)#	0	Control	None
039	4	Female	N.D (66)#	0	Control	None

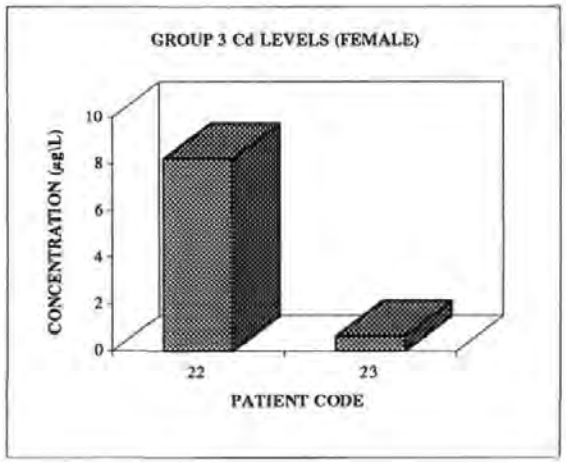
* Patients also participiated in the preliminary lead trials.

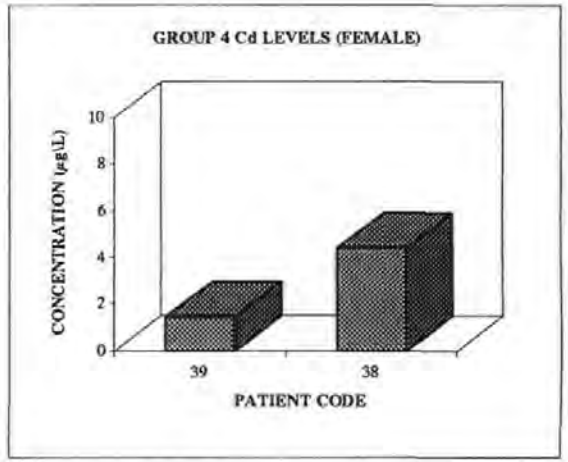
N.D Not Declared

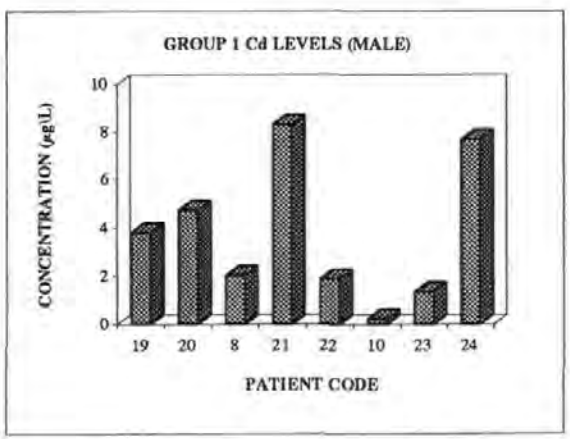
Patients age not declared but age of spouse given in brackets, giving an indication of the possible age.

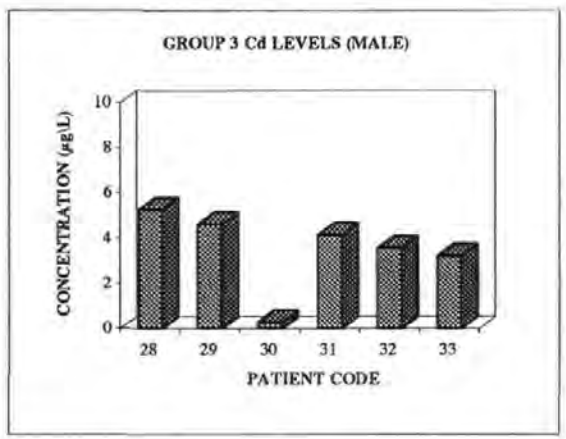
Figure 7.7: Graphical presentation of the initial blood cadmium levels for females and males in each group. (Each graph plotted in increasing age order).

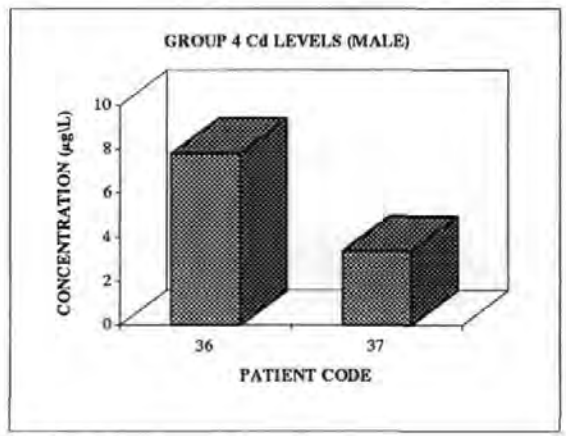










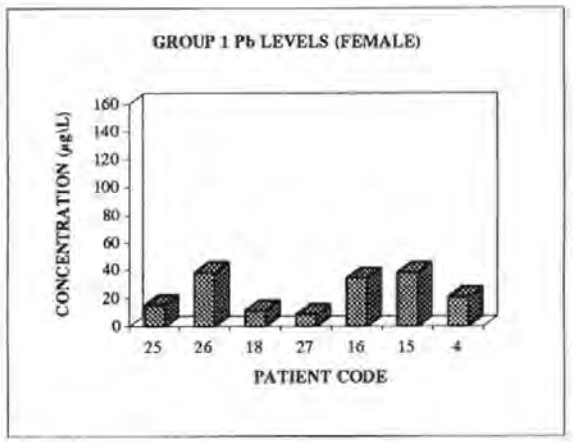


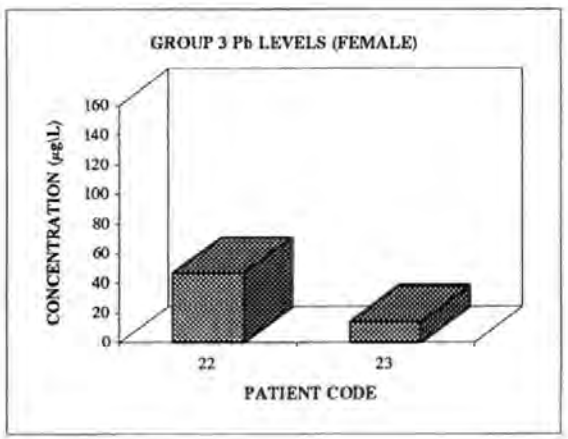
metastases). Similarly Figure 7.8 summarises the initial blood lead levels. The bulk of the experimental results gave blood leads of less than 70 $\mu g\ l^{-1}$, with only 4 out of the 27 patients (15%) with higher blood lead levels. All these patients were males in the group 1 category. This could be attributed to the release of stored lead from the skeletal system, or it could be due to higher exposure levels to lead, for example, occupational exposure. However if this was so, then similar trends should be observed in the other groups. This factor may not be apparent due to the small numbers studied. Alternatively it could be speculated that these two factors are linked in that due to occupational exposure their bone lead store was higher, hence upon bone breakdown the amounts released were greater compared to non exposed individuals. The differences were therefore much more discernable.

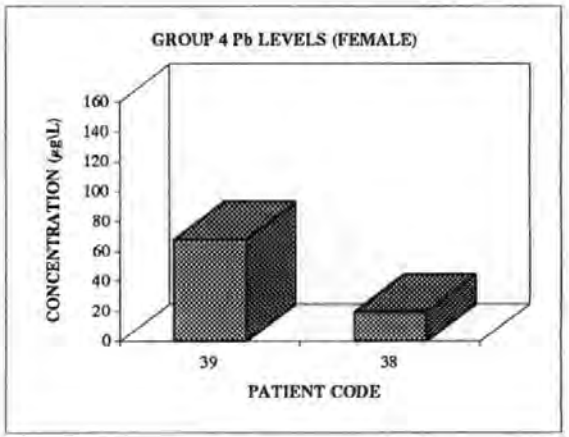
Figure 7.9 summarises the initial blood strontium values for each patient in all groups. The initial blood strontium levels were in the range of 5 - 30 $\mu g\ l^{-1}$, and fairly constant across all the groups, with the exception of one (patient code 038), which had an initial level of 60 $\mu g\ l^{-1}$. Again due to the lack of information about the individual patient, reasons for this elevated level can only be speculated, with diet being the most likely cause.

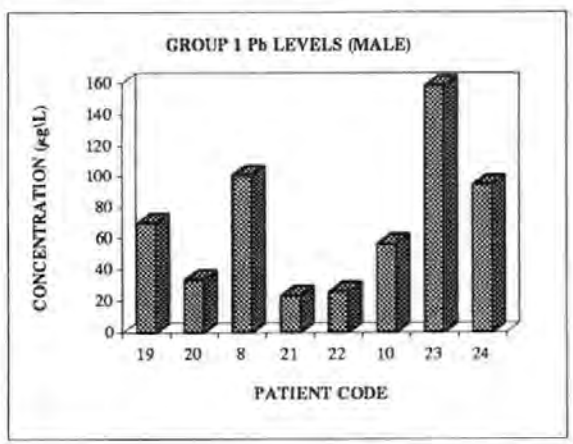
The initial urinary cadmium levels, summarised in Figure 7.10, were similar to the blood levels, with fairly constant values observed in the range 1 - 6 $\mu g\ l^{-1}$. Adjustment of the values using creatinine levels did effect the relative concentrations of cadmium in each group, and therefore will have an effect on any subsequent interpretation of the data. The urinary lead concentrations were around the normal expected values of 10 $\mu g\ l^{-1}$ or less,

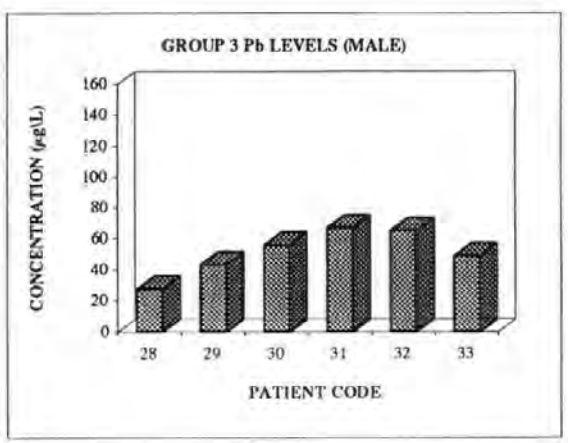
Figure 7.8: Graphical presentation of the initial blood lead levels for females and males in each group. (Each graph plotted in increasing age order).











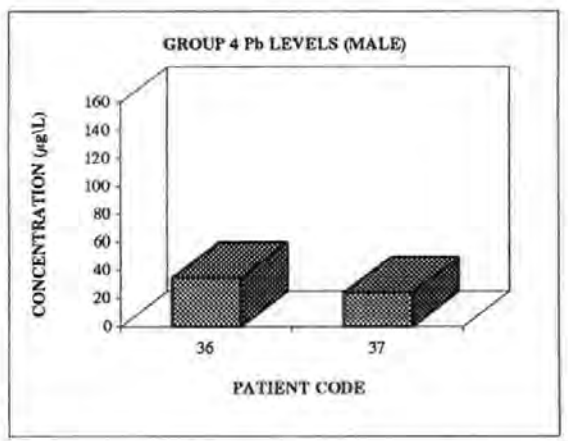
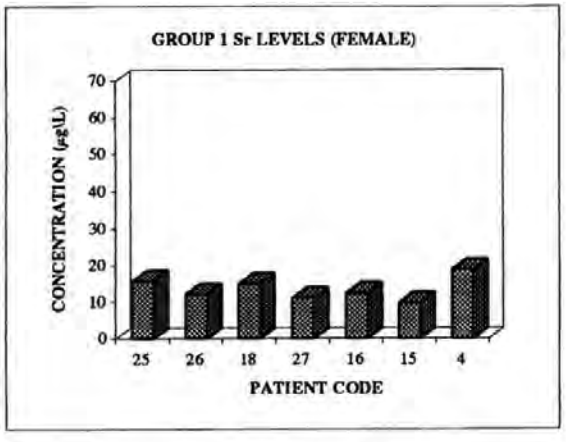
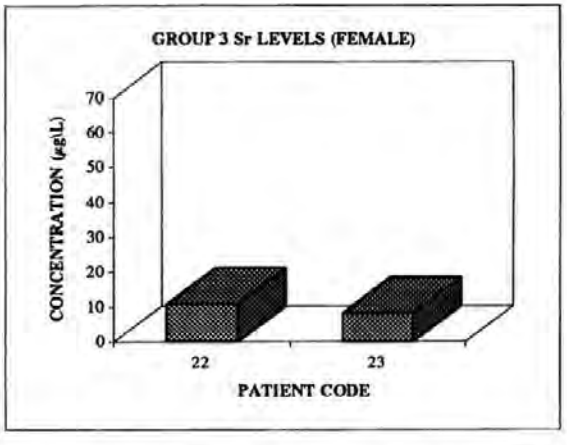
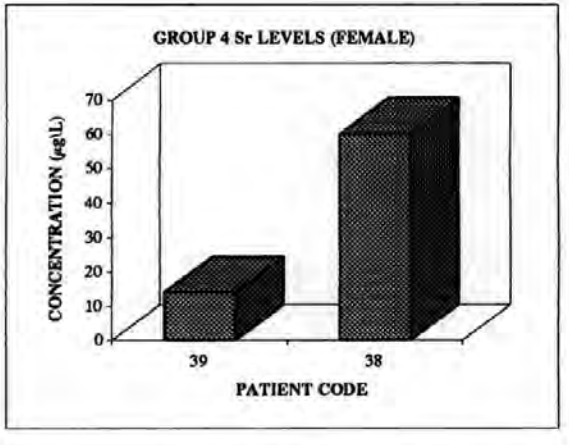
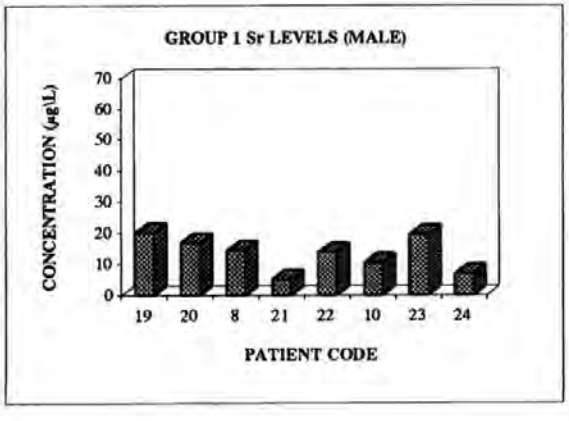


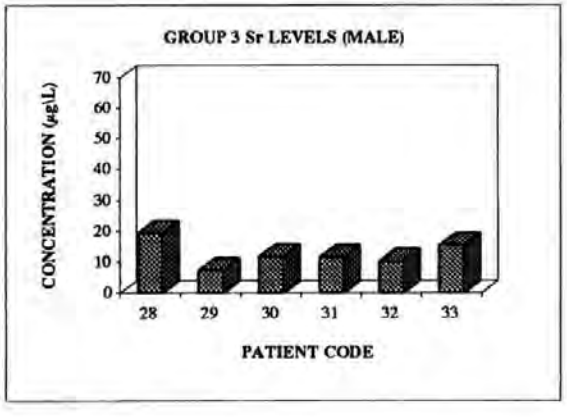
Figure 7.9: Graphical presentation of the initial blood strontium levels for females and males in each group. (Each graph plotted in increasing age order).











