TITLE

AN INVESTIGATION OF THE PHARMACOKINETIC INTERACTION BETWEEN XIPAMIDE AND TRIAMTERENE WHEN ADMINISTERED ALONE OR IN FIXED COMBINATION

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SIGNED 

ABSTRACT

A double blind placebo controlled, 4-way crossover study was carried out to investigate the pharmacokinetics of single oral doses of xipamide (10mg), triamterene (30mg) and Trirexan (10mg xipamide + 30mg triamterene) in a group of 12 healthy male volunteers. HPLC assays were developed for the measurement of both drugs in plasma. Plasma levels of xipamide and triamterene were measured using these validated HPLC methods. The individual plasma level data are presented as tables and the mean plasma levels are presented graphically. Plasma xipamide levels measured over a 24 hour period after dosing with xipamide alone and in a fixed combination as Trirexan were analysed using the JANA curve stripping program. The estimates of the pharmacokinetic parameters thus obtained were then used in curve fitting analysis using an IBM-PC compatible version of NONLIN 84. Plasma triamterene levels after dosing with triamterene and Trirexan were likewise analysed.

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DEDICATED TO DENISE

RECENT ADVANCES IN BIOPHARMACEUTICAL ANALYSIS

INTRODUCTION

Biopharmaceutical analysis is the analysis of drugs and their metabolites in biological fluids. There are a number of reasons why it is important to measure drug levels in such samples. Before a new drug can be administered to patients it is necessary that it be rigorously tested in a number of animal species followed by testing in normal healthy volunteers. As well as measuring parent drug levels in tissues , blood , urine and faeces it is also necessary to measure levels of the drug metabolites which may also be present. While it is generally assumed that drug metabolites are inactive and more readily excreted by the kidneys than the parent drug there are many examples of drugs whose metabolites are active or even toxic (1). Major inactive metabolites although they may not be of clinical importance may be of extreme importance from an analytical point of view as they may interfere with drug assay methods because of structural similarity with the parent compound. Careful control of the analytical conditions can allow for seperation of structurally similar compounds in some assay systems. It may not however always be possible to alter assay conditions to allow differentiation between parent drug and structurally similar compounds. In radioimmunoassay for example the specificity of the

antibody determins the specificity of the assay.

New formulation procedures and drug delivery systems are continuously being developed and it is necessary to monitor drug levels in order to demonstrate bioequivalence with standard formulations which have been in use and found to be effective. Associated with these developements in pharmaceutical research, pharmacokinetics and pharmacodynamics as well as drug metabolism have become established as scientific disciplines in their own right. Clinical pharmacokinetics has been discribed as " a health science discipline which deals with the application of pharmacokinetics to the safe and effective therapeutic management of the individual patient " (2). The large inter subject variations which occur in response to drugs are apparent to all who work in this field and often optimisation of individual dosage regimens is only possible after close monitoring of plasma drug level data.

The illicit use of drugs has become a major worldwide problem and the use of definative assays for drugs in biological fluids and other materials in forensic science departments has grown in line with the drug abuse problem. For obvious reasons it is extremely important that the methods used in this area need to be

highly specific , very reproducible and rapid (suspects can invariably only be held in custody for short periods of time).

Many of the methods that have been developed are used to detect trace quantities of drug in the very small amounts of biological materials which are available (3).

A variety of analytical techniques are used in clinical laboratories for the measurement of drugs in biological materials. Chromatographic techniques using a variety of detection systems, spectroscopic analysis and competitive protein binding asseys have been most frequently employed. Drug level monitering is based on analytical techniques suitable for the quantitative detection of drugs and their metabolites in biological samples. A survey of the analytical techniques for drug analysis in biological samples published in the litrature will reveal a long list of techniques. These include:-

High performance liquid chromatography (HPLC)

Gas chromatography (GC)

Mass spectrometry (MS)

Combinations of HPLC and GC with Mass spectrometry

Thin layer chromatography (TLC)

Ultraviolet spectrophotometry.

Flourescence spectrophotometry.

Colorimetry.

Polarography.

Radioimmunoassay (RIA).

Enzyme immunoassay (EIA, ELISA and EMIT).

Protein binding assays.

Microbiological assays.

The current trend is undoubtedly directed towards even more sensitive, specific and reproducibal techniques for the quantitation of drugs and their metabolites in biological specimens.

The measurement of drugs and their intermediates in fermentation broths and during chemical synthesis are other areas where the methods used in biopharmacutical science can be applied, this area is however considered to be outside the scope of this review.

The most commonly used metods will be reviewed in turn with specific emphasis on new developments.

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CHROMATOGRAPHIC TECHNIQUES.

Chromatographic techniques (HPLC , GC AND TLC) combined with a variety of detection systems are the most widely used for drug measurements in biological materials. This has been brought about by the availability of sensitive and selective detection systems as well as significant advances in column technology (4). The recent popularity of HPLC assays for drugs in biological samples is based on the versatility, sensitivity, specificity and speed of the technique. Reverse phase packing materials show great potential for the analysis of drugs in biological materials, as polar compounds elute quickly and do not therefore interfere with the analysis of lipophilic drugs. Most drugs are by design weak electrolytes with varying molecular weights. Metabolic transformation of these compounds generally results in the formation of metabolites having increased polarity and decreased lipophicity. It is in the simultaneous measurement of drugs and their metabolits that chromatographic methods realise their greatest potential. The chrometographic techniques which will be considered here are HPLC and GC.

RECENT DEVELOPMENTS IN HPLC

MICROBORE COLUMNS

The last ten years have seen many publications and much discussion on microbore columns. Three types of column have been discribed, narrow bore packed (5,6), microcapillary packed and open tubular (7,8). These publications have quoted internal diameters ranging from 10 u to 2 mm. The terminology has become further confused by the use of additional terms such as low dispersion liquid chromatography (LDLC), fast microbore and capillary liquid chromatography.

Although there are a large number of reports of the advantages of this type of chromatography (5,6,7,8 and 9) their use for routine pharmaceutical analysis has not been as widespread as might be expected. One reason for this is that microbore LC is not compatible with all conventional HPLC Systems. This situation will be improved by the development of new equipment which is compatible with both conventional and microbore technology. Many conventional HPLC systems may also be adapted at minimal cost to accomodate microbore columns (10). The main advantages associated .pa with the use of microbore columns are the considerable reduction in running costs that can be achieved by the use of low

solvent flow rates and smaller amounts of column packing materials, the high effeciencies and mass sensitivities that can be achieved and the fact that the low flow rates used make the coupling of liquid chromatography with mass spectrometry as a detection system a much easier proposition (11). Like any analytical technique micro column HPLC is not without it´s difficulties, these include the necessity for low dead volume injector and detector systems, difficulties associated with packing of such columns and column overload when too large a sample is injected onto the column.

Apart from the economic advantages of narrow bore HPLC there are three other general areas where advances are being made. These include increased mass sensitivity, linking of columns to give high plate counts and the application of new detection systems which are made possible because of the low flow rate. The most interesting of these are flame based detectors which in general have little application in biopharmaceutical analysis and mass spec. detectors. The potential increases in mass sensitivity may not always be achieveable in practice however and the difficulties in achieveing the theoretical improvements are outlined by Gill in his article on the subject (12). Briefly for UV detectors using small volume flow cells e.g. 0.5ul. the

theoretically expected improvements will be seen when reducing the column bore.With electrochemical detectors flow cell volumes of down to 1n litre have been described (13). and the use of these detectors would be expected to produce massive increases in .pa sensitivity, however, the flow rate in such detectors also affects the performance and increases of 5-6 fold are as much as are achieveable in practice with such systems(12).

Experimental work with microbore columns has shown that coupling of a number of such columns can result in great increases in plate count. For example greater than 40,000 theoretical plates at optimum velocity has been quoted (12).Such high plate counts can be useful in the analysis of complex mixtures of drugs in biological fluids and are particularly useful in forensic work.

INTERNAL SURFACE REVERSE PHASE COLUMNS

As recently reported the development by Prof. T. Pinkerton of Purdue University , Indiana, of HPLC columns which allow for the direct injection of serum samples has greaty reduced the sample preparation time for pharmaceutical analysis (14). Injection of serum directly on to conventional columns is not possible and removal of protein by some mechanism such

as percipitation or on or off line solid phase extraction is necessary. If these precautions are not taken back pressure quickly builds up and further analysis is not possible. The developement of a new packing material called internal surface reverse phase (IRSP) allows for direct injection of untreated serum or plasma or other protein containing materials.

Conventional silica particles are derivitised with glycerylpropyl groups forming a bonded hydrophilic phase that is non adsorbtive to protein. To provide the hydrophobic partitioning phase that is also needed, polypeptides such as glycine-l-phenylalanine-l-.pa phenylalanine are covalently bonded to the glyceropropyl groups. The derivitised silica is then treated with carboxypeptidase which cleaves the phenylalanine moieties from the external surface of the particles but not from the internal surfaces as it cannot enter the particles on account of it´s size. When serum samples are injected on to these ISRP columns the proteins do not adsorb on to the external surfaces and elute rapidly. The analytes do however penetrate the internal surfaces and interact with the polypeptides. Tests of this new packing material have shown that as many as 240 direct injections were possible without the build up of excessive back pressure. This development has been licenced to a manufacturing company in the United States

of America who are currently adapting the idea for mass production and developing applications methodology.

CARTRIDGE COLUMN SYSTEMS

Many companies have now produced cartridge columns which allow for the insertion of prepacked cartridges into HPLC assemblies. This allows different lengths of column to be inserted into the HPLC system and thus reduce column cost and solvent consumdption Low cost, easily interchangeable columns are ideal for method development. It is possible to have on hand a wide variety of packing materials e.g. 3 centimetre columns for method development . When a suitable solvent has been selected and higher resolution is required a longer cartridge with the same packing material is chosen (15).

Most laboratories spend more per year on HPLC grade solvents than they do on columns. The use of cartridge systems allow for the use of 2.1mm internal diameter microbore columns with conventional HPLC appparatus. These microbore systems use only 20% of the solvent that a normal 4.6mm internal diameter column would use.

These modular column systems are connected into the HPLC using finger tightened couplings which it is claimed will not leak at pressures of up to 7000 psi. These

couplings allow for direct connection of two or more columns this has the advantage that seperations can be achieved using combinations of columns that could not be achieved with any of the columns on their own.

DETECTOR SYSTEMS

The function of any detector is to measure accurately the concentration or amount of the sample components eluted from the column and generally the following requrements are necessary

(a) ability to detect 1ppm or less of solute

(b) no remixing of components as they pass through the detector

(c) a wide linear dynamic range to ensure that quantative analysis can take place in a straight-forward manner.

Detector characterstics have been covered in detail by Scott. (15). The most important parameters being low noise, high sensitivity and wide linear response.

The detector type most commonly used in biopharmaceutical analysis is the UV or UV/visable detector. For highly absorbing species a detection limit of as low 1ng/ml may be achieved but generally a limit of detection of 5-10 ng/ml would be considered good for

The original UV detectors were fixed wavelength detectors. Low pressure mercury lamps were used to give a series of discreet wavelengths and 254 nm was very commonly used. More modern detectors use deuterium lamps which provide a continous source and used in conjunction with good quality filters, species absorbing in the range 180-400 nm can be detected.

The improvements that have taken place in microcomputer technology over the past few years have led to a new breed of rapid scanning UV spectrophotometers that permit simultanious multiwavelength detection. These detectors are based on optical multichannel analysers that use either a silicon intensified vidicon tube (SIT) or a linear diode array (LDA) for the rapid capture of UV light (16).

An alternative approach is the use of a microprocessor controlled variable wavelength detector which will monitor the absorbtion at a specific wavelength for a pre-programmed amount of time, then switch to another wavelength re zero the instrument and monitor absorbtion at that new wavelength. This will allow for the simultaneous measurement of a number of compounds with markedly different absorbtion maxima in a single assay.

PIEZOELECTRIC CRYSTAL DETECTION SYSTEMS

A voltage can be obtained from some materials e.g. quartz crystals and certain ceramics by compressing them. Conversely if a voltage is appllied to these materials the crystal expands or contracts. This is known as the piezoelectric effect. Recently several groups of workers have tried to link these types of device to HPLC systems. Some pleminary work has been reported on the applications of piezoelectric crystals as mass detectors in liquid chromatography however, limits of detection were poor and the crystals became saturated after a small number of injections. Clearly the routine use of such detectors in biopharmaceutical analysis is a long way off (17).

MASS SPECTROMETRIC DETECTORS.

Over the past ten years a considerable research effort has been expended on the production of an LC-MS system. GC-MS systems have been in use for many years now and the interfacing of GC-MS was a logical step as the eluent from a GC was in the gaseous phase and this fits the sample requirements for the MS. With LC the solutes are in the liquid phase and LC & MS are not directly compatible. Vapourisation of the HPLC eluent is necessary and different approaches to this problem have

been adopted.

One of the first LC-MS systems produced incorporated a moving wire for collection of the solute (18). This was later replaced by a moving belt system (19). The disadvantages encountered include restricted flow rate (max 1ml/min) and eluents containing buffers or polar modifiers produce a drastic reduction in sensitivity. To extend the range of HPLC applications microbore columns are being used as these operate with very small amounts of eluent. Although different types of transport systems have been used it is generally agreed that microbore systems should be interfaced using techniques that do not use transport devices i.e. direct liquid injection systems.

SAMPLE PREPARATION.

