FRUSEMIDE IN THE HORSE AND ITS EFFECTS ON THE METABOLISM, PHARMACOKINETICS AND DETECTABILITY OF CO-ADMINISTERED DRUGS

by

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ABSTRACT

The diuretic frusemide is used in race horses for the treatment of oedema and for the prophylaxis of epistaxis. The increasing popularity of frusemide treatment in recent years has led to widespread concern over its effect on other drugs. The studies described in this thesis investigate the disposition of frusemide and its effect on the metabolism, pharmacokinetics and "detectability" of an acidic drug and a basic drug. Salicylic acid and theophylline were the two drugs chosen, in view of their therapeutic use and occasional occurrence as drugs of abuse.

A new analytical method for the determination of frusemide was developed. The fluorescence properties associated with the ring structure make this drug particularly amenable to this form of detection, permitting the development of a selective method of assay using solid phase extraction and reverse phase HPLC.

In the first two experiments three horses received on separate occasions in a randomized cross-over design frusemide (1 mg/kg; i.v.) alone, the radioactive test drug ([^{14}C]-salicylic acid or [^{14}C]-theophylline) alone and the test drug followed by frusemide (1 mg/kg; i.v.). Serial venous blood samples were collected from an indwelling jugular cannula for 24 h and urine was collected as voided for up to 96 h.

Plasma pharmacokinetic parameters show that the peak salicylate concentration was significantly higher after administration of frusemide. After absorption was complete, frusemide administration resulted in persisting salicylate concentration differences in plasma for up to 12 h. Frusemide had an immediate but short-lasting diuretic effect, flow rates of 200 ml/min being obtained with 7-9 litres of urine produced in the first hour. It significantly reduced the excretion of salicylate in the first 4 h after administration. The higher plasma levels of salicylate after frusemide result from haemoconcentration as a consequence of diuresis. The delay in salicylate excretion is likely to be due to the competition between these compounds for the organic acid transport system. Competition between drugs for the organic acid transport system and the mechanism of excretion of frusemide via this transport system was further studied

after the administering of frusemide with probenecid, a drug used clinically to delay the excretion of other acidic drugs.

In the frusemide/theophylline study the diuretic was administered 4 h after theophylline to ensure that absorption was complete and the effect of frusemide on the elimination of the drug was being observed. Frusemide did not appear to have any effect on plasma concentration of theophylline. The concentration of radioactivity in urine was considerably diluted by the increased fluid output, but the rate of excretion was unaffected, indicating that frusemide did not affect the elimination of theophylline.

Unchanged theophylline and three metabolites were separated using a highly selective HPLC method. 42.8% of the dose was excreted as 1,3-dimethyluric acid and 15.9% as unchanged theophylline. Two metabolites not previously reported in the horse were also detected, 8.9% of the dose as 1-methyluric acid and 1.6% as 3-methyluric acid. This pattern of metabolism and elimination was very similar after frusemide treatment.

The disposition of frusemide was studied following the administration of [^{14}C]-frusemide by three different routes of administration- intravenously, intramuscularly and orally. The plasma levels of frusemide after i.v. injection were well fitted by a 2-compartment open model, the elimination half-life was 1.6 h. Plasma concentrations of frusemide after i.m. injection peaked very rapidly with a T_{max} of 0.17 h and then declined over the next 12 h. The longer plasma half-life of frusemide by the i.m. route (2.8 h) correlates well with the prolonged and enhanced diuretic response observed, 13.5 litres of urine was produced compared with 8-9 litres after i.v. administration. Oral administration resulted in a very slight diuretic effect due to the drug being very poorly absorbed. Total recovery of frusemide in urine was only 4.0%, faecal excretion accounted for 82.6%. Cumulative radioactivity recovery was 78.9% and 91.9% for i.m. and i.v. administration respectively. Urinary concentrations of frusemide were much higher than plasma concentrations and the drug was detected in urine for up to 40 h, long after it was no longer detectable in plasma. Using the HPLC method outlined earlier, unchanged frusemide and one metabolite, 2-amino-4-chloro-5-sulphamoylanthranilic acid (CSA) were separated. 62.2% (i.m.) and 69.1% (i.v.) of

the dose was excreted as unchanged frusemide while the metabolite accounted for 13.2 (i.m.) and 17.4% (i.v.) of the dose.

The findings are discussed in terms of the possible "diluting", "masking" or "flushing out" effects of frusemide and how this diuretic may hinder the forensic detection of illicit drugs in routine screening tests. The implications of these results for presently held opinions within the racing fraternity are addressed.

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This thesis is dedicated to my mother
Ophilda D'Souza

I hope you find in this some reward and compensation
for the sacrifices of the last 22 years

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Chapter 1

GENERAL INTRODUCTION

1.1. HISTORICAL INTRODUCTION TO RACING

Horse racing antedates the Christian era by many centuries. The horse has served man as a beast of burden, for transportation and recreation. As early as the fifteenth century B.C., horses and riders participated in the competitions of the Olympiads. From earliest times, when man first domesticated the horse, he recognised that the speed and the endurance of individual horses vary, and consequently he pitted one horse against another, often with the fortune or the life of the riders dependent upon the outcome.

It was not until the reigns of the Tudor and Stuart kings that the nobility developed horse racing into the sport we know today. Henry VIII (1509-1547) was so fond of horses and horse racing that he promoted legislation prohibiting the grazing of stallions on the public lands of England ("the Age of the Gelding") in order to ensure the improvement of the horse population through the controlled use of well-bred stallions. Under the Stuart kings, James I (1603-1625) and particularly Charles II (1660-1685), the sport continued to thrive. During the years of the Commonwealth (1649-1660), however, there was a break during which both kings and horse racing were banished from the islands. Although Cromwell, the Lord Protector, had a great love for horses, his supporters, the Puritans, were so rigid in their opposition to worldly pleasures that the racetracks were closed. They did not reopen until the restoration of the Stuarts to the throne in 1660 under Charles II.

The racing stock of this time, which was descended from the primitive strains of horses in early England with heavy infusions of blood from the horses brought by the Romans and later by the Norman French, possessed great stamina but lacked the combination of speed and endurance that make for great racehorses. To remedy this major defect, English breeders brought in horses of Oriental breeding- Barbs, Turks, and Arabians -to cross with the native stock. Although many of these Oriental Stallions were used, only three left their mark, and it is to them that all modern Thoroughbreds - the most famous of the racing breeds, trace their lineage in the male line. They were the Byerly Turk (the Herod line), the Godolphin Barb (the Matchem line), and the most

influential of all, The Darley Arabian (the Eclipse line). There are over 200 recognized breeds all belonging to the same species, *Equus callabus*.

During this period, the Jockey Club of Newmarket (founded c.1750) established the rules under which English racing has been governed ever since. Initially the races were run over any convenient open space, and it was not until the mid-seventeenth century that the Jockey Club laid out a circular track at Newmarket and appointed racing stewards to supervise and conduct the meets. Although racing at this time was a bit primitive, these early enthusiasts did recognize differences in the ability of horses and used weight handicaps to minimize some of the advantages of the superior horse.

1.2. DOPING - "ILLEGAL MEDICATION"

The term "doping" emerged towards the end of the 19th century and first appeared in an English dictionary in 1899, defined as a mixture of opium and narcotics used for horses. The origin of this word stems from the Dutch language, where the word "doop" means to dip. The word also made its way into American slang, where it was used to describe a gypsy habit of using tobacco contaminated with the seeds of *Datura stramonium* to stupefy wayfarers before robbing them. Other common expressions widely used to describe the doping of racehorses are "hopping" which refers to stimulatory doping and "nobbling" used to describe either depressant doping or the disabling of a horse.

The general public tends not to differentiate between the disturbing picture of stimulant doping and the beneficial effects of legitimate medication, thus American racing authorities prefer to avoid the word "dope" as an inappropriate term. In America "doping" is "illegal medication", in contrast with legal or controlled or permitted medication, which encompasses legitimate therapeutic agents whose use on, or close to race day may be approved. On the other hand, the racing authorities in Britain, where

no medication whatsoever is allowed, class any substance that is not a "normal nutrient" in equine urine as "dope" and use this term freely.

In the U.K. the official definition of doping defined by the Horse Racing Anti-Doping Committee states that "the detection in its tissues, body fluids or excreta of any quantity of any substance which is either a prohibited substance or a substance the origin of which cannot be traced to normal and ordinary feeding and which by its nature could affect the performance of the horse." For the purpose of this definition a 'prohibited substance' is any substance originating externally.

An Historical Background

Classical mythology is full of incidents of doping being used to produce useful effects, for example Diomedes, son of Aries and Cyrene, fed his horses on human flesh to make them savage and unbeatable. It does appear that both the concept and practice of pharmacological modification of animal behaviour were well established in ancient times. Given this background, it is not surprising that the use of drugs in an attempt to improve performance in sports is as old as competitive sports themselves.

Much of the historical background to doping in racehorses has been described by Tobin (1981). In the 3rd Century B.C., Roman chariot horses were doped with a mixture of honey and water called "hydromel", an offence punishable by death. The first report of doping in racing horses in England occurred at Worksop, where a decree in 1666 banned the use of "exciting substances". This was unusual, as most of the doping at this time was extremely crude. Unsophisticated poisoning of horses competed with outright laming as a means of stopping or "nobbling" horses. The first documented trial of a horse doper took place in Cambridge Assizes in 1812. Donald Dawson was hanged, having been found guilty of poisoning horses with arsenic.

Serious stimulant doping appeared in England about the year 1900, with the arrival of an American-financed trainer called Wishard. Wishard and his backers brought with them something they believed would bring them instant success in England -drugs, especially the newly introduced cocaine. In the four years that this team operated it is estimated that they made approximately two million dollars. This

period was known as the era of the "Yankee alchemists"; Wishard trained fifty-four winners in England in 1900, which made him the leading trainer that year.

The usual routine for this group was typified by their success with a horse called ROYAL FLUSH. Wishard bought ROYAL FLUSH for 450 guineas at six years of age when he was considered well over the hill. However, following the administration of a suitable quantity of cocaine ROYAL FLUSH finished first to win the Royal Hunt Cup at Ascot, and with a slightly higher dose went on to win the Steward's Cup at Goodwood a few months later. This was the last coup brought off by this gang before their departure *en bloc* for France. This was due to George Lambton, the leading English trainer, having finally convinced the Jockey Club to take action to prevent the administration of illegal stimulants.

The veterinary profession was concerned that doping would ruin the horse breeding industry and should be stopped, however the Jockey Club declined to take any action. Lambton's solution was to demonstrate to all what cocaine could do. He quite openly doped his own horses, and after he achieved remarkable success with horses that had never won previously, the Jockey Club were finally convinced, and doping was made a criminal offence in 1903.

Racing authorities throughout Europe soon followed the English lead, but the ban had little effect and doping became widespread throughout the continent. In 1910 the Austrian racing authorities called upon the services of a Russian chemist, Bukowski, who claimed to be able to detect the presence of various drugs in the saliva of horses, but refused to divulge his techniques. It is probable that Bukowski was detecting contamination following oral dosing rather than the secretion of drugs in saliva. However, these tests were adequate for the small number of drugs (strychnine, morphine, cocaine, caffeine) in use at the time and the situation was partially resolved. In 1912 BOURBON ROSE won the Gold Cup in France, but was subsequently disqualified when it yielded the first positive dope test.

In 1932 a change in the law in the United States permitted betting on the racecourse to be legal in 28 States. This led to a great revival in racing and to a comparable increase in the amount of stimulant medication being used. Tack rooms

were reportedly equipped like pharmacies, complete with scales, measures, test tubes, and a vast selection of drugs. Because the racing authorities did not enforce the medication rules, an honest trainer had little option but to adopt similar tactics.

The situation was reaching ridiculous dimensions and eventually some action was taken. Convictions were finally obtained, but these were under the United States narcotic regulations, and not under any rules imposed by the racing authorities. The only effect of this was to make the dopers more secretive. Finally the Florida Racing Commission decided to deal with the problem, and after a visit to France to learn about saliva testing, routine drug testing was started. The situation was gradually brought under control as more and more States set up there own Racing Laboratories. In 1947 these men formed the Association of Official Racing Chemists (AORC). Membership of the Association was limited to official racecourse analysts, and there was an air of secrecy surrounding the members' work. Members were barred from disclosing the methods by which they had obtained their results.

Meanwhile in England there was a revival in doping partly due to the military use of amphetamines during World War II. There were several notorious doping conspiracies and discontentment with the system grew until in 1960, the Jockey Club appointed a committee to investigate the whole affair. A number of noteworthy recommendations emerged. Among these was the suggestion that there should be an advisory scientific board, this resulted in the setting up of the Horse Racing Anti-Doping Committee (HADC). A second suggestion was that the Jockey Club should set up their own forensic laboratory to do both routine analysis of racecourse samples and basic research on the action of drugs in horses. This laboratory, now known as the Horseracing Forensic Laboratory (formerly Racecourse Security Services) was originally under the control of the Animal Health Trust, but has since become independent. Routine dope testing was introduced into Britain in 1963. On the whole doping incidents in the 1960's were on a pharmacologically much sounder basis than previously, a more sophisticated twentieth-century version of the eighteenth-century horse-poisoning scene, with much less danger of permanent damage to the horse.

1.3. TYPES OF DOPING

An incident of doping detected in equine blood or urine may arise in a number of ways. Doping was divided into various categories by the late Professor E.G.C. Clarke of the Horse Racing Anti-Doping Committee. (Table 1.1.)

Medication to Win

Acute stimulant doping involves the administration of a short acting stimulant such as amphetamine or caffeine shortly before the race. This type of short term medication required good knowledge of the compound as well as of the horse. Thus detection of a stimulant is usually assumed to be the result of an "inside job," i.e. a drug administered by the trainer.

Chronic doping is typified by the use of anabolic steroids, which are given for weeks or months before the event. Drug use is stopped days before the race, and although the beneficial effects will persist, the compound may well be undetectable at the time of racing. An anabolic steroid will not always show up in a test even if it is present in a horse, the release of the substance not being continuous. This was illustrated by a dope test on SOUTHERNAIR who showed traces of testosterone after a race. The Jockey Club were satisfied that the horse had received no treatment from the trainer, but that it had been given the drug some months before in a sustained release preparation and then sold (The Guardian, 16th November, 1988). It is a problem of recent years that these drugs can be used in yearlings to increase aggressiveness, vigour and muscle mass. The success of these horses will result in them subsequently being used for breeding. This type of interference will, in the long term, lead to a weakening in the quality of the thoroughbred blood line.

Paradoxically tranquillisers may also be used to improve a horse's performance. A small dose of a tranquilliser can be sufficient to "take the edge off" a horse, i.e. calm a hyperexcitable horse. Obviously if the dose given is too large, the effect can be highly detrimental.

Table 1.1. Categories of medication in performance horses

1. Medication to Win

a) Acute: short acting stimulants e.g. cocaine amine, or small amounts of tranquillisers e.g. acetylpromazine to calm an excitable horse.

b) Chronic: repeated dosing for weeks or months e.g. anabolic steroids.

2. Medication to Lose

Depressants: large doses of a tranquilliser or sedative e.g. acetylpromazine.

3. Medication to Restore Normal Performance

a) Non-steroidal anti-inflammatory drugs: often permitted under controlled medication rules in certain States in America. e.g. phenylbutazone.

b) Corticosteroids: administered intra-articularly to control joint pain; occasionally permissible in certain States of America. e.g. betamethasone.

c) Local anaesthesia: nerve or joint blocks either temporarily to numb or freeze an area e.g. lignocaine, or permanently by destruction of the nerve e.g. alcohol injections.