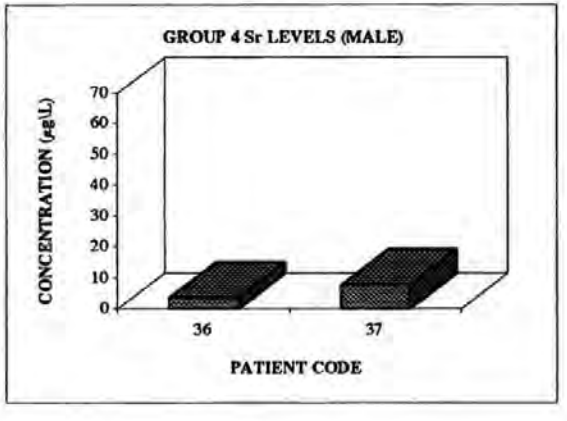
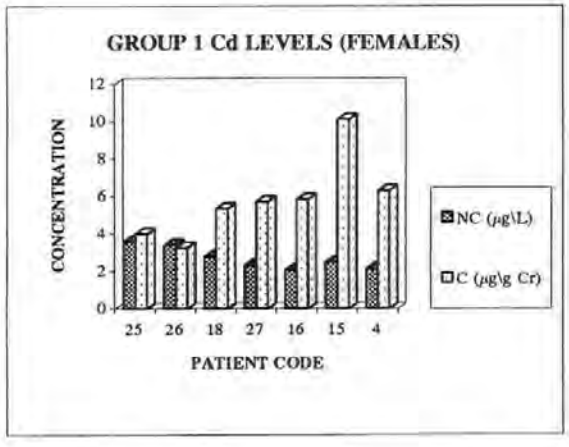
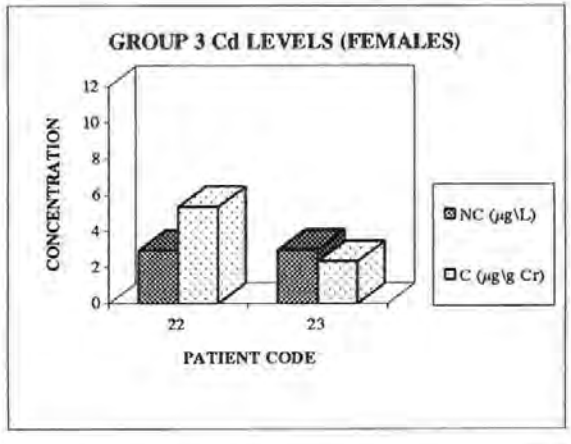
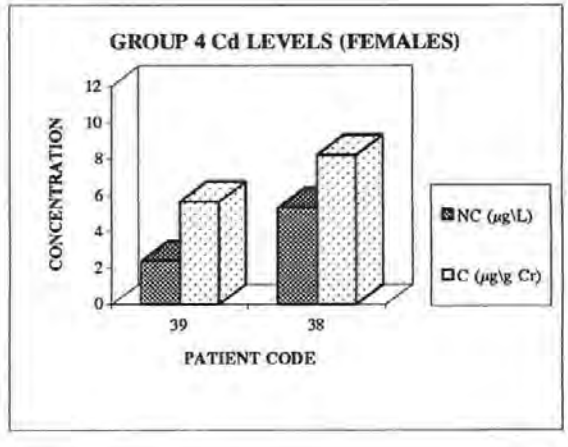
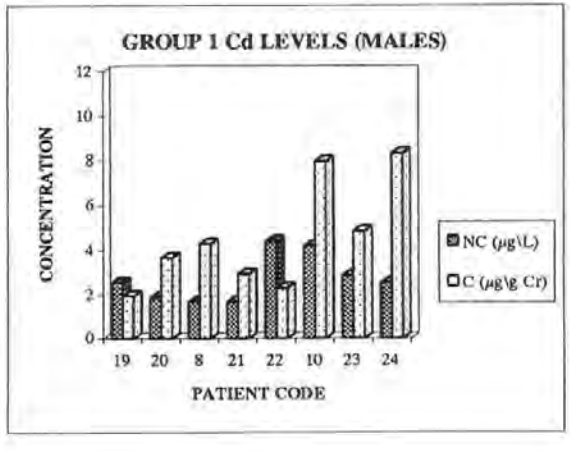


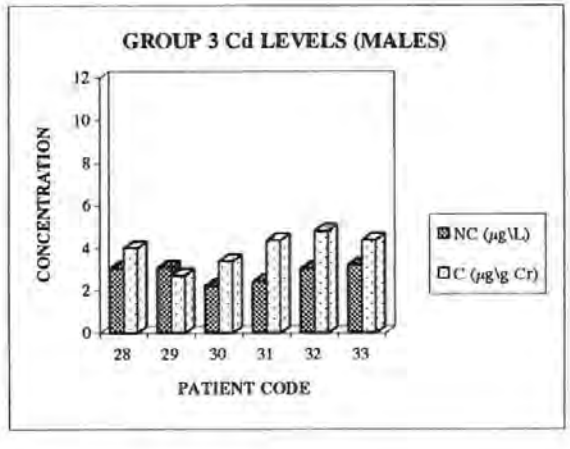
Figure 7.10: Graphical presentation of the initial urinary cadmium levels, uncorrected (NC) and corrected with creatinine (C), for females and males in each group. (Each graph plotted in increasing age order).

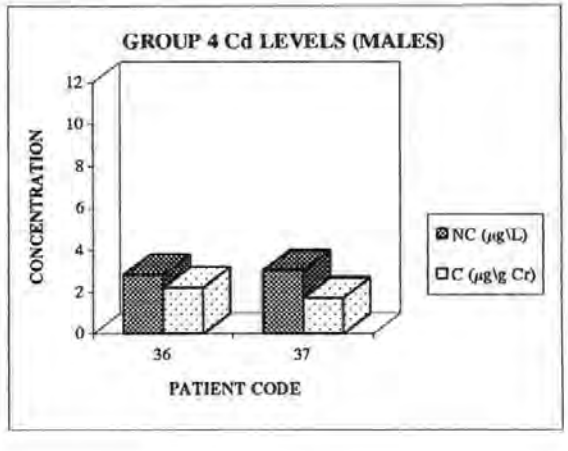










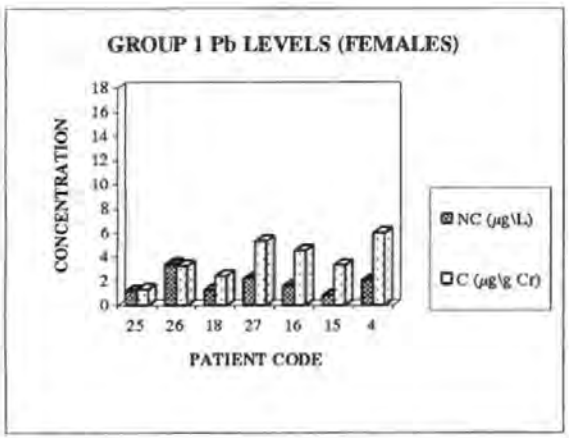


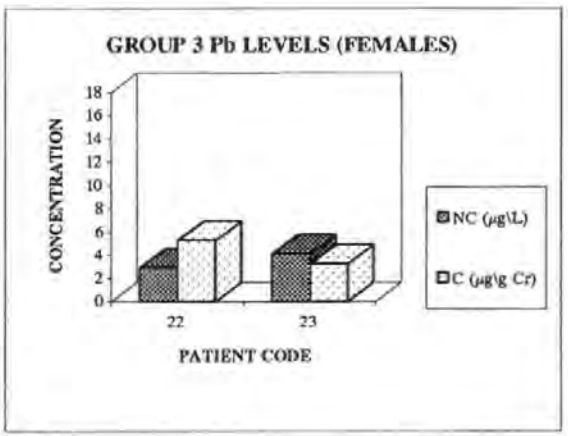
as summarised in Figure 7.11, with only a few patients showing slightly higher initial levels. Again as in the blood analyses these were all males in the group 1 category. As with the cadmium results, adjustment with creatinine had influenced the results.

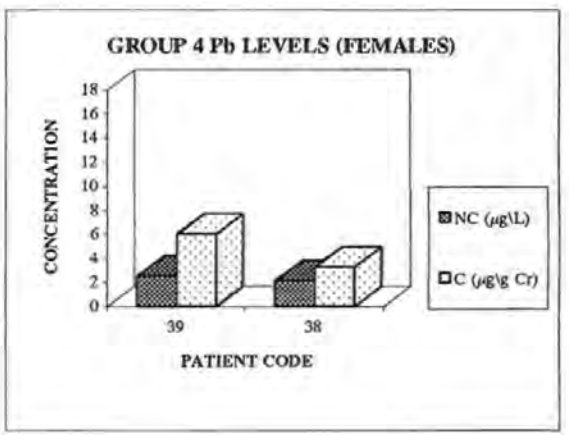
The urinary strontium levels were generally higher and more variable compared to the blood levels, with concentrations ranging from 15 - 754 $\mu g\ l^{-1}$ (Figure 7.12). Again the highest strontium level in urine corresponded to the patient with the elevated blood-Sr level. Adjustment with creatinine again made some difference to the overall end results. These observations suggest that adjustment with creatinine can change results and trends, therefore influencing any interpretation of the data. However whether adjustment offers any advantage over absolute values is not clear, and therefore both corrected and non corrected values will be assessed throughout the discussions.

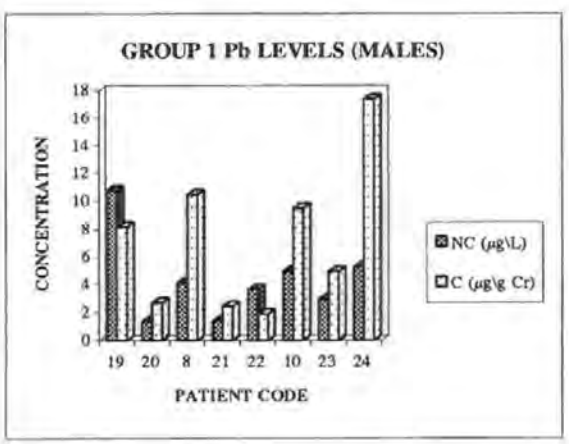
Figure 7.13 summarises the initial DPYD levels for each patient in each group. In group 1 60% of the patients had levels above the normal upper limit (7.4 nM\mM Cr for females aged 25-44 years, and 5.4 nM\mM Cr for males aged 25-55 years) (435), compared to 25% and 50% for groups 3 and 4 respectively. Since the majority of the patients were above these age limits, higher normal levels could be expected, hence the slightly higher levels seen in all the groups was not unexpected. Taking this into account, any major increases in levels can be considered representative of the extent of bone resorption, as expected in those patients diagnosed with skeletal metastases (group 1), although the extent of bone breakdown will be dependant on the effectiveness of their treatment. Figure 7.13 shows that as expected the highest levels were seen in the group 1 category, particularly the male participants. The higher levels observed for patients 030

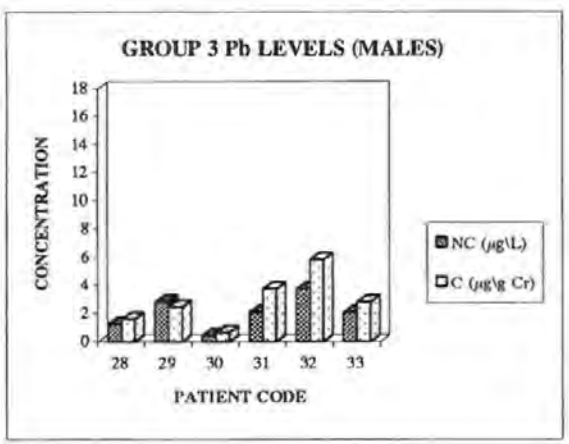
Figure 7.11: Graphical presentation of the initial urinary lead levels, uncorrected (NC) and corrected with creatinine (C), for females and males in each group. (Each graph plotted in increasing age order).











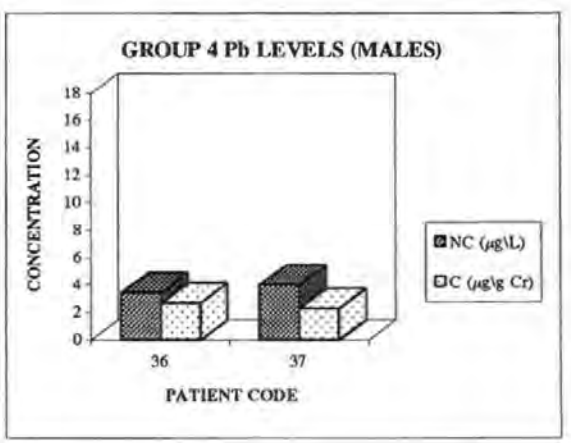
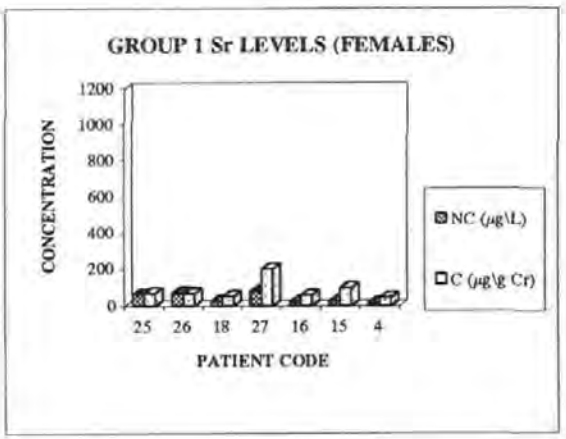
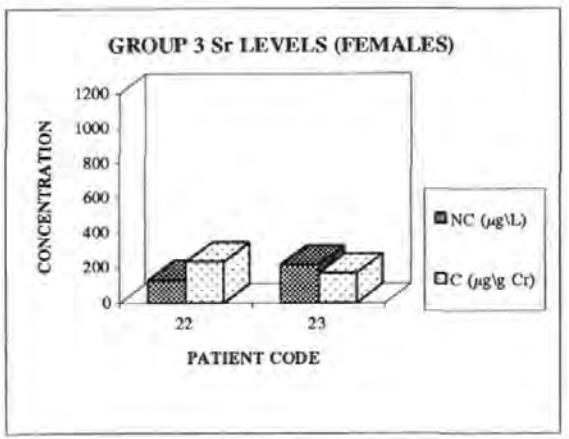
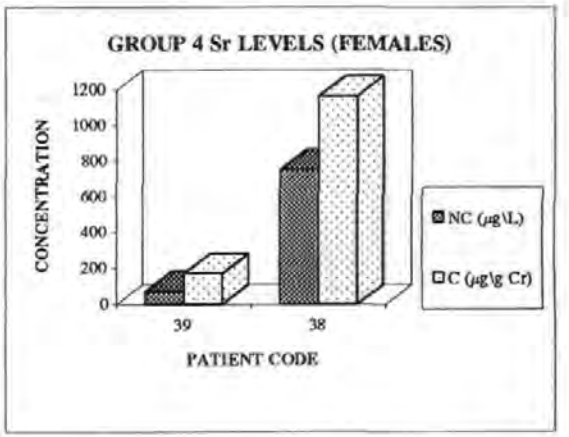
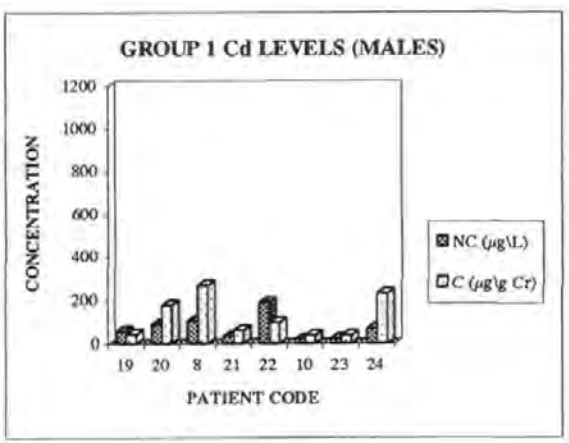


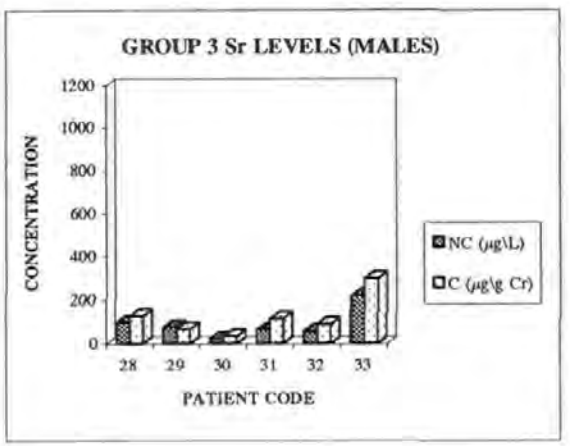
Figure 7.12: Graphical presentation of the initial urinary strontium levels, uncorrected (NC) and corrected with creatinine (C), for females and males in each group. (Each graph plotted in increasing age order).