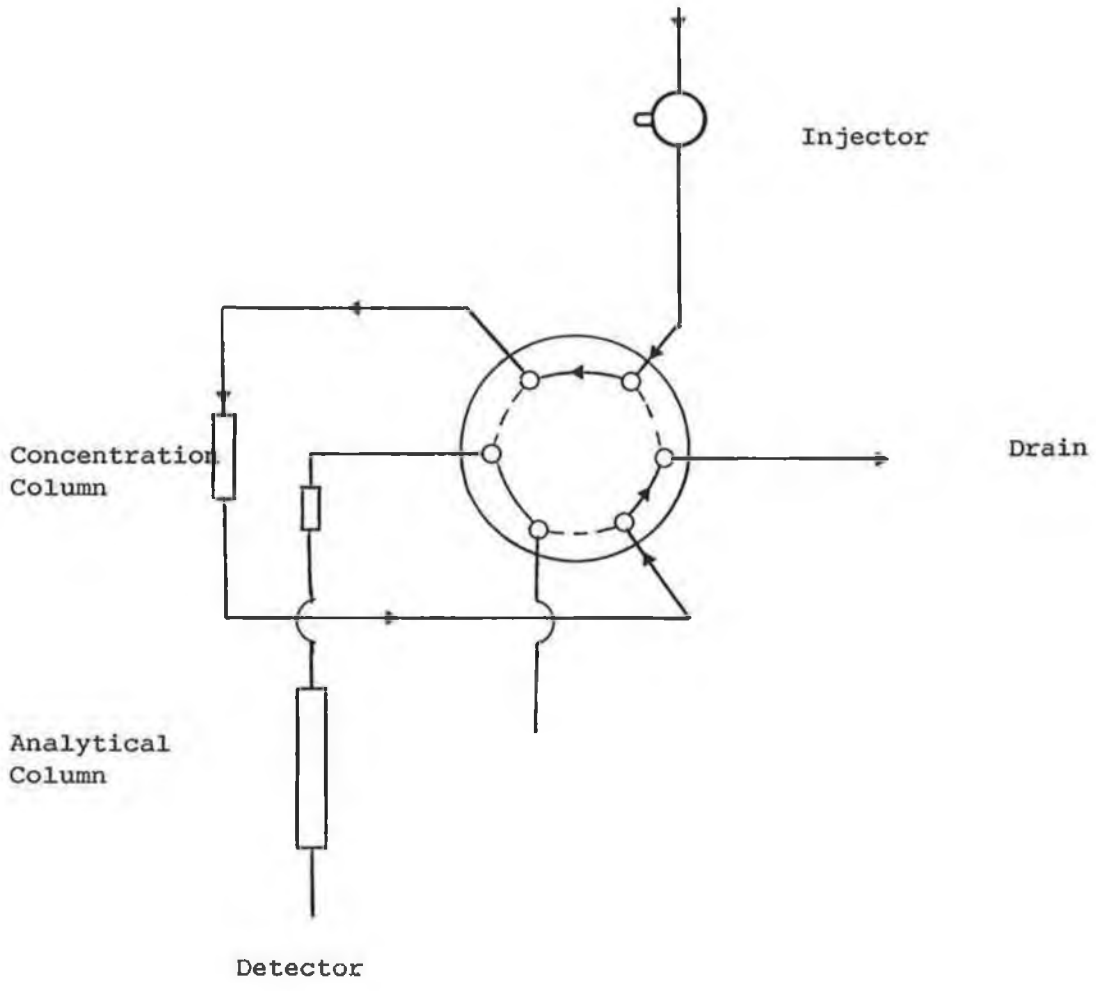
The analysis of drugs in biological fluids by HPLC can often be troubled by interferences from endogenous compounds and several different approaches can be applied to tackle this problem. The solution may involve the use of a more selective detector, a change of the HPLC eluent or column packing material or even a chemical derivitasation in order to enhance the relative detector response of a drug. An alternative approach involves the modification of the sample preparation

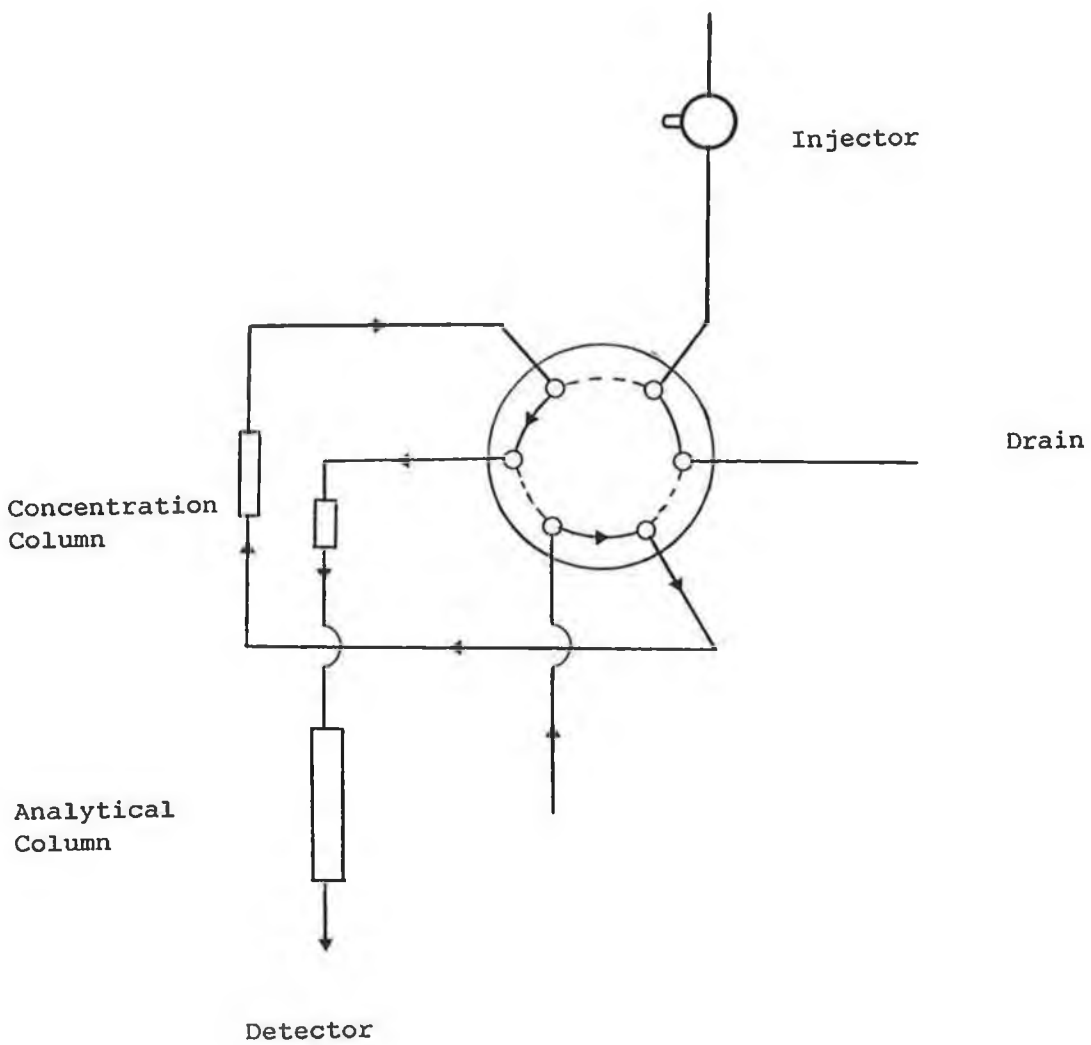
procedure such that the interfering compounds are eleminated before chromatography.

Protein precipitation using either an acid solution such as trichloroacelic acid or using solvent preciptation with miscible organic solvents such as methanol or acetonitrile can be employed. Liquid - liquid extractions however have remained the most popular method of sample preparation for a number of years. Liquid-solid extraction either "on" or "off line" have recently become very popular.

On line solid extraction involves the use of column switching technology to selectively remove interfering biological material while holding the analyte of interest on a pre column before it´s application to the analytical column.

The Rheodyne 7000 six port switching valve is typical of those used for column switching applications. During this procedure the sample is injected directly onto a preconcentration column with an aqueous mobile phase flowing through it (Fig.1). This concentration column is typically packed with a packing material similar to that of the analytical column but of a larger particle size (for example 40u particle size for the concentration


Injector
Concentration
Column
Drain
Analytical
Column
Detector


Injector
Drain
Concentration
Column
Analytical
Column
Detector

column and 5 or 10 u particle size for the analytical column . When serum components have been removed to waste ,the valve is switched and the eluent flows in the backflush mode through the concentration column and on to the analytical column (Fig.2).

Such switching techniques allow for the direct injection of biological samples on to HPLC systems without the need for sample pretreatment. Column switching techniques can also be used for zone cutting. This is a technique where only the zone of interest is switched on to the analytical column (20). Much greater sensitivity is achieveable using this technique as interfering substances have been removed and greater detector sensitivity settings can be used.

Off line liquid solid sample preparations have also become very popular and the use of cartridges such as sep pack or bond elute for sample pretreatment to remove interfering substances from biological material is becoming widespread.

Small columns similar to syringe barrels (sep pack) or to micro filters (bond elute) filled with column packing material (C8 ,C18 and CN are

most commonly reported in the litrature) are firstly conditioned by washing with methanol followed by a wash with aqueous solution. The aqueous samples are then applied to the column followed by a number of washes with an aqueous solution to remove interfering substances.The bound analyte is then removed with a suitable eluent which may be evaporated followed by reconstitution in a suitable fluid for application onto the column (21).

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GAS CHROMATOGRAPHY (GC)

The utility of gas liquid chromatography in drug level analysis is attested to by the large number of drugs that can be assayed using this technique. There are two basic modes of gas chromatography, gas liquid chromatography and gas solid absorbtion. Absorbtion GC employed an absorbtive solid column packing material and is mainly applied to gases and highly volatile compounds, for example volatile anasthetics and ethanol. The gas solid chromatography of volatile compounds is usualy performed using head space analysis of biological samples without organic solvent extraction. However, the predominant GC mode is by partitioning gas liquid chromatography. The liquid stationary phase consists of silicone plastomers or long chain hydrocarbons with low vapour pressure. These materials are actually solid at room temprature and melt at tempratures around 100C. Seperation of components of a mixture occurs by partitioning between the liquid stationary phase and the mobile gas phase. Retention in the column is a function of the physicochemical interactions of the compound and the liquid phase the temprature and the flow rate of the gas phase. Partitioning GC is performed either on columns packed with an inert microparticulate support material that is coated with the stationary liquid

phase, or on capillary tubes coated with the liquid phase on their inside wall. These latter capillary GC columns are highly effecient since turbulence of the carrier gas is minimised. With a column length of in excess of 100m. capillary GC offers the greatest seperation potential at present. Packed columns on the other hand are quite versatile and are sufficient for most applications in drug level monitoring.

Biological samples contain large amounts of polar non volitile materials such as peptides, sugars and amino acids which have to be removed prior to analysis by GC in order to prevent column deterioration. Organic solvent extractions are the most commonly used sample purification procedures for GC.

Gas chromatography is one of the most versatile techniques available to the analyst. The technique is finding it´s way into an increasing number of industries, and is widening it´s application within many of those industries. Gas chromatographs now range from sophisticated process control instruments costing tens of thousands of pounds, to the simple gas chromatographs used for teaching purposes. The spread of gas chromatography has been matched by changes in both instrumentation and peripherals. The main areas where

advances have been made include gas supplies injection systems, columns, ovens, detectors and data handling systems (24).

GAS SUPPLIES

Perhaps the most basic of requirements for gas chromatography are gases. A few years ago the gases were usually industrial grade and were supplied by cylinders standing alongside the instrument. Very often the gas streams required cleaning and purifying before being piped to the instruments. Today gas suppliers recognise the needs of chromatographers and can supply high grade gases requiring no further purificiation for chromatography (it is advisible however always to clean gases required for electron capture detectors).

Automatic cylinder changeover valves are now often fitted to cylinders remote from the laboratory this avoids sudden loss of carrier gas flow when cylinders are empty.

An interesting new development in gas supply systems is the "liquid cylinder " supplied by some companies. These cylinders combine high gas capacity with low volume, and would appear to be ideal for establishments running a number of chromatographs.

It is now possible to run gas chromatographs without the use of any cylinder gases. In this case the gases are obtained from laboratory scale gas generators. Hydrogen generators for flame ionisation detector gas chromatographs have been available for some years. These operate by electryolysing water, and produce very high quality gas. A number of manufacturers, produce instruments specifically suitable for chromatography use. Air can be obtained from a simple compressor, but varying concentrations of impurities lead to detector instability, or baseline drift. Air purifier units, can overcome these difficulties by remoiving all impurities from the compressed air supply.

INJECTORS

The primary purpose of a chromatograph injection system is to place a homogeneous bolus of sample, in the vapour phase, at the start of the column. Whilst this a relatively simple matter when using liquid samples and packed columns, both gas analysis and capillary columns present problems of scale. Large volumes of sample are required for gas analysis, and it is often difficult to reproducibly inject these volumes manually. Specially designed gas sampling valves are available from most gas chromatograph manufacturers. These ensure that a reproducible volume of sample is injected provided both

temperature and pressure are constant; this is relatively easy to arrange.

The analysis of very low concentrations of gases or pollutants requires the use of sample volumes of the order of several tens of litres. These volumes are far too large for commercial chromatographs. Adsorption techniques are often used for these analyses. Gas is drawn through tubes filled with an adsorbent such as charcoal or porous polymer beads. The compounds of interest will adsorb on the material in the tubes. The analytes can then be washed into a small volume of solvent, or flash evaporated onto the chromatograph. Unfortunately the latter technique, whilst more rapid will cause some differentiation because of the time lag required to heat the relatively large mass of adsorbent and sample tube.

Small sample volumes are generally required for capillary chromatography. Sample splitters are often used to route a proportion of the injected sample to atmosphere. Some sample differentiation by molecular weight is inevitable. Splitless and on-column injection techniques are also available.

COLUMNS

Column tehcnology has changed greatly in the past few years. Packed columns in glass or metal are still widely used for routine analyses. In general there have been few major imporvements in support or stationary phase materials during the last few years. On the other hand high resolution gas chromatography has become a much more routine technique. PLOT columns (porous layer open tube) are now available for gas analysis applicaions. The major advantage of small bore columns is that they provide high resolution from a relatively short analysis. This allows either more intensive instrument use, or more detailed analysis.

OVENS

Column ovens have also changed greatly. The replacement of the early linear proportional temperature controllers with logic and later microprocessor technology allowed very close control of oven temperature both in isothermal operation and during temperature programming. Microprocessors also facilitate the use of multiple ramp rates, a technique that can greatly speed up complete analyses.

The thermal mass of chromatography ovens is a very important factor. A high thermal mass tends to produce a

stable temperature environment within the oven. Unfortunately high thermal mass also reduces the maximum ramp rate of the oven, and prolongs the cool-down time. Very low thermal mass ovens have fast ramp and cool rates but tend to have poor isothermal temperature stability.

High power heaters are needed to achieve the fast ramp rates required; these are cycled on and off by the digital circuitry to maintain either a constant temperature, or one that is changing at a constant rate. Unfortunately stable isothermal operation normally requires very small amounts of additional head to maintain the set temperature. As it is very difficult to obtain a small amount of heat from a high power heater there is inevitably some temperature cycling within the oven. The effects of this can be minimised by using a large fan to circulate air rapidly around the oven, and a dump system to dispose of surplus heat. Unfortunately the energy of the fan also enters into the oven performance equation. Most gas chromatograph ovens are therefore a compromise between all of the factors and it is a tribute to the designers that most gas chromatograph ovens work extremely well with nearly all column configurations.