4. Accidental or Inadvertent Doping

a) Procaine from procaine penicillin.

b) Dietary components: e.g. caffeine and theobromine from cocoa husks in food pellets.

c) Botanical positives or false positives e.g. perloline an alkaloid derived from rye grass.

5. Medication to Hinder the Detection of Other Drugs

a) Administration of various compounds, thought to interfere with the detection of illegal medication e.g. thiamine, dipyrone.

b) Administration of diuretics to dilute the concentration of other drugs in the urine

e.g. frusemide, ethacrynic acid.

c) Administration of basic or acidic compounds to alter urinary pH e.g. "bicarbonate doping".

6. Miscellaneous Mechanisms

Blood doping.

Medication to Lose

Doping to lose, is an attempt to diminish a horse's performance. This is typified by the administration of a large dose of tranquilliser or depressant, and is generally considered to be an "outside" job, usually by someone who is trying to manipulate the post-race payoff. This was highlighted recently at the Doncaster St. Leger meeting where two fancied horses BRAVEFOOT and NORWICH were almost certainly nobbled with a fast-acting tranquilliser shortly before the race. There were also signs of a doping scandal from the betting ring, where both horses lengthened in price to a notable degree (The Times, 24th September 1990; The Independent, 24th September 1990).

Medication to Restore Normal Performance

This is the use of medication to treat a condition usually of musculo-skeletal origin, which would otherwise impede performance. Thus arthritic conditions may be treated with phenylbutazone, or a corticosteroid may be injected into a joint to control inflammation and pain. This type of controlled medication is permitted in certain States of America, but is illegal in Western Europe.

Accidental or Inadvertent Doping

Inadvertent doping is often encountered and accounts for many of the positives reported. Procaine, a potent local anaesthetic, may be used to block a nerve or joint thus enabling an unsound horse to run. The use of this drug is therefore prohibited. Unfortunately procaine is also a product of the long acting antibiotic procaine penicillin, and may be excreted in urine for up to two weeks after a legitimate therapeutic dose. The pH of horse urine varies between 4.5 and 10 (Tobin and Woods, 1979), this results in procaine (pK_a 8.7) levels in equine urine being highly unpredictable and differing by as much as 10,000-fold. These large variations can give rise to accidental positives (Tobin 1981). Procaine was the most commonly reported drug between 1947 and 1973, and it seems likely that a substantial proportion of these were inadvertent.

Another source of inadvertent positives is from the ingestion of substances accidentally, usually from compounds present in the diet. In the early 1970's there was a spate of positives for caffeine and theobromine, and this was eventually traced to the inclusion of cocoa husk in horse rations by a feedstuff manufacturer. Recently a trainer was fined after a post-race urine sample from his horse GLADSTONIAN revealed the presence of caffeine, theobromine and theophylline. The Jockey Club was satisfied the source was a feed, mixed by a local supplier (Daily Mail, 7th June 1989). In 1979 the horse NO BOMBS was disqualified when both caffeine and theobromine were detected. This was traced to the horse having snatched and eaten a Mars bar on the way to the starting line. A similar event made front page news "Mars bars DE RIGEUR from £10,000 win" (The Guardian 7th January 1987) after a Mars bar was given to a horse as a treat before it won a big race at Ascot.

Inadvertent doping may result from an increase in the sensitivity of analytical methods. An increase in the sensitivity of the test will result in an increased clearance time, and consequently a previously established "safe" therapeutic dose between races will now give rise to a positive. The calling of positives on these obviously non-effective levels of drugs, has led to proposals for quantification of drug levels, and the setting of a threshold level, below which no action would be taken.

Medication to Hinder the Detection of Other Drugs

Doping to prevent or hinder the detection of other drugs is occasionally undertaken, and various tactics have been attempted.The administration of large quantities of a permitted substance is thought by some to mask the presence of an illegal medicant. Thiamine (Vitamin B_1) was often used for this purpose in the past. The ability of thiamine to mask drugs presumably depends on its ability to absorb ultraviolet light, thus interfering with UV spectrometry, but today with modern analytical techniques this is not difficult to overcome. The elimination of some acidic or basic drugs can be enhanced or diminished simply by altering the urinary pH.

Amphetamine abusers have been known to co-administer bicarbonate to reduce its renal elimination (Hirom and Smith, 1975) thereby prolonging its effect and

decreasing the urinary concentration. The use of diuretics such as frusemide or ethacrynic acid will "dilute" other drugs or drug metabolites in urine and may render their detection more difficult. This phenomenon will be discussed later.

Miscellaneous Mechanisms

In recent years there has been considerable interest in enhancing performance, by inducing changes in the blood especially induced erythrocythemia, commonly termed blood doping or blood boosting. This manipulation gained notoriety in the sports world because of rumours of blood doping by athletes in endurance events in recent Olympiads (Beckett 1986). The method involves the removal of a volume of blood weeks before an event, the red blood cells are separated and stored, and then shortly before the race the blood cells are infused. Although relatively straightforward with human athletes, the much greater circulating volume in the horse would presumably require the transfusion of substantial amounts of blood, in order to have a similar effect. This in itself could be a means of controlling blood doping. The amount of blood one would need to collect, store and re-infuse would be difficult to conceal, also to prevent the blood from clotting during storage an anticoagulant is required. When the blood is re-infused into the horse, these substances are likely to be detected in either the blood or urine and thus point towards a recent blood transfusion.

The physiological basis for the effectiveness of blood doping in the horse is a contentious issue (Hamlin 1978). An improved performance is a balance between the beneficial effects of an increased packed cell volume and the adverse effect of the increased viscosity of the blood reducing the efficiency of its flow to working muscles.

Controlled Medication Programmes

Drugs such as phenylbutazone and frusemide are clearly beneficial therapeutic agents for horses. Phenylbutazone is an aid to training in that it helps to condition a horse, and frusemide appears to aid horses with respiratory problems. These drugs are thought to allow horses to run "up to form" but not to exceed their "form", and they also appear to allow horses to run more consistently from day to day. The popularity of

these agents led to pressure on racing authorities to allow their use in the late 1950's and early 1960's, and by the mid-1970's many States had adopted controlled medication programmes.

A controlled medication programme is one in which certain drugs are approved for use in horses at times most often including the last day before racing and occasionally including medication on race day (Table 1.2.). The programme called for detailed reporting of all medications administered, dispensed or prescribed within the racing enclosure. If any unreported drug was detected, it was considered an improper medication.

This served as the prototype for many medication programmes in the United States, such programmes represented one approach to the considerable technical and regulatory problems that are involved in medication control, and they were welcomed among the racing community becoming widespread in the States during the 1970s. Recently, however, these programmes have come under strong attack from individuals and groups who feel it is basically wrong to allow horses access to modern therapeutic agents during a competitive event. On one side are veterinary surgeons and national horse federations who are demanding either a ban or severe limitations on the use of phenylbutazone. Ranged against them are the riders, owners and trainers who are concerned for their livelihood, the value of their horses and sponsorships.

Sweden is leading the anti-drug initiative. In 1988, the Swedish parliament outlawed all drugs for horses in competition. Another influential group is pushing for the International Equestrian Federation (FEI) to slash permitted levels of phenylbutazone by more than half. The current level of 5 μg/ml in plasma was set 10 years ago. What concerns the vets is the long-term damage the drug can cause.

The English and French Jockey Clubs are cracking down hard on the countries who use drugs in racing. The sport is now so international, that for true competition to exist horses must be allowed to run on their own merits in whichever country they meet. Thus some action had to be taken to bring the Americans in line with drug free racing as practiced in Europe. At the International Racing Conference in Paris in 1988 it was proposed that the sport should be cleaned up. This was supported by the New

Table 1.2. A Controlled Medication List from a Midwestern State, 1974.

A. The administration of only the following list of drugs, classes of drugs, and medicaments is permitted by this rule and considered for the purposes of this rule to be controlled medication:-

1. Vitamins
2. Minerals & Electrolytes
3. Sugars
4. Proteins & Protein Hydrolysates
5. Sex Hormones
6. Anabolic Hormones
7. Salicylates
8. * Dipyrone
9. * Antibiotics & Sulphonamides
10. * Furosemide
11. * Chlorothiazide
12. ACTH (adrenocorticotropic hormones)
13. Adrenocorticosteroids (natural & synthetic)
14. * Phenylbutazone
15. * Oxyphenbutazone
16. * Indomethacin

B. Controlled medication may be administered to a horse that is entered to race on a particular day later than 4 hours prior to post time of the first race of the day.

NOTE: While this may look like a very extensive list, in actual fact most of these substances are endogenous to the horse. The asterisks (*) mark the substances "foreign to the natural horse" which most laboratories can test for and are thus amenable to control.

York Jockey Club which, unlike those in many states in America, agrees with the Europeans.

Henry Cecil, Britain's number one trainer created a sensation in racing circles here and abroad when he decided to take on the Americans on their own terms by running his filly INDIAN SKIMMER on "Bute" in the 2 million dollars Breeders' Cup Turf event in Kentucky in 1988. Bute (PBZ) is on the Jockey Club's list of banned drugs. Trainers and vets can only use it out of racing time. If traces are found in a dope test analysis the horse involved will automatically be disqualified anywhere in Europe. But this of course, does not apply in America - except for New York - so there would be no disqualification dangers at the home of the Kentucky Derby. The Jockey Club and other European authorities were totally against Cecil flouting their rules and taking advantage of an American system which is so castigated in Europe. Following considerable pressure from the racing authorities, Cecil decided against running his horse on the pain killer. Only 17 of the 78 horses in the Breeder's Cup events ran without medication, these included the three British horses.

The Changing Pattern of Doping

The most complete statistics on drugs detected in horses are those of the AORC, which has records of all drugs reported in horses by members of this group from 1947. However this is a very closed association and the only data available are the Medication Case Reports which they release. These simply list the number of times various drugs have been detected. The incidence of a reported positive does not necessarily reflect the incidence of doping with that compound. An indication of the trends in the pattern of drug abuse can be obtained by comparing the data from the returns of the AORC for 1947-1973 and 1987-1988.

139 compounds were reported as positives in 1987 and 1988, compared with just 88 compounds in the 26 years between 1947 and 1973. This increased diversity of drugs reflects both the increasing number of compounds now available to horsemen, as well as improvements in analytical methodology. GCMS together with extensive data bases are now routinely used. The Horseracing Forensic Laboratory has a library of

over 900 drugs, and with access to the joint Environmental Protection Agency - National Institute of Health (EPA-NIH) library, a positive identification of over 33,000 compounds can be obtained from a mass spectrum. Although the number of drugs of abuse has increased, the incidence of finding a positive has remained fairly constant at approximately 0.20%.

In Table 1.3. the drugs used for doping have been classified (as far as possible) on the basis of their pharmacological actions into 9 groups. Distinct trends are obvious. The incidence in the detected abuse of local anaesthetics and CNS stimulants has decreased sharply. Also, procaine alone accounted for 6.4% of the incidence of anaesthetics, and it is likely that many of these positives are accidental. The reporting of CNS sedatives and narcotic analgesics has remained fairly constant. The incidence of non-steroidal anti-inflammatory drugs (NSAIDs), diuretics and steroids has increased substantially. The increase in the appearance of NSAIDs has been documented (Moss 1972). By far the most important of these are the pyrazolones -phenylbutazone and its active metabolite oxyphenbutazone, accounting for 26.5% of all positives reported. The appearance of diuretics is due mainly to the advent of controlled medication programmes. Frusemide made up the majority of the positives (4.4%).

A large number of drugs (22%), come under the heading of "miscellaneous". This includes the many drugs used in the treatment of infections. These are unlikely to be used as doping agents in the true sense i.e. for the improvement of performance. Polyethylene glycol, the second most commonly reported compound (9%), has no pharmacological effect of its own, but is often given to horses as a vehicle in the treatment of azoturia.

Doping in the Future

The technology required in the fight against dope is quite considerable. Before a laboratory is accredited by the IOC Medical Commission it must have the following equipment:- thin layer chromatography (TLC), high performance liquid

Table 1.3. Comparison of the types of positive samples found in 1947-1973 and in 1987-1988

		% incidence	
		1947-1973	1987-1988
1.	**Anaesthetics** Procaine, lidocaine, butacaine, tetracaine, lignocaine etc.	21.0	8.0
2.	**Methylxanthines** Caffeine, theobromine, theophylline etc.	18.9	10.3
3.	**CNS Stimulants** Methylphenidate, amphetamine, ephedrine, apomorphine etc.	27.0	5.9
4.	**CNS Sedatives** Promazine, reserpine, xylazine, phenobarbitone, phenothiazine etc.	6.7	6.3
5.	**Non-steroidal anti-inflammatory drugs**		
	a) **Pyrazolones:-** Phenylbutazone, oxyphenbutazone etc.	10.2	26.5
	b) **Others:-** Meclofenamic acid, naproxen, dipyrone, flunixin, salicylic acid, indomethacin etc.	5.1	7.9
6.	**Narcotic analgesics** Etorphine, morphine, codeine, butorphanol, pentazocine etc.	3.6	3.6
7.	**Steroids** Prednisolone, testosterone, dexamethasone, androstanediol etc.	0.6	3.8
8.	**Diuretics** Frusemide, ethacrynic acid, bumetanide, thiazides etc.	-	5.7
9.	**Miscellaneous** Anti-bacterials, anti-fungals anthelminthics, anti-histamines.	-	8.2
	Polyethylene glycol, thiamine, nicotinamide, arsenic etc.	6.9	13.8

chromatography (HPLC), capillary gas chromatography (GC), mass spectrometry in combination with gas chromatography (GCMS) and computer evaluation. The scope and sensitivity of these techniques have advanced significantly, however the discovery of endogenous neuropeptides such as enkephalin and endorphin, and the development and use of synthetic peptide hormones and analogues such as corticotrophin (ACTH) and growth hormone (HGH), will present enormous problems for the analyst, since not only is the active amount minute but also, present techniques are not applicable for the separation and identification of these compounds.

Probably no category of doping agent has achieved the notoriety of the anabolic steroids due in part to the problem of differentiating between natural variations in endogenous levels and the exogenous administration of these compounds, solely on the basis of urinary concentrations. The abuse of anabolic steroids in horseracing dates from the early 1970's. The steroid that enjoyed by far the greatest popularity was 19-nortestosterone (19-NT) administered as the phenylpropionate ester Nandrolin, or the laurate ester, Laurabolin.

In 1981 the three-year colt VAYRANN, owned by the Aga Khan, won the Champion Stakes at Newmarket. A post-race urine sample was found to contain oestranediol. After the report on this sample and before the resultant stewards' enquiry, many urine samples from male horses were also analysed. These showed that oestranediol and 19-NT were in fact present in low concentration, albeit sporadically, in the urine of some apparently normal, untreated colts. The presence of this steroid was also age-linked, first appearing around the age of three years. These observations posed a challenging question for the Horseracing Forensic Laboratory: "How might administered 19-NT be detected in a horse already producing the steroid?". A possible solution to this problem may be to measure both drug and metabolite(s) levels in urine so as to obtain a ratio. It is possible that after drug administration this ratio will change if, for example, the higher (exogenous) dose leads to saturation of a particular metabolic pathway. This proved to be the case (Moss and Houghton, 1987). Following further investigations, it was found that colt urine contained a further steroid, closely related to oestranediol, namely its unsaturated counterpart 5(10)-oestrene 3,17-diol.

Also, the ratio of oestranediol to oestrenediol was less than unity. However, because oestranediol, but not oestrenediol, is a metabolite of 19-NT, it was found that administered 19-NT distorted the ratio of these two steroids enough to provide a reliable diagnosis of 19-NT administration. From this data it is possible to detect the administration of 19-NT not only to fillies and geldings, but also to colts.