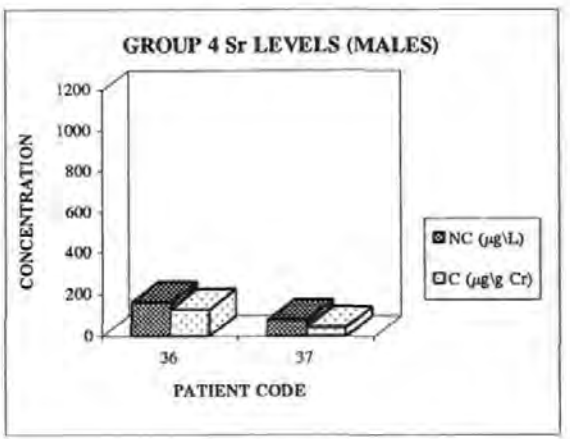
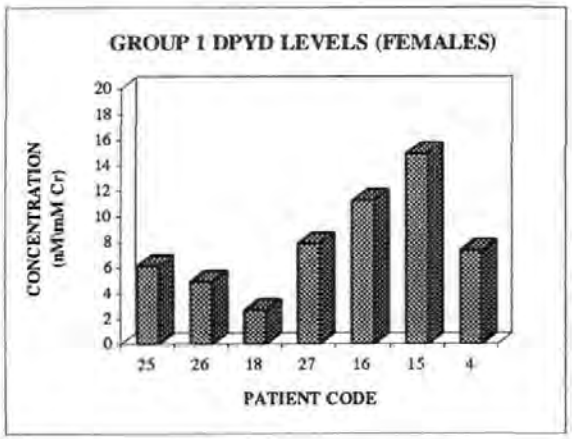
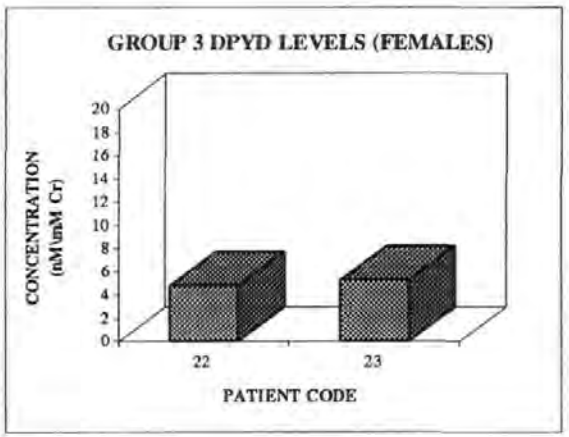
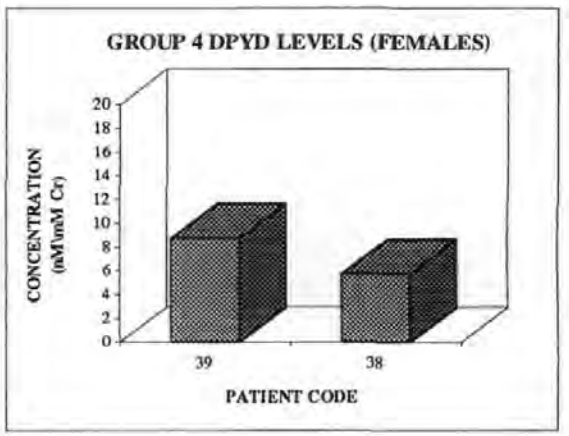
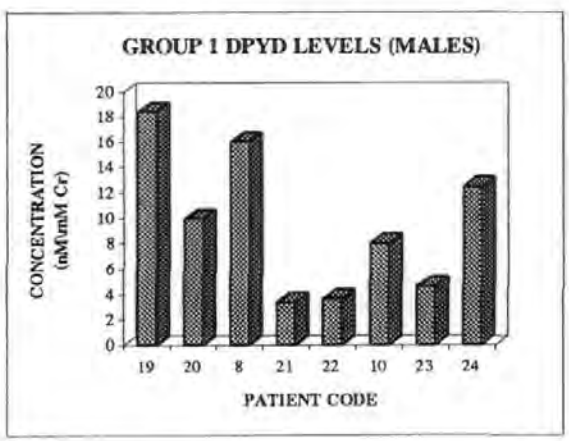


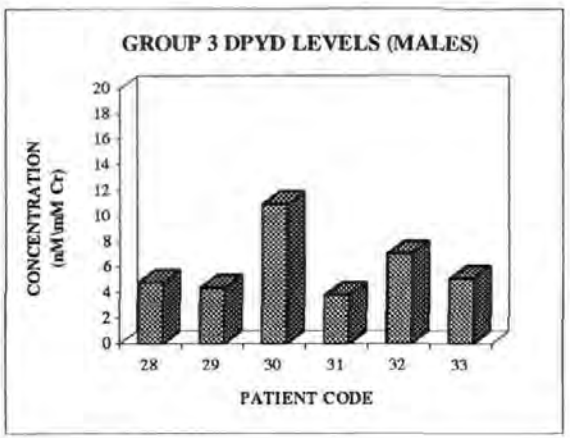
Figure 7.13: Graphical presentation of the initial urinary deoxypyridinoline crosslink levels, uncorrected (NC) and corrected with creatinine (C), for females and males in each group. (Each graph plotted in increasing age order).

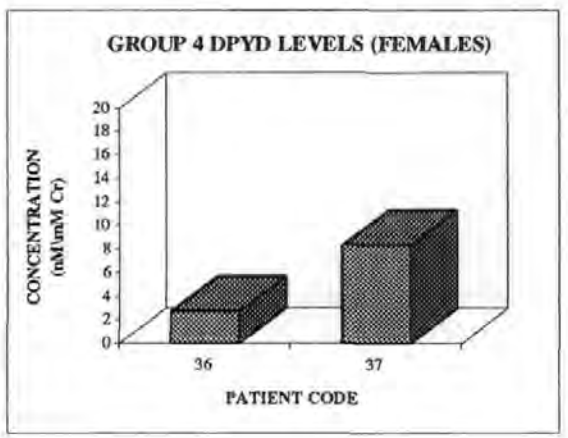












and 032, may be attributed to undiagnosed skeletal metastases. Only continued assessment will determine the predictive capability of this parameter.

7.3.2.2: Comparison of trace metal levels in blood and urine.

Although comparisons between the groups was difficult, as highlighted in the previous sections, comparisons between the analyte levels within the individual patients was possible. Those patients whose levels followed a similar pattern for all but one point plotted, were considered as showing a good similarity between levels. Those patients whose levels followed a similar pattern for at least half the points plotted were considered to show some similarities.

Table 7.6 summarises the results for each individual patient where the concentrations of the trace metals in blood were compared and correlated with the trace metal levels measured in the urine samples over time. Further details and full graphical presentations for individual patients can be found in Appendix 2.1.

Only a few patients in group 1 showed any similarity between the blood cadmium and urinary cadmium results, whereas all of group 3 and all the female patients in group 4 showed at least some degree of correlation. Only a few patients overall did not show any similarities between the blood lead and urine lead values. In contrast to lead, very few similarities were observed between the strontium levels measured in blood and urine. Correction of the urine values with creatinine also made some difference to the overall end results.

Table 7.6: Summary of trends in the trace metal levels measured in blood and urine.

Patient code	Group	B-Cd vs U-Cd		B-Pb vs U-Pb		B-Sr vs U-Sr	
		NC	C	NC	C	NC	C
004	1	Y		Y			
008	1			P	P		
010	1			Y	P	Y	P
015	1		P		Y		
016	1		Y	Y	Y		
018	1					P	
019	1	Y	P	P	P		
020	1			P	P		
021	1						
022	1	P		P	P		
023	1					Y	
024	1	Y		Y	Y		
025	1			Y	Y		
026	1		Y				
027	1						
028	3	Y	Y	P	P	P	P
029	3	Y	Y				
030	3	Y	Y	P	P		
031	3		P	Y	Y		
032	3	Y			Y		Y
033	3		P	P	P		
034	3		Y	P	P		
035	3		P	P	P		

Table 7.6: Continued.

Patient code	Group	B-Cd vs U-Cd		B-Pb vs U-Pb		B-Sr vs U-Sr	
		NC	C	NC	C	NC	C
036	4			P	P		
037	4			Y	P	Y	P
038	4	P	Y	P	P		
039	4		Y	P	P		

B-Cd Blood cadmium

U-Cd Urine cadmium

B-Pb Blood lead

U-Pb Urine lead

B-Sr Blood strontium

U-Sr Urine strontium

NC Not Corrected

C Corrected with creatinine

Y Good similarity shown

P Some similarity shown

These results indicate that in normal bone resorption (as depicted in groups 3 and 4) lead and cadmium levels in blood and urine correlate, whereas in general there is no correlation in the levels of strontium measured in blood and urine samples. However in the patients with bone metastases and therefore increased bone resorption (group 1), the lead levels maintain this good correlation, whilst the number of patients whose blood and urine cadmium levels correlate decreases. This could be attributed to possible renal or hepatic damage incurred during the course of cancer treatment or through some other medical complication. Again without the full medical and life history of the patient explanations can only be speculative.

7.3.2.3: Comparison of trace metal and deoxypyridinoline levels in urine.

Table 7.7 summarises the results for each patient where the trends in the trace metal levels in urine were compared with the urinary DPYD levels over time. Further details and full graphical presentations for individual patients can be found in Appendix 2.2. The best results were obtained for lead, followed by cadmium, and finally strontium. In all cases the extent of the correlation with DPYD was less than that for the respective blood metal values (refer to Section 7.3.2.4).

All group 3 patients and some of the group 1 and 4 patients showed at least some degree of correlation between the urinary levels of cadmium and lead with DPYD, whilst only a few group 1 patients showed some similarity between the urinary strontium and DPYD levels. Again adjusting the values with creatinine appeared to make some difference to the overall end result.

Table 7.7: Comparison of trends in the urinary trace metal levels with the urinary deoxypyridinoline crosslink levels.

Patient code	Group	U-Cd vs DPYD		U-Pb vs DPYD		U-Sr vs DPYD	
		NC	C	NC	C	NC	C
004	1	Y		Y		Y	
008	1			Y	P		
010	1						
015	1		Y		Y		
016	1					P	Y
018	1						
019	1	Y	Y	P	Y	P	P
020	1	P	P				
021	1	P		P			
022	1			P			
023	1			Y	Y		
024	1						
025	1						
026	1		Y				
027	1						
028	3	P	P	P	P		
029	3	Y	Y	Y	Y		
030	3			Y	Y		
031	3	P		Y	Y		
032	3	Y		Y			
033	3		P		P		
034	3				Y		
035	3		P	P	P	P	

Table 7.7: Continued.

Patient code	Group	U-Cd vs DPYD		U-Pb vs DPYD		U-Sr vs DPYD	
		NC	C	NC	C	NC	C
036	4		P				
037	4			Y			
038	4						
039	4		Y		P		

U-Cd	Urinary cadmium	NC	Not Corrected
U-Pb	Urinary lead	C	Corrected with creatinine
U-Sr	Urinary strontium	Y	Good similarity shown
DPYD	Deoxypyridinoline	P	Some similarity shown

7.3.2.4: Comparison of the trace metal levels in blood with urinary DPYD levels.

Table 7.8 gives a summary for each patient where the trends in the trace metal levels in blood were compared with the urinary DPYD levels over time. Further details and full graphical presentations for individual patients can be found in Appendix 2.3. The blood lead measurements gave the best results with only a small proportion showing no correlation with the DPYD levels. Contrary to the urinary results the blood strontium levels also corresponded with the DPYD levels in the majority of cases. Both lead and strontium are stored in the bone, and subsequently released into the blood during bone breakdown. These results also agreed to a large extent with the current reference marker for bone breakdown (*i.e.* the DPYD level), hence these results are particularly encouraging and offer potential for further and more detailed investigations.

The cadmium levels showed some correlation with the DPYD levels. However due to the low levels present in the blood and the fact that cadmium accumulates in the liver and kidney, rather than the skeleton, this trace metal is probably not as suitable as lead or strontium to measure bone resorption.

The interpretation of data obtained over a short time scale does not give any indication of whether the patient is showing any improvement or deteriation in their clinical condition. A much clearer picture can be attained by studying patients over a longer period. For the majority of patients recruited this was not possible, however 8 out of the 27 patients studied had blood samples collected for several months prior to the start of the extended clinical trial. Figures 7.14, 7.15 and 7.16, show the complete history of analyte concentrations measured for these patients. Throughout this study the blood lead

Table 7.8: Comparison of trends in the trace metal levels measured in blood with the urinary deoxypyridinoline crosslink levels.