Advances in electronics will doubtless lead to greater refinement with respect to both uniformity and stability of temperature, and it may well be that future generations of gas chromatographs will have different oven software/hardware configurations for different column types and arrangements.

One interesting technique this is becoming increasingly important is multi-dimensional chromatography. This uses multiple columns in two or more ovens and is especially useful for complex mixture analysis. Many instruments now allow the storage, and instant recall of a number of specific methods.

DETECTORS

Detectors are probably the components in which there has been most development in recent years. Research appears to be concentrated on two major areas; sensitivity and selectivity. Sensitivity has been enhanced both by careful design of the detector hardware, and better electronics in both amplifiers and power supplies. The advent of capillary chromatography has made great demands on detector technology, and the various detector manufacturers have not been slow to respond. Detector and amplifier response times have fallen significantly, and a new generation of thermal conductivity detectors have been developed.

Detector selectivity is becoming more important as resolution increases. The correct choice of detector can help to isolate specific compounds of interest from the general clutter of complex matrix. A new " turntable " detector called the thermionic ionisation detector has recently been developed and this is a variant of the catalytic nitrogen phosphorous detector in which the catalyst, it´s temperature, and it´s gas phase environment can be changed. The effect of these changes is to enhance response to certain goups of compounds, while supressing the response to others. This is potentially one of the most exciting detectors now

available. Especially interesting is the fact that in halogen mode it becomes sensitive at the point where the electron capture detector becomes non-liner.

Virtually any physical or chemical property of a compound or group of compounds can be used as the basis of a chromatographic detector. One interesting development is the sorbtion detector system being developed by Dr. Brian Buffham and colleagues at Loughborough University. The detector responds initially to the adsorption of the sample at the head of the column by producing a negative peak whose area is relative to the volume of the sample. The desorption of each individual component from the column is then recorded as a positive peak in the usual way. Column and oven defects cause predictable baseline perturbations. The area of the negative peak should equal the sum of the areas of the positive peaks. This detector however is still in the experimental stage.

Gas chromatography is a mature, well established technique for biopharmaceutical analysis (22). In the search for positive peak identifications in GC, instrument manufacturers have given attention to fourier transformed infrared spectrometry (FTIR) as an alternative to the now well established GCMS systems.

Gas chromatograms have recently been combined with matrix isolation sprectroscopy (23) . Matrix isolation is a well established technique of trapping ions, molecules or free radicles in a crystilline cage at tempratures near to absolute zero, basically to permit improved spectral resolution by the lack of intramolecular interaction and the absence of molecular rotation.

DATA HANDLING SYSTEMS

With the development of storage devices such as the Winchester disc-drive, the range of applications of computer systems has increased so that information storage and retrieval are now as important as numerical computation. With the advent of knowledge based systems computing has brought analytical chemistry to a stage where method development and data interpretation can now be handled by the instrumentation thus assisting the chemist in ways previously unforseen.

The most common form of knowledge based system is the expert system (25) which contains encoded expert knowledge and is capable of exploiting this knowledge for problem solving purposes One of the earliest implementations of an expert system designed to solve a chemical problem was the Dendral system, developed at Stanford University (26). This system uses data from

mass specrometry to predict the structure of target molecules by using rules about possible structures and fragmentation processes in the spectrometer. These rules have been obtained from two main sources. They have either been supplied by an expert, or been derived by an associated program which analyses the mass spectra data produced from a particular class of compounds to suggest probable fragmentation rules (27).

An expert system is a knowledge based system and may consist of one or more programs. It is characterised by three essential features :-

(1) a knowledge base containing modules of organised knowledge derived from an expert's skill.

(2) an inference engine that uses the knowledge from the knowledge base to supply intelligent advice or to assist with decision making on a specialised topic.

(3) an explanation facility that can be invoked to explain the line of reasoning which has been followed to arrive at the advice given.

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COMPETATIVE PROTEIN BINDING ASSAYS

The saturable, high affinity binding of a drug to proteins represents the basis for competative binding assays.The nature of the protein can be used as a criterion for classifying these assays into immunoassays, which employ drug-specific antibodies and account for the major proportion of protein binding assays,and into assays utilising other proteins, for example, drug target enzymes, receptors, and carrier proteins. Immunoassays are further subdivided according to the analytical technique by which the fraction of free and protein bound drug is measured; radioimmunoassay, enzyme immunoassay,flourescence immunoassay etc.(1).

The major determinant of assay sensitivity and specificity is the nature of the ligand-protein binding interaction.The affinity, or dissociation constant, limits the assay sensitivity.Extraction and seperation of the drug from non-specific serum protein binding may

be necessary to achieve maximum assay sensitivity. Furthermore, the cross-affinity of the binding protein or other compounds in the sample determins assay specificity for a drug. Many of the proteins selected for competitive binding assays are highly specific; yet drug metabolites and endogenous substrate analogs may be chemically very similar to the drug of interest and can interfere with the drug-protein binding process. It is therefore mandatory to carefully test for assay interferences by drug analogs which may be present at much higher concentrations than is the active parent drug and a cross-affinity of the protein to a drug metabolite of only 1% may cause significant assay interference.

RADIOIMMUNOASSAY

Radioimmunoassay (RIA) procedures are widely used for drug analysis in biological samples. The ligand S* is a radioactively labeled tracer drug which binds to a drug-specific antibody as the protein. The nature of the antibody is responsible to a large extent for the quality of the RIA method, and antibody production therefore represents an important factor in the assay development. Drugs of low molecular weight (<1000) are normally inactive as antigens and do not produce antibodies. However, when coupled to a suitable protein

via a chemical spacer bridge, drugs act as immunologically active haptens. The antibodies raised against a drug hapten-protein conjugate also recognise the unbound drug; good antibodies have affinity constants of >10 9/M toward the drug hapten.

ENZYME IMMUNOASSAY

Immunoassays involving enzyme-labeled antigens, haptens, or antibodies have been recently developed for a large number of macromolecules and small molecular weight substances serving as haptens. Enzyme immunoassays (EIAs) are increasingly used in clinical laboratories for diagnositc tests and for therapeutic drug level monitoring. The advantageous features of the EIA include speed, simplicity, ready automation, and versatility, which render it suitable for routine clinical applications and rapid drug screening. Initial lack of sensitivity of the EIA, relative to the RIA , has been largley overcome; for example, the EIA assay for progesterone is sensitive to 15 pg/sample, using B-galac-tosidase as the enzyme label and a heterogeneous EIA (28).

Thus the sensitivity and specificity of the EIA are comparable to those of the RIA, while the EIA offers advantages over the RIA in avoiding radioactive tracers and in speed of analysis. Many articles in the litrature

include comparison studies performed with the EIA, RIA, and specific physiochemical assays. The results indicate that the EIA and RIA are usually equivalent and specific for the test drug. However, the enzyme label introduces additional potential assay interferences by several mechanisms, including differential binding of free drug and enzyme label to the antibody, presence of enzyme inhibitors, and presence of enzyme activity in the test sample. These factors have to be eliminated or minimized for clinically useful EIA methods.

FLUORESCENCE IMMUNOASSAY

Fluorescence immunoassays are based on changes in the fluorescent properties of fluoresceine caused by binding to a macromolecule; either fluorescence quenching or fluorescence polarization induction is measured, the latter requiring a specialized fluorimeter.

Fluorescence immunoassays are rather sensitive; the major disadvantage may be the potential for assay interference by nonspecific fluorescence quenching or background fluorescence by other compounds in the biological sample.

MONOCLONAL ANTIBODIES IN IMMUNOASSAYS

The field of drug immunoassay has greatly benefited from the production of monoclonal antibodies (29). These antibodies are obtained by harvesting spleen cells

instead of serum from the immunized animal and then fusing these cells with myueloma cells. The generated hybrid cells retain the ability to produce antibodies and can be grown indefinitely without the growth limitations of differentiated cells.

POLAROGRAPHY

Polarography is based on microelectrolysis, with the generated current proportional to the drug concentration. The general term " voltammetry " is used for all current-voltage recording methods with microelectrodes, while polarography is normally performed with the dropping mercury electrode and the saturated calomel electrode as the auxiliary electrode. The drug potentials are usually given relative to the saturated calomel electrode. Dissolved oxygen is reduced to H202 at - 0.1V and therefore has to be removed from the sample before polarographic analysis of many drugs. This can be achieved by bubbling nitrogen through the sample. The sensitivity of regular polarography can be as good as mMolar to uMolar for suitable samples, which is sufficient for some drugs at therapeutic serum levels. A more sensitive technique is pulse polarography (30). The main advantages of using differential-pulse instead of d.c. polarography for trace drug analysis are

increased detection limits and more convenient measurement.

The most favourable comopounds for determination by differential-pulse polarography are those containing a nitro group (31). This group is reduced at small negative potentials and the reduction usually involves at least four electrons. Further, some nitro compounds are adsorbed quite strongly on mercury and as this results in a concentration process between pulses it increased the differential-pulse signal and improves determinations made directly in plasma samples.

It should be noted that some progress has been made in using cathodic stripping voltammetry to determin drugs, such as thioamines, that form insuluble salts with mercury(II).(32). Compounds particularly amenable to oxidation at carbon are phenols and amines. Particularly successful and important is the determination of

catecholamines. Structurally related to these is morphine, which is determined specifically in forensic applications. The paper by Peterson (33) shows the complementary use of spectrophotometric and voltammetric detectors.

Electrochemical sensors are particularly suited to invivo work. Ion-selective electrodes have been applied increasingly in this field as have voltammetric oxygen

sensors. A recent application of a voltammetric sensor is that for the determination of catecholamines in situ, based on a graphite-loaded epoxy resin.(34).

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PART II

AN INVESTIGATION OF THE PHARMACOKINETIC INTERACTION BETWEEN XIPAMIDE AND TRIAMTERENE WHEN ADMINISTERED ALONE OR IN COMBINATION

GENERAL SUMMARY

A double blind placebo controlled, 4-way crossover study was carried out to investigate the pharmacokinetics of single oral doses of xipamide (10mg), triamterene (30mg) and Trirexan (10mg xipamide + 30mg triamterene) in a group of 12 healthy male volunteers . HPLC methodologies were developed for the measurement of both drugs in plasma. Plasma levels of xipamide and triamterene were measured using these validated HPLC methods . The individual plasma level data are presented as tables and the mean plasma levels are presented graphically.

The plasma level data obtained from these analyses were subjected to analysis using curve stripping (JANA) and the results from this procedure were further analysed using PC NONLIN in order to estimate half lives and area under the time/concentration curves. area under the curve was also measured using the trapezoidal method for the periods 0-4, 0-6 and 0-24 hrs. This was done in order to acertain if there was any difference in the absorption rates of both compounds administered alone or in combination.

ABOUT THE DRUGS UNDER INVESTIGATION

Xipamide is a diuretic derived from salicylic acid and has a structural resemblance to chlorihalidone. It´s pharmacodynamic profile shows a diuretic efficacy is similar to that of frusemide (furosemide) at doses up to 40mg, but the onset and duration of action are comparable to those of hydrochlorothiazide. Xipamide has been studied mostly in the treatment of mild to moderate essential hypertension with few controlled studies of it´s use in oedematous states. The efficacy of xipamide 20 to 40mg once daily in patients with mild to moderate hypertension is comparable to that of bendrofluazide 5mg. bumetanide 1mg. or hydrochlorothiazide 50mg. when used alone in newly treated or previously treated patients. The addition of xipamide 20 to 40mg. daily to regimens containing B-blockers,adrenergic neuron-blocking drugs and/or methyldopa has resulted in a further reduction in blood pressure. A few studies in odemalous states suggest that xipamide 40 to 80 mg. is comparable in efficacy to equal doses of frusemide, and that the side effects of hypokalaemia, hyperuricaemia and increased blood glucose in diabetics or latent diabetics are similar to those of other diuretics. Thus, xipamide is a suitable alternative to other diuretics in the treatment of mild to moderate hypertension and combines the efficacy of frusemide with a less abrupt action in the treatment of oedema. Although structurally related to chlorthalidone xipamide is not a thiazide.

Following oral administration of xipamide 20mg to 14 healthy vonunteers, the drug appeared to be completely absorbed, with maximum plasma concentrations of about 3mg/L occurring within 1 hour (knauf and Mutschler, 1984). In an earlier study using labelled xipamide, peak plasma radio-activity representing 13% of the dose per litre occurred 0.75 to 2 hours after oral administration of 42mg to 3 healthy volunteers (Hempelmann and Dieker, 1977).

A comparison of the area under the plasma concentration-time curve (AUC) after intravenous and oral administration of xipamide 20 mg to healthy subjects indicated an absolute bioavilablity of 73%. The volume of distribution of xipamide as calculated from the total plasma clearance and elimination half-life was 21L (Knauf and Mutschler, 1984).

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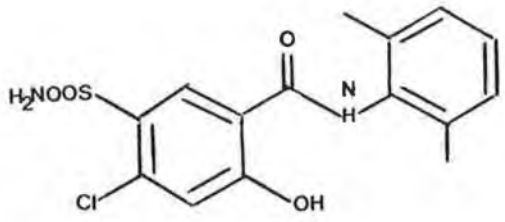
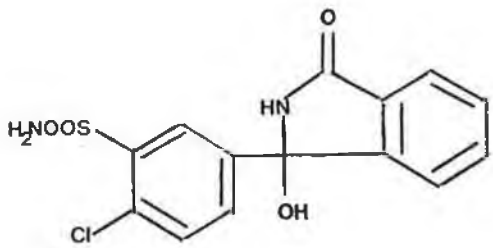
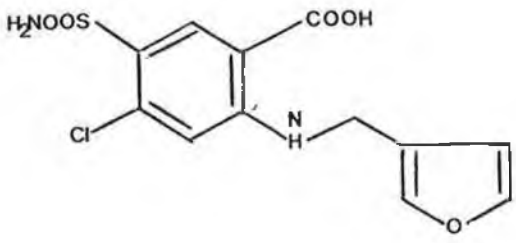


XIPAMIDE



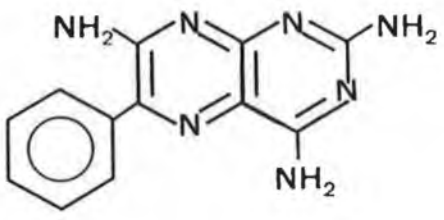
CHLORTHALIDONE



FRUSEMIDE

Triameterene is a mild diuretic which appears to act mainly on the distal renal tubules. It produces a diuresis in about 2 to 4 hours, reaching a maximum effect in about 6 hours. The full effect may be delayed until after several days of treatment.