As well as differences in urinary pH and volumes, individual differences between horses will also give rise to variations. As Tobin points out "the testing of some 10,000 horses a year is a good way of finding the biochemically atypical horses in the population". This was illustrated by the case involving HILL HOUSE, the winner of the Schweppes Gold Cup at Newbury in 1967. The urine sample taken after his unexpected victory was found to contain abnormally high concentrations of cortisol; the horse was disqualified, and the owner and trainer subsequently suspended. However, the use of steroids was denied and the owner proposed that the high levels of cortisol, although biochemically atypical, were normal for this individual horse. The horse was thus removed from the trainer, and the concentrations of cortisone periodically measured while being securely housed at the Equine Research Station in Newmarket. The data obtained supported the trainer's case, the horse was re-awarded the race, and the case dismissed, presumably on the assumption that HILL HOUSE was a biochemically atypical horse and that the "positive" was the horse's "fault". A possible way around this problem is to establish the normal range of concentration of such substances in urine such that when concentrations are found well in excess of this it can be confidently attributed to be from an external source.

1.4. WHAT IS A DIURETIC ?

The kidney has a variety of functions, all directed towards the preservation of homeostasis. It is the primary function of these organs to maintain the constancy of the internal environment. The kidneys possess multiple mechanisms that can maintain a constant extracellular fluid volume, but if renal function is compromised, life-

threatening alterations in extracellular fluid volume can result. Diuretics find their greatest usefulness when alterations in renal excretory function occur such that the extracellular fluid volume is expanded.

Diuretics are agents that increase the rate of urine formation and excretion. By common usage the term diuresis has two separate connotations; one refers to the increase in urine volume *per se*, the other to the net loss of solute and water. This definition would therefore include water, the physiological diuretic, but clinically it is customary to restrict the term to substances which cause a net loss of sodium and water from the body and to exclude such substances as water, digitalis and ethanol.

Under some conditions, the need to maintain an adequate urine volume in itself justifies the use of diuretic agents. However, in many pathological conditions there is a progressive impairment of the ability of the kidney to excrete excess sodium. This retention of sodium results in oedema due to the increased extracellular fluid needed to contain it. The aim of diuretic therapy is the mobilization of oedema fluid, that is, the production of a negative fluid balance to facilitate the contraction of this pathologically large extracellular space.

One of the most powerful and frequently used clinical tools in the physician's armamentarium is the class of diuretic drugs (Table 1.4.). They are used in the treatment of patients with conditions such as congestive heart failure or hepatic, renal or pulmonary disease, when salt and water retention have resulted in oedema or ascites. Diuretics may also be used to counter salt and water retention produced by other drug treatments, to enhance the effects of drugs given to reduce the blood pressure, and as an adjunct to the treatment of drug overdosage or poisoning, by enhancing the elimination of drugs or poisons excreted by the kidneys. It is possible to increase the formation of urine by increasing glomerular filtration, diminishing tubular absorption or by increasing tubular secretion, though in practice only the first two mechanisms are important.

Table 1.4. Classes of diuretics

The principal groups of diuretics are:-

1. **OSMOTIC DIURETICS**
   - eg. mannitol
   - isosorbide
   - urea

2. **MERCURIAL DIURETICS**
   - eg. mersalyl
   - dimercaprol

3. **XANTHINE DIURETICS**
   - eg. theophylline
   - caffeine

4. **THIAZIDE DIURETICS**
   - eg. chlorothiazide
   - bendrofluazide
   - mefruside

5. **LOOP DIURETICS**
   - eg. frusemide
   - bumetanide
   - ethacrynic acid

6. **POTASSIUM-SPARING DIURETICS**
   - eg. triamterene
   - amiloride

7. **ALDOSTERONE ANTAGONISTS**
   - eg. spironolactone
   - canrenone

8. **CARBONIC ANHYDRASE INHIBITORS**
   - eg. acetazolamide
   - ethoxzolamide

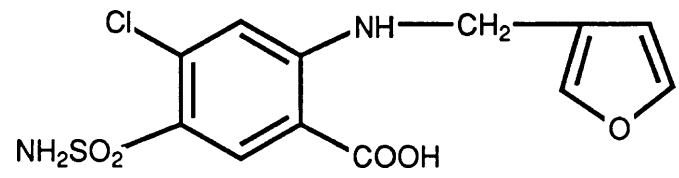
1.5. FRUSEMIDE AND OTHER HIGH-CEILING DIURETICS

The term high-ceiling has been used to denote a group of diuretics that have a distinctive action on renal tubular function. The main site of action is the thick ascending limb of the loop of Henlé. The agents are thus sometimes referred to as loop diuretics. Due to the large NaCl absorptive capacity of this segment, agents which act at this site produce a diuretic effect which is much greater than that seen with any other diuretic group. Three drugs of this class are in common clinical use: frusemide, ethacrynic acid, and bumetanide (Fig.1.1.). There are a number of other such compounds e.g. muzolimine and etozolin.

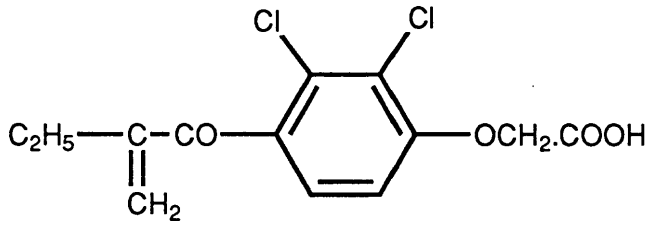
These drugs share few structural features, and they constitute a pharmacological rather than a chemical class. Of the five agents mentioned above, three are carboxylic acids and a fourth, etozolin, is an ester that is hydrolyzed to an active carboxylic acid *in vivo*. Only two of the drugs are sulphonamides. Ethacrynic acid contains an α,β-unsaturated ketone moiety, which confers on it a high degree of reactivity toward sulphydryl groups. It was synthesized in an attempt to mimic the sulphydryl reactivity of the mercurial diuretics (Schultz *et al*. 1962). Frusemide is one of a series of anthranilic acid derivatives, although it has only been available since the early 1960's, it is now the most widely used member of this group. Congeners differ in milligram potency but exhibit the same pharmacological spectrum. Several analogues, which have various substituents, are about equally active in test animals (Feit 1971). Bumetanide, a 3-aminobenzoic acid derivative, has a higher milligram potency than frusemide but in other respects these compounds are similar (Flamenbaum and Friedman, 1982).

In general, the time of onset and the duration of diuresis achieved with these agents is very short. The brevity of action is determined in large part by pharmacokinetic factors (discussed later), the intensity of diuresis also brings compensatory mechanisms into play.

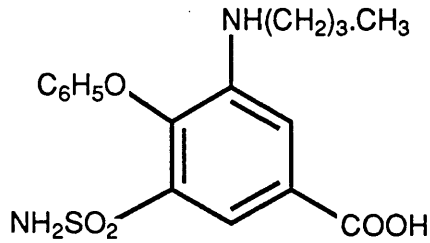
Fig.1.1. Structures of some high-ceiling diuretics



FRUSEMIDE



ETHACRYNIC ACID



BUMETANIDE

Evidence that the thick ascending limb of the loop of Henlé is the site of action for these drugs comes from micropuncture experiments which demonstrate a greatly enhanced delivery of sodium and chloride to the beginning of the distal tubule, and also from *in vitro* microperfusion experiments which show complete inhibition of sodium chloride transport in the thick ascending limb at luminal concentrations of drug in the range expected to occur *in vivo* (Reineck and Stein, 1981; Flamenbaum and Friedman, 1982). These drugs inhibit the co-transport mechanism for the entry of sodium and chloride (Stoner and Trimble, 1982). The effect has been localized to a transport site at the luminal face of the epithelial cells and probably corresponds to a coupled entry mechanism for Na^+, Cl^- and possibly K^+.

The thick ascending limb plays an important role in divalent cation reabsorption in the kidney (Suki *et al.* 1980; Shareghi *et al.* 1982). Inhibition of active NaCl transport causes an associated increase in magnesium and calcium excretion (Dirks 1983). Chronic use of high-ceiling diuretics has been associated with magnesium wasting and severe hypomagnesemia (Duarte 1968). The calciuric action of these agents is the basis for their use in the acute management of hypercalcaemia (Suki *et al.* 1970).

Loop agents tend to increase renal blood flow without increasing filtration rate, especially after intravenous injection. When frusemide increases the renal blood flow, there is a redistribution of flow from medulla to cortex and within the cortex. The haemodynamic effects appear to involve the renin-angiotensin system and vasodilatory prostaglandins. The renal secretion of these substances is increased by the high-ceiling diuretics. However, the haemodynamic effects have not been directly linked to the diuretic response. In patients with pulmonary oedema frusemide has been shown to improve pulmonary congestion by increasing venous capacitance, thereby reducing left ventricular filling pressure. This is an acute haemodynamic effect, and it can be of benefit before any measureable increase in urinary output occurs (Dikshit *et al.* 1973).

Changes in renal haemodynamics reduce fluid and electrolyte reabsorption in the proximal tubule and may augment the initial diuretic response. The increase in renal blood flow is relatively short-lived. With the reduction of extracellular fluid volume

which is induced by diuresis, there is a tendency for renal blood flow to decrease; this sets the stage for increased reabsorption from the proximal tubule. The latter phenomenon may be thought of as a compensatory mechanism that limits delivery of solute to the thick ascending limb, thereby diminishing the diuresis. The question of a minor direct action of high-ceiling diuretics on the proximal tubule remains controversial (Reineck and Stein, 1981). Both frusemide and bumetanide are inhibitors of carbonic anhydrase (both are sulphonamides), but these activities are too weak to contribute to a proximal diuresis except when massive doses are employed (Ostergaard *et al*. 1972). Actions of high-ceiling diuretics in segments distal to the thick ascending limb have not been firmly established. However, the magnitude of the diuresis engendered by these drugs suggests that there may be multiple secondary sites of action.

1.6. THE CLINICAL USE OF FRUSEMIDE IN THE HORSE

Frusemide was introduced into equine medicine in Colorado in the 1960's, and has since been the drug of choice for the treatment of various oedematous syndromes:-

Congestive oedema with venous congestion due to cardiac insufficiency or obstruction of venous or lymphatic circulation by thrombosis, pressure or trauma seen as generalised or localised oedema of the skin, thorax, abdomen and cerebrum.

Protein deficiency oedema (hypoproteinaemia) due to renal proteinuria, liver lesions with disorders of protein metabolism, parasitism or malnutrition, seen as generalised skin oedema and ascites.

Oedema due to vascular lesions of allergy, toxic, traumatic or inflammatory origin as localised oedema of the skin at site of wounding, operation, contact with allergic or noxious agents, or as pulmonary or cerebral oedema.

For supportive treatment in the elimination of fluid from body cavities (ascites, hydrothorax), from hollow organs (bronchopneumonia, hydropericardium), from joints, tendon sheaths or bursae and for supportive treatment in laminitis.

For about the last 15 years frusemide administration has been the treatment of choice in the prophylaxis of epistaxis in racing horses (discussed in further detail below). Frusemide is particularly useful in cases of pulmonary oedema, and its use for this condition has been approved by the Food and Drug Administration in America. The mechanism by which frusemide produces an effect on the pulmonary system is unclear, since the drug is primarily a diuretic. However, frusemide has clearcut actions on the pulmonary system in most species. The drug causes renal and extra-renal releases of prostaglandins which affect the pulmonary vasculature and airways. Volume shifts and fluid losses may play a role, but the effects of frusemide occur very rapidly, even before it causes any significant increase in the rate of urine formation. Frusemide's ability to increase the volume of urine may also be used to dilute substances and prevent kidney damage. Tying-up disease is a muscle disorder that occurs in racehorses under heavy exercise, because of the depletion of muscle stores of glycogen and the accumulation of metabolic waste products. The urine may be coffee-coloured because of the myoglobin released from damaged cells, which can damage the kidneys. In such a situation, the increased urine volume due to frusemide acts to dilute the pigment and greatly reduces the possibility of renal damage.

1.7. EXERCISE-INDUCED PULMONARY HAEMORRHAGE

Definition

The history of bleeders is at least as old as Thoroughbred racing itself. Horses which bleed have been recorded amongst the earliest Thoroughbreds and are still with us to this day. Confusion surrounding bleeding, known technically as "epistaxis" has arisen because two false analogies have been drawn between epistaxis in the horse and

man. Firstly, the term itself needs clarification. Nasal haemorrhage in man results in a flow of blood from the nostrils and the word epistaxis was coined to describe this, from the Greek epi meaning "on" and stazo meaning "to fall in drops". By contrast, when pulmonary haemorrhage occurs in man, the blood is coughed or hawked up and is finally spat out of the mouth, generally in a frothy state, as this is the only way it can be moved up the vertical human trachea.

It had been assumed, though without evidence, that when a racehorse "broke a blood vessel" the haemorrhage originated in the nasal cavity. Small blood vessels in the turbinate region or on the nasal septum were suspected of rupturing under the influence of a raised blood pressure. However, if this was correct, bleeding should be predominantly unilateral. In fact, bleeding is bilateral and the majority of horses are thought to bleed from the lungs. Because of the horse's long soft palate, blood is discharged at the nostrils and not at the mouth. This is what is considered the classic "bleeder". Since the classic pattern of pulmonary bleeding occurs after exercise, this syndrome has been defined by Pascoe *et al.* (1981) as exercised-induced pulmonary haemorrhage (EIPH).

A medical adage teaches that upper respiratory tract haemorrhage can be distinguished from lower respiratory tract haemorrhage by the blood in the latter case being foamy. This rule does not apply to the horse (Cook 1966, 1968). Blood from a lung haemorrhage is not foamy because the respiratory tract in the horse is horizontally disposed and there is no need for the blood to be coughed up as in man. With the head lowered, blood flows easily to the nostrils by gravity. Cook (1974) has published a detailed review of epistaxis in the racehorse.

Pathophysiology

On the basis of autopsy evidence, Mahaffey (1962) believed that haemorrhage in "bleeders" originated in the lungs. He commented on histological evidence of the rupture of alveolar capillaries. Supporting evidence is provided by Rooney (1970), together with an opinion to the effect that "pulmonary haemorrhage may well be the most important and common cause of epistaxis". There is only limited understanding of

the pathophysiological mechanism of EIPH, one hypothesis being that it may be triggered by the asphyxia that develops during breath-holding after horses leave the starting gate (Rooney 1970). Alternative explanations have assumed that EIPH only occurs if there is an underlying disease process such as a defect of haemostasis (Franco 1969); upper airway obstruction and asphyxia (Cook 1988; Rooney 1970); chronic lung disease, especially chronic obstructive pulmonary disease (COPD) (Cook 1974) and hypertension (Littlejohn *et al.* 1984). In a recent study (O'Callaghan *et al.* 1988), extensive small airway disease was observed in close association with the vascular changes in lungs of horses with EIPH. Clarke (1985) proposed that EIPH is the result of physiological mechanical stress which can occur in otherwise healthy horses.

All structures within the lung are interconnected and movement of one part of the lung exerts forces on adjacent structures. Bronchi and bronchioles are pulled open by inflation of alveoli. When one region of the lung has limited mobility and fails to move with adjacent lung tissue, additional forces are applied to the region with limited mobility during respiration. This interaction of structures is termed interdependence. Limited mobility or non-homogeneous inflation of portions of the lung is thought to be responsible for exercise-associated haemorrhage (Robinson and Derksen, 1980). These authors suggest that local lesions in the lung tissue such as scar tissue or pleural adhesions are critical factors in the incidence of bleeders.