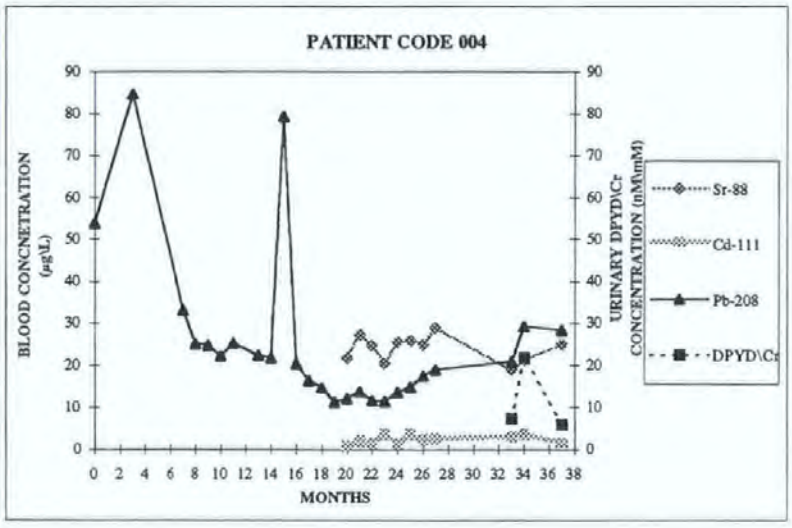
Patient code	Group	B-Cd vs DPYD	B-Pb vs DPYD	B-Sr vs DPYD
004	1	P	Y	
008	1	P	P	P
010	1	P	Y	P
015	1		Y	
016	1		P	P
018	1		Y	P
019	1		Y	Y
020	1	P	P	P
021	1			
022	1	P	Y	Y
023	1	Y	P	Y
024	1	P	P	P
025	1	Y	Y	Y
026	1	Y		
027	1		Y	Y
028	3			
029	3			P
030	3		Y	P
031	3	Y	Y	Y
032	3	Y	P	P
033	3		P	
034	3			P
035	3	P	P	

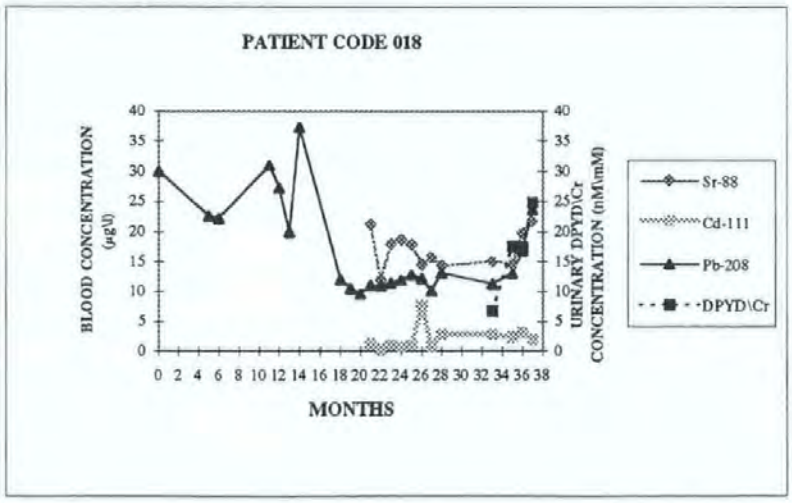
Table 7.8: Continued.

Patient code	Group	B-Cd vs DPYD	B-Pb vs DPYD	B-Sr vs DPYD
036	4		Y	Y
037	4	P	P	
038	4	Y	Y	
039	4	Y	P	P

B-Cd	Blood cadmium	Y	Good similarity shown
B-Pb	Blood lead	P	Some similarity shown
B-Sr	Blood strontium		
DPYD	Deoxypyridinoline		

Figure 7.14: Graphical presentation of the trace metal and DPYD values for patients 004, 018 and 027, studied over the longer time period.





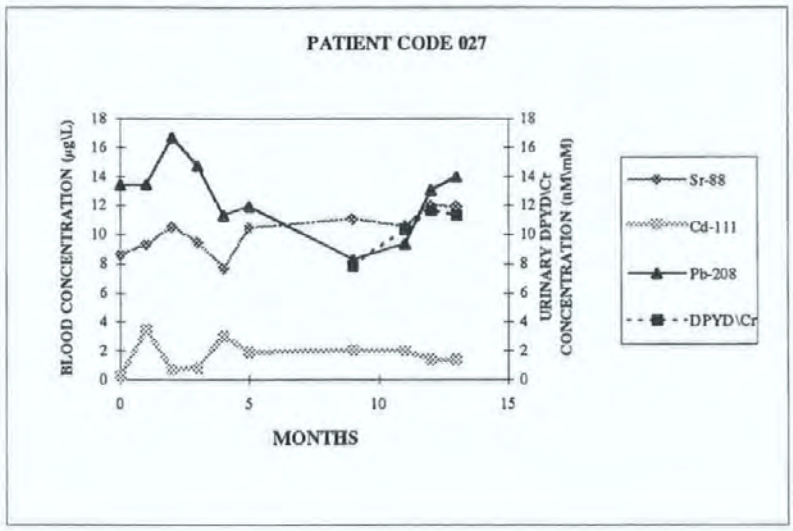
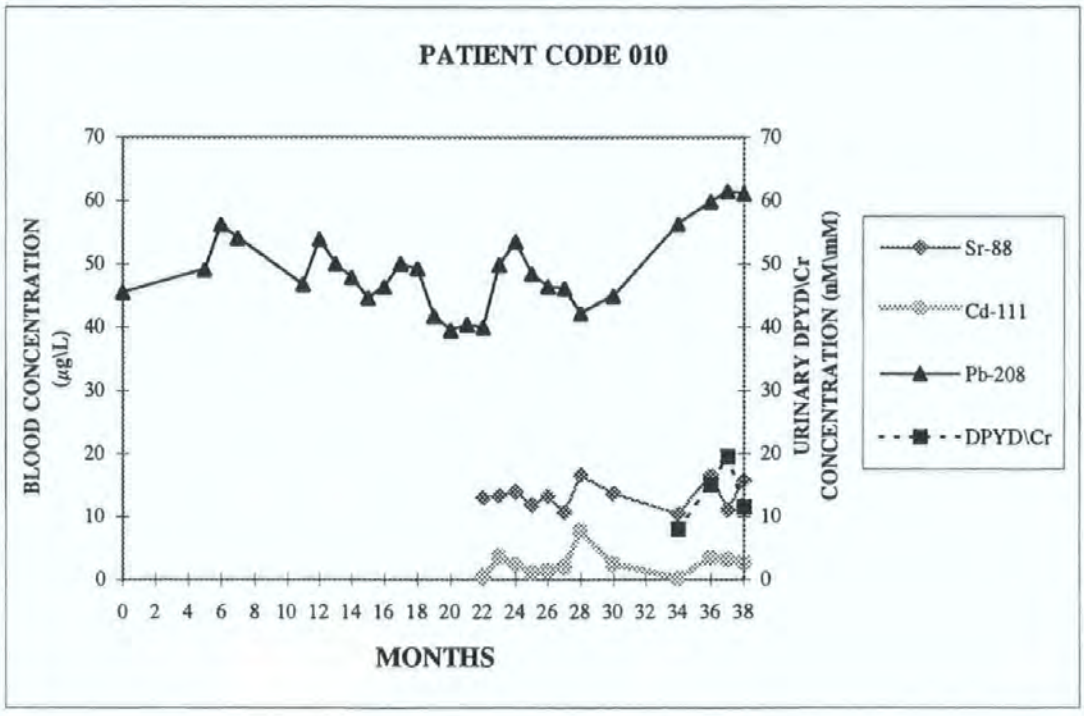


Figure 7.15: Graphical presentation of the trace metal and DPYD values for patients 010 and 016, studied over a longer time period.



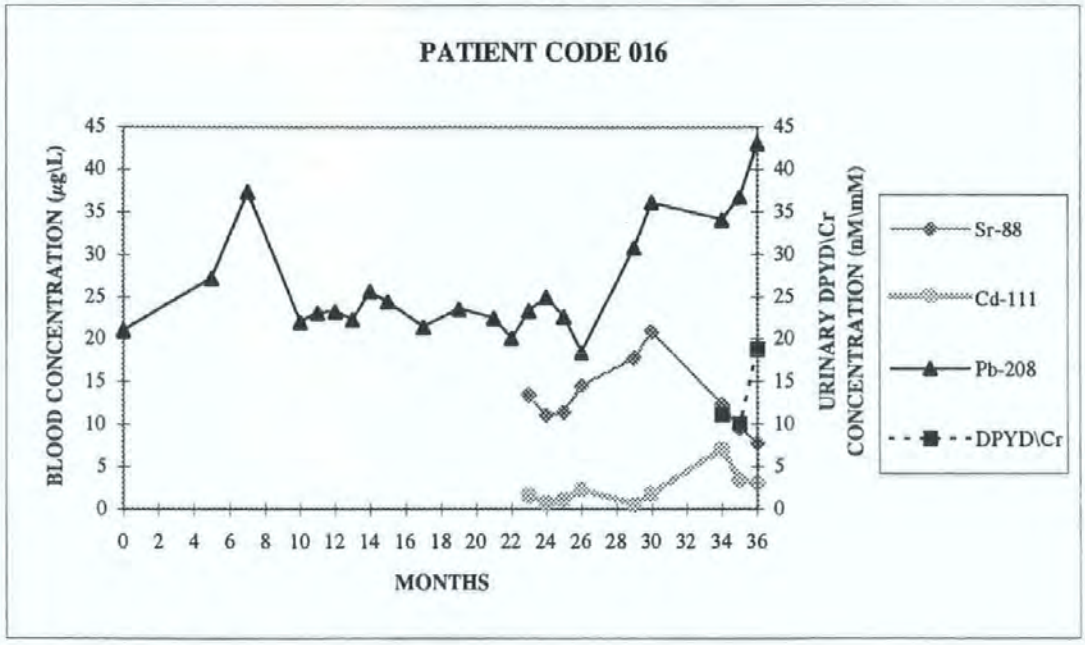
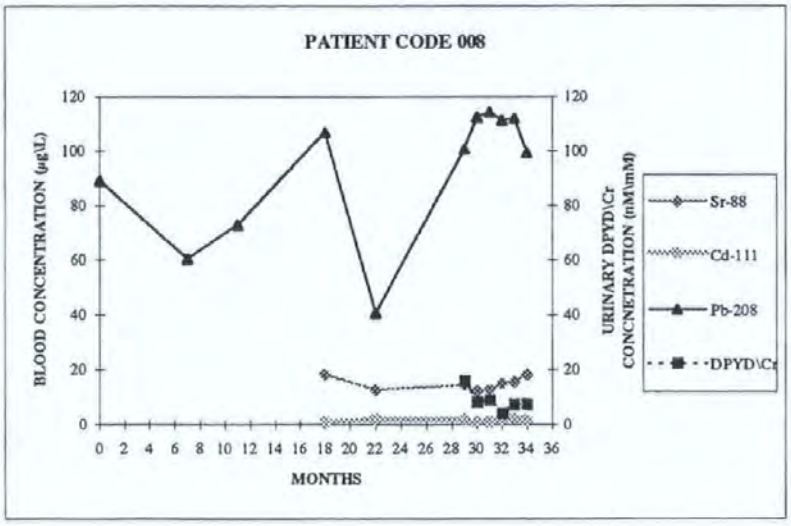
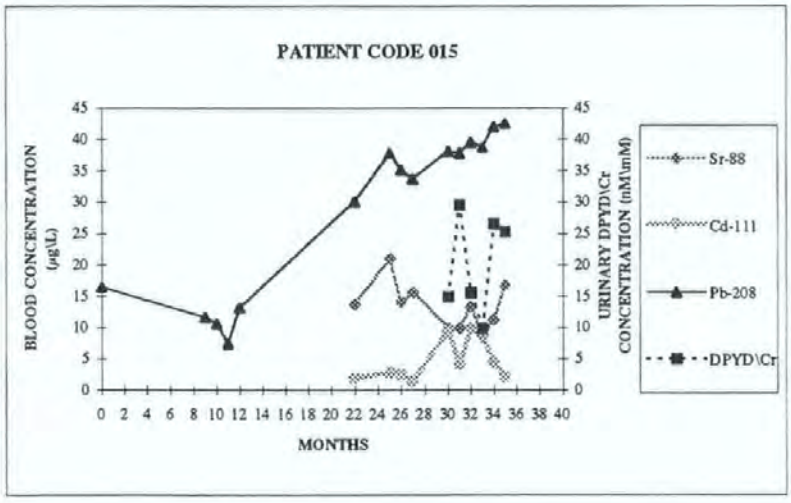
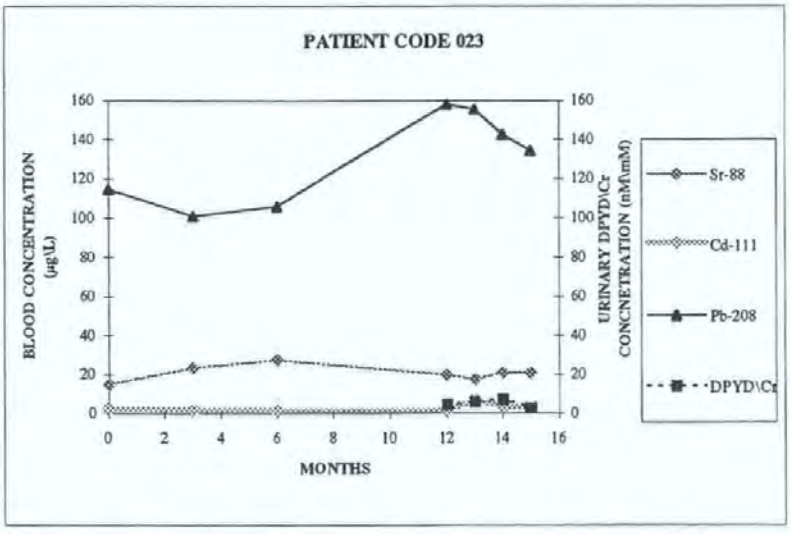


Figure 7.16: Graphical presentation of the trace metal and DPYD values for three patients who showed variable results over the longer time period.







values were measured, however cadmium and strontium were only measured in the latter stages, and of course the DPYD values were only measured during the extended clinical trial stages at the very end. In Figure 7.14 patient 004 initially showed a decrease in their blood lead levels upon the commencement of treatment. The lead levels steadily decrease over a period of 23 months, corresponding to the fact that this patient was responding well to treatment. The blood lead levels then started to steadily increase and at the very end tending to plateau or decrease slightly. This could be attributed to the patient's clinical condition no longer responding to the treatment, followed by a positive response, if the treatment was changed. Only careful evaluation with all the clinical records could confirm this hypothosis. Both the blood strontium and DPYD values correlated with the blood lead measurements.

Similarly patient 018 also showed an overall downward trend in blood lead levels over the first 20 months. The levels then remained fairly constant for a further 13 months, as the patient continued to respond well to treatment. A sharp increase in the blood lead levels occurred, which again could be attributted to a deteriation in the patients clinical condition as a result of either no longer responding to treatment, or that treatment had been stopped for whatever reason. However confirmation can only be achieved by consulting with the medical personnel and records. Both the blood strontium and DPYD values correlated well with the blood lead measurements. In patient 027, a decrease in the blood lead levels was initially observed over the first 9 months. This was followed by a sharper increase in the blood lead levels and again the blood strontium and DPYD values compared well with the blood lead values. Similar reasons to those given above can be used to interpret these observed trends.