Triamterene is incompletely but fairly rapidly absorbed from the gastro-intestinal tract. It has been extimated to have a plasma half-life of about 2 hours. It is extensively metabolised and is mainly excreted in the urine in the form of metabolites with some unchanged triamterene; variable amounts are also excreted in the bile. Animal studies have indicated that triamterene crosses the placental barrier and is excreted in milk. Following administration of triamterene 100 or 200 mg by mouth to 7 healthy subjects and intravenous administration to 2, rapid and extensive metabolism occured, the metabolite 2,4,7-trimino-6-p-hydroxyphenylpteridine being found in the plasma as soon as 30 minutes after a dose and at a concentration of up to 12 times that of the parent drug, and in the urine at 1.5 hours (again at a much higher concentration than the parent drug). Excretion also occurred in the bile after either oral or intravenous administration, with faecal excretion being the primary route in 1 subject.



TRIAMTERENE

XIPAMIDE METHODOLOGY

PLASMA XIPAMIDE ASSAY

Reagents :

- Methanol. H.P.L.C. Grade.
- Nanopure Water.
- Disodium Hydrogen Orthophosphate, anhydrous. Analar Grade.
- Orthophosphoric acid (conc.) Analar Grade.
- Helium Gas.
- Internal Standard Chlorothalidone.
- Sodium Bicarbonate. Analar Grade.
- Ethyl Acetate Analar Grade.

Instruments :

- High pressure pump : M6000 A Waters.
- Automatic sample injector : Waters WISP 710B
- Column uBondapak : C18 (25cm x 4.6mm).
- Pre-Column uBondapak : C18 Guard Column.
- U.V. Detector : Spectra Physics PU8480 XR.
- Integrator : Shimadzu C-R3A Chromatopac.

Working Conditions :

- Mobile Phase.

  Methanol : Na HPO (0.05M pH 6.4)

  35 : 63

- Flow Rate 1.4 ml/min.
- Wavelength 240 nm.
- Chart Speed 2.5 cm/min.
- Retention Time Xipamide 10.0 min.

  Chlorotalidone 8.0 min.

- Sensitivity : 50 ng/ml of xipamide.

Standard Solutions :

Xipamide stock solution : 1.0 mg/ml (in Methanol).

Xipamide working stock : 100 ug/ml (1 ml stock + 9 ml methanol/water (50:50))

Preparation of Spiked Plasma Standards

9.5 ml of fluoride oxalate plasma was pipetted into labelled 20ml glass vials : Standards were spiked as follows, the volume adjusted with (50:50) methanol/water.

Concentration in Plasma ng/ml	Xipamide 100 ug/ml	MEOH/H 50:50
50	5 ul	495 ul
100	10 ul	490 ul
250	25 ul	475 ul
500	50 ul	450 ul
1000	100 ul	400 ul
2500	250 ul	250 ul
5000	500 ul	0 ul

The glass vials were vortexed well.
.op

Internal Standard

A working solution of 7.5 ug/ml was prepared in methanol from a stock solution of 100 ug/ml.

Assay Procedure

Plasma xipamide assay was carried out as outlined below. The method used was a modification of one previously developed at this laboratory. The extraction procedure

was based on that used at Whickham laboratories (1). Briefly ,after the addition of sodium bicarbonate , Xipamide is extracted from plasma with ethyl acetate. After evaporation the solvent the residue is reconstituted in methanol and analysed by reverse phase HPLC.

Chromatography was carried out on a uBondapak C18 column with UV detection at 240 nm.

All standards and QC samples were assayed in duplicate and the mean values reported. Likewise, 10% of plasma samples were assayed in duplicate and the mean values reported.

1. Pipette 1.0 ml of plasma (spiked or unknown) into culture tube.
2. Add 50 ul of internal std. solution.
3. Add 100 mg of sodium bicarbonate and 4.0 ml of ethyl acetate to each tube.
4. Vortex for 30 seconds.
5. Mix on a rotary mixer for 15 minutes.
6. Centrifuge for 15 minutes at 4000 rpm.
7. Remove supernatant and dry under a nitrogen stream at 60 ^{o}C.
8. Reconstitute the residue in 200 ul of methanol
9. Inject 10-30 ul supernatant onto HPLC system.

REFERENCES

1. Whickam laboratories , Unpublished data , Personal communication.

CALIBRATION DATA

CALIBRATION DATA

ASSAY No. 1

Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
50	56.7	+ 13.4
100	103.8	+ 3.8
250	227.5	- 9.0
500	482.9	- 3.4
1000	941.5	- 5.9
2500	2500.8	0.0
5000	5096.5	+ 1.9
QC 200	197.4	- 1.3
QC 600	589.6	- 1.7
QC 1200	1164.6	- 3.0
QC 4000	4252.0	+ 6.3

CALIBRATION DATA

ASSAY No. 2

Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
50	52.9	+ 5.8
100	98.9	- 1.1
250	239.1	- 4.4
500	493.3	- 1.3
1000	1000.3	0.0
2500	2580.0	+ 3.2
5000	4940.0	- 1.2
QC 200	205.6	+ 2.8
QC 600	586.1	- 2.3
QC 1200	1211.2	+ 0.9
QC 4000	4212.1	+ 5.3

CALIBRATION DATA

ASSAY No. 3

	Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
	50	*	
	100	108.3	+ 8.3
	250	241.4	- 3.4
	500	503.6	+ 0.7
	1000	951.7	- 4.8
	2500	2443.8	- 2.2
	5000	5108.3	+ 2.2
QC	200	208.1	+ 4.1
QC	600	621.0	+ 3.5
QC	1200	1189.2	- 0.9
QC	4000	4192.4	+ 4.8

* Insufficient sample for analysis.

CALIBRATION DATA

ASSAY No. 4

	Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
	50	46.9	+ 6.2
	100	111.4	+ 11.4
	250	250.9	+ 0.4
	500	488.5	- 2.3
	1000	986.9	- 1.3
	2500	2504.0	+ 0.2
	5000	5013.4	+ 0.3
QC	200	200.2	+ 0.1
QC	600	511.8	- 14.7
QC	1200	1149.9	- 4.2
QC	4000	4145.1	+ 3.6

CALIBRATION DATA

ASSAY No. 5

Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
50	51.9	+ 3.8
100	98.5	- 1.5
250	250.3	+ 0.1
500	492.7	- 1.5
1000	983.8	- 1.6
2500	2493.4	- 0.3
5000	5030.0	+ 0.6
QC 200	199.2	- 0.4
QC 600	579.9	- 3.4
QC 1200	1136.3	- 5.3
QC 4000	3978.7	- 0.5

CALIBRATION DATA

ASSAY No. 6

Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
50	55.3	+ 10.6
100	106.0	+ 6.0
250	230.5	- 7.8
500	483.5	- 3.3
1000	874.8	- 12.5
2500	2508.3	+ 0.3
5000	5169.3	+ 3.4
QC 200	215.7	+ 7.9
QC 600	667.0	+ 11.2
QC 1200	1351.8	+ 12.7
QC 4000	4490.6	+ 12.3

CALIBRATION DATA

ASSAY No. 7

	Xipamide Added ng/ml	Xipamide Found ng/ml	% Difference
	50	55.9	+ 11.8
	100	113.2	+ 13.2
	250	252.7	+ 1.1
	500	486.8	- 2.6
	1000	840.7	- 15.9
	2500	2490.9	- 0.4
	5000	5198.6	+ 4.0
QC	200	220.5	+ 10.3
QC	600	614.4	+ 2.4
QC	1200	1283.2	+ 6.9
QC	4000	4229.7	+ 5.7

PLASMA XIPAMIDE LEVELS

PLASMA XIPAMIDE CONCENTRATION

TIME SUBJECT	1	2
PRE DOSE	ND	ND
0.5 hrs.	228	1320
1.0 hrs.	388	1102
1.5 hrs.	516	818
2.0 hrs.	564	644
3.0 hrs.	1047	456
4.0 hrs.	742	353
6.0 hrs.	446	207
8.0 hrs.	338	147
10.0 hrs.	262	117
12.0 hrs.	196	77
18.0 hrs.	121	ND
24.0 hrs.	75	ND

(ng/ml) POST DOSING WITH XIPAMIDE

3	4	5	6
ND	ND	ND	ND
335	1010	1570	1115
1316	620	1204	1074
926	439	862	777
720	316	643	665
461	198	423	448
340	115	304	364
175	68	144	203
141	ND	123	154
117	ND	114	127
72	ND	79	96
ND	ND	ND	48
45	ND	ND	ND

PLASMA XIPAMIDE CONCENTRATION (ng/ml)

TIME SUBJECT	7	8
PRE DOSE	ND	ND
0.5 hrs.	352	275
1.0 hrs.	810	1608
1.5 hrs.	891	1315
2.0 hrs.	909	1240
3.0 hrs.	787	951
4.0 hrs.	723	719
6.0 hrs.	518	449
8.0 hrs.	412	349
10.0 hrs.	347	245
12.0 hrs.	322	207
18.0 hrs.	171	118
24.0 hrs.	101	ND

POST DOSING WITH XIPAMIDE

9	10	11	12
ND	ND	ND	ND
747	108	1464	283
1279	299	1248	908
991	933	1069	1098
867	818	904	994
528	583	684	714
427	461	488	500
274	271	304	350
223	227	206	279
159	185	163	189
139	129	112	136
82	50	68	79
56	ND	ND	ND

PLASMA XIPAMIDE CONCENTRATION (ng/ml) POST DOSING WITH TRIREXAN

TIME SUBJECT	1	2	3	4	5	6
PRE DOSE	ND	ND	ND	ND	ND	ND
0.5 hrs.	96	1334	919	449	818	823
1.0 hrs.	1202	1232	1329	409	1042	1138
1.5 hrs.	1208	929	1021	506	1279	782
2.0 hrs.	1201	647	760	568	662	591
3.0 hrs.	882	471	511	291	486	429
4.0 hrs.	632	331	389	186	342	319
6.0 hrs.	348	221	228	89	214	211
8.0 hrs.	281	185	189	57	148	120
10.0 hrs.	222	135	146	ND	111	94
12.0 hrs.	176	101	112	ND	84	46
18.0 hrs.	113	ND	60	ND	ND	ND
24.0 hrs.	72	ND	ND	ND	ND	ND

PLASMA XIPAMIDE CONCENTRATION (ng/ml) POST DOSING WITH TRIREXAN

TIME SUBJECT	7	8	9	10	11	12
PRE DOSE	ND	ND	ND	ND	ND	ND
0.5 hrs.	1414	1266	1020	ND	902	844
1.0 hrs.	1340	1424	1377	387	1275	891
1.5 hrs.	1078	1000	979	1037	1166	1051
2.0 hrs.	922	848	774	1042	914	1038
3.0 hrs.	641	628	571	620	602	570
4.0 hrs.	562	464	428	460	458	414
6.0 hrs.	411	311	286	281	270	314
8.0 hrs.	313	211	226	223	192	232
10.0 hrs.	241	174	177	158	152	188
12.0 hrs.	195	106	139	107	134	166
18.0 hrs.	123	ND	81	97	ND	52
24.0 hrs.	ND	ND	ND	ND	ND	ND

ASSAY VALIDATION

INTER ASSAY VALIDATION

Xipamide Concentration ng/ml

Added	Found in 4 batches				Mean
50	54.4	54.4	54.5	50.5	53.5
100	102.2	100.4	95.8	90.2	97.2
250	227.5	239.0	241.6	266.6	243.7
500	488.9	488.5	512.2	498.6	497.1
1000	1026.4	995.1	976.3	1026.9	1006.2
2500	2513.1	2479.9	2464.2	2590.3	2511.9
5000	4990.9	5044.3	5058.4	4885.5	4994.8

SD	CV %	Accuracy %
2.0	3.7	107.0
5.4	5.5	97.2
16.5	6.8	97.5
11.1	2.2	99.4
24.9	2.5	100.6
56.1	2.2	100.5
78.4	1.6	99.9

INTRA ASSAY VALIDATION

Xipamide Concentration ng/ml

Added	Found in 4 batches				Mean
50	45.9	57.6	56.1	53.3	53.2
100	93.0	93.1	103.2	92.6	95.5
250	229.9	247.5	259.2	262.3	249.7
500	515.0	503.3	510.0	489.5	504.5
1000	970.8	975.2	990.5	983.4	980.0
2500	2469.3	2450.6	2474.9	2563.6	2489.6
5000	5083.2	5021.9	5037.9	4971.3	5028.6

SD	CV %	Accuracy %
5.2	9.8	106.4
5.2	5.4	95.5
14.7	5.9	99.9
11.1	2.2	100.9
8.7	0.9	98.0
50.4	2.0	99.6
46.2	0.9	100.6

TRIAMTEREN METHODOLOGY

PLASMA TRIAMTERENE ASSAY (HPLC)

Reagents :

- Methanol HPLC Grade.
- Dichloromethane HPLC Grade.
- Internal Standard Cianopramine.
- Diethyl ether HPLC Grade.
- Helium Gas.
- Isopropan-2-ol HPLC Grade.
- Nanopure Water.

Instruments :

- Spectra Physics Labnet H.P.L.C. System.
  (Integrator, pump, automatic injector).
- Fluorescence spectrometer LS-3. Perkin Elmer.
- Column Spherisorb 3 Silica (15 x 4.6 cm).
- Pre column - Guard Pak Silica.

Working Conditions

- Mobile Phase :

  70 : 30 *

  Dichloro Methane : Methanol plus 0.1% Ammonia.

  Flow Rate : 1.6 ml/min.

  Fluorescence detector. Excitation 315 nm.

  Emission Filter 420nm.

  Chart Speed. 0.5 cm/min.

  Retention time Internal standard 1.9 minutes

  Triamterene 2.9 minutes

  Sensitivity. 5ng/ml triamterene.

*Following the use of a new replacement column, the mobile phase ratio was changed to 80 : 20 so as to obtain closely related retention times.

Standard Solutions :

A stock solution (0.1mg/ml) was prepared in methanol.

A first working solution of 10 ug/ml was prepared as follows :

1 ml stock solution + 4 ml methanol + 5 ml nanopure water.

A further series of working standards were prepared in methanol/water (1:1) from working solution (10 ug/ml) and these were 100, 200, 500, 1000, 2000, 4000, 6000

50 ul of each standard was taken and a 1 ml aliquot of plasma was spiked to give the following standard concentrations :

5, 10, 25, 50, 100, 200, 300 ng/ml.