While scars are one cause of limited lung motion, asynchronous ventilation of pulmonary lobules may be a more important cause. The obvious causes of local variation in the resistance of peripheral airways and compliance of lobules are diseases such as heaves in which there is extensive bronchiolar obstruction or pneumonia. The degree of bronchitis and emphysema is probably minimal, especially in the racehorse, however, competitive exercise is thought likely to exacerbate a tendency to bronchospasm, which leads to lung distension and rupture of bronchial or alveolar capillaries. This situation in the horse may be analogous to exercise-induced airways constriction in the asthmatic human subject.

Robinson's equine epistaxis hypothesis suggests that the reason the horse suffers from epistaxis more than other species is anatomical (Robinson 1979). In

addition to the bronchi and bronchioles which normally supply air to alveoli, there are collateral pathways for ventilation between lobules. These collateral pathways may be alveolar pores or anastomosing respiratory bronchioles but physiologically they behave like bronchioles. Species with unlobulated lungs such as dogs have extensive collateral pathways and the time constants for collateral ventilation are fairly rapid. The horse has little collateral ventilation and the time constant for collateral ventilation is long (5 sec). In the horse, it is therefore unlikely that collateral pathways are effective in ventilating alveoli distal to an obstructed airway. The poor collateral ventilation of the horse may allow extreme fluctuation in alveolar pressure in the asynchronous region resulting in capillary rupture. In contrast in the dog, the short collateral time constants (0.2 sec) may prevent extreme alveolar pressure changes distal to an airway obstruction.

A predisposing factor of genetic origin has been suggested by Robertson (1913) who attributed epistaxis amongst racehorses to inbreeding, tracing all "bleeders" back to "Herod", a horse born in 1758. The possibility that inbreeding may be a factor influencing the occurrence of epistaxis is not denied, though it is thought unlikely that the problem traces back to only one ancestor. Unlike haemophilia, which is attributable entirely to genetic factors, epistaxis in the racehorse- if it is inherited at all- is probably only partly genetic in origin, other factors being physiological, pathological or environmental. It is feasible, that a genetic influence could manifest itself by rendering a given horse more susceptible to chronic bronchitis and pulmonary emphysema.

Incidence and Diagnosis

Most horses suffer EIPH without clinical signs although repeated swallowing has been observed immediately after exercise in a bleeding horse. One swallow after a gallop is thought to be physiological (Cook 1965) but frequent swallowing may be an indication that the horse is swallowing blood which is flowing into the pharynx from the trachea.

Historically the incidence of bleeders has been reported to be less than 5%, but until recently there was no experimental evidence to support this. However a study of haemorrhage in the upper respiratory tract of horses post-race has come up with some

results that throw a completely new light on the incidence of bleeders in racing horses (Pascoe *et al.* 1981). The introduction of the flexible fibreoptic endoscope has facilitated the clinical examination of the equine upper respiratory tract. Of 235 thoroughbred racehorses examined within 2 hours of racing to determine the frequency of EIPH, 43.8% had various degrees of haemorrhage in the tracheal lumen. The incidence of overt bleeding from the nostrils post-race was about that which has been reported over the years i.e. 2 to 3%.

More recent studies have shown an even higher incidence of EIPH, 75% of 191 horses examined after racing and 66% of 107 horses examined after breezing had blood in their tracheo-bronchial airways (Raphel and Soma, 1982). The limitations of the endoscope must not be forgotten, since using it to grade haemorrhage is difficult but repeated endoscopy on 76 horses indicated that EIPH is not a random observation (Raphel and Soma, 1982), and consecutive examinations of horses breezing under similar conditions have shown good agreement for EIPH scores (Pascoe 1985). Statistical analysis of frequency data for 191 horses which finished in first, second and third places did not show any relationship between EIPH and horse's age, sex or finishing position. However, a trend towards an increased frequency of EIPH with age was shown, by a greater proportion of horses of 5 years and older having EIPH. This trend is thought to reflect the chronicity of the pulmonary lesions and an inability of the lung to repair damaged regions while training and racing continued.

Despite its occurrence in 45 to 86% of racing horses, data are not available on the death rate of horses with EIPH. Sudden death in seemingly healthy horses most often is attributed to diseases of the cardiovascular system, such as myocarditis (Fregin 1982; Platt 1982), rupture of the heart, aorta or large arteries (Daneilus 1941; Rooney 1977) and atrial dysrhythmia (Deem *et al.* 1982). Nevertheless, in a recent study of 25 horses, 21 of which died during racing or training, only 7 of the 21 (33%) had lesions sufficient to explain the sudden death (Gelberg *et al.* 1985). Gunson *et al.* (1988) carried out a study between January 1981 and July 1983 to determine the incidence and cause of sudden death in exercising horses. Their data indicated that EIPH was the cause of death in 82% of the thoroughbred race horses that died suddenly (while

exercising) of causes unrelated to musculoskeletal trauma. Thus, EIPH should be considered as the most common cause of sudden death in exercising horses.

Therapy

Because of the stringent regulations for bleeders, there has long been a search for a drug that, when administered pre-race, will prevent bleeding. However, many therapeutic approaches to EIPH have either been empirical or based on unproven hypothesis. The efficacy of available medications has been anecdotal and unsupported by clinical trials. Among the drugs that have been used for this have been drugs that are thought for various reasons to increase blood coagulability, even though there is no known coagulation defect in bleeders. The use of coagulants should be regarded as placebo medication.

Oxalic and malonic acids, which bind calcium ions in the blood, have historically been used for bleeders. The rationale behind this approach was that if one produced a small reduction in free calcium ion in the blood, one could accelerate the clotting process and thus prevent bleeding. While small amounts of oxalic and malonic acid will indeed tie up calcium ions, there is no good reason to think that this will affect the incidence of bleeders in any way as clotting defects have been discarded as likely aetiological factors in EIPH (Cook 1974; Johnson *et al.* 1973; Pascoe 1983). Similarly the continued use of preparations such as vitamin K and tranexamic acid is suspect, as vitamin K deficiency is associated with a clotting defect and there is no reason to suppose that there is a clotting defect in these animals.

While no rationale for the use of oestrogens has been presented nor clinical trials reported, recommended dosage regimes have been published (Pascoe 1983). Premarine^{R}, which is a preparation of conjugated oestrogens (principally oestrone sulphate) from mare's urine has been used. This has been reported to accelerate blood clotting in dogs and is presumably used in horses for this reason.

The use of hesperidin-citrus bioflavinoids is based on the assumption that capillary weakness is an underlying factor of EIPH. There have been reported reductions in capillary and venous bleeding resulting from pre-operative use of citrus

bioflavinoids (Strully 1958). However, clinical trials using these agents at 28 g/day for 90 days did not alter the prevalence of EIPH (Sweeney and Soma, 1984).

Another used therapy has been bloodletting, presumably on the hypothesis that reducing blood volume will tend to reduce blood pressure. The withholding of water from a horse for about nine hours before a race has been another approach. This will have the effect of reducing fluid volume in the horse, it is likely that the reported success with this approach first gave rise to the use of frusemide in an attempt to control the bleeder problem.

Based on clinical experience with the drug, a large number of veterinarians and trainers came to believe that frusemide was an effective drug in the treatment of epistaxis. According to such clinical reports, 200 mg of Lasix given intravenously or intramuscularly within one to three hours before racing either prevents or reduces the risk of epistaxis. Based on this knowledge and the known small incidence of bleeders, many Racing Commissions approved frusemide for use in racing horses as prophylaxis against epistaxis. However, while only a small percentage of horses suffer from epistaxis, much larger proportions of horses were being run on frusemide in jurisdictions where frusemide was a permitted medication. Prior to a study on the pharmacology of frusemide in the horse (Tobin *et al.* 1978), there was a multitude of theories about what frusemide did to horses (Tobin 1981).

Frusemide's mechanism of action in minimizing haemorrhage is not clearly understood, but an effect on haemodynamics and lung mechanics has been suggested (Muir *et al.* 1976; Robinson and Derksen, 1980). Frusemide increases the capacity of pulmonary blood vessels to store blood; therefore, the blood pressure in the pulmonary capillaries is reduced. Such a reduction makes less likely the rupture of capillaries into the alveoli and may, therefore, be one mechanism why frusemide is prophylactic against epistaxis.

During the 1970's, frusemide treatment became very popular. In some cases, it was given not only on the day of the race but every day for seven to eight weeks during the racing season. Veterinarians who administer frusemide for epistaxis felt that frusemide was extremely efficacious and estimated that at least 85% of the horses with

epistaxis have the condition either reduced in frequency and severity, or abolished completely. Other than these testimonies stating that the agent is useful, no objective data existed at the time.

Clinical trials using several different regimes have shown frusemide to be of limited value in preventing EIPH. The efficacy of frusemide thus remains a contentious issue. In a survey carried out over 15 consecutive racing days at the Del Mar Thoroughbred Club, California of the 56 frusemide-treated horses examined, 30 (53.6%) showed evidence of EIPH after racing (Pascoe *et al.* 1981). These results create doubts about the treatment, but it is not known, if frusemide reduced the amount of haemorrhage these horses may have otherwise experienced.

In another study, Sweeney and Soma (1984) found that when comparing medicated and unmedicated groups, the effect of frusemide was not statistically significant in reducing the prevalence of EIPH. However, the criteria for determining the efficacy of frusemide in preventing EIPH was absence of blood in the trachea, so no assessment of partial reduction of haemorrhage was made. In a study using a numerical system to grade the degree of EIPH (Pascoe *et al.* 1981) when the frusemide treatment score was compared with the untreated score it indicated that although frusemide did not stop EIPH it did reduce the EIPH score in 28 (64%) horses (Pascoe *et al.* 1985).

Many of the recent therapeutic approaches to EIPH have been based on Cook's hypothesis that chronic obstructive pulmonary disease (COPD), is the underlying lesion of EIPH (Cook 1974). Cook (1974) recommended good ventilation and alternative beddings to straw and soaking hay. Mason *et al.* (1984) found that the use of paper bedding instead of straw did not decrease the incidence of EIPH although the incidences of other cases of respiratory disease were decreased.

Drugs used in the management of COPD and bronchoconstriction have now begun to be considered for a possible role in the prevention of EIPH these include disodium cromoglycate, atropine and ipratropium (Pascoe 1983; Sweeney *et al.* 1984). Sweeney *et al.* suggest that the bronchodilator effects of atropine and ipratropium may have inhibited EIPH, but only one endoscopic examination was carried out 1 hour after

exercise thus the results were inconclusive, also the failure of certain regimes in this study may simply reflect an inappropriate dosage regime.

Treatment with water vapour-saturated air (WVSA) is claimed to promote clearance of mucus by preventing airway water loss, promoting mucociliary clearance and preventing EIPH in racehorses. Sweeney *et al.* (1988) report that if WVSA is to prevent bronchospasm and small airway obstruction, it would have to be delivered during exercise. There was no reported benefit from the WVSA before exercise. Therefore, it seems unlikely that WVSA could prevent EIPH by preventing small-airway obstruction.

Advances in the treatment of EIPH will require a greater understanding of respiratory pathophysiology in the horse and more thorough pharmacological and clinical studies in this species.

1.8. CONTROVERSY OVER THE USE OF FRUSEMIDE

After the NSAID phenylbutazone, frusemide is the most controversial drug in racing. As mentioned earlier the incidence of EIPH in horses has been reported as low as 2.5 to 5%, but large numbers of horses are being run on frusemide where it is a permitted medication. This widespread use of frusemide has led to many questions concerning its actions, the key one being, "why is the use of frusemide so popular in horses that are not bleeders?".

A tremendous number of "reasons" have been put forward for the use of this drug. Frusemide it was rumoured could "move horses up" i.e. improve their performance, and this was the reason that it was widely used in horses. It was supposed to be very potent in this regard and could make "pulmonary cripples" run like the wind. It was also believed that frusemide increased blood pressure, concentrated the blood and calmed the horse. All these effects apparently make horses run better.

These varying reports about frusemide have led to a series of studies since the mid-1970's on the pharmacology of frusemide, as a result of which this drug is now

well characterised in the horse (Roberts *et al.* 1978; Tobin *et al.* 1977, 1978; Soma *et al.* 1984).

Studies on the performance effects of frusemide are difficult to investigate due to the large number of variables which have to be accounted for. In two studies in which standardbreds ran simulated races after being given either a placebo or frusemide, changes in performance were not detected (Tobin *et al.* 1978; Milne *et al.* 1980). A retrospective analysis performed by Tobin *et al.* (1978) on racing times in 58 horses run at Louisville Downs in 1977, revealed no significant differences in racing times with or without frusemide. However, the analysis of racing times of horses with EIPH, with and without frusemide treatment have indicated that frusemide was effective in returning race times to a level of performance attained before the detection of EIPH in some horses (Soma *et al.* 1985). This suggests that while frusemide may not improve the performance of a racing horse, it may be able, under some circumstances, to restore performance that has declined due to EIPH or some other frusemide-treatable condition.

However, the results of recent research by Sweeney *et al.* (1990) have aroused considerable curiosity and controversy in the racing industry in America resulting in such headlines as "Yank boffins blow the lid on Lasix" (Kentucky Sporting Life Weekender, 10th May 1990), "Lasix: Opposing sides consult the evidence" (The New York Times, 8th May 1990), "Lasix increases speed of horses" (Daily Racing Form, Hightstown N.J. 9th May 1990), "Improving qualities of Lasix" (Racing Post, 7th May 1990), and "The Lasix debate gathers momentum" (Racing Post, 10th May 1990). This is because the widely held assumption among horsemen that frusemide makes horses run faster appears to have been certified in a prestigious medical journal. The study commissioned by the Jockey Club (American) and conducted by the University of Pennsylvania from July 1988 to February 1989 shows conclusively that frusemide improves the racing performance of Thoroughbred horses who do not suffer from EIPH. Because of the prevalence of EIPH in Thoroughbreds, 665 horses had to be screened for the study to identify 79 horses that were free of the disorder after three races.

Briefly the study found that the 79 horses studied (non-bleeders) raced one mile an average of 0.48 sec (2.4 lengths) faster when given frusemide. Geldings showed the greatest improvement in racing time on the drug, averaging 1.08 sec (5.4 lengths) faster. In older geldings who had seen plenty of racing, the improvement could be as much as nine lengths in a race. Investigating the diuretic's effect on 52 bleeders, the study found that frusemide also improved the racing times of the horses with EIPH, but the improvement was not as great (average 0.26 sec or 1.3 lengths). The group report that frusemide failed to stop bleeding in a large number of cases. 62% of horses treated with frusemide continued to bleed. Frusemide also failed to prevent the development of bleeding after racing, in horses previously regarded as non-bleeders. The study thus also brings into question the efficacy of frusemide; the use of frusemide as a bleeding remedy was called a "common misconception". Sweeney *et al.* (1990) also provide evidence indicating that the improved performance was not simply a training effect.

This data has led to a lot of controversy in the States as results in big races are questionable if the winners have been run on frusemide. For example, in this year's Kentucky Derby (5th May 1990) all except one of the 15-strong field were declared as being on Bute, and five were on Lasix including the winner UNBRIDLED, the runner-up SUMMER SQUALL and the horse in fourth place. Two of the previous five Kentucky Derby winners, including ALYSHEBA in 1987 who won by less than 1 length, have also raced on Lasix. No one knows whether Lasix is keeping these horses from bleeding, making them run faster, neither or both.

A second issue concerning frusemide is a widely held belief that this diuretic affects the detection of other drugs in horses. It was suggested that frusemide might cause a "flushing" of drugs out of the body of the horse by increasing the volume of urine. Secondly, frusemide might mask or interfere with the detection of other drugs. A third and more likely effect is that the diuresis following frusemide treatment may result in the dilution of illegal drugs and drug metabolites in post-race urine samples, thereby rendering their detection more difficult.