In Figure 7.15, both patients 010 and 016, showed small fluctuations in their blood lead levels for the first 28 and 25 months respectively, as they both responded well to treatment. Thus the blood lead levels gave a good indication of the stability of their clinical condition. This was then followed by an increase in the blood lead levels, and in the case of patient 010, a levelling off in the blood lead values was shown. Again similar reasons to those given above, describing the observed trends may apply. The DPYD values corresponded with the blood lead levels, although the very last measurement for patient 010 showed more of a decrease rather than a plateau effect. In the case of the blood strontium values, for patient 010, if the measurement at month 37 was low due to an experimental error, then this parameter too corresponded well with the blood lead and the DPYD values. However if this lower value was due to natural fluctuation, then blood-Sr shows only some agreement with the blood lead and DPYD trends. The blood strontium values for patient 016 showed a constrasting decrease in levels.

In all cases shown in Figures 7.14 and 7.15, the DPYD values correlated well with the blood lead levels, and trends could be observed in the blood lead levels during the course of the study. Whilst the clinical performance of the patient was known at the beginning and in the early stages of the study, it would be extremely useful to know if the observed increased levels of blood lead and DPYD corresponded with a deteriation in the patients condition. This information is essential to ascertain the potential of the blood lead levels with regard to their use as a biochemical marker to measure bone resorption.

However as in all clinical trials there are patients who show more variable or even contrasting results as shown in Figure 7.16. Patient 008 demonstrates rather erratic blood

lead measurements, although it is also evident that the collection of blood samples every 3 or 6 months rather than once a month is not satisfactory. The blood lead measurements were in poor agreement with the DPYD or strontium values. The blood lead levels for patient 015 decreased over the first 11 months, and this was followed by an increase in the blood lead levels over the remaining period. However both the DPYD and strontium levels although rather erratic did show some correlation with each other, but did not agree with the blood lead values. In the case of patient 023, the blood lead levels tended to increase over 12 months, followed by a steady decline over the last 3 months. However neither the DPYD nor the blood strontium values showed any correlation with one another or the blood lead values.

7.4: CONCLUSIONS.

Proper interpretation of the results from this extended clinical trial is limited by the small number of patients recruited, and the fact that each patient was studied over a very short time scale (*i.e.* about 3 months). This was also compounded by the lack of information on each patient concerning their full personal and medical history, which would have provided essential information on their lifestyle, habits, occupational and geographical history etc. Consequently conclusions concerning the analtye levels measured between the different clinical categories cannot be made, and only provisional comments can be made on the information gleaned from comparing the cadmium, lead, strontium and DPYD levels in individual patients.

In terms of a preliminary feasibility study, the results obtained are more than satisfactory to warrant further and more detailed clinical studies. The results indicate that the blood

lead levels show the greatest potential as biochemical markers to measure bone breakdown. Differences in lead concentrations can be clearly distinguished between blood samples collected each month from the same patient. The blood lead levels also clearly demonstrated the best correlation with the DPYD values. The blood strontium values may also be determined, and also show reasonable correlation with the DPYD values and therefore should not be discounted from any further studies. However the blood cadmium levels were generally too low to observe any changes in concentration and hence offer only poor correlation with the DPYD values.

In general the results for the urinary levels of all the trace metals were not as good as the blood results. The urinary lead values although lower than the blood levels again gave the best results when compared to the DPYD values. The urinary cadmium levels were also low and again made differentiating differences very difficult, although some correlation was observed. The urinary strontium levels were higher, making measurement and hence differentiating differences easier, however there was little correlation between strontium and the DPYD values.

CHAPTER 8

GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK.

CHAPTER 8: GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK.

The purpose of this study was to establish if trace metals measured in blood and\or urine samples showed potential as biochemical markers to measure bone resorption in patients diagnosed with skeletal metastases. To evaluate their potential, the trace metal levels were correlated against an established reference marker, known to be both sensitive and specific for the measurement of bone resorption. Currently the best available markers are the pyridinium crosslinks, which are unique maturation products from the breakdown of mature collagen. Chapters 3 and 4 details the investigations into the two analytical methods (HPLC and ELISA) available to measure these pyridinium crosslinks: pyridinoline (PYD) and deoxypyridinoline (DPYD). Chapter 3 provided clear evidence that the use of high performance liquid chromatography was far from ideal for the routine measurement of the crosslinks and confirmed the findings published by various other establishments. The technique gave excellent qualitative information for both crosslinks. Unfortunately the sample preparation procedure, whilst selective in extracting the crosslinks from the urine sample, is very time consuming and tedious. It has also been shown in this work to be responsible for the quantitative variations observed. Thus it is the "Achilles heel" when using the crosslinks as biomarkers of bone resorption in routine clinical assessments. With improvements in the field of automation, availability of internal standards etc, the problems associated with this sample preparation procedure may be overcome to facilitate the use of HPLC on a routine basis as well as in research laboratories.

In the last few years, whilst research has focused on the pyridinium crosslinks, an immunological method has been successfully developed and became commercially available. Chapter 4 evaluated this enzyme linked immunoasorbant assay (ELISA) with respect to the measurement of the bone specific deoxypyridinoline crosslink in urine samples. The results compared well with the literature, demonstrating excellent accuracy and precision. The technique was simple, quick and easy to use, and proved more robust and less prone to interferences when compared to the HPLC methods. All these factors favour the ELISA technique for the analysis of the crosslinks, plus it offers a higher sample throughput and is easily introduced into clinical laboratories for routine assessments. The only major disadvantage is that unlike the HPLC method, ELISA cannot simultaneously measure both PYD and DPYD crosslinks. However for the purpose of the clinical trials to be conducted in this research programme the ELISA method was selected as the best and most suitable technique available. The development of this ELISA method has resulted in increased research activities with respect to clinical studies into a wide range of metabolic diseases.

Chapter 5 describes the development of a simple dilution method to analyse blood lead levels in a preliminary clinical trial. The results obtained using the method agreed closely with a certified reference material, gave excellent % RSD's and remained under control throughout the preliminary clinical trials. The results demonstrated that changes in the blood lead levels in individual patients could be identified and the measured levels relate to the clinical performance of the patients in the majority of cases. These results warranted further and more detailed clinical trials.

In the extended clinical trials a number of other elements (in addition to lead) were selected to investigate their potential as biochemical markers to measure bone breakdown. Chapter 6 emphasises the need for a thorough investigation into all aspects of the analytical methodology, when measuring the trace metals in biological fluids in conjunction with clinical trials. The need for a robust, accurate and precise method must be emphasised alongside the needs and praticalities of the clinical trials, the patients needs, as well as the medical and scientific requirements. This is often difficult to achieve and a number of compromises may be necessary. The results of the initial investigations identified seven elements (aluminium, barium, cadmium, cerium, lead, strontium and zirconium) to be incorporated into the extended clinical trials. Unfortunately Al, Ba and Ce had to be excluded from the trial due to major contamination from the glass Vacutainers used to collect the blood samples (the hospital was unable to collect the blood samples by alternative means). High blank levels were consistently attained for Zr and after extensive investigations it was concluded that this contamination could probably be attributed to the ICP-MS instrumentation, and hence Zr was also excluded from the clinical trial. Investigations into Cd, Pb and Sr demonstrated no contamination problems during the sample collection procedure. Calibration using aqueous standards compared well with matrix matched standards. An interference study evaluating the effects of the major potential interferants found in biological samples (K, Na, Mg, Ca, Cl, PO_4^{3-} and SO_4^{3-}) showed that any suppression or enhancement of the analyte signal was satisfactorily corrected by use of the internal standards. The limits of detection were also at acceptable levels, between 0.1 - 0.5 $\mu g\ l^{-1}$ (based on the average value + 3 S.D, n=10). Hence, after all the analytical and clinical considerations were taken into account only cadmium, lead and strontium were finally selected to be measured in the subsequent extended

clinical trials.

Chapter 7 discusses the results of the extended clinical trial where the blood and urinary levels of cadmium, lead and strontium were compared with the reference marker, urinary deoxypyridinoline (DPYD). The blood lead levels showed the greatest potential as a marker of bone resorption, with values closely related to the DPYD values over time for the majority of patients studied. The blood strontium values showed less of a correlation with the DPYD values, but demonstrated sufficient promise not to be discounted from this feasibility study. The blood cadmium levels along with the urine cadmium levels were generally too low to distinguish any changes in the concentration levels over time. The urinary lead levels showed some correlation with the DPYD values but to a lesser extent than the blood levels. This could be attributed to the fact that the urinary lead levels were lower and therefore any changes in concentration over time were much more difficult to observe. In contrast to the blood results the urinary strontium levels did not correlated well the DPYD levels, despite showing higher concentration values.

Since both lead and strontium accumulate in the skeleton, and cadmium accumulates in the liver and kidney, it was not surprising that cadmium should show the least potential as a biochemical marker of bone breakdown. The results for this feasibility study are encouraging with the measurement of blood lead and to a lesser extent blood strontium levels showing the greatest potential as biochemical markers to assess bone breakdown. However more conclusive evidence is required, and hence more detailed clinical trials should be conducted to explore this more fully. However the results presented in this thesis suggest that the lead and strontium stored in the skeletal system, and subsequently

released into the blood upon bone resorption can be used as potential biochemical markers to measure bone breakdown in skeletal metastases and any other bone diseases.

It is essential that any future clinical trials should encompass a much larger number of patients, and that each patient is studied for a much longer period of time (minimum of six months). It is also essential that a full patient history is obtained, where details concerning their dietary, drinking and smoking habits, as well as their occupational, geographical and medical history can be incorporated into the full statistical evaluation of the results. Only on this basis can a viable statistical evaluation be conducted, from which clearer evidence and firmer conclusions can be drawn.

The wider implications of this research, suggest these results could provide a valuable insight into bone metabolism, trace metal metabolism, the behaviour of bone metastases, as well as relevancy to other conditions, such as osteoporosis and trace metal toxicology. The improvements suggested for the analytical techniques and methodologies, along with a greater understanding and awareness with respect to contamination control, has enabled sensitive, accurate and precise measurement of many trace elements in complex matrices, including biological samples to be made. Clearly quality control\assurance measures are also important. The measurement of trace elements has helped to study, understand and evaluate their biological significance and the essential and\or toxic roles they play in human health and disease. As technology and our understanding improves, greater opportunities will exist to explore the relevance of other potentially essential and\or toxic elements, and how these element interact with one another. Further metals may be used in drug formulations and treatments. Research will also continue in order to ascertain

how and why elements and their species, which constitute the foundations of all living biological processes, dictate health and the pathology of disease. These exciting advances will therefore place greater emphasis and responsibility on the analyst, clinicians and the scientific and medical fraternity in general.

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APPENDICES.

Appendix 1.1: Further details on UK cancer incidence, for males and females.

MALES

Site	Number of new cases registered in: England	Scotland	Wales	N. Ireland	UK	UK rate per million pop.	UK Rank
All neoplasms	116144	13193	8246	2718	140301	5044	
All malignant neoplasms	112643	12828	7824	2661	135956	4888	
Bladder	7608	862	598	117	9185	330	4
Bone	274	22	39**	10	243	12	27
Brain	1672	162	125	39	1998	72	13
Breast	175	13	11	6	205	7	32
Colon	7254	814	419	210	8697	313	5
Connective tissue	539	59	47	13	658	24	22
Eye	193	43	9	1	246	9	30
Gallbladder	508	68	40	16	632	23	24
Hodgkin's Disease	643	81	52	22	798	29	20
Kidney	2160	259	173	61	2653	95	12
Larynx	1592	195	121	46	1954	70	14
Leukaemia	2660	260	188	65	3173	114	11
Lip	188	44	21	17	270	10	28
Liver	694	103	92	24	913	33	18
Lung	24908	3040	1618	600	30166	1085	1
Melanoma	1405	203	92	16	1716	62	15
Mouth	511	94	37	15	657	24	23
Multiple Myeloma	1335	130	95	32	1592	57	16
Non-Hobgkin'sLymphoma	3071	287	203	74	3635	131	8
Oesophagus	2843	319	206	76	3444	124	10
Pancreas	2847	304	226	93	3470	125	9
Penis & other genital organs	350	43	19	8	420	15	26
Pharynx	656	90	53	21	820	29	19
Pleura	580	107	9	19	715	26	21
Prostate	11761	1225	746	242	13974	502	3
Rectum	5345	479	487	140	6451	232	7
Skin (non-melanoma)	15749	1860	941	279	18829	677	2
Small intestine	170	23	9	4	206	7	31
Stomach	6437	721	530	152	7840	282	6
Testis	1169	148	74	27	1418	51	17
Tongue	383	51	36	8	478	17	25
Thyroid	213	31	11	9	264	9	29
Population (in thousands)	23189.5	2462.3	1386.5	774.5	27812.8		

** Bone cancer cases for Wales are currently being evaluated

Source: Cancer Research Campaign Factsheet 1.2 1994 (32)

FEMALES

Site	Number of new cases registered in: England	Scotland	Wales	N. Ireland	UK	UK rate per million pop.	UK Rank
All neoplasms	135510	16993	9854	3068	165425	5655	
All malignant neoplasms	111840	13339	7951	2747	135877	4645	
Bladder	3033	388	245	50	3716	127	10
Bone	175	21	39**	8	243	8	28
Brain	1266	145	125	39	1575	54	17
Breast	24777	2665	1738	690	29870	1021	1
Cervix	4010	460	390	83	4943	169	8
Carcinoma in situ of cervix	17513	2883	1366	225	21987	752	
Colon	8831	1069	515	224	10639	364	4
Connective tissue	468	50	41	13	572	20	23
Eye	181	28	9	3	221	8	30
Gallbladder	655	93	54	13	815	28	19
Hodgkin's Disease	516	62	38	16	632	22	21
Kidney	1347	207	94	29	1677	57	16
Larynx	334	48	34	6	422	14	24
Leukaemia	2225	227	158	49	2659	91	14
Lip	55	14	3	0	72	2	32
Liver	435	62	73	24	594	20	22
Lung	11080	1579	699	269	13627	466	3
Melanoma	2256	300	138	28	2722	93	13
Mouth	294	64	26	12	396	14	26
Multiple Myeloma	1260	118	88	38	1504	51	18
Non-Hobgkin'sLymphoma	2773	327	170	76	3346	114	12
Oesophagus	1953	282	175	54	2464	84	15
Ovary	4825	579	340	88	5832	199	5
Pancreas	2984	319	195	80	3578	122	11
Pharynx	350	32	29	10	421	14	25
Placenta	7	1	0	0	8	0	33
Pleura	114	17	4	4	139	5	31
Rectum	4176	453	403	113	5145	176	6
Skin (non-melanoma)	14501	1859	823	272	17455	597	2
Small intestine	180	19	17	8	224	8	29
Stomach	3991	508	346	130	4975	170	7
Tongue	233	27	15	8	283	10	27
Thyroid	579	68	54	14	715	24	20
Uterus	3564	348	226	41	4179	143	9
Population (in thousands)	24346.8	2631.7	1470.5	803.6	29252.6		

** Bone cancer cases for Wales are currently being evaluated

Source: Cancer Research Campaign Factsheet 1.3 1994 (33)

Appendix 1.2: Further details on UK cancer mortality rates for males and females.