Internal Standards :

Cianopramine - 12.5 ug/ml in methanol/water (1:1).

Assay Procedure :

Plasma triamterene assay was carried out as outlined below.

Briefly, 1.0 ml plasma spiked with 50 ul internal standard solution (in methanol/water) was extracted with diethylether/isopropan-2-ol. After centrifugation, the organic layer (6 ml) was separated, dried and reconstituted with 200 ul methanol prior to injection onto the HPLC column.

Chromatography was carried out on a Spherisorb 3 silica column with fluorescence detection at 420 nm.

All standards and QC samples were assayed in duplicate and the mean values reported. Likewise, 10% of plasma samples were assayed in duplicate and the mean values reported.

METHOD

1. Take 1 ml plasma. Spiked or unknown.
2. Add 50 ul of Internal standard.
3. Vortex.
4. Add 7 ml diethyl ether : Isopropan-2-ol (19:1).
5. Rotate for 15 minutes.
6. Centrifuge at 3000 rpm for 5 minutes.
7. Remove 6 ml of organic layer and evaporate under a stream of Nitrogen at 35-37 C.
8. Reconstitute residue in 200 ul Methanol. Vortex well.
9. Inject 50 ul onto column.

ASSAY VALIDATION

INTER ASSAY VALIDATION

Triamterene concentration ng/ml

Added	Found in 4 batches				Mean
5	3.8	4.2	4.6	4.1	4.2
10	12.1	12.0	11.4	12.7	12.1
25	25.9	24.5	23.4	24.1	24.5
50	49.9	50.7	49.8	53.9	51.1
100	99.1	96.8	99.7	91.8	96.9
200	204.8	206.6	203.3	193.9	202.1
300	296.4	296.3	297.2	312.3	300.6

SD	CV %	Accuracy %
0.3	7.9	84.0
0.5	4.2	121.0
1.1	4.4	98.0
1.9	3.8	102.2
3.6	3.7	96.9
5.7	2.8	101.1
7.9	2.6	100.2

INTRA ASSAY VALIDATION

Triamterene Concentration ng/ml

Added	Found in 4 batches				Mean
5	4.6	4.7	4.9	3.4	4.4
10	11.6	10.8	11.7	13.7	12.0
25	23.6	23.8	23.5	24.5	23.4
50	48.3	50.9	49.4	48.9	49.4
100	99.6	101.2	104.6	99.0	101.1
200	201.5	198.3	202.0	211.7	203.4
300	301.7	286.9	295.3	301.7	296.4

SD	CV %	Accuracy %
0.7	15.5	88.0
1.2	10.4	120.0
0.6	2.6	93.6
1.1	2.3	98.8
2.5	2.5	101.1
5.8	2.8	101.7
7.0	2.4	98.8

CALIBRATION DATA

CALIBRATION DATA

ASSAY NO. 1

	Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
	5	4.5	-10.0
	10	12.7	+27.0
	25	22.3	-10.8
	50	48.6	- 2.8
	100	102.9	+ 2.9
QC	15	13.8	- 8.0
QC	40	41.3	+ 3.3
QC	75	69.9	- 6.8

CALIBRATION DATA

ASSAY NO. 2

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	4.4	-12.0
10	12.2	+22.0
25	23.5	- 6.0
50	48.4	- 3.2
100	102.0	+ 2.0
QC 15	12.6	-16.0
QC 40	35.1	-12.3
QC 75	75.3	+ 0.4

CALIBRATION DATA

ASSAY NO. 3

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.4	+ 8.0
10	9.9	- 1.0
25	25.2	+ 0.8
50	49.1	- 1.8
100	85.1	-14.9
QC 15	14.4.	- 4.0
QC 40	38.4	- 4.0
	28.3	-29.3
QC 75	78.7	+ 4.9
	74.1	- 1.2

CALIBRATION DATA

ASSAY NO. 4

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.7	+14.0
10	9.8	- 2.0
25	22.6	- 9.6
50	50.4	+ 0.8
100	104.3	+ 4.3
QC 15	12.5	-16.7
	19.4	+29.3*
	17.0	+13.3
QC 40	39.7	- 0.8
	44.2	+10.5
QC 75	75.6	+ 0.8
	82.0	+ 9.3

CALIBRATION DATA

ASSAY NO. 5

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.7	+14.0
10	10.7	+ 7.0
25	21.6	-13.6
50	48.0	- 4.0
100	99.9	- 0.1
QC 15	15.0	0
QC 40	37.9	- 2.1
QC 75	76.3	+ 1.7

CALIBRATION DATA

ASSAY NO. 6

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.4	+ 8.0
10	10.2	+ 2.0
25	23.5	- 6.0
50	49.0	- 2.0
100	97.6	- 2.4
QC 15	16.1	+ 7.3
QC 40	41.2	+ 3.0
	33.6	-16.0*
QC 75	77.6	+ 3.5
	72.7	- 3.1

CALIBRATION DATA

ASSAY NO. 7

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.0	0
10	11.6	+16.0
25	22.2	-11.2
50	48.1	- 3.8
100	103.9	+ 3.9
QC 15	14.4	- 4.0
QC 40	40.8	+ 2.0
QC 75	76.5	+ 2.0

CALIBRATION DATA

ASSAY NO. 8

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	4.3	-14.0
10	13.1	+31.0
25	22.0	-12.0
50	51.3	+ 2.6
100	100.4	+ 0.4
QC 15	14.5	- 3.3
QC 40	39.5	- 1.3
QC 75	86.3	+15.1

CALIBRATION DATA

ASSAY NO. 9

Triamterene Added (ng/ml)	Triamterene Found (ng/ml)	% Difference
5	5.2	+ 4.0
10	10.2	+ 2.0
25	23.2	- 7.2
50	54.0	+ 8.0
100	94.5	- 5.5
QC 15	15.5	+ 3.3
QC 40	40.5	+ 1.3
QC 75	71.0	- 5.3
	77.7	+ 3.6

PLASMA TRIAMTERENE LEVELS

PLASMA TRIAMTERENE CONCENTRATION
TRIAMTERENE

TIME	SUBJECT 1	2
PRE DOSE	ND	ND
0.5 hrs.	18	76
1.0 hrs.	23	56
1.5 hrs.	17	45
2.0 hrs.	20	33
3.0 hrs.	12	17
4.0 hrs.	7	9
6.0 hrs.	ND	ND
8.0 hrs.	ND	ND
10.0 hrs.	ND	ND
12.0 hrs.	ND	ND
18.0 hrs.	ND	ND
24.0 hrs.	ND	ND

(ng/ml) POST DOSING WITH

3	4	5	6
ND	ND	ND	ND
36	36	73	47
30	31	83	53
20	17	68	35
14	19	64	28
9	7	45	16
ND	ND	30	9
ND	ND	12	5
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND

PLASMA TRIAMTERENE LEVELS (ng/ml)

TIME	SUBJECT 7	8
PRE DOSE	ND	ND
0.5 hrs.	64	43
1.0 hrs.	43	52
1.5 hrs.	29	39
2.0 hrs.	22	29
3.0 hrs.	10	16
4.0 hrs.	5	11
6.0 hrs.	ND	ND
8.0 hrs.	ND	ND
10.0 hrs.	ND	ND
12.0 hrs.	ND	ND
18.0 hrs.	ND	ND
24.0 hrs.	ND	ND

POST DOSING WITH TRIAMTERENE

9	10	11	12
ND	ND	ND	ND
36	42	27	92
30	30	53	43
39	24	52	28
28	20	43	26
13	18	19	14
9	5	10	8
ND	ND	ND	6
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND

PLASMA TRIAMTERENE LEVELS (ng/ml)

TIME	SUBJECT 1	2
PRE DOSE	ND	ND
0.5 hrs.	ND	47
1.0 hrs.	39	43
1.5 hrs.	24	35
2.0 hrs.	22	29
3.0 hrs.	9	18
4.0 hrs.	ND	12
6.0 hrs.	ND	ND
8.0 hrs.	ND	ND
10.0 hrs.	ND	ND
12.0 hrs.	ND	ND
18.0 hrs.	ND	ND
24.0 hrs.	ND	ND

POST DOSING WITH TRIREXAN

3	4	5	6
ND	ND	ND	ND
55	20	70	56
42	11	101	45
34	21	75	29
22	16	56	19
13	11	38	13
8	9	24	7
ND	7	22	ND
ND	ND	11	ND
ND	ND	ND	ND
ND	ND	ND	6
ND	ND	ND	ND
ND	ND	ND	ND

PLASMA TRIAMTERENE CONCENTRATION

TIME	SUBJECT 7	8
PRE DOSE	ND	ND
0.5 hrs.	59	44
1.0 hrs.	39	32
1.5 hrs.	28	38
2.0 hrs.	21	32
3.0 hrs.	10	19
4.0 hrs.	5	11
6.0 hrs.	ND	ND
8.0 hrs.	ND	ND
10.0 hrs.	ND	ND
12.0 hrs.	ND	ND
18.0 hrs.	ND	ND
24.0 hrs.	ND	ND

(ng/ml) POST DOSING WITH TRIREXAN

9	10	11	12
ND	ND	ND	ND
64	ND	97	79
56	26	63	39
34	50	55	28
23	26	31	22
11	15	16	11
5	11	12	10
ND	6	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	ND	ND	ND
ND	6	ND	ND
ND	ND	ND	ND

PHARMACOKINETIC DATA ANALYSIS

Pharmacokinetic Data analysis

Plasma Xipamide levels measured over a 24 hour period post dosing with Xipamide alone and in a fixed combination as Trirexan were analysed using the JANA curve stripping prgram. The estimates of the pharmacokinetic parameters thus obtained were then used in curve fitting analysis using an IBM-PC compatible version of NONLIN 84. Plasma triamterene levels post dosing with Triamterene and Trirexan were likewise analysed.

Xipamide

The mean AUC values after dosing with Xipamide and Trirexan were 5554 and 5255 ng.h/ml respectively, and the mean elimination half-life values were 3.82 and 3.14 h. The mean observed Cmax values were 1222 and 1203 ng/ml, and the mean observed Tmax values were 1.13 and 1.21 h. These mean values were not significantly different ($p > 0.05$).

Triamterene

The mean AUC values post dosing with Triamterene and Trirexan were 138 and 145 ng.h/ml respectively, and the mean elimination half-life values were 1.28 and 1.47 h. The mean observed Cmax values were 54 and 59 ng/ml, as

the mean observed Tmax values were 0.79 and 0.75 h. These mean values were not significantly different ($p > 0.05$).

There were no statistically significant differences between the pharmacokinetic parameters studied after administration of either formulation.

THE JANA PROGRAM

THE JANA COMPUTER PRIOGRAM FOR EXPONENTIAL STRIPPING OF PHARMACOKINETOC DATA

This program is used for the statistical analysis of pharmacokinetic data. The model used is the polyexponential equation

$$Y = A,\exp(-B, (t-L))$$

where Y is the response variable e.g. plasma drug concentration at time t following drug administration, m is the number of exponential terms with exponents B,and L is the lag time if one is required.

Anlysis of pharmacokinetic data generally involves a parameter estimation or curve fitting step which is frequently accomplished by a nonlinear least squares regression program e.g. PCNONLIN (1). However, nonlinear least squares programs require initial estimates of the parameters and the success of the least squares analysis depends on how good the initial estimates are . A number of graphical/numerical techniques have been described (2-7) for the provision of initial parameter estimates for polyexponential models . The most commonly used of these is curve stripping (8). This method assumes that the terminal data points can be described by a single exponential term and its coefficient and exponent are estimated by fitting a straight line to these points on a semi-logarithmic plot. The value of this exponential term at all of the earlier times is estimated using these parameter estimates and subtracted from the original data to yield a set of so-called residuals. The terminal residuals are assumed to described by a

single exponential term whose parameters are estimated by fitting a straight line to them on a semi-logarithmic plot. This procedure is repeated until the desired number of exponential terms have been accounted for and their parameters estimated. This method has been automated for digital computers (9-11).

The curve stripping technique makes the assumption that the exponential terms do not significantly overlap one another and consequently their parameters may be estimated one term at a time. This assumption is approximately true if the ratios of the exponents are large i.e.

$$B1 >> B2 >> B3 \ldots\ldots\ldots\ldots >> Bm$$

However , violation of this assumption would be expected to yield biased parameter estimates (12-14).

JANA is based on an approach to curve stripping which does not make any assumption regarding the ratios of the exponents. This technique iteratively corrects for overlap of the exponent terms (15-18).

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MODELLING, NONLINEAR REGRESSION AND PHARMACOKINETICS

The value of mathematical models is well recognised in all of the sciences - physical , biological , behavioral and others .Models are often used in the quantitative analysis of all types of observations or data and with the power and availability of computers , mathematical models provide convenient and powerful ways of looking at data. Models can be used to help interpret data , to test hypotheses and to predict future results. We are concerned in pharmacokinetics with "fitting" models to data ; that is , finding a mathematical equation and a set of parameter values such that values predicted by the same model are in some sense "close" to the observed values.

The most commonly encountered of these models are linear models in which the dependent variable can be expressed as the sum of products of the independent variables and parameters. The simplest example being the equation of a line :-

$$Y = A + B*X$$

where Y is the dependent variable , X is the independent variable , A is the intercept and B is the slope. Other commonly used examples are polynomials such as :-

$$Y = A1 + A2*X + A3*X^2 + A4*X^3$$

and multiple linear regression :-

$$Y = A1 + A2*X1 + A3*X2 + A4*X3$$

These examples are all "linear models" since the

parameters appear only as coeffecients of the indepfndent variables.

In non linear models , at least one of the parameters appears as other than a coeffecient . A simple example is the decay curve

$$Y = YO*\exp(-B*X)$$

This model can be linearised by taking the log of both sides but as written it is nonlinear. There are many models which cannot be made linear by transformation. One such model is the sum of two or more exponentials, such as

$$Y = A1*\exp(-B1*X) + A2*\exp(-B2*X).$$

These models are called nonlinear models or nonlinear regression models. All pharmacokinetic models derive from a set of basic differential equations. Given a model of the data and a set of data some criteria of best fit is needed and the most frequently used is the least squares method. In the least squares method the best estimates are those which minamise the sum of the squares of the deviations between the observed values and the values predicted by the model.