There is no evidence in the scientific literature that frusemide "washes" or "flushes" drugs out of a horse, since frusemide does not move very much fluid out of

horses. Although the diuretic response appears dramatic to the casual observer, the actual volume of urine is only a few percent of the total body water of the horse. Thus in its brief period of action, frusemide is unlikely to lead to appreciable elimination of a drug from the horse. However, the diuresis following frusemide treatment can dilute drugs and their metabolites in post-race urine samples, and studies detailing examples of this are outlined below.

1.9. FRUSEMIDE AND ITS EFFECT ON OTHER DRUGS

Many drugs have the potential for altering the pharmacokinetic properties of other co-administered drugs. These interactions can be evaluated by investigating the individual pharmacological and pharmacokinetic properties of the drugs involved. As frusemide is very commonly administered in a variety of clinical conditions, it is often given together with other drugs. As such there are numerous examples in the literature of clinically significant drug interactions. For example, frusemide with the aminoglycosides (Thomsen *et al.* 1976; Lawson *et al.* 1976), with cephaloridine (Dodds and Foord, 1970; Kleinknecht *et al.* 1974), phenytoin (Ahmad 1974), digoxin (Tsutsumi *et al.* 1979, Semple *et al.* 1975) and lithium (Hurtig and Dyson, 1974).

Frusemide may affect the pharmacological response to and disposition and/or urinary elimination of other drugs. A number of studies have determined the effects of frusemide on plasma and urine levels of other drugs from a doping point of view. In the horse the most detailed information is available on frusemide's effect on phenylbutazone, this is due to this drugs' forensic importance, being the most commonly encountered doping agent in racehorses. The data available for some of the other drugs, however, is rather sparse and qualitative in nature.

Outlined below is a summary of the data on the influence of frusemide on the excretion and metabolism of doping agents in the horse.

Frusemide + Doping Agent Interactions

(a) Phenylbutazone

Roberts *et al.* (1976) and Tobin *et al.* (1977) challenged their horses with frusemide (1 mg/kg; i.v.) 2.5 h after having administered phenylbutazone (6.6 mg/kg; i.v.). Frusemide produced an immediate 18-fold increase in urine volume, but it had no clinically significant effect on either plasma levels or plasma half-life of phenylbutazone. In contrast to the lack of effect of frusemide on plasma levels urinary concentrations of phenylbutazone were markedly decreased after frusemide. Urinary concentrations dropped forty-fold within 15 minutes of administration of the diuretic and remained depressed for up to 12 hours. This very substantial reduction is sufficient to interfere with the drug's detection in routine screening tests. Phenylbutazone and frusemide are both acidic, highly protein bound drugs so that a possible point of interaction is at the level of the organic acid secretory system. These drugs can theoretically interfere with each other's renal transport and thus, urinary levels. A fall in the urinary excretion rate of phenylbutazone after frusemide administration suggests that such an effect may be occurring (Tobin *et al.* 1977). In another study, by Combie *et al.* (1981), a lower dose of frusemide (0.385 mg/kg) was given following phenylbutazone (4.4 mg/kg). Urinary concentrations of phenylbutazone initially fell sharply but quickly recovered, there being no significant difference between the treated and untreated horses from 2 h after the horses had been given the diuretic.

The principal effect of frusemide on urinary levels of phenylbutazone, appears to be a diluting one, however the experimental data suggests that under practical conditions the degree of interference with drug detection by frusemide is small and likely negligible if the dose usually used in the treatment of epistaxis i.e. 0.5 mg/kg is given three or more hours before sample taking. If there is no time interval to allow the effects of the diuresis to be negated then phenylbutazone concentrations can only be accurately determined by taking a blood sample, this will ensure that phenylbutazone medication rules are effectively and fairly enforced.

(b) Pentazocine

Pentazocine is rapidly and essentially completely metabolised to glucuronides in the horse. Miller *et al.* (1977) have investigated the pentazocine-frusemide interaction. Horses pre-treated with pentazocine (0.33 mg/kg; i.v.) were injected with frusemide (1 mg/kg; i.v.) thirty mins later. At peak diuretic effect urinary concentrations of pentazocine glucuronides fell rapidly from about 100 µg/ml pre-frusemide to less than 2 µg/ml. This effect was transient, peaking at 45 mins post-frusemide, thereafter, urinary pentazocine concentrations returned towards control levels.

Many drugs used in illegal medication of race horses are detected in equine urine primarily as conjugates with glucuronic acid (Maylin 1974). Because these conjugates are highly water soluble, they are concentrated by the kidney resulting in urinary concentrations many times higher than plasma levels. As sensitive analytical methods generally do not exist for drug glucuronides, this urinary concentrating mechanism is critical in that it allows chemists to identify these drugs during routine forensic screening (Miller *et al.* 1976). The observed dilution of glucuronide metabolites is a result of the diuretic acting on the renal concentration mechanisms, but other mechanisms exist by which frusemide may act to reduce urinary concentrations. Glucuronides contain acidic carboxyl groups, thus they are "pumped" into the urine by the organic acid transport system. By binding to receptor sites on this transport system, frusemide is likely to inhibit the transport of glucuronides and so further reduce their concentration in equine urine.

Miller *et al.* (1977) had no difficulty in detecting the diluted concentrations of pentazocine metabolites in frusemide treated horses, and it may be argued that these dilution effects are not forensically significant. However, it is important to realize that these experiments were performed under optimized conditions, with sensitive detection methods. Under routine forensic conditions, however, circumstances are likely to be considerably less than ideal and a 50-fold dilution would be significant. Also, it is likely that the diluting effect applies to other drugs, such as apomorphine, the phenothiazines, fentanyl and other narcotic drugs, since these drugs are effective at very low concentrations there is likely to be much less margin for dilution as the analyst

is already operating close to the detection limits. Indeed, the concentrating mechanism is vital for the forensic detection of apomorphine in equine urine, as the routine detection of concentrations of this drug found in plasma is very difficult. There have been reports of substantial reductions in concentrations of urinary apomorphine in frusemide-treated horses (Ozog 1977), supporting the hypothesis that urinary concentrations of many other drugs excreted as glucuronide metabolites are susceptible to diuretic dilution (Tobin *et al.* 1977).

(c) Fentanyl

Combie *et al.* (1981) dosed horses with 0.001 mg/kg of the potent narcotic analgesic fentanyl and administered frusemide (0.5 mg/kg) 0.5 h later. The diuretic caused urinary concentrations of fentanyl to fall about 18-fold within 30 mins of dosing, but urinary levels returned to control within 2.5 h of dosing. A more detailed study has been carried out by Soma *et al.* (1984). The dose of frusemide usually given in the treatment of epistaxis (0.5 mg/kg; i.v.) was used, as the diluting effect of this dose should be both smaller and of shorter duration than the effect of a 1 mg/kg dose. The plasma concentration of fentanyl was unaltered during the period of diuresis though an increase in packed cell volume and total protein was detected. In contrast however, frusemide produced a 17-fold increase in urine volume, a 2.8% reduction in urine specific gravity, and an 80% decrease in the urinary fentanyl concentration within one hour. Urinary fentanyl concentrations returned to control levels by 3 hours after injection.

Despite the reduction of urinary fentanyl concentrations, the rate of excretion of this drug increased more than 30%. This is due to the increase in glomerular filtration rate during the first post-injection hour which results in an increase in the filtered load of fentanyl. It is also likely that frusemide is having some direct tubular effect, either inhibiting tubular reabsorption or increasing tubular secretion. The fractional reabsorption of fentanyl decreased from a control of 70.3 ± 6.2% to 25.2 ± 2.3% during the first hour, returning to values not statistically different from controls 3 hours post-frusemide.

(d) Procaine

Two groups of horses were given procaine hydrochloride intramuscularly (10 mg/kg), and one group was challenged with frusemide intravenously (1 mg/kg) 2 hours later (Tobin *et al.* 1977). In terms of plasma levels, no statistically significant differences between groups were noticed. This supports the observation of others that the possible reduction in plasma water does not alter the concentration of a drug, and that the plasma concentration is an accurate representation of drug levels following diuresis.

The rate of excretion of procaine after frusemide injection rose transiently to 20-fold or more than control values and returned to normal 4 hours after frusemide treatment. Procaine readily distributes throughout the body, being a lipid soluble drug at physiological pH (Tobin *et al.* 1977a). It is thus likely that, as urine volume increases after frusemide therapy, procaine effortlessly enters this expanding compartment and is excreted at a rate proportional to urine flow. In agreement with this, Evans and Lambert (1974) have shown that urinary concentrations of procaine are closely associated with urinary pH changes, consistent with a rapid equilibration of procaine across renal tubules. Thus frusemide substantially enhances the urinary excretion rate of procaine. The maintenance of a steady state plasma concentration despite increases in rate of excretion can be explained by a possible shrinkage of the central compartment due to a loss of plasma water during the period of diuresis. A reported increase in packed cell volume (PCV) after frusemide, found by some workers (Fregin *et al.* 1977; Soma *et al.* 1984) may indicate a loss of plasma water. Increases in PCV have been found to be proportional to the frusemide dose in the horse. The increase in PCV and total protein indicate a haemoconcentration and a possible reduction of plasma volume. The constant infusion into a smaller compartment would maintain the plasma levels, despite the increased elimination of drug by the kidneys.

(e) Methylphenidate

Frusemide (1 mg/kg; i.v.) administered 2 hours after methylphenidate (0.33 mg/kg; s.c.), did not affect the plasma levels, but reduced the plasma half-life of

methylphenidate in the horse; this however does not appear to be of sufficient magnitude to be clinically or forensically important (Tobin *et al.* 1977). Frusemide was found to decrease the concentration of the drug in urine. However, this change in urinary concentration after frusemide was small, presumably due to the lipid nature of methylphenidate, and urinary output of the drug was therefore increased more than 10-fold.

(f) Morphine

Combie *et al.* (1981) administered morphine (0.1 mg/kg) intravenously to 6 horses and followed this 1 hour later with frusemide (0.4 mg/kg; i.v.). Urinary concentrations of free morphine plus morphine released by β–glucuronidase i.e. total morphine, fell 13-fold within 15 min after the diuretic was given. Total morphine concentrations in urine then rose rapidly from this low point until the levels were not significantly different from control values some 3 hours after frusemide.

(g) Multiple Drug Study

An extensive study by Stevenson *et al.* (1990) was performed to examine the influence of two doses of frusemide (150 mg or 250 mg; i.v.) on the plasma and urinary concentrations of nine drugs in standardbred horses. Frusemide reduced the plasma concentrations of codeine compared to control 2-6 h after frusemide administration. However, the plasma concentrations of theophylline, flunixin, phenylbutazone, pentazocine and guaifenesin were not altered by frusemide. Data obtained for acepromazine, clenbuterol and fentanyl were inadequate to state with certainty whether or not frusemide had an effect.

Urinary data indicated that there was a significant reduction in the urinary concentrations of guaifenesin, flunixin, acepromazine, clenbuterol, phenylbutazone, fentanyl and pentazocine within 1-4 h of frusemide dosing, and up to 8 h for theophylline. The influence of frusemide on urinary morphine concentrations appeared to be biphasic, horses pre-treated with codeine resulted in depression of morphine concentrations 2-4 h and 9-12 h after frusemide injection. Stevenson *et al.* (1990)

concluded that frusemide affects urinary and plasma concentrations of co-administered drugs but not in a predictable fashion, thus extrapolation of data to untested drugs may not be reliable.

1.10. THE MISUSE OF DRUGS IN SPORT

Doping in sport must be considered within the context of misuse of drugs in society. In countries throughout the world, authorities are concerned that the escalation of drug misuse is undermining the very fabric of their society. Increasingly society has come to believe that there are drugs available to cope with most ills, diseases and problems. Inevitably sport, being an integral part of society, has not escaped the drug abuse problem.

The misuse of drugs to enhance performance is an abhorrent practice, widely recognised by individual sportsmen and women, as well as their governing bodies, as a major threat to the successful development of sport. The use of performance-enhancing drugs is contrary to the spirit of fair competition and is intended to confer unfair advantage on the user. Regrettably, the use of drugs is growing - not only among top level sportsmen and women but also among the many people who use local health and fitness clubs.

Social, economic and even national pressures are imposed on athletes to win, sometimes at any cost. Some athletes use drugs in an attempt to improve performance, reduce stress, increase muscle strength, lessen fatigue etc. Athletes who embark upon the path of drug misuse are in danger of introducing ethical as well as health problems for themselves. Misuse of some drugs may have the immediate effect of impairing judgement and jeopardizing the safety of individuals and other competitors. Even death in sport may be the consequence. Birgit Dressel, the West German heptathlete, moved from 33rd in the world rankings to 6th within one year. Then, aged 26, she collapsed and died from the horrifying number of drugs she had taken. Her 12-month rise had been stimulated by 400 injections of various drugs e.g. steroids and stimulants (The

Guardian, 28th September 1988). Misuse of other drugs, especially the anabolic steroids, can induce long term effects causing serious health problems, reducing both the quality and the span of life.

The abuse of drugs is no longer a shallow problem affecting one or two competitors; it can involve officials, coaches, doctors, administrators, promoters, and dealers illegally trading in drugs. In Britain the problem was at its worst in the decade prior to the advent of random testing. Drug-taking became progressively more refined during this time period. From dosages that were often hit and miss, and in some cases high enough to be dangerous, the procedures evolved into a far more scientific approach. Doctors advised, and in a number of cases supplied the drugs, while coaches wrote the schedules around the drug intake. The advice included the drug to take, the dosage cycles, when to decrease training and how to avoid detection during high-level competition. This situation led Sir Arthur Gold, the chairman of the British Olympic Association, to comment that "Only the stupid or ill-advised should be caught during competition" (Daily Mail, 28th September, 1989).

The suspected growth in the use of performance enhancing drugs is reflected in the increase in the number of drug tests thought necessary to detect and deter since the testing of sportsmen and women first took place. Table 1.5. details the number of tests each year since 1979 and the incidence of banned substances, which is unchanged over time.

The International Olympic Committee (IOC), as custodians of the Olympic Games, has drawn-up internationally recognised guidelines on drug abuse and banned substances. The IOC via its Medical Commission, has played a major role in the fight. This involves education emphasising the dangers of drug misuse and explaining the positive aspects of sports physiology and the ethics of sport. Controls to deter misuse are also an essential part of this campaign. The IOC published its first "banned substances" list in 1967; in 1986 the list was revised to include two further classes of substances (Table 1.6.). The first dope controls to be carried out during sports competitions took place during the Olympic Winter Games in Grenoble in 1968 and since this time the work of the Commission has expanded considerably.

Table 1.5. Summary of drug testing in the United Kingdom in sports governed by the International Olympic Committee

Substance Detected	Number of Positives/year							
	1979	1980	1981	1982	1983	1984	1985	1986
A. Stimulants	5	6	4	10	10	12	15	21
B. Narcotics	0	0	0	1	0	3	0	2
C. Anabolic Steroids	0	1	0	1	4	1	2	11
D. β-Blockers	0	0	0	0	0	0	0	7
Total No. of UK Tests	497	557	643	780	647	1060	1523	2545
Positives found	5	7	4	12	14	16	17	41
% of Total	1.0	1.2	0.6	1.5	2.2	1.5	1.1	1.6

Table 1.6. International Olympic Committee List of Doping Classes and Methods

1. DOPING CLASSES

A. Stimulants
B. Narcotics
C. Anabolic Steroids
D. β-Blockers
E. Diuretics

2. DOPING METHODS

A. Blood doping
B. Pharmacological, chemical and physical manipulation

3. CLASSES OF DRUGS SUBJECT TO CERTAIN RESTRICTIONS

A. Alcohol
B. Local anaesthetics
C. Corticosteroids

The doping definition of the IOC Medical Commission is based on the banning of pharmacological classes of agents. The definition has the advantage that also new drugs, some of which may be especially designed for doping purposes, are banned. Unless indicated all substances belonging to the banned classes may not be used for medical treatment. If substances of the banned classes are detected in the laboratory the IOC Medical Commission will act. The presence of a drug in the urine is an offence, irrespective of the route of administration.