MALES

Site	Number of new cases registered in: England & Wales**	Scotland	N. Ireland	UK	UK rate per million pop.	UK Rank
All neoplasms	73689	7770	1883	**83342**	2915	
All malignant neoplasms	72835	7652	1854	**82341**	2880	
Bladder	3140	295	51	**3486**	122	6
Bone	133	6	4	**143**	5	24
Brain	1479	167	41	**1687**	59	12
Breast	88	3	0	**91**	3	28
Colon	5107	532	156	**5795**	203	3
Connective tissue	313	30	6	**349**	12	19
Eye	38	5	0	**43**	2	30
Gallbladder	219	30	4	**253**	9	21
Hodgkin's Disease	156	19	7	**182**	6	23
Kidney	1587	161	36	**1784**	62	11
Larynx	635	76	18	**729**	25	15
Leukaemia	1888	153	56	**2097**	73	10
Liver	982	110	38	**1130**	40	14
Lung	21114	2645	511	**24270**	849	1
Melanoma (skin)	647	57	14	**718**	25	16
Mouth	272	45	6	**323**	11	20
Multiple Myeloma	1064	87	31	**1182**	41	13
Non-Hohgkin'sLymphoma	2001	177	69	**2247**	79	9
Oesophagus	3539	403	87	**4029**	141	5
Pancreas	2778	241	70	**3089**	108	8
Penis & other genital organs	114	10	5	**129**	5	25
Pharynx	479	69	13	**561**	20	17
Pleura	371	52	24	**447**	16	18
Prostate	8686	740	209	**9635**	337	2
Rectum	2827	349	60	**3236**	113	7
Skin (non-melanoma)	257	23	9	**289**	10	22
Small intestine	108	11	5	**124**	4	26
Stomach	4636	432	130	**5198**	182	4
Testis	77	8	5	**90**	3	29
Thyroid	91	5	6	**102**	4	27
Population (in thousands)	25303.6	2486.2	801.8	**28591.6**		

** Provisional data

Source: Cancer Research Campaign Factsheet 3.2 1995 (34)

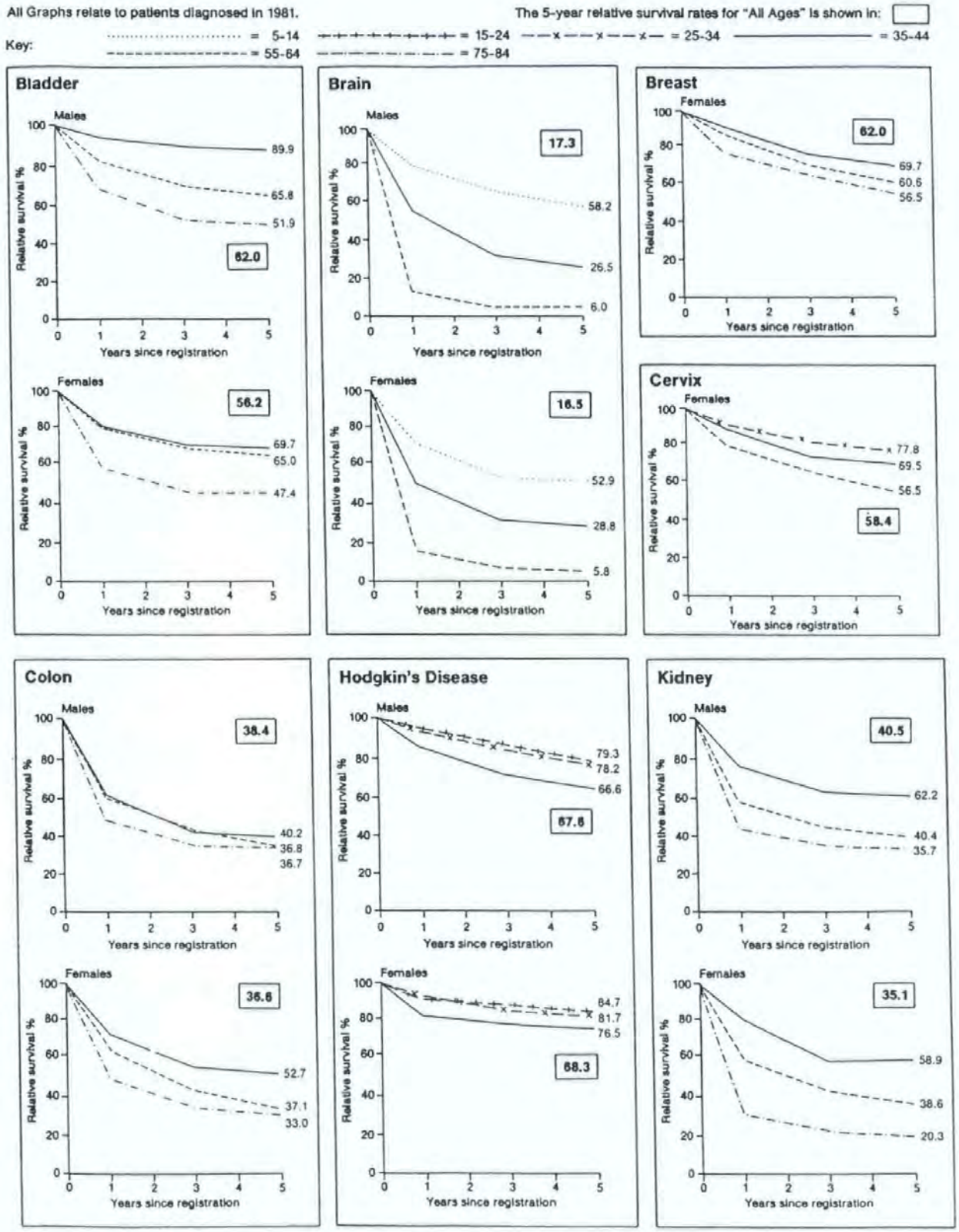
FEMALES

Site	Number of new cases registered in: England & Wales**	Scotland	N. Ireland	UK	UK rate per million pop.	UK Rank
All neoplasms	67928	7624	1782	**77334**	2595	
All malignant neoplasms	66877	7512	1741	**76130**	2554	
Bladder	1625	166	32	**1823**	61	10
Bone	71	12	4	**87**	3	28
Brain	1110	135	33	**1278**	43	13
Breast	12830	1275	338	**14443**	485	1
Cervix	1369	154	38	**1561**	52	12
Colon	5781	607	168	**6556**	220	3
Connective tissue	331	35	12	**378**	13	20
Eye	49	11	0	**60**	2	30
Gallbladder	420	56	19	**495**	17	19
Hodgkin's Disease	120	21	5	**146**	5	26
Kidney	976	129	21	**1126**	38	15
Larynx	183	31	8	**222**	7	22
Leukaemia	1617	137	38	**1792**	60	11
Liver	781	78	41	**900**	30	16
Lung	11009	1592	257	**12858**	431	2
Melanoma	731	50	22	**803**	27	18
Mouth	187	28	3	**218**	7	24
Multiple Myeloma	1054	114	34	**1202**	40	14
Non-Hohgkin'sLymphoma	1820	206	44	**2070**	69	9
Oesophagus	2182	312	56	**2550**	86	7
Ovary	3858	445	90	**4393**	147	4
Pancreas	3027	310	79	**3416**	115	5
Pharynx	224	35	6	**265**	9	21
Pleura	62	8	0	**70**	2	29
Rectum	2195	253	54	**2502**	84	8
Skin (non-melanoma)	179	19	2	**200**	7	25
Small intestine	88	17	3	**108**	4	27
Stomach	2951	322	71	**3344**	112	6
Thyroid	192	20	7	**219**	7	23
Uterus	705	112	17	**834**	28	17
Population (in thousands)	26316.9	2646.2	839.9	**29803**		

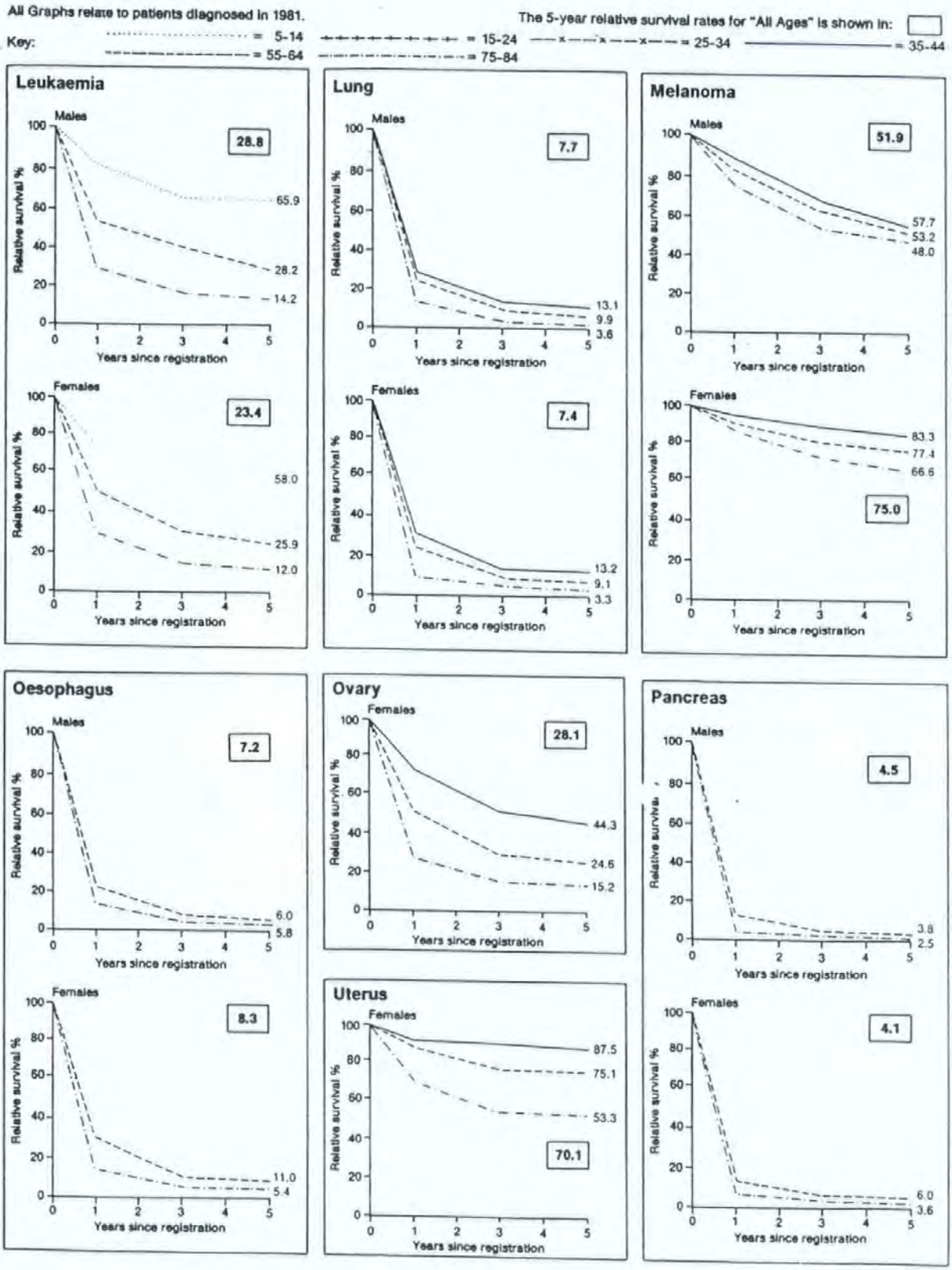
** Provisional data

Source: Cancer Research Campaign Factsheet 3.3 1995 (35)

Appendix 1.3: Further details on cancer survival rates in England and Wales.

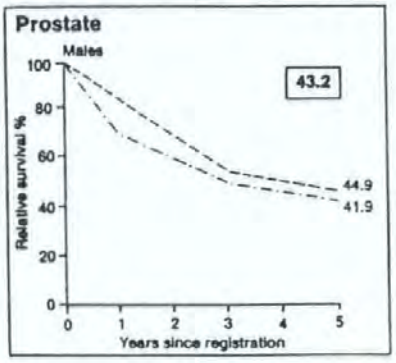


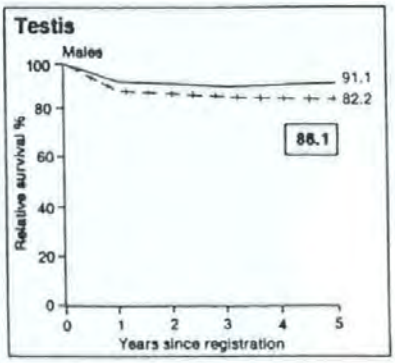
Appendix 1.3: Continued.

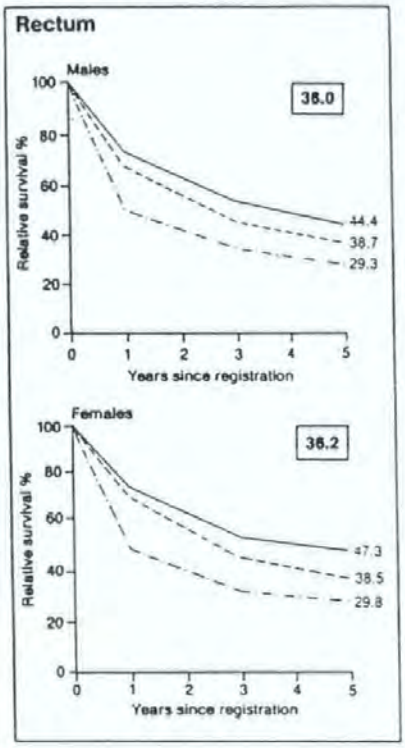


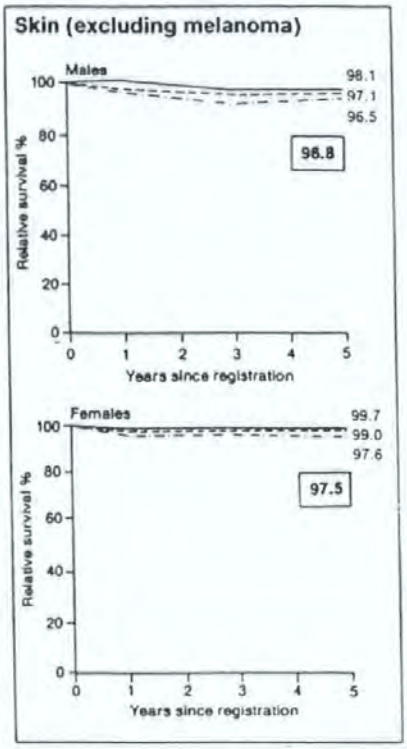
All Graphs relate to patients diagnosed in 1981. The 5-year relative survival rates for "All Ages" is shown in: ☐

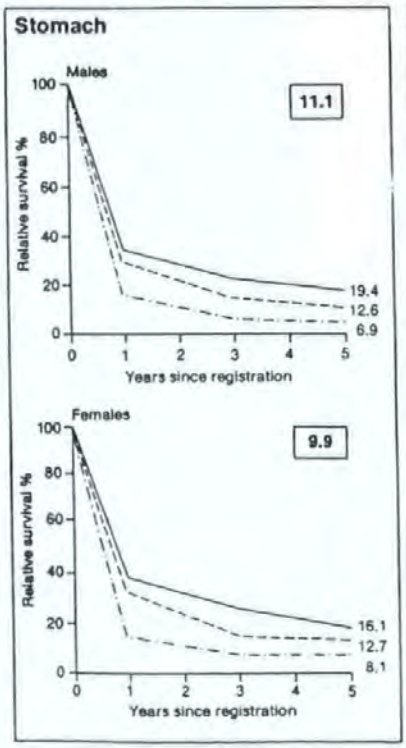
Key: ····· = 5-14 +-+-+-+-+-+-+-+ = 15-24 —×—×—×— = 25-34 ——— = 35-44 ——— = 55-64 —·—·—·—·— = 75-84