XIPAMIDE
PHARMACOKINETICS

ELIMINATION HALF-LIFE AND AUC VALUES
2-3 EXPONENTIAL MODELS
(using PC NONLIN Curve Fitting)

Drug: Xipamide/Trirexan

	XIPAMIDE			TRIREXAN		
Subj.	r Squared	Elim. t-1/2 h	AUC ng.h/ml	r Squared	Elim. t-1/2 h	AUC ng.h/ml
1	0.810	3.64	6920	0.834	2.83	6464
2	0.994	3.36	4645*	0.998	4.94	5115*
3	0.964	1.23	3588	-	cd	cd
4	-	cd	cd	0.949	1.61	1988
5	0.998	4.13	4565*	0.931	1.66	3830
6	0.998	4.12	4635*	0.960	1.86	3432
7	0.976	2.86	6402	0.998	5.91	7920*
8	-	cd	cd	-	cd	cd
9	0.953	2.50	5151	-	cd	cd
10	-	cd	cd	-	cd	cd
11	1.000	5.96	6551*	0.978	2.06	4839
12	0.953	2.69	5163	0.953	2.69	5184
Mean		3.39	5291		2.95	4847
Std.Dev.		1.32	1106		1.61	1830
N		9	9		8	8

cd = Parameter values cannot be determined with reasonable accuracy.

* = Parameter values determined using 3 exponential model.

Initial estimates of derived parameters
2-3 Exponential Terms (JANA Curve Stripping)

Drug: Xipamide

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.782	1144	-1145	NA	0.1244	0.6422	NA
2	0.999	669	1492	-2161	0.1808	1.0418	4.999
3	0.808	3078	-3080	NA	0.5740	1.5251	NA
4	0.982	511	1579	-2090	0.3429	1.6476	4.1753
5	0.999	392	2169	-2561	0.1359	0.9214	5.4899
6	0.997	443	1517	-1959	0.1257	0.7878	3.6030
7	0.951	1027	-1027	NA	0.0999	2.0930	NA
8	0.857	621	3819	-4415	0.0924	0.5554	1.0884
9	0.786	870	-870	NA	0.1323	5.1300	NA
10	0.682	923	3895	-4818	0.1623	1.7980	1.4505
11	0.999	315	1446	-1760	0.0878	0.4054	9.2251
12	0.843	1098	-1099	NA	0.1629	3.2061	NA

NA = Not applicable to 2 exponential model.

Final estimates of derived parameters
2-3 Exponential Model (PCNONIN Curve Fitting)

Drug: Xipamide

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.810	1534	-1714	NA	0.1907	1.5259	NA
2	0.994	724	1535	-2256	0.2064	0.9198	4.2510
3	0.964	3050	-3070	NA	0.5658	1.7034	NA
4	-	cd	cd	cd	cd	cd	cd
5	0.998	477	2082	-2559	0.1680	0.9510	5.5228
6	0.998	636	2201	-2837	0.1682	1.1957	2.8802
7	0.978	1610	-1599	NA	0.2421	6.4414	NA
8	-	cd	cd	cd	cd	cd	cd
9	0.953	1541	-1577	NA	0.2773	3.8969	NA
10	-	cd	cd	cd	cd	cd	cd
11	1.000	423	1356	-1779	0.1162	0.4339	8.3812
12	0.953	1507	-1508	NA	0.2575	2.1900	NA

NA = Not applicable to 2 exponential model.

Initial estimates of derived parameters
2-3 Exponential Terms (JANA curve stripping)

Drug: Trirexan

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.729	1648	-1650	NA	0.1489	1.9046	NA
2	0.997	488	2545	-3032	0.1292	1.0224	3.0821
3	0.973	425	3077	-3505	0.1091	0.8705	2.0627
4	0.944	967	-967	NA	0.3788	1.8297	NA
5	0.873	1160	-1160	NA	0.2396	3.4097	NA
6	0.934	1097	-1097	NA	0.2650	4.1441	NA
7	0.997	749	2474	-3224	0.1053	1.0722	2.9313
8	0.991	852	2630	-3482	0.1692	1.0951	2.4205
9	0.975	489	3123	-3612	0.1011	0.8682	1.8005
10	0.700	185	1849	-2035	0.0408	0.4412	1.2705
11	0.935	1304	-1304	NA	0.2152	3.1596	NA
12	0.917	1078	-1078	NA	0.1717	4.0055	NA

NA = Not applicable to 2 exponential model

Final estimates of derived parameters

2-3 Exponential Terms (PCNONLIN Curve Fitting)

Drug: Trirexan

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.834	2030	-2248	NA	0.2450	1.2337	NA
2	0.998	513	2549	-3064	0.1402	1.0471	3.1226
3	-	cd	cd	cd	cd	cd	cd
4	0.949	1212	-1199	NA	0.4315	1.4616	NA
5	0.931	2056	-2090	NA	0.4187	1.9321	NA
6	0.960	1469	-1494	NA	0.3736	2.9930	NA
7	0.998	800	2380	-3180	0.1173	1.0994	2.9714
8	-	cd	cd	cd	cd	cd	cd
9	-	cd	cd	cd	cd	cd	cd
10	-	cd	cd	cd	cd	cd	cd
11	0.978	1922	-1942	NA	0.3363	2.2179	NA
12	0.953	1516	-1508	NA	0.2575	2.1503	NA

Paired t-Test Result

Drugs : Xipamide and Trirexan.

	Cmax ng/ml	Tmax h
Mean		
Xipamide	1222	1.13
Trirexan	1203	1.21
Std. Error	70	0.25
t-value Probability	0.792	0.746
N	12	12

Elim. t 1/2 h	AUC ng.h/ml
3.82	5554
3.14	5255
0.92	409
0.512	0.504
7	7

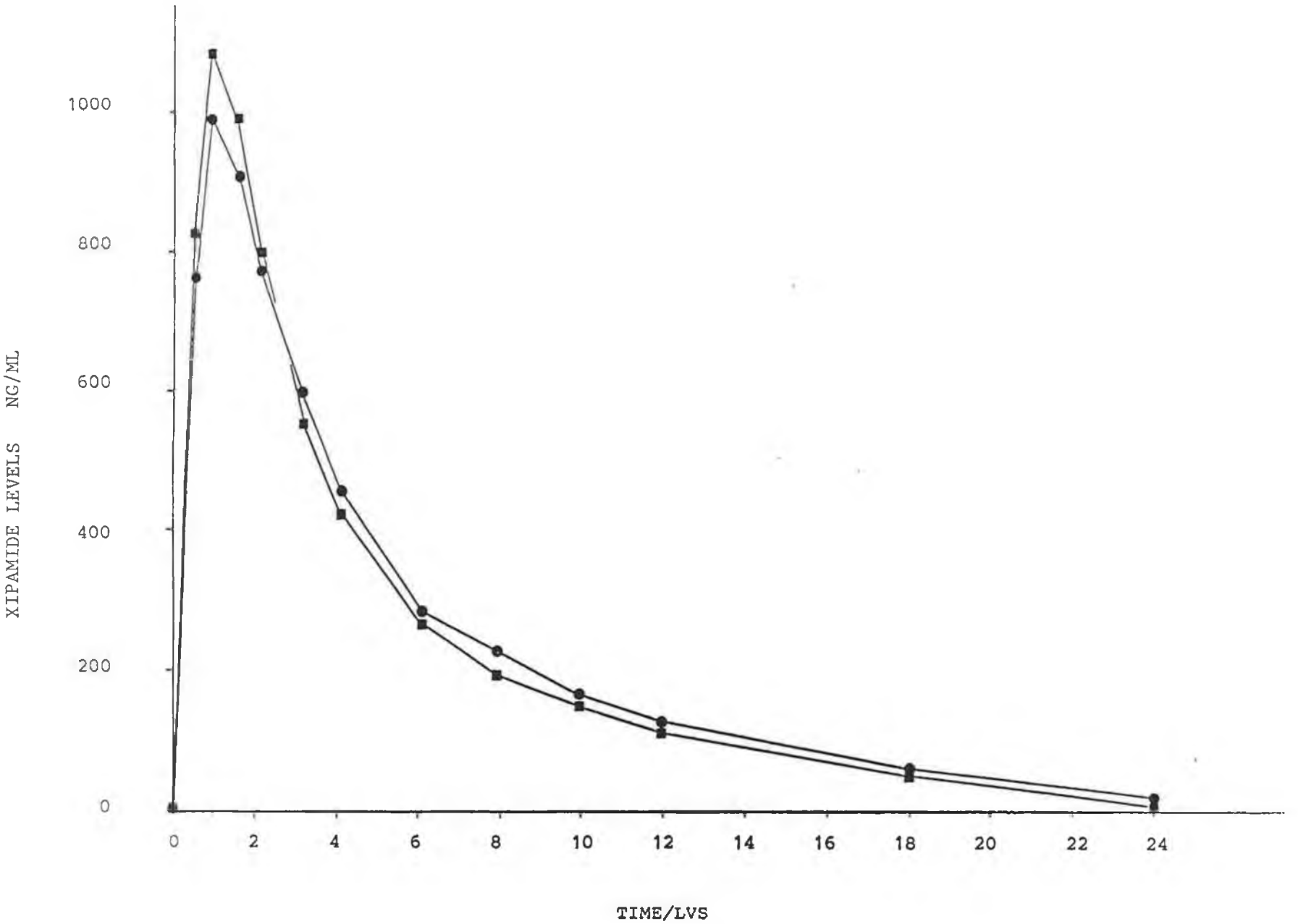
MEAN PLASMA XIPAMIDE LEVELS (N=12)

Drug: Xipamide and Trirexan

Time	Xipamide ng/ml	Trirexan ng/ml
Pre Dose	0	0
0.5 hr Post Dose	733	824
1 hr " "	988	1087
1.5 hr " "	886	1003
2 hr " "	774	831
3 hr " "	607	559
4 hr " "	461	415
6 hr " "	284	265
8 hr " "	217	198
10 hr " "	169	150
12 hr " "	130	114
18 hr " "	61	44
24 hr " "	23	6

For determination of mean value ND = 0 ng/ml.

MEAN PLASMA XIPAMIDE LEVELS (ng/ml) POST DOSING WITH XIPAMIDE (●) AND TRIXEXAN (■) N = 12



TRIAMTERENE
PHARMACOKINETICS

ELIMINATION HALF-LIFE AND AUC VALUES

2-3 EXPONENTIAL MODELS

(using PC NONLIN Curve Fitting)

	TRIAMTERENE			TRIREXAN		
Subj.	r Squared	t-1/2 Elim. h	AUC ng.h/ml	r Squared	t-1/2 Elim. h	AUC ng/h/ml
1	0.972	1.35	72	-	cd	cd
2	0.998	1.15	161	0.994	1.41	132
3	0.992	0.98	71	0.996	1.14	118
4	0.976	0.90	70	0.878	2.72	89
5	0.994	1.81	298	0.964	1.56	288
6	0.988	1.31	132	0.994	0.98	109
7	-	cd	cd	0.996	0.90	104
8	0.988	1.30	135	0.966	1.80	135
9	0.956	1.45	116	0.998	0.76	116
10	0.978	1.41	102	0.701	1.82	129
11	0.951	1.21	154	0.992	1.01	185
12	0.998	1.60	170 *	-	cd	cd
Mean		1.32	135		1.41	141
Std.Dev.		0.26	65		0.59	57.8
N		11	11		10	10

* = Parameter values determined using 3 exponential model.

cd = Parameter values cannot be determined with reasonable accuracy.

Initial estimates of derived parameters
2-3 Exponential Terms (JANA curve stripping)

Drug: Triamterene

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.958	34.8	-34.8	NA	0.3783	2.3414	NA
2	0.997	110.3	-110.3	NA	0.6222	6.2765	NA
3	0.984	50.4	-50.4	NA	0.5932	7.0478	NA
4	0.975	59.4	-59.4	NA	0.6903	4.5719	NA
5	0.992	129.8	-129.8	NA	0.3833	2.6686	NA
6	0.979	72.5	-72.5	NA	0.4723	3.9116	NA
7	0.878	1326.9	-1326.9	NA	2.3809	2.7264	NA
8	0.982	85.1	-85.1	NA	0.5277	2.6714	NA
9	0.948	63.5	-63.5	NA	0.4864	3.0563	NA
10	0.974	57.1	-57.1	NA	0.5351	6.9923	NA
11	0.935	145.6	-145.7	NA	0.6849	1.8309	NA
12	0.996	44.0	350.9	-394.8	0.3577	3.3544	7.284

NA = Not applicable to 2 exponential model.

Final estimates of derived parameters
2-3 Exponential Terms (PCNONLIN Curve Fitting)

Drug: Triamterene

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.972	51.7	-51.8	NA	0.5122	1.7788	NA
2	0.988	106.8	-106.9	NA	0.6053	6.9352	NA
3	0.992	58.9	-58.5	NA	0.7104	4.7994	NA
4	0.976	67.8	-67.9	NA	0.7699	3.8252	NA
5	0.994	134.8	-134.4	NA	0.3839	2.5126	NA
6	0.988	82.4	-82.4	NA	0.5279	3.4177	NA
7	-	cd	cd	cd	cd	cd	cd
8	0.988	89.5	-89.7	NA	0.5329	2.6809	NA
9	0.956	69.0	-68.5	NA	0.4779	2.4242	NA
10	0.978	52.9	-52.7	NA	0.4925	8.9543	NA
11	0.951	152.8	-155.5	NA	0.5742	1.3842	NA
12	-	cd	cd	cd	cd	cd	cd

cd = Parameter values cannot be determined with reasonable accuracy.
NA = Not applicable to 2 exponential model.

Initial estimates of derived parameters
2-3 Exponential Terms (JANA curve stripping)

Drug: Trirexan

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	0.601	118.9	-119.0	NA	0.8938	1.8701	NA
2	0.992	67.8	-67.8	NA	0.4349	4.3650	NA
3	0.996	73.7	-73.7	NA	0.5650	9.8845	NA
4	0.818	25.7	-25.7	NA	0.2353	1.6861	NA
5	0.937	107.4	-107.4	NA	0.2930	3.1045	NA
6	0.976	236.6	-236.6	NA	1.2870	2.4604	NA
7	0.960	81.9	-81.9	NA	0.6983	3.8568	NA
8	0.964	55.3	-55.3	NA	0.3759	6.8367	NA
9	0.995	116.2	-116.2	NA	0.7897	4.1922	NA
10	0.699	65.2	-65.3	NA	0.4354	1.2863	NA
11	0.977	114.4	-114.4	NA	0.5961	9.1615	NA
12	0.685	14.6	56.6	-71.3	0.0953	0.8719	3.2200

NA = Not applicable to 2 exponential model.