Seoul's Catalogue of Shame

The 1988 Olympic Games in Seoul will be remembered as the games of the drugs cheats with such front page headlines as "The Olympics wallow in a vat of urine" and "Cheats can't kill the flame" (Sunday Times, 2nd October, 1988).

The IOC set up in Seoul the most sophisticated drug detection laboratory in the history of the games in an effort to stamp out drug abuse. In a speech just before the games, the president of the IOC Juan Antonio Samaranch pointed to the dangers and said: "Doping equals death." 11 competitors were disqualified for taking drugs, the same as in Moscow in 1980 and one less than in Los Angeles in 1984. However, the exposure of Ben Johnson for taking stanozolol, an anabolic steroid used for back problems and repairing damaged muscles, was acclaimed as the long-overdue victory of the drug-testers over a superstar cheat. For the first time in Olympic history, "a big fish", and not just an East European weightlifter or shot putter had been caught.

The International Weightlifting Federation launched its own investigation into drug taking in an attempt to restore the credibility of the sport following the number of positive tests at Seoul. Drug abuse in weightlifting is endemic and the IOC may soon be forced to throw the sport out of the games altogether. Even before the Games began it was obvious that drugs were as prevalent as ever. Four Canadian weightlifters were thrown out of the seven-man Olympic squad for taking drugs, inspiring one Canadian wit to change the sign in the weightlifting headquarters from "Canadian weightlifters, Clean and jerk", to "Canadian weightlifters, Three Clean, Four jerks".

The entire Bulgarian team, the premier force in the sport in recent years, withdrew from the games because two of its gold medal winners, Anguelov Guenchev and Mitko Grablev, were tested positive for frusemide. This may have been used either to reduce weight drastically, allowing them to drop into a desired weight division, or to help eliminate traces of hormone drugs from the kidneys. Britain's judo bronze winner, Kerrith Brown, was also found guilty of using frusemide without his doctors' knowledge to reduce the swelling in a knee injury. Other drugs detected during the games were testosterone, amphetamines, propranolol, caffeine and ephedrine.

In the fight against drugs there is still a hard road ahead, of the 37,882 samples taken from athletes worldwide in 1987, only 854 proved positive. No one believes for a moment that this figure represents the true proportions of the problem. The battle against drugs can never be won; the most one can hope is that the cheats are forced further on to the defensive. As Prince Alexandre de Merode, the Belgian chairman of the IOC's medical commission said: "A fight of this type is never-ending. Cheating will come to an end when the world comes to an end." (Sunday Times, 2nd October, 1988.)

1.11. AIMS OF THIS STUDY

The widespread use of frusemide treatment in sport as highlighted in the last Olympic Games and its increasing popularity in recent years for the prophylaxis of epistaxis in racehorses has led to widespread concern over its effect on other drugs. It is hoped that by investigating the influence of frusemide on the metabolism, pharmacokinetics and "detectability" of an acidic and a basic drug that a greater knowledge of the actual drug interaction of frusemide can be established. It is beneficial to do metabolic and kinetic studies so that an assessment of the possible "diluting", "masking" or "flushing out" effects of frusemide can be made; and we can determine whether there is a need for concern, and whether racing officials are justified in their fears over the use of this diuretic as far as its ability to hinder the forensic detection of illicit drugs. In this study, it is intended to obtain information on the following:-

1. The metabolism and pharmacokinetics of frusemide following different routes of administration.

2. How frusemide affects the pharmacokinetics of salicylic acid, and its urinary elimination and detectability in plasma and urine when frusemide is coadministered.

3. How frusemide affects the pharmacokinetics of theophylline, and its urinary elimination and detectability in plasma and urine when frusemide is coadministered.

4. The metabolism and disposition of theophylline in the horse.

5. The mechanism of excretion of frusemide via the organic acid transport system by investigating the frusemide-probenecid interaction.

6. The effects brought about as a consequence of the diuresis, i.e. the influence of frusemide on normal plasma and urine constituents and the subsequent haemoconcentration and renal effects.

By investigating frusemide's effect on different classes of compounds we hope to gain a better understanding of the actual drug interaction thus allowing us to draw some conclusions on how frusemide affects these different classes of compounds.This will enable us to predict the behaviour of other co-administered drugs.

Thus we hope to examine the credibility of presently held opinions within the racing fraternity concerning the actions of this potent diuretic and so clarify the controversy concerning frusemide's effect on other drugs.

Chapter 2

THE DETERMINATION OF FRUSEMIDE IN BIOLOGICAL FLUIDS

2.1. INTRODUCTION

The earliest methods for analysis of frusemide were based on spectrophotofluorimetry (Hajdu and Haussler, 1964) and spectrophotometry (Haussler and Hadju, 1964) following extraction from biological fluids with organic solvents. Subsequently a variety of different assays have been developed which distinguish between frusemide and its possible metabolite, 4-chloro-5-sulphamoylanthranilic acid (CSA). These are essentially modifications of the original method, i.e. additional extraction steps have been added (Andreasen and Jakobsen, 1974; Forrey *et al.* 1974) or a thin layer chromatographic step follows extraction prior to spectrophotofluorimetry (Mikkelsen and Andreasen, 1977).

Chromatography techniques are superior, so gas chromatography being sensitive and reproducible has also been used for the detection of frusemide. The methods are based on that developed by Lindstrom and Molander (1974) who used an extractive alkylation technique using THAHS (tetrahexylammonium hydrogen sulphate) and electron capture detection. The variations are generally in the column packing material and in the internal standards used. Packing materials include 3% JXR (Aranda *et al.* 1978), 3% OV-101 (Roberts *et al.* 1978) and 1% SE-30 (Beermann *et al.* 1977). ^{63}Ni electron capture detection of frusemide is used in all the GC methods.

HPLC analyses are by far the most extensively used techniques for the determination of frusemide (Table 2.1.), due mainly to their accuracy, precision and reliability. Methods are available for the quantitation of frusemide in both plasma and urine. The majority of the assays involve acidification of the sample prior to extraction into an organic solvent. Reverse-phase C-18 columns are generally employed but ion-exchange columns have been used (Blair *et al.* 1975; MacDougall *et al.* 1975). A number of different internal standards have been utilized and detection may be by UV absorption or fluorescence. The methods vary depending on the purpose they are required for, for example Nation *et al.* (1979) have used an assay which is particularly suitable for measuring frusemide in infants, as only very small volumes of plasma (0.1 ml) are required. Most methods require the use of 1 to 2 ml of plasma to achieve

TABLE 2.1. HPLC assays for frusemide in biological fluids

Biological Fluid	Stationary Phase	Sample Treatment	Comments	Reference
Plasma/ Urine	RP C-18	Acidification and ether diether extraction	The mobile phase consisted of 0.02M buffer (pH 2) mixed with acetonitrile, could damage stainless steel HPLC fittings	Lindstrom 1974
Serum/ Urine	Cation-exchange resin column	Sample acidified to pH 2.5	No internal standard used, 5 µl aliquots of sample used	Blair *et al.* 1975
Plasma/ Urine	RP C-18	Ether extraction from acidified samples	Methyl ester of frusemide used as an internal standard, fluorimetric detection	Carr *et al.* 1978
Plasma/ Urine	RP C-18	No prior extraction and/or derivation	Na - cephalathin as an internal standard, 280 nm fixed wave-length UV absorption detector	Lin *et al.* 1979

Table 2.1. continued.

Biological Fluid	Stationary Phase	Sample Treatment	Comments	Reference
Plasma/ Urine	RP C-18	No prior extraction and/or derivation	Na - phenobarbital as an standard, 254 and 280 nm dual-channel fixed wavelength UV detector	Lin *et al.* 1979
Plasma	RP C-18	Protein precipitation with acetonitrile	Suitable for frusemide determination in small volumes of plasma collected from infants	Nation *et al.* 1979
Plasma/ Urine	RP CH-10	Acid extraction into diethyl ether and back extraction into aqueous sodium hydroxide	N-benzyl-4-chloro-5-sulphamoyl-anthranilic acid as an internal standard, one sample chromatographed in 8-9 mins	Swezey *et al.* 1979
Plasma/ Urine	RP C-18	Sodium phenobarbitone as an internal standard, protein precipitation with acetonitrile.	Samples were not acidified	Smith *et al.* 1980a

Table 2.1. continued.

Biological Fluid	Stationary Phase	Sample Treatment	Comments	Reference
Plasma/ Urine	RP C-18	Acidified with HCl and extracted into ether, reconstituted in pH phosphate buffer	Phenyl analogue of frusemide as the internal standard, fluorescence detection	Martin *et al.* 1984
Plasma/ Urine	10μ Pell ODS	Sample acidified and extracted with anhydrous diethyl ether, reconstitute with glycine buffer (0.02M pH 11).	Hydroflumethiazide as internal standard	Waller *et al.* 1985
Urine	RP C-18	Sample acidified with NaH_2PO_4 and extracted with ethyl acetate, further washing	UV detection at 271 nm, method suitable for screening 12 diuretics in urine	Fullinfaw *et al.* 1987
Plasma/ Urine	Ion-pairing	Minimal sample preparation	Rapid microscale method 7 minutes/sample	Sood *et al.* 1987

quantitation of frusemide in plasma with a lower limit between 0.1 and 1 μg/ml (Lindstrom 1974; MacDougall *et al.* 1975; Carr *et al.* 1978).

New techniques are continuously being evaluated for monitoring frusemide as well as ethacrynic acid and bumetanide, potent diuretics which may be illegally substituted for frusemide in racing horses (Wood *et al.* 1988,1990). Wood *et al.* (1988) have developed a one step enzyme-linked immunosorbent assay (ELISA) test and a particle concentration fluoroimmunoassay (PCFIA) test to determine the illegal medication of frusemide in racing horses. The principal utility of these tests lies in rapid screening of samples for compliance with regulations governing the use of frusemide. Their applicability to metabolic and pharmacokinetic studies is limited.

In this study, thin-layer chromatography (TLC) of frusemide was used as a screening technique to detect the presence of the diuretic in urine samples. However, the pharmacokinetic studies were performed using HPLC. Initially a liquid-liquid extraction method was used but this was excessively time consuming for routine application, so we subsequently developed a new assay using solid phase extraction. Both methods however had the required sensitivity and specificity for our purposes.

2.2. METHODS

Compounds

Frusemide (4-chloro-5-sulphamoyl N-furfurylanthranilic acid) (Lasix 5% solution; Hoechst; Milton Keynes, Bucks. U.K.). N-benzyl-4-chloro-5-sulphamoylanthranilic acid was a gift from Hoechst (Frankfurt, F.R.G.). Furosemide (5-[Aminosulphonyl]-4-chloro-2-[(2-furanylmethyl)amino] benzoic acid) (Solid; No. F-4381 Sigma Chemical Company, St. Louis U.S.A.). HPLC grade solvents were purchased from Rathburn Chemicals Ltd., (Walkerburn, England) or May and Baker Ltd., (Dagenham, England). Other materials were reagent-grade chemicals purchased from usual U.K. commercial sources.

1. Thin-Layer Chromatography

a. Sample preparation

For thin layer chromatographic detection of frusemide, 10 ml of urine (2x5 ml) was acidified to a pH of 1-2 with a few drops of concentrated hydrochloric acid. 4 ml of chloroform (2x2 ml) were added to the sample which was then vortexed for 5 min. Following centrifugation at 1500 x g for 10 min, the urine layer was aspirated and discarded and the chloroform layers were pooled and evaporated to dryness under a gentle stream of nitrogen. The residue was then taken up in 100 µl of chloroform prior to TLC.

b. Chromatography

Thin layer chromatography was carried out using silica gel F_{254} plates, layer thickness 0.2 mm, 20 x 20 cm on aluminium supports (Catalogue no. 5554; E.Merck A.G., Darmstadt, F.R.G.). The residue together with standard solutions of frusemide were spotted on to the TLC plates. The solvent system used to develop the plates was chloroform:cyclohexane:glacial acetic acid (6:4:1.5 by vol.). After developing to 16 cm from the origin (approx. 2 h), the plates were air-dried and immediately examined under short-wave ultraviolet light.

2. High Performance Liquid Chromatography

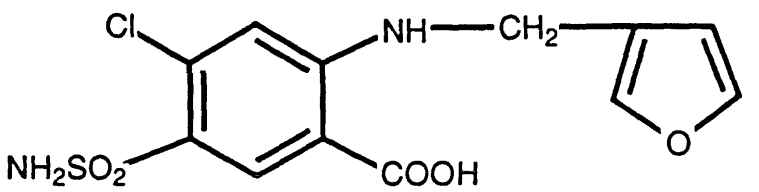
Method 1 Liquid-Liquid Extraction

a. Sample preparation

Standard solutions of frusemide and the internal standard (N-benzyl-4-chloro-5-sulphamoylanthranilic acid) (Fig.2.1.) were made up in sodium carbonate-bicarbonate buffer (0.1M; pH 10.2) and stored at 4°C. Aluminium foil was used to cover the vessels, as degradation of frusemide occurs when it is exposed to light.

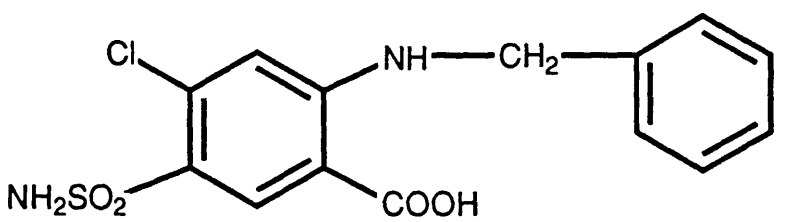
To 1 ml of plasma or urine was added the internal standard (60 µl; 10 ng/ml working solution), hydrochloric acid (100 µl 6M HCl) and distilled water (500 µl).