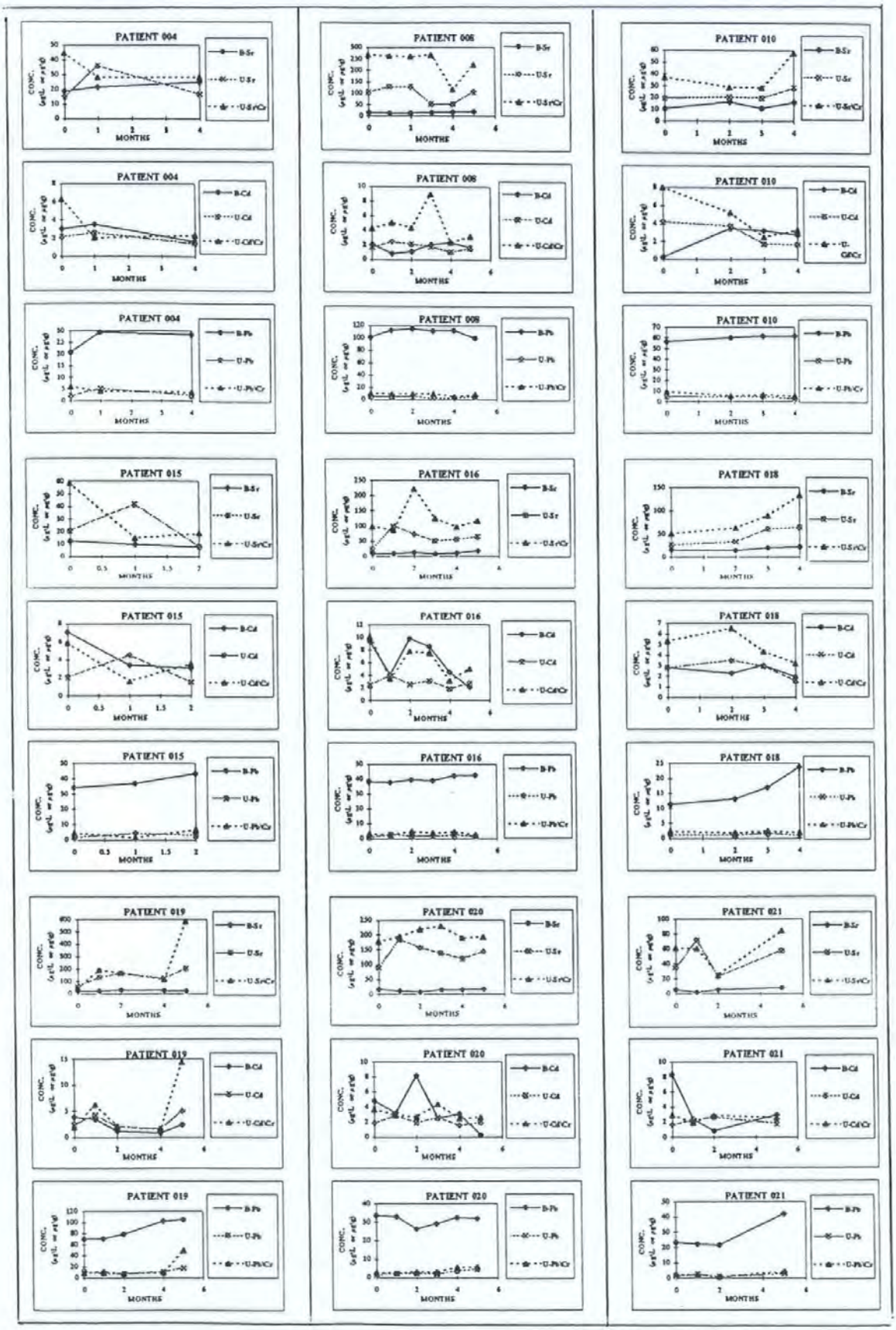




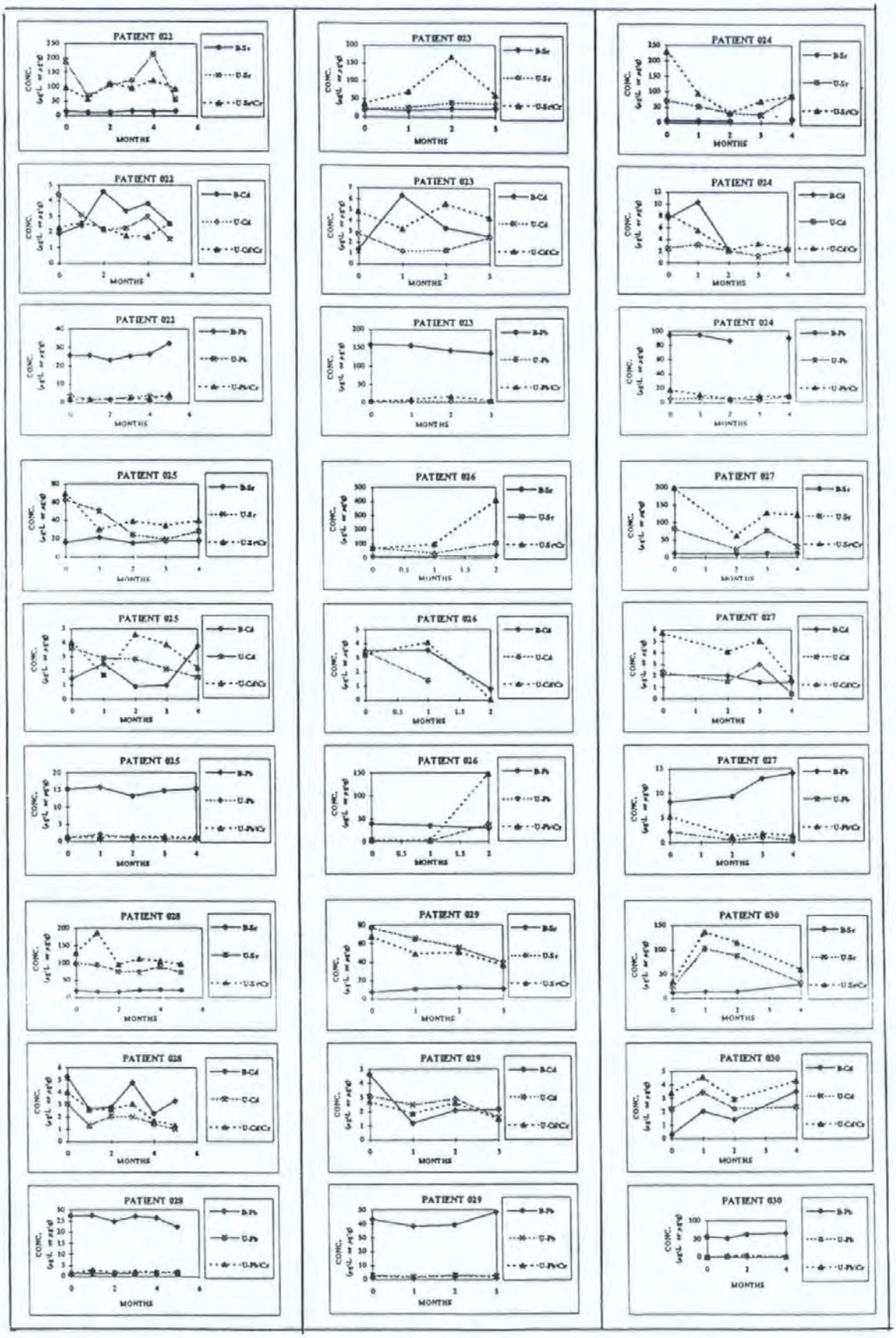
England & Wales 1981
5-Year Relative Survival Rates

Site	Males	Females
Bone	43.2	36.9
Connective tissue	50.4	54.4
Eye	71.9	60.9
Gallbladder	10.0	7.9
Larynx	65.3	53.0
Liver	4.1	5.3
Mouth: gum	40.7	58.2
floor of mouth	39.0	52.3
Multiple myeloma	17.9	19.5
Pharynx: oropharynx	26.3	36.4
nasopharynx	31.1	39.8
hypopharynx	15.1	18.5
Placenta	—	92.5
Pleura	5.6	4.1
Small Intestine	21.2	30.8
Thyroid	58.9	64.9

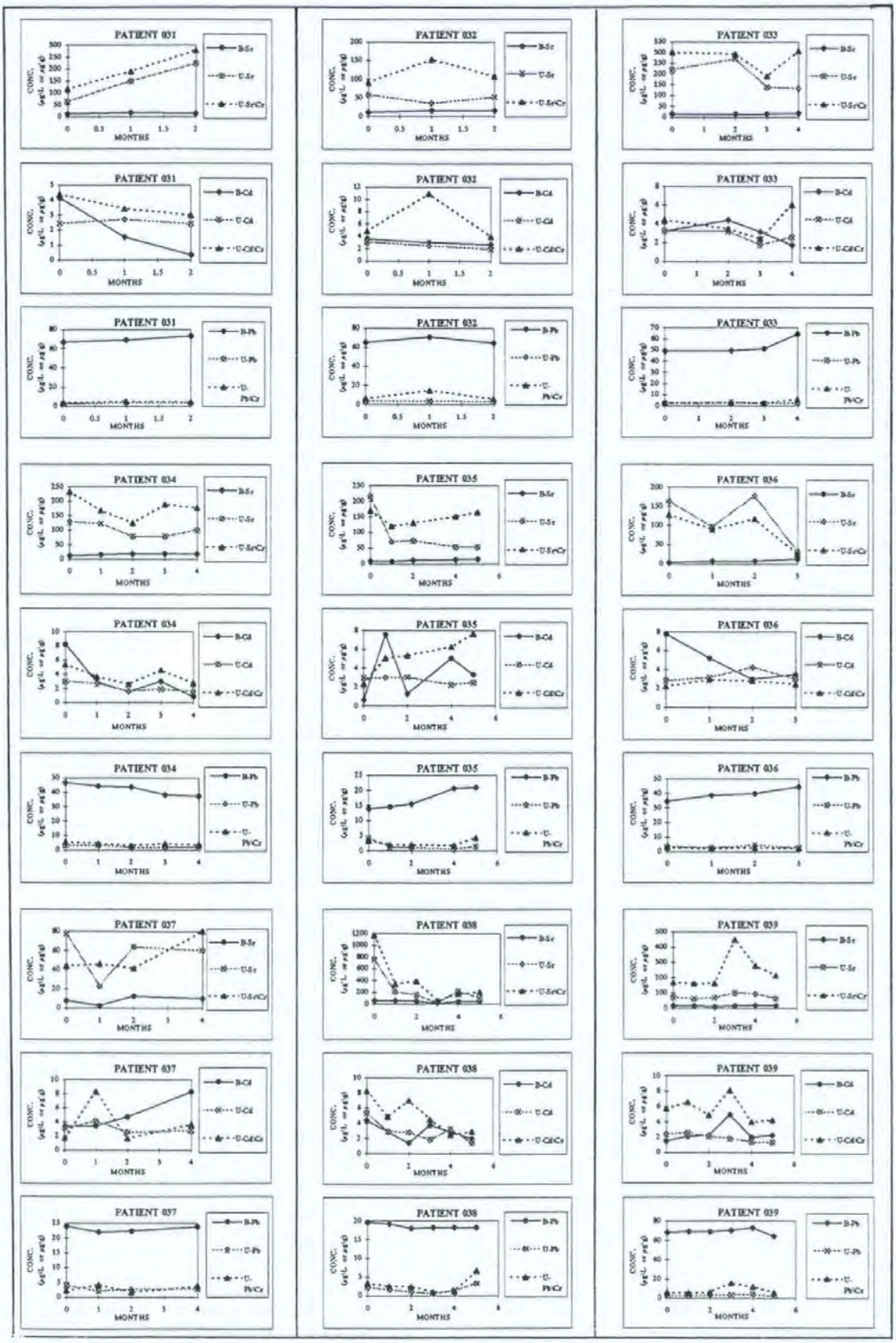
Appendix 2.1: Graphical presentation of individual patient results - comparison of the trace metal levels in blood and urine.



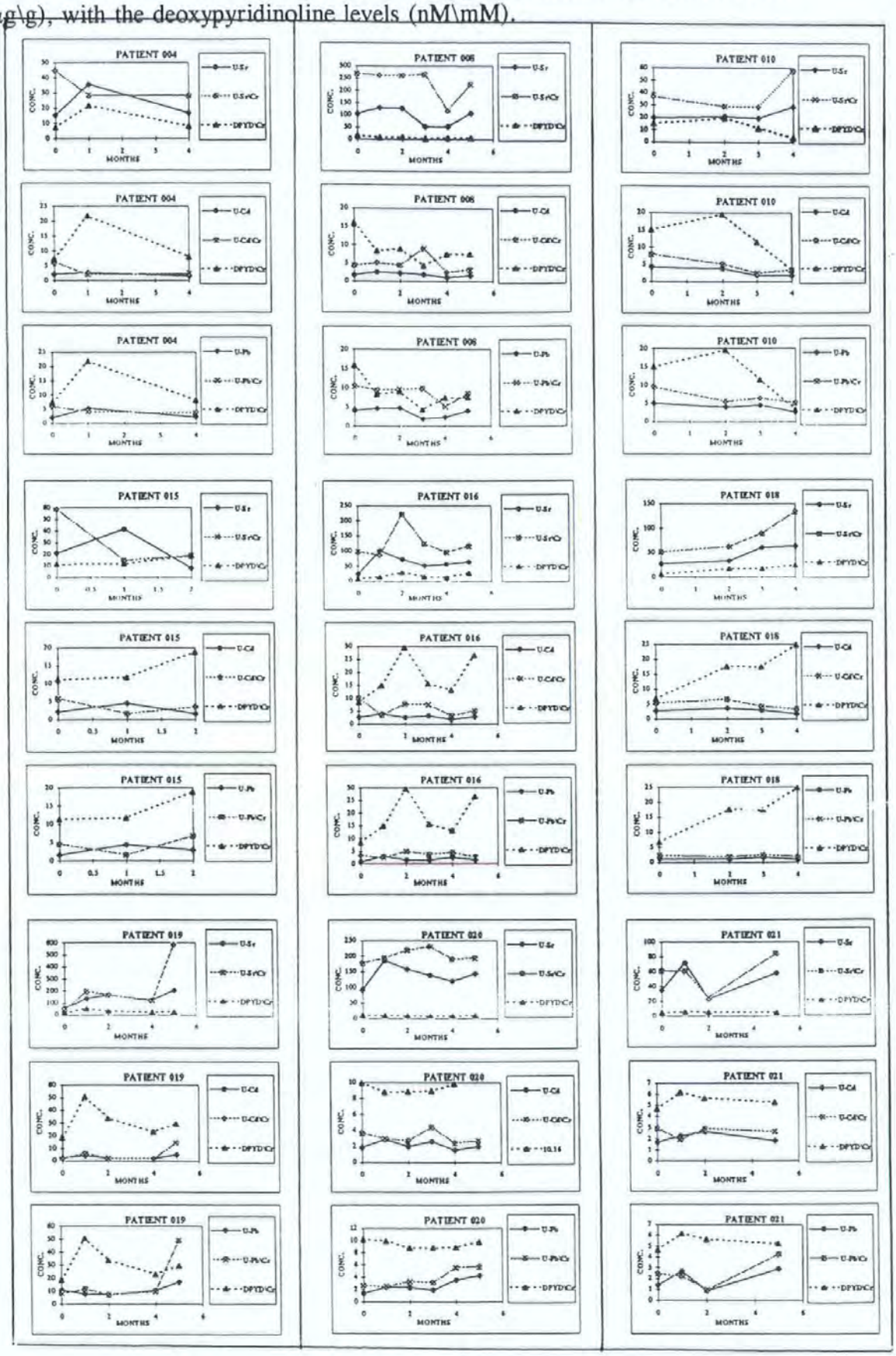
Appendix 2.1: Continued.