Final estimates of derived parameters
2-3 Exponential Terms (PCNONLIN Curve Fitting)

Drug: Trirexan

Subj. No.	r Squared	Derived Parameters A1	A2	A3	B1	B2	B3
1	-	cd	cd	cd	cd	cd	cd
2	0.994	74.7	-74.7	NA	0.4908	3.6949	NA
3	0.996	79.3	-79.3	NA	0.6063	6.1346	NA
4	0.878	23.3	-23.5	NA	0.2546	9.1060	NA
5	0.964	157.5	-158.0	NA	0.4447	2.3735	NA
6	0.994	88.3	-88.4	NA	0.7074	5.4445	NA
7	0.996	89.8	-89.1	NA	0.7661	6.7459	NA
8	0.996	54.9	-54.9	NA	0.3849	7.1201	NA
9	0.998	146.9	-146.9	NA	0.9093	3.2603	NA
10	0.701	74.6	-79.8	NA	0.3800	1.1749	NA
11	0.992	135.5	-135.3	NA	0.6889	11.6576	NA
12	-	cd	cd	cd	cd	cd	cd

cd = Parameter values cannot be determined with reasonable accuracy.
NA = Not applicable to 2 exponential model.

OBSERVED Cmax AND Tmax VALUES

Drug: Triamterene and Trirexan

	TRIAMTERENE		TRIREXAN	
Subj. No.	Cmax ng/ml	Tmax h	Cmax ng/ml	Tmax h
1	23	1.00	39	1.00
2	76	0.50	47	0.50
3	36	0.50	55	0.50
4	36	0.50	21	1.50
5	83	1.00	101	1.00
6	53	1.00	56	0.50
7	64	0.50	59	0.50
8	52	1.00	44	0.50
9	39	1.50	64	0.50
10	42	0.50	50	1.50
11	53	1.00	97	0.50
12	92	0.50	79	0.50
Mean	54	0.79	59	0.75
Std. Dev.	21	0.33	23	0.40
N	12	12	12	12

Paired t-Test Result

Drugs : Triamterene and Trirexan.

	Cmax ng/ml	Tmax h
Mean		
Triamterene	54	0.79
Trirexan	59	0.75
Std. Error	6	0.17
t-value Probability	0.605	0.803
N	12	12

Elim. t 1/2 h	AUC ng.h/ml
1.28	138
1.47	145
0.24	9
0.533	0.550
9	9

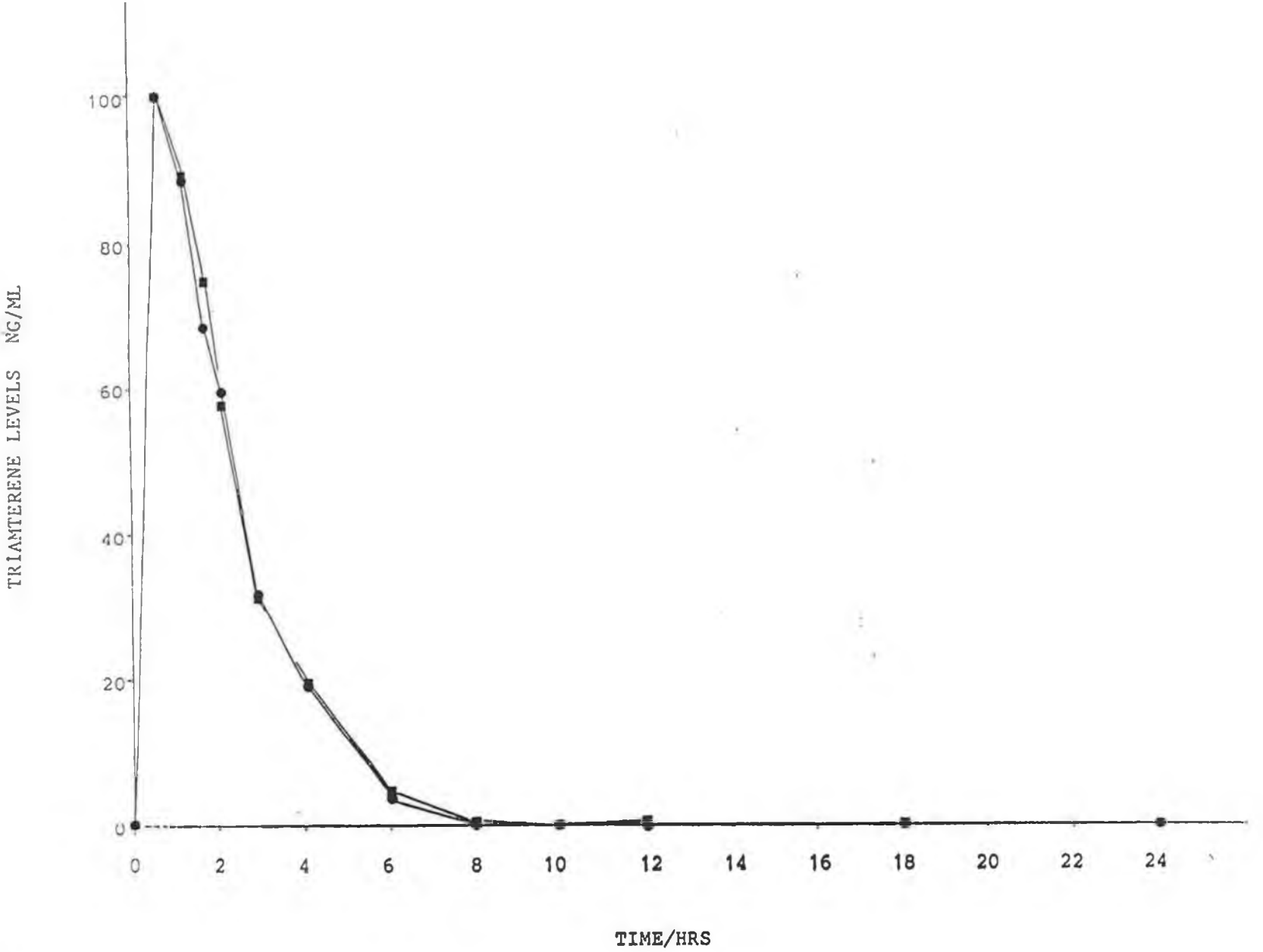
MEAN PLASMA TRIAMTERENE LEVELS (N=12)

Drug : Triamterene and Trirexan.

Time	Triamterene ng/ml	Trirexan ng/ml
Pre dose	0	0
0.5 hr. post dose	49	49
1.0 hr.	44	45
1.5 hr.	34	38
2.0 hr.	29	27
3.0 hr.	16	15
4.0 hr.	9	10
6.0 hr.	2	3
8.0 hr.	0	1
10.0 hr.	0	0
12.0 hr.	0	1
18.0 hr.	0	1
24.0 hr.	0	0

For the determination of mean values ND = 0 ng/ml.

MEAN PLASMA TRIAMTERENE LEVELS POST DOSING WITH TRIAMTERENE ( ) AND TRIREXAN ( ) (N=12)



DETERMINATION OF THE AREA UNDER THE TIME/CONCENTRATION CURVE USING THE TRAPEZOIDAL METHOD

Determination of the area under the curve (AUC) using the trapezoidal method

Plasma xipamide and triamterene levels after dosing with xipamide, triamterene and Trirexan were also used to determine AUC values by the trapezoidal method over the following periods : 0-4, 0-6 and 0-24 hours.

a) Xipamide

The mean xipamide AUC values post dosing with xipamide and Trirexan over 0-4 hour period were 2721 (range 1523-3840) and 2846 (1492-3595) ng.h/ml respectively ; those over the 0-6 hour period were 3467 (1706-5008) and 3527 (1767-4503), and over the 0-24 hour period 5510 (1774-8751) and 5225 (1970-7540 ng.h/ml). Paired t-test analysis showed that the AUC values for each of these periods were not significantly different ($p = 0.320$, 0.645 and 0.310 respectively). The AUC values obtained for subject 4 were consistently low (approx. 2 standard deviations or more from the mean) irrespective of the drug formulation.

Over the 0-24 hour period, the AUC ratio (Trirexan/xipamide) ranged from 0.70 to 1.26 with a mean value of 0.98 ; only 1 value was just outside

the lower limit range (subject 8 : 0.69). The AUC ratio was essentially the same over the 3 periods studied.

b) Triamterene

The mean triamterene AUC values post dosing with triamterene and Trirexan over 0-4 hour period were each 106 ng.h/ml, and the AUC ranges were 60-220 and 54-215 ng.h/ml respectively. Those over 0-6 hour period were 117 and 118 ng.h/ml respectively and the corresponding ranges were 63-262 and 57-261 ng.h/ml : those over 0-24 hour period were 119 and 128 ng.h/ml and the corresponding ranges were 63-274 and 57-305 ng.h/ml. Paired t-test analysis indicated that the AUC values for each of these periods were not significantly different (p = 0.950, 0.847 and 0.251).

The AUC ratio over 0-24 hour period (Trirexan/triamterene) ranged from 0.54-1.66 with a mean value of 1.10. One subject (number 3) was above the upper limit (1.70).

The AUC values indicated that triamterene absorption was complete 4 hours following triamterene and Trirexan administration, except in 2 subjects (No 3, 10) where a longer absorption process was apparent.

AUC VALUES (Trapezoidal Method)

DRUG: XIPAMIDE

AUC ng.h/ml

Subject	0-4 hr.	0-6 hr.	0-24 hr.
1	2407	3595	6976
2	2736	3296	4339
3	2460	2975	4440
4	1523	1706	1774
5	2875	3323	4257
6	2612	3179	4616
7	2857	4098	8751
8	3840	5008	8181
9	2900	3601	5855
10	2096	2828	4739
11	3497	4289	6187
12	2854	3704	6008
Mean	2721	3467	5510
S.D.	599	821	1908
n	12	12	12

AUC VALUES (Trapezoidal Method)

DRUG: TRIREXAN (XIPAMIDE)

AUC ng.h/ml

Subject	0-4 hr.	0-6 hr.	0-24 hr.
1	3352	4332	7284
2	2869	3421	4686
3	2910	3527	5233
4	1492	1767	1970
5	2723	3279	4347
6	2403	2933	3756
7	3530	4503	7540
8	3341	4116	5621
9	3054	3768	5902
10	2344	3085	5138
11	3188	3916	5410
12	2949	3677	5807
Mean	2846	3527	5225
S.D.	559	732	1490
n	12	12	12

AUC VALUES (Trapezoidal Method)

DRUG: TRIAMTERENE

AUC ng.h/ml

Subject	0-4 hr.	0-6 hr.	0-24 hr.
1	60	67	67
2	135	144	144
3	63	63	63
4	63	63	63
5	220	262	274
6	109	123	128
7	97	102	102
8	110	121	121
9	91	100	100
10	84	89	89
11	122	138	144
12	118	132	138
Mean	106	117	119
S.D.	44	54	57
n	12	12	12

OBSERVED Cmax AND Tmax VALUES

Drug: Xipamide and Trirexan

Subj. No.	XIPAMIDE		TRIREXAN	
	Cmax	Tmax	Cmax	Tmax
1	1047	3.0	1208	1.5
2	1320	0.5	1334	0.5
3	1316	1.0	1329	1.0
4	1001	0.5	568	2.0
5	1570	0.5	1279	1.5
6	1115	0.5	1138	1.0
7	909	2.0	1414	0.5
8	1608	1.0	1424	1.0
9	1279	1.0	1377	1.0
10	933	1.5	1042	2.0
11	1464	0.5	1275	1.0
12	1098	1.5	1051	1.5
Mean	1222	1.13	1203	1.21
Std. Dev.	241	0.77	238	0.50
N	12	12	12	12

AUC VALUES (Trapezoidal Method)

DRUG: TRIREXAN (TRIAMTERENE)

AUC ng.h/ml

Subject	0-4 hr.	0-6 hr.	0-24 hr.
1	57	57	57
2	108	120	120
3	99	107	107
4	54	70	77
5	215	261	305
6	96	103	127
7	91	96	96
8	103	114	114
9	108	113	113
10	78	95	137
11	153	165	165
12	106	116	116
Mean	106	118	128
S.D.	43	52	62
n	12	12	12

PAIRED T-TEST : AUC VALUES (TRIAMTERENE/TRIREXAN)

	TIME PERIODS		
	0-4 hour	0-6 hour	0-24 hour
Mean Triamterene (30mg)	106	117	119
Trirexan*	106	118	128
Diff. between means	-	1	9
Standard Error	5	6	7
t-value probability	0.950	0.847	0.251

* Trirexan containing 30mg triamterene.

PAIRED T-TEST : AUC VALUES (XIPAMIDE/TRIREXAN)

	TIME PERIODS		
	0-4 hour	0-6 hour	0-24 hour
Mean Xipamide(10mg)	2721	3467	5510
Trirexan*	2846	3527	5225
Diff. between means	125	60	286
Standard Error	120	126	268
t-value probability	0.320	0.645	0.310

*Trirexan containing 10mg xipamide.

RESULTS

RESULTS

a) Xipamide

The mean AUC values after dosing with xipamide and Trirexan were 5554 and 5255 ng.h/ml respectively, and the mean elimination half-life values were 3.82 and 3.14 h. The mean observed Cmax values were 1222 and 1203 ng/ml, and the mean observed Tmax values were 1.13 and 1.21 h. These mean values were not significantly different ($p > 0.05$).

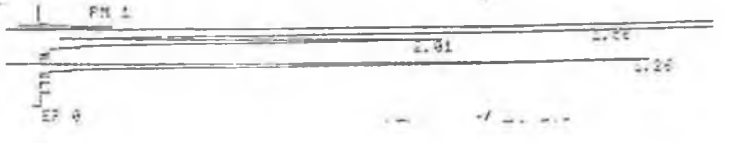
b) Triamterene

The mean AUC values post dosing with triamterene and Trirexan were (138 and 145 ng.h/ml respectively, and the mean elimination half-life values were 1.28 and 1.47 h. The mean observed Cmax values were 54 and 59 ng/ml, and the mean observed Tmax values were 0.79 and 0.75 h. These mean values were not significantly different ($p > 0.05$).

In summary, the pharmacokinetic profiles of xipamide and triamterene obtained when administered as a single oral dose in a fixed combination (Trirexan), were similar to the corresponding profiles obtained when each drug was administered alone.