Fig.2.1. Structure of frusemide and the internal standard



FRUSEMIDE

(4-chloro-5-sulphamoyl N-furfurylanthranilic acid)



INTERNAL STANDARD

(N-benzyl-4-chloro-5-sulphamoylanthranilic acid)

Diethyl ether (8 ml) was then added and the samples were vortexed for 30 sec in 13 ml glass tubes with teflon-lined screw caps. The tubes were then placed in an electronic rotor-rack for 10 min. Following centrifugation at 3000 rpm for 5 min using an MSE Centaur 2 bench-top centrifuge, the upper organic layers were transferred into clean glass tubes and the solvent evaporated to dryness either under a stream of nitrogen, or with an evaporating centrifuge. The residue was reconstituted in 500 µl of distilled water prior to chromatography.

b. Chromatography

The reconstituted extracts were injected on to a Lichrosorb RP-18 analytical HPLC column (E. Merck, Darmstadt, F.R.G. Cat. 15539), 250 mm x 4 mm i.d. 7 µm particle size; using a Rheodyne 7125 valve loop injector or a WISP autoinjector (model 710B, Waters/ Millipore U.K. Ltd., Harrow, Middlesex). A reciprocating HPLC pump (Model-45, Waters/Millipore U.K. Ltd., Harrow, Middlesex) and a filtered mobile phase of acetonitrile: water: acetic acid (30:65:5 by vol.) were used to elute frusemide and the internal standard at ambient temperature and a flow rate of 2 ml/min. The solvent filtering system used was Millipore HA (0.45 µm), Millipore Ltd., Harrow, London. A spectrofluorimeter (model RC 200 Ratiometric, Baird Fluoricord; Spex Industries U.K. Ltd., Braintree, Essex) set at an excitation wavelength of 354 nm and an emission wavelength of 404 nm was used to detect frusemide and the internal standard. The mobile phase is sufficiently acidic to render frusemide fluorescent. A 25 µl silica HPLC flow cell (Cat. RC126; Spex Industries U.K. Ltd., Braintree, Essex) with stainless steel inlet and outlet tubes (0.25 mm and 0.5 mm bore) was used, to allow continuous monitoring of the column effluent. Both monochromators were set with 10 nm slit widths.

c. Validation of the method

i. Chromatography

Blank urine and plasma were spiked with frusemide and internal standard to obtain HPLC traces where the peaks were well separated and free from any interfering peaks. Quantitation of frusemide was achieved by the peak height ratio method, with respect to the internal standard.

ii. Linearity of response

Fresh calibration curves representing frusemide concentrations over a wide range 0-8000 ng/ml in plasma, and 0-200 ng/ml and 0-8000 ng/ml in urine were prepared each time the assay was used.

iii. Inter-assay variation

New calibration curves in plasma for the range 0-5000 ng/ml were prepared on seven separate occasions. Similarly curves in urine for the range 0-8000 ng/ml were prepared. Day to day variation in the slope of the peak height ratio vs. concentration line was determined.

iv. Reproducibility

The reproducibility of the method was investigated by spiking blank plasma with frusemide at three different concentrations, viz. 10 ng/ml, 50 ng/ml, and 100 ng/ml. Six identical samples were prepared and analysed. Similarly for urine, two concentrations were analysed; 25 ng/ml and 50 ng/ml. The coefficient of variation (%) of the peak height ratio was determined as standard deviation/mean.

v. Accuracy

The accuracy of the assay was determined by having a second operator spike three urine samples with frusemide and internal standard. These samples were then analysed blind and concentrations of frusemide determined from a calibration curve.

vi. Extraction efficiency

The extraction efficiency of frusemide and the internal standard was assessed by spiking six tubes of 480 µl of distilled water with frusemide equivalent to a frusemide concentration of 200 ng/ml, i.e. the total volume was 500 µl equal to the reconstituted volume following extraction. These six tubes were analysed directly by making 50 µl injections on to the HPLC system, this represented 100% extraction of frusemide. Another six tubes containing 480 µl of plasma were spiked in the same way and carried through the extraction procedure before analysis. The same method was followed for the internal standard. Six tubes containing the equivalent of 600 ng/ml of internal standard and 440 µl of water were prepared. These were analysed by injecting directly on to the column, and six tubes of spiked plasma were extracted and reconstituted with 500 µl of water prior to injection. Following HPLC analysis the extraction efficiency was determined from the peak heights as:-

$$\frac{\text{mean peak height of frusemide / mean peak height of internal standard after extraction}}{\text{mean peak height of frusemide / mean peak height of internal standard after direct injection}} \times 100\%$$

The extraction efficiency from plasma samples spiked with frusemide 100 ng/ml and internal standard 500 µg/ml was obtained from the ratio of the peak height ratio:-

$$\frac{\text{mean peak height ratio after extraction}}{\text{mean peak height ratio after direct injection}} \times 100\%$$

Method 2 Solid Phase Extraction

a. Sample preparation

To 500 µl of plasma or urine was added the internal standard (as above) (80 µl; 10 ng/ml solution), hydrochloric acid (100 µl; 6M HCl) and distilled water (920 µl). The samples were vortexed briefly prior to solid phase extraction. Disposable solid phase C18 endcapped cartridges (100mg/ml) were used, purchased either from BondElut (Analytichem International) or Worldwide Monitoring Services (supplied by Technicol, Part No. CEC18111, Cheshire, U.K.). The cartridges were twisted on to the Luer endfittings of a VacElut SPS 24 manifold (Jones Chromatography, Mid-Glamorgan, U.K.). A sidearm flask was connected between the VacElut and a vacuum pump. The VacElut system functioned as a solvent reservoir during the washing steps and allowed collection of sample during the elution step. Sufficient negative pressure (5-10 inches of Hg) was applied to discharge the fluid at a steady rate. Additional positive pressure was also applied by hand, using a syringe plunger if it appeared that the flow was inconsistent or reduced in any of the columns.

The procedural steps (Fig.2.2.) were followed sequentially for each cartridge. Firstly the cartridge was conditioned with methanol (1 ml) and water (1 ml). The plasma or urine sample (1 ml) was then applied to the cartridge within 30 sec to make sure that the sorbent did not dry out. The cartridge was then washed with 1 ml aliquots of water, 10% methanol, 20% methanol, 30% methanol and pH 7.4 buffer consisting of 0.1M monosodium hydrogen phosphate with 0.1M disodium hydrogen phosphate. Frusemide and the internal standard were eluted from the cartridge with 1 ml of 15% acetonitrile. In this step the effluent was collected directly into clean glass HPLC vials. These were then capped and either kept at 4°C or analysed by HPLC immediately.

Fig.2.2. **Procedure for solid-phase extraction of frusemide and the internal standard from plasma and urine samples**

(1) Condition column

↓ 1 ml 100% methanol

1 ml distilled water

(2) Load sample

↓ 1 ml plasma or urine sample

spiked with internal standard

(3) Washing steps

↓ 1 ml distilled water

1 ml 10% methanol

1 ml 20% methanol

1 ml 30% methanol

1 ml PO_4 buffer pH 7.4

(4) Elute column

↓ 1 ml 15% acetonitrile

(5) Inject sample (10-100 µl) on column

b. Chromatography

The HPLC system consisted of a LC-6A solvent delivery system, a RF-535 fluorescence HPLC monitor set at an excitation wavelength of 354 nm and an emission wavelength of 404 nm, linked to a C-R6A Chromatopac integrator (all Shimadzu, Kyoto, Japan; supplied by Dyson Instruments, Hetton-le-Spring, U.K.).

The extracts were injected on to a Lichrosorb RP-18 analytical cartridge (E. Merck, Darmstadt, F.R.G. Cat. 612081), 125 mm x 4 mm i.d. 5 μm particle size; using a WISP autoinjector (model 710B, Waters/Millipore U.K. Ltd., Harrow, Middlesex). An isocratic system was used to elute the compounds, consisting of water: acetonitrile: acetic acid (67:28:5 by vol.) filtered and degassed with a Millipore HA (0.45 μm) solvent filtering system (Millipore Ltd., Harrow, London). Frusemide and the internal standard were eluted at ambient temperature and a flow rate of 2 ml/min. The run time for each sample was 12 min.

c. Validation of the method

This was analogous to that described above for the liquid -liquid extraction method of frusemide.

d. Correlation between the liquid-liquid extraction method and the solid phase extraction method

Urine samples obtained from one subject who had received frusemide intravenously in a pharmacokinetic study, were split and extracted by both methods. The resulting concentrations were derived from their respective standard curves and the correlation coefficient between the two techniques was determined.

2.3. RESULTS

Thin Layer Chromatography

Frusemide was visualized under UV light on a fluorescent background as a brightly coloured spot. The spot was identified as frusemide following TLC, as it had an essentially identical R_F value to an authentic standard (R_F = 0.32). Frusemide could be detected in horse urine for approximately 16 hours after a 1 mg/kg intravenous dose.

High Performance Liquid Chromatography

Method 1 Liquid-Liquid Extraction

Chromatography

The liquid-liquid method gave well separated peaks which were not affected by any interfering peaks. Baseline resolution was obtained for frusemide and the internal standard, (N-benzyl-4-chloro-5-sulphamoylanthranilic acid). Typical HPLC traces are shown in Fig.2.3. The retention times of frusemide and the internal standard were approximately 4.0 and 7.4 min respectively.

Linearity of response

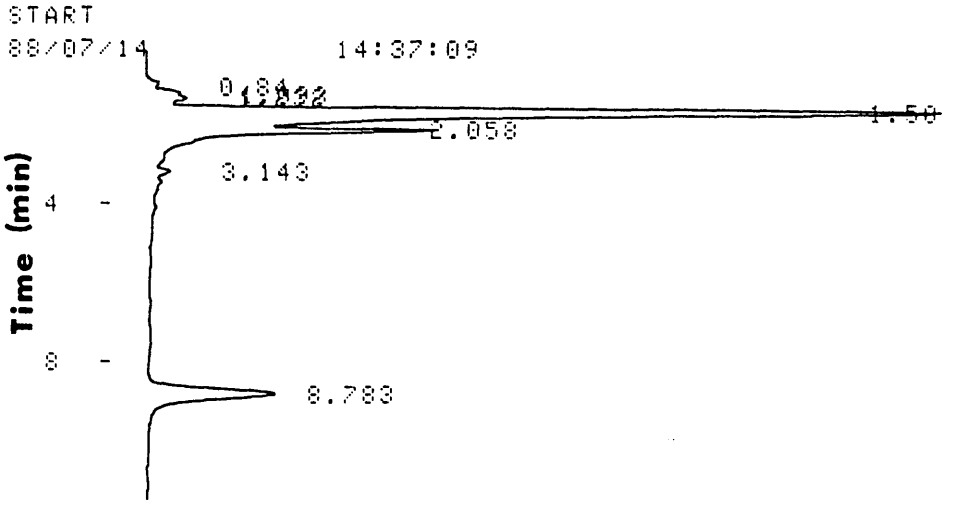
Figs.2.4. and 2.5. show typical calibration curves from plasma and urine of peak height ratio of frusemide to internal standard vs. concentration. All calibration curves were linear over the range investigated with correlation coefficients determined by regression analysis to be greater than 0.990.

Inter-assay variability

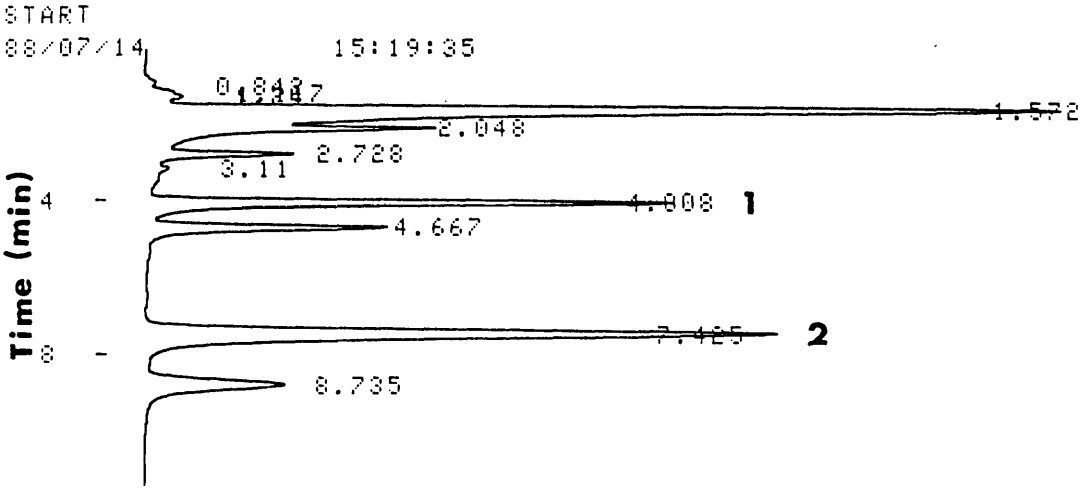
The inter-assay variation in the slope of the peak height ratio vs. concentration was 8.5% for plasma and 4.0% in urine. Even though this was sufficiently replicable, a new calibration curve was constructed whenever the assay was used.

Fig.2.3. Typical HPLC radiochromatograms of frusemide and and internal standard recovered from plasma using liquid-liquid extraction

(a) Blank plasma



(b) Plasma sample following frusemide (1 mg/kg; i.v.) administration



Peak	Compound	Retention time
1	FRUSEMIDE	4.0 min
2	INTERNAL STANDARD	7.4 min

Fig.2.4. Calibration curve for frusemide (0-5000 ng/ml) in plasma following liquid-liquid extraction

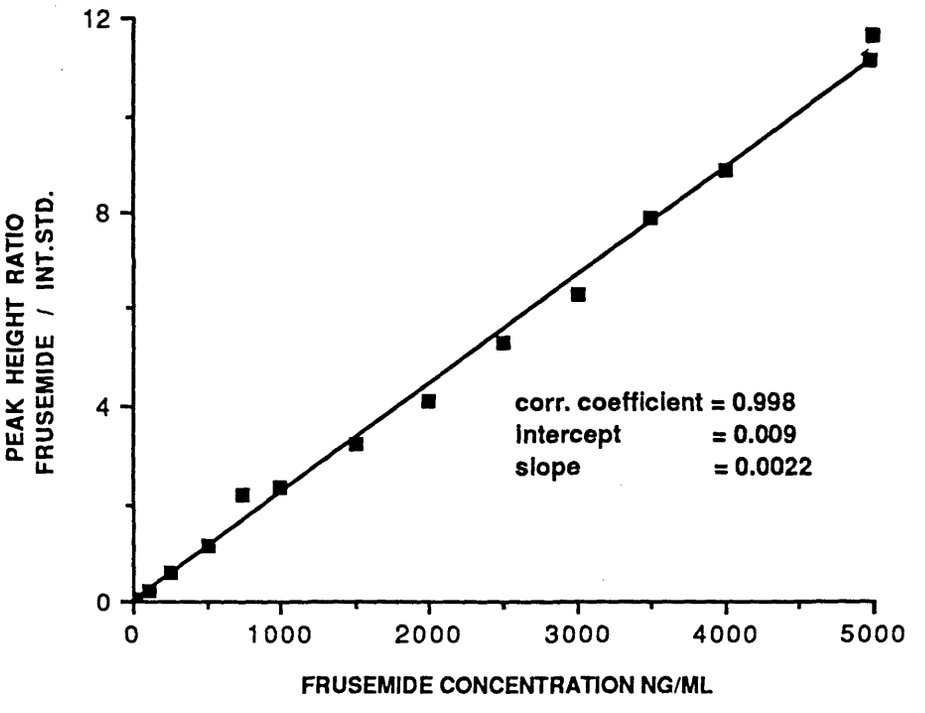
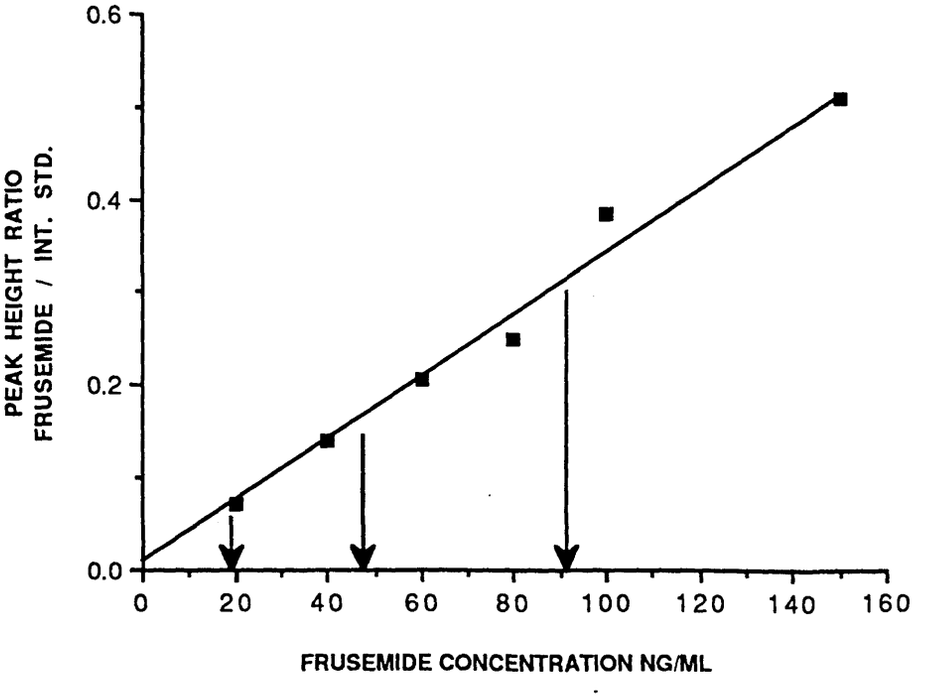


Fig.2.5. Calibration curve for frusemide in urine illustrating the accuracy of the liquid-liquid extraction method



Reproducibility

For plasma the coefficient of variation determined from peak height ratios at the three concentrations chosen were 2.1%, 2.4% and 3.2% respectively. In urine the coefficient of variation was greater, 3.9% at 25 ng/ml and 11.4% at 50 ng/ml.