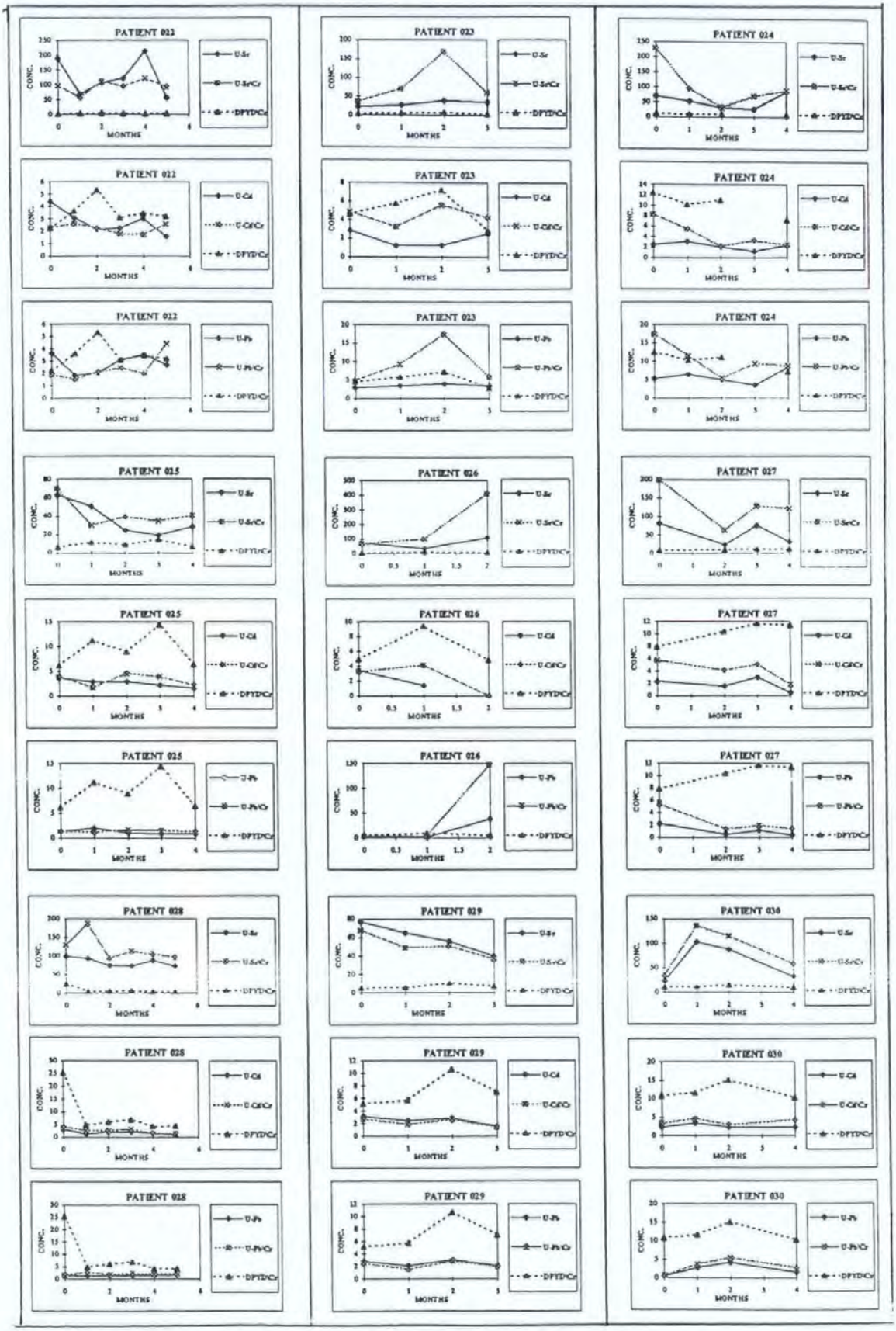
Appendix 2.1: Continued.



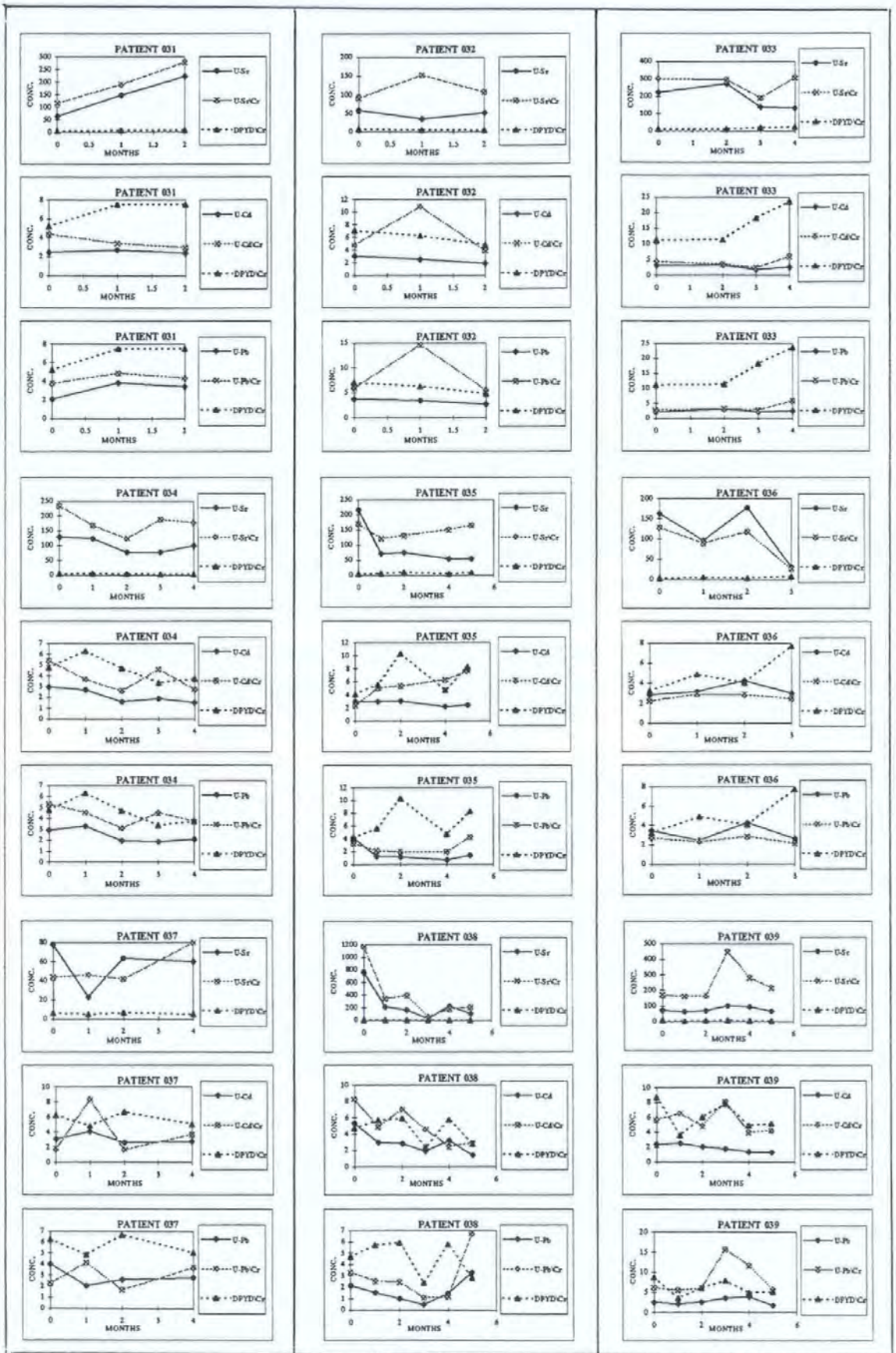
Appendix 2.2: Graphical presentation of individual patient results - comparison of the trace metal levels in urine, uncorrected (μg\L) and corrected with creatinine (μg\g), with the deoxypyridinoline levels (nM\mM).



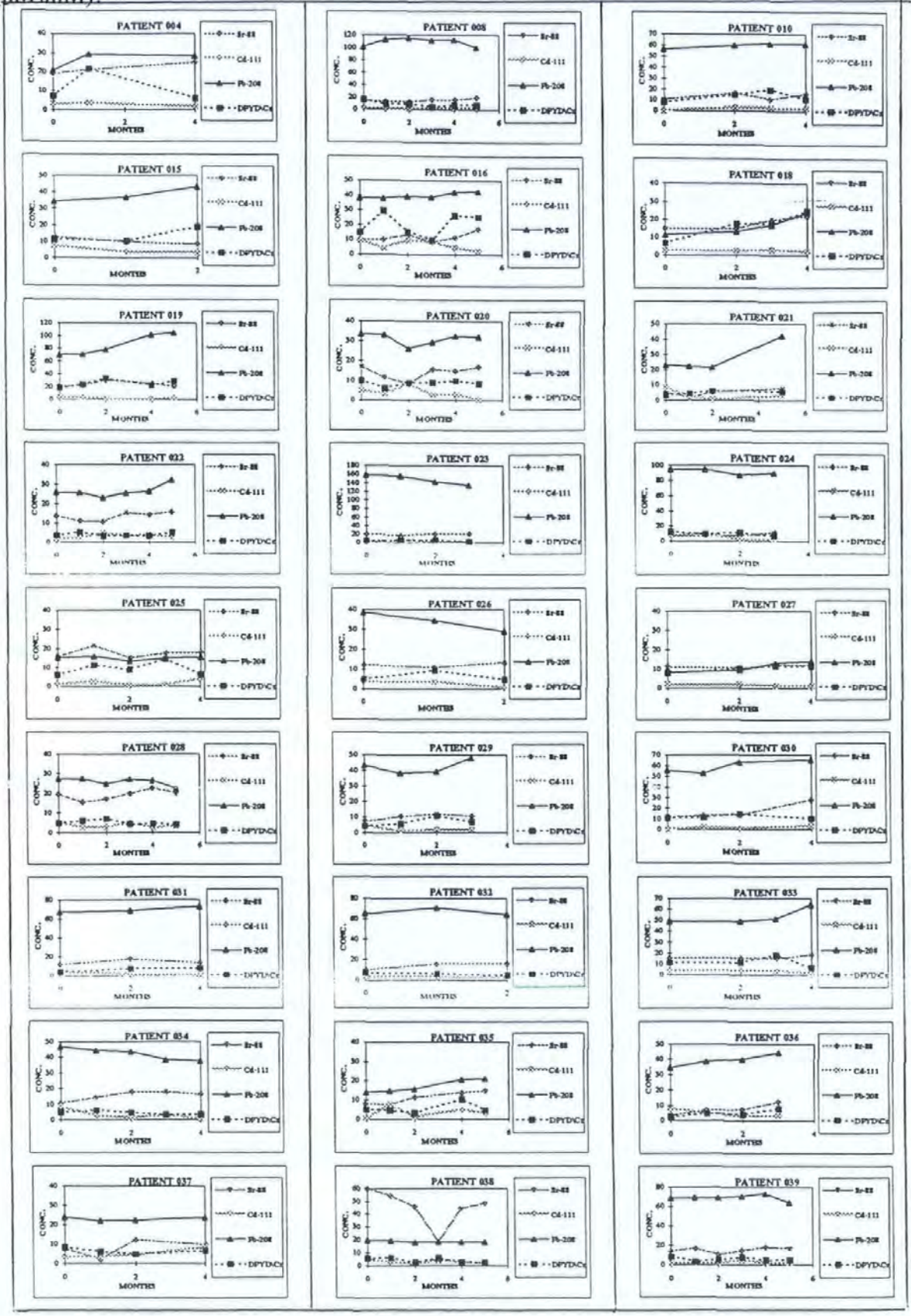
Appendix 2.2: Continued.



Appendix 2.2: Continued.



Appendix 2.3: Graphical presentation of individual patient results - comparison of the trace metal levels in blood (μg\L), with the deoxypyridinoline levels (nM\mM).



PRESENTATIONS AND MEETINGS ATTENDED.

1) Research and Development Topics in Analytical Chemistry, University of Bradford, 16 - 17 July 1993.

2) Fisons Elemental (VG) users group meeting, Northampton, 16 - 17 November 1993.

3) *"The determination of trace metals in whole blood, as potential markers of bone breakdown in patients with bone metastases"*. Poster presented at Research and Development Topics in Analytical Chemistry, University of Hertfordshire, 18 - 19 July 1994.

4) *"Trace metal levels in blood as markers of bone breakdown in patients with bone metastases"*. Poster presented at the 7th Biennial National Spectroscopy Symposium (BNASS), University of Hull, 20-22 July 1994.

5) *"Determination of trace metal levels in whole blood by ICP-MS as potential markers of bone breakdown in patients with bone metastases"*. Poster presented at the European Winter Conference in Plasma Spectrometry, Cambridge, 8 - 13 January 1995.

6) Atomic Spectrometry Updates, Royal Society of Chemistry Meeting "Applications of atomic spectrometry in trace element speciation", University of Bristol, 30th March 1995.

7) *"A clinical evaluation into the potential of trace metals as biochemical markers to measure bone breakdown in patients with bone metastases"*. Poster presented at Research and Development Topics in Analytical Chemistry, University of Hull, 10 - 11 July 1995.

8) *"The diagnostic potential of trace metal profiles in blood and urine, measured by inductively coupled plasma-mass spectrometry, as markers of bone resorption in patients with skeletal metastases"*. Lecture presented at the 8th Biennial National Spectroscopy Symposium (BNASS), University of East Anglia, Norwich, 17 - 19 July 1996.

9) *"A clinical evaluation of trace metals in blood and urine as potential biomarkers of bone resorption in patients with skeletal metastases"*. Lecture presented at Research and Development Topics in Analytical Chemistry, Nottingham Trent University, 22 - 23 July 1996.

10) *"A clinical evaluation of trace metals in blood and urine as potential biomarkers of bone resorption in patients with skeletal metastases"*. "Runner-up" paper presented for the 1996 Pharmaceutical Analysis Science Group (PASG) Award.

11) Royal Society of Chemistry lectures and lectures by invited speakers, at the University of Plymouth, 1993-1996.

12) Various weekly departmental research lectures at the University of Plymouth, 1993 - 1996.