SAMPLE CHROMATOGRAMS

SAMPLE CHROMATOGRAMS
SUBJECT 1 18.0 AND 24.0 hrs. post dosing
AND 25 ng/ml Standard

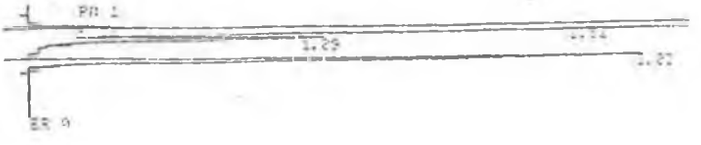


SAMPLE CHROMATOGRAMS
SUBJECT 4 , 6.0, AND 12.0 hrs. post dosing

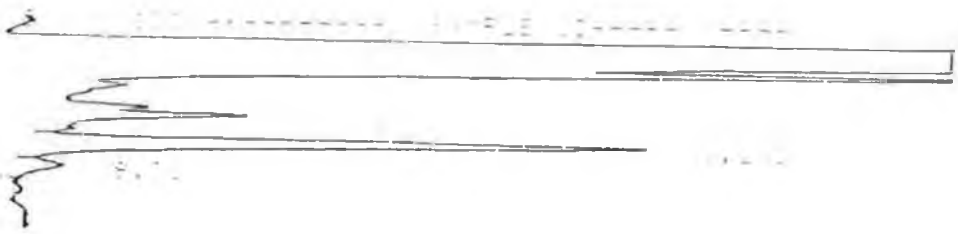
SAMPLE CHROMATOGRAMS
SUBJECT 1 , 1.5, 2.0 AND 3.0 hrs. post dosing

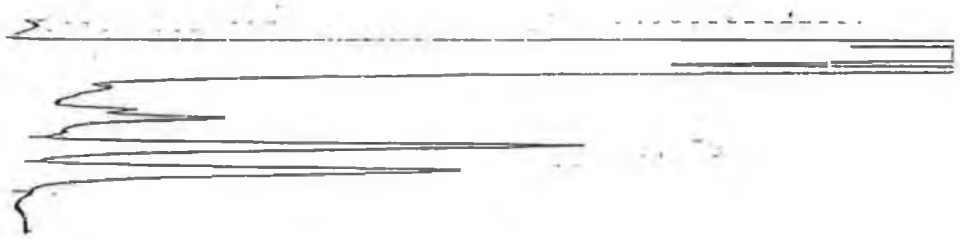
SAMPLE CHROMATOGRAMS STANDARDS
0 AND 5 AND 10 (ng/ml)

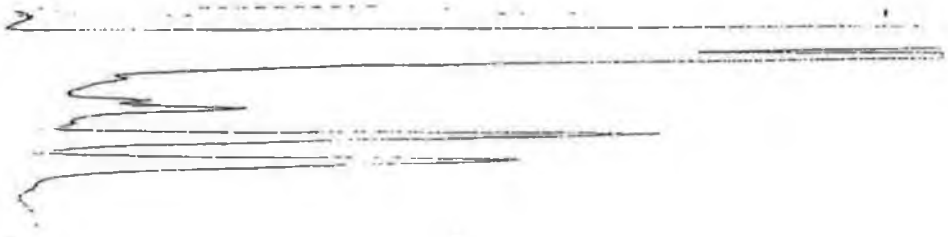
SAMPLE CHROMATOGRAMS STANDARDS
50 AND 100 AND QC 25 (ng/ml)



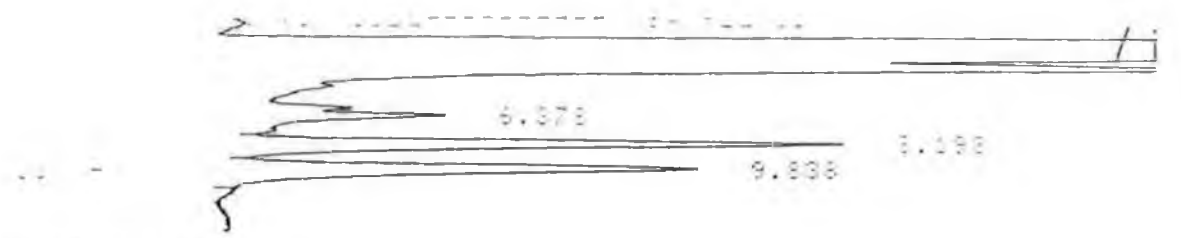
SAMPLE CHROMATOGRAMS
SUBJECT 3 0.5 ,1.0 AND 1.5 hrs. post dosing

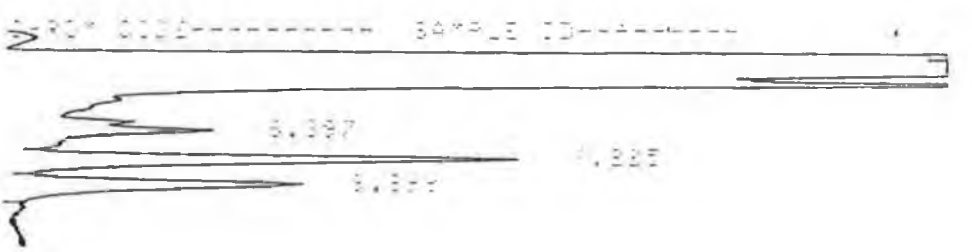


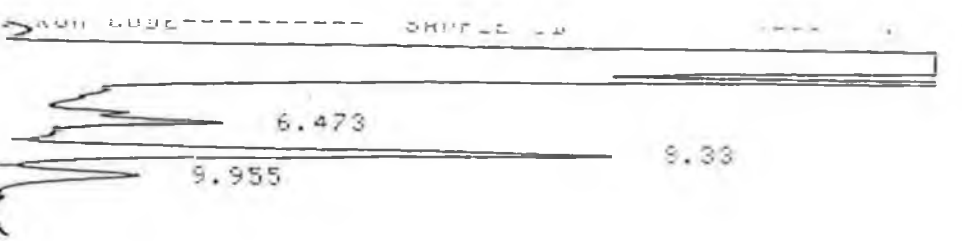




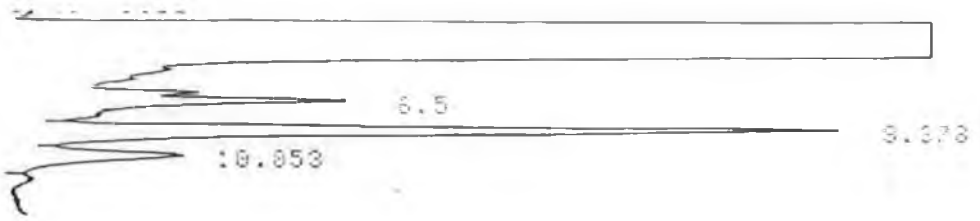
SAMPLE CHROMATOGRAMS
SUBJECT 3 2.0, 3.0 AND 6.0 hrs. post dosing

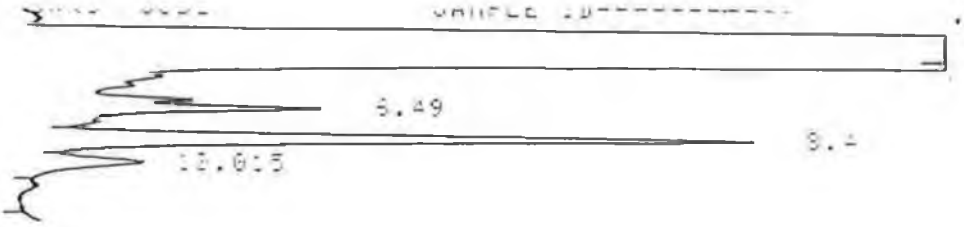


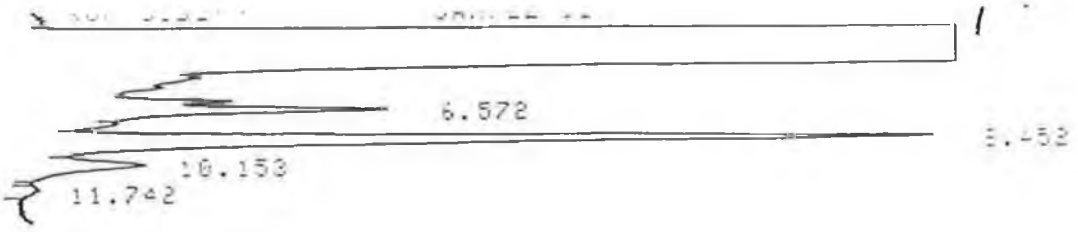




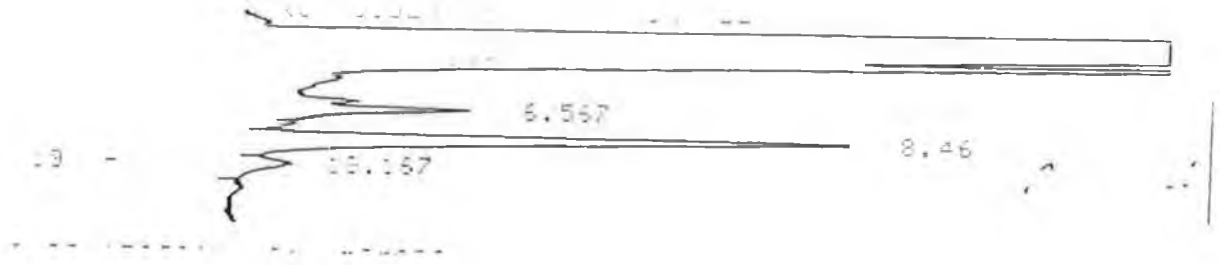
SAMPLE CHROMATOGRAMS
SUBJECT 3 8.0, 10.0 AND 12.0 hrs. post dosing

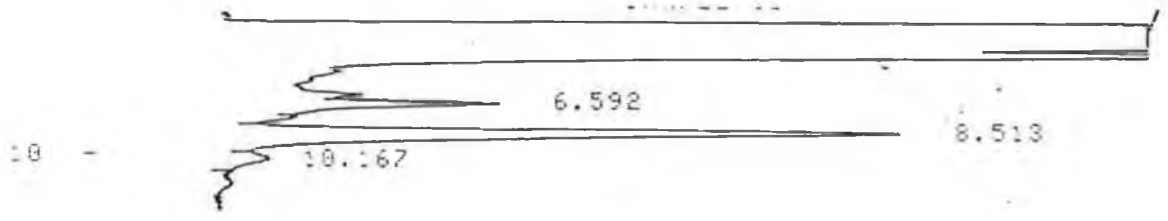




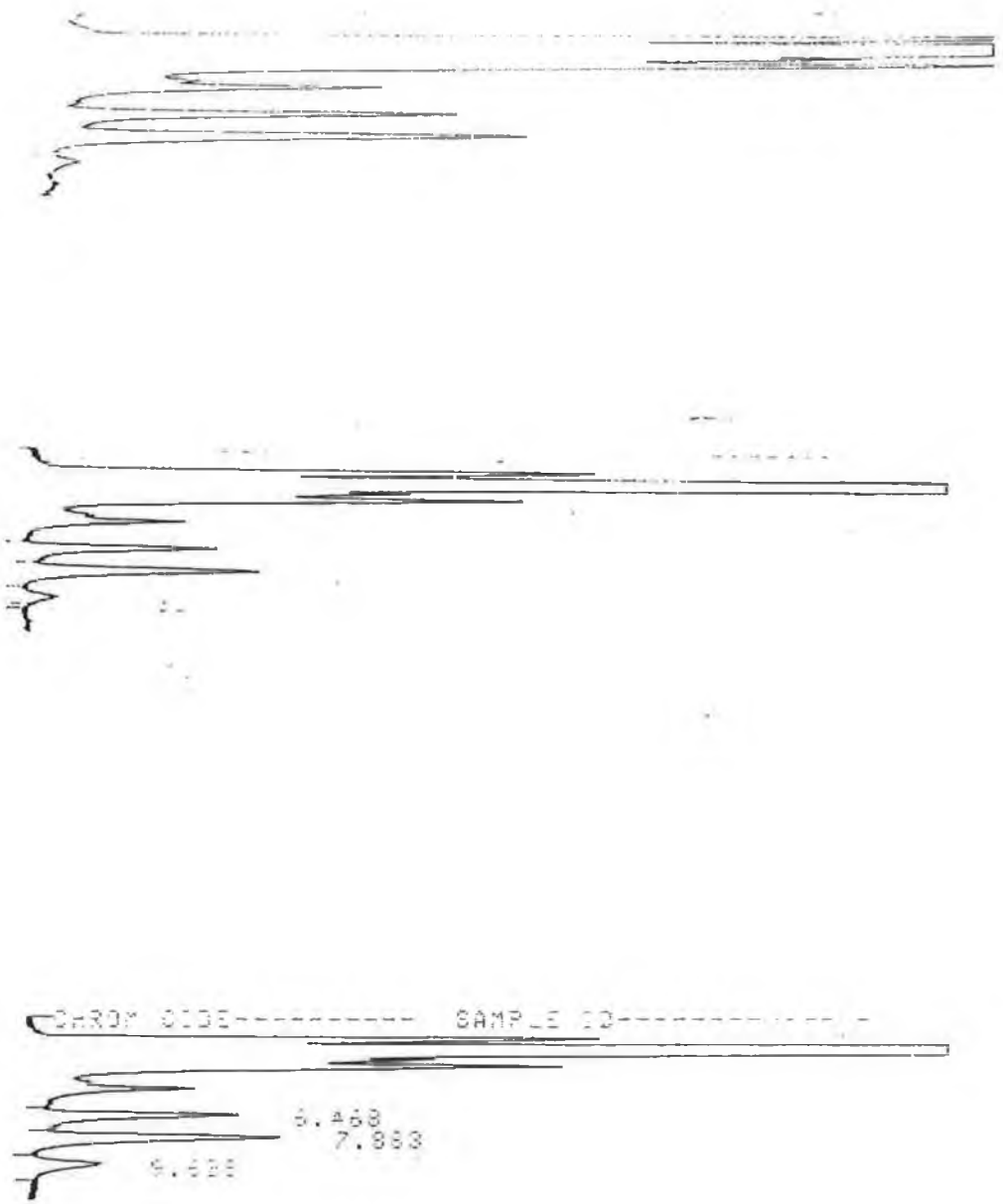


SAMPLE CHROMATOGRAMS
SUBJECT 3 18.0, AND 24.0 hrs. post dosing





SAMPLE CHROMATOGRAMS
STANDARDS 100 , 250 AND 500 ng/ml



SAMPLE CHROMATOGRAMS
STANDARDS 1000 , and 2500 ng/ml