Accuracy

The spiked concentrations chosen were 20 ng/ml, 46 ng/ml and 92 ng/ml. Analysis found these samples to contain (A) 19.2 ng/ml (B) 45.7 ng/ml and (C) 91.1 ng/ml respectively (Fig. 2.5.).

Extraction efficiency

Absolute recovery of frusemide and the internal standard was good, the extraction efficiency being 90.2% for frusemide and 81.3% for the internal standard. The relative extraction efficiency obtained from the peak height ratio was 86.8%.

Method 2 Solid Phase Extraction

Chromatography

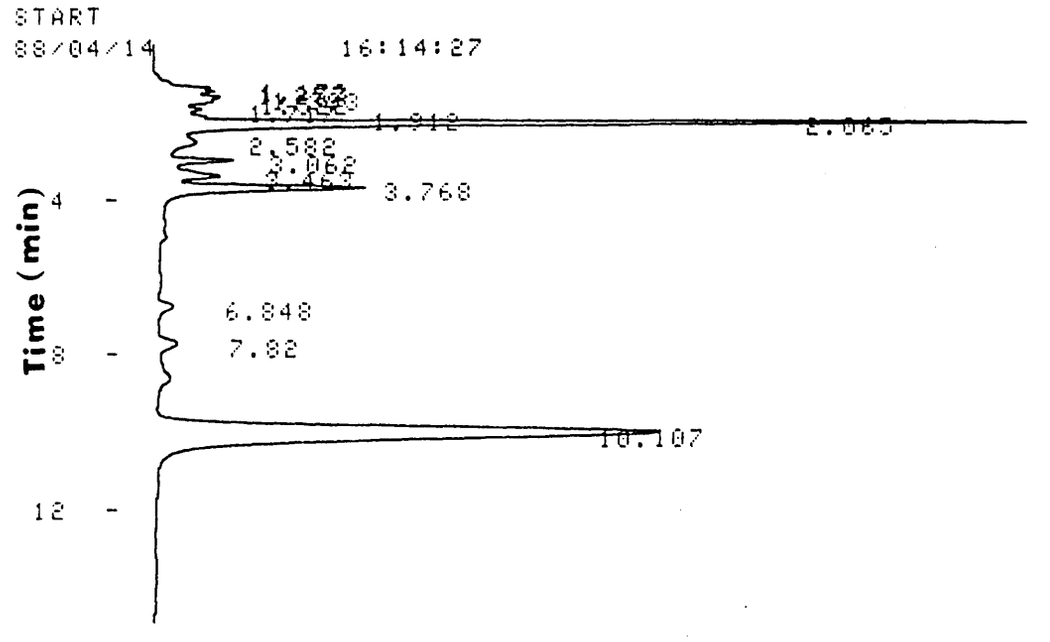
The solid phase extraction method afforded baseline resolution for frusemide and the internal standard. Peak shape was good, sharper peaks were obtained than with liquid-liquid extraction (Fig.2.6.). The retention times of frusemide and the internal standard were 4.4 and 8.4 min respectively.

Linearity of response

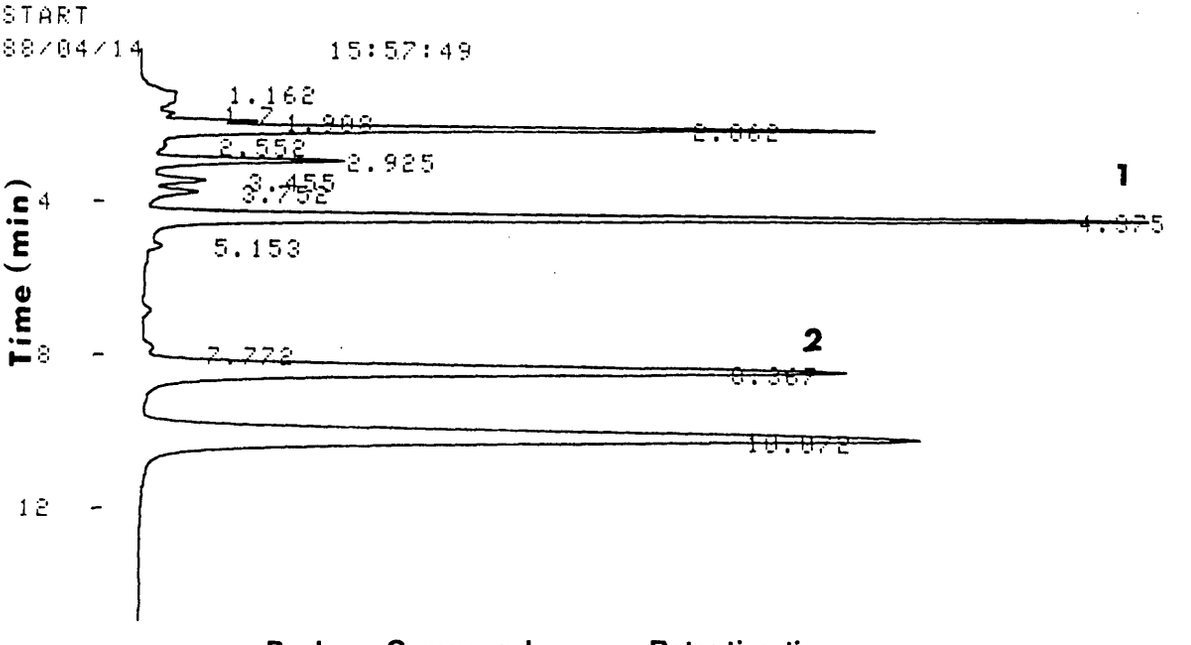
Fig. 2.7. shows typical calibration curves of peak height ratio of frusemide to internal standard vs. concentration. The calibration curves were linear over the range investigated (0-8000 ng/ml) in plasma and in urine. Correlation coefficients were greater than 0.995.

Fig.2.6. Typical HPLC radiochromatograms of frusemide and internal standard recovered from urine using solid phase extraction

(a) Blank urine

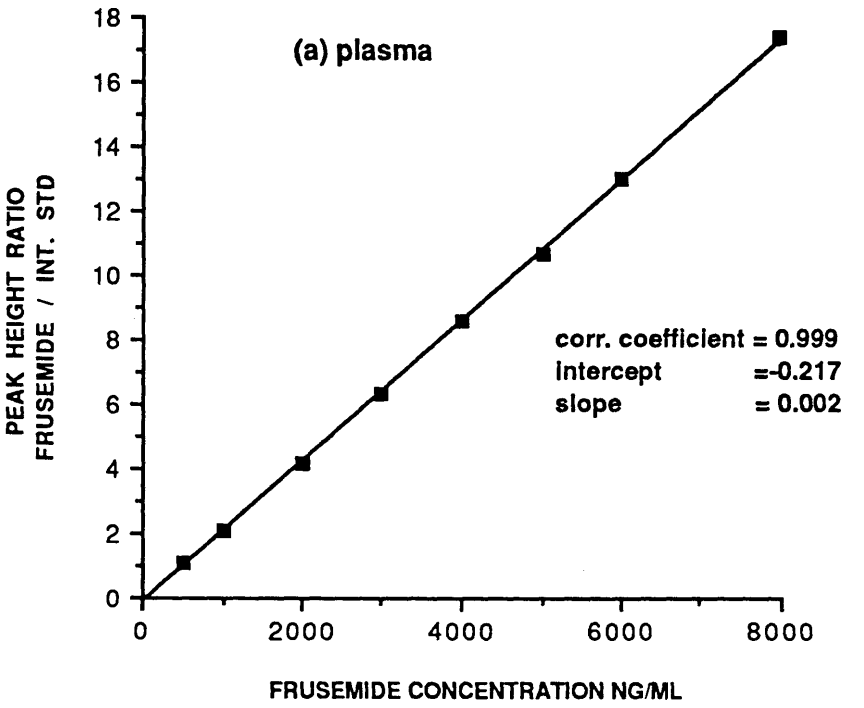


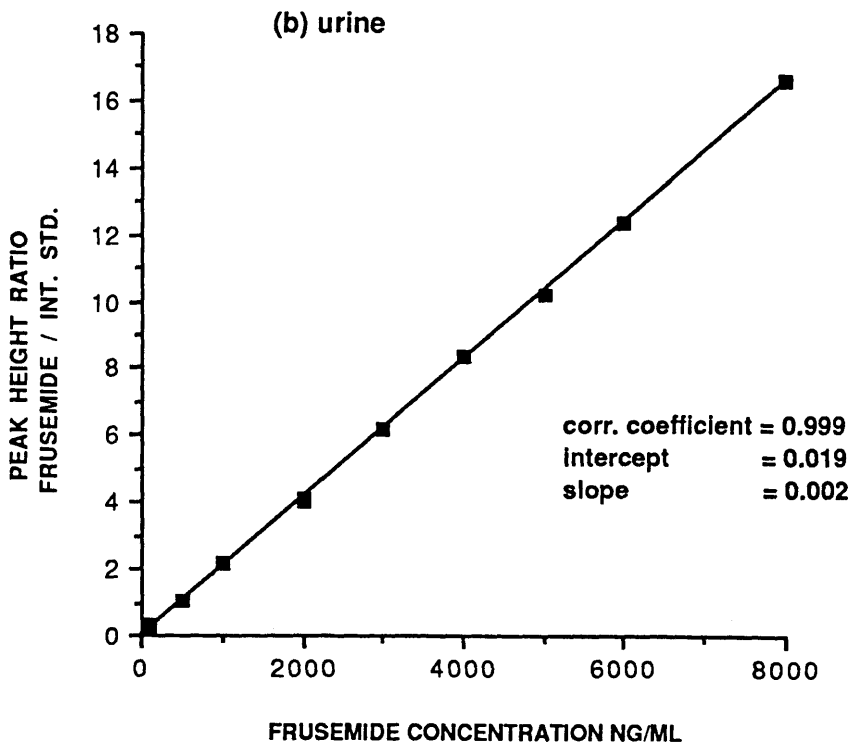
(b) Urine sample following frusemide (1 mg/kg; i.v.) administration



Peak	Compound	Retention time
1	FRUSEMIDE	4.4 min
2	INTERNAL STANDARD	8.4 min

Fig.2.7. Calibration curves for frusemide (0-8000 ng/ml) using solid phase extraction in (a) plasma and (b) urine





Inter-assay variability

The inter-assay variation in the slope of the peak height ratio vs. concentration was 3.6% for plasma and 5.3% for urine. This is slightly superior to that obtained by our initial method.

Reproducibility

The intra-assay coefficient of variation for frusemide was less than 7.9% at the concentrations investigated, n=6 for each concentration.

Extraction efficiency

Absolute recovery of frusemide and the internal standard was very good with solid phase extraction. The extraction efficiency being 95% for frusemide and approximately 100% for the internal standard.

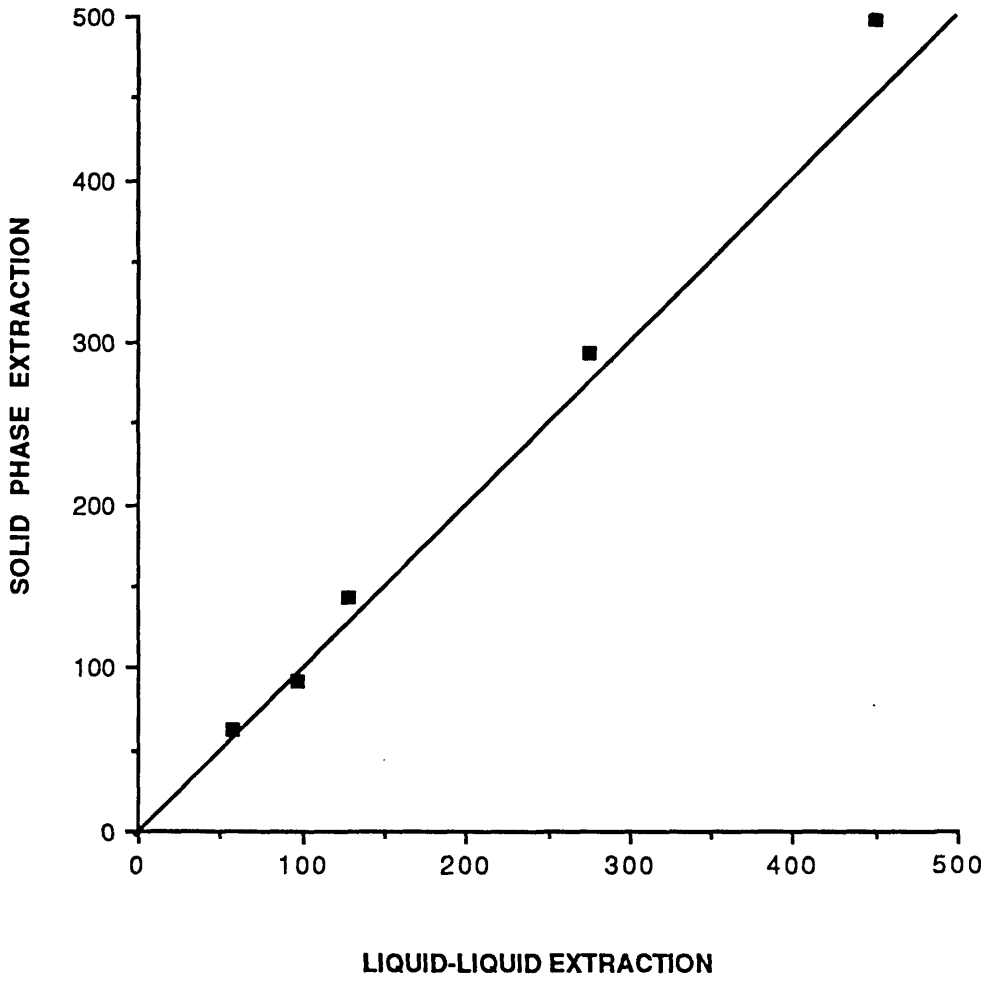
Correlation between the liquid-liquid extraction method and the solid phase extraction method

Fig.2.8. illustrates the correlation curve obtained when the two methods for the quantitation of frusemide were compared. Each data point in the scatterplot represents one sample, for which the x-coordinate is the concentration obtained by liquid-liquid extraction sample preparation, and the y-coordinate is the corresponding value from solid phase sample preparation and HPLC analysis. The 45^{o} line drawn in Fig.2.8. is the line of identity (y=x). With perfect correlation, all points would be on the line. The small degree of random scatter of data points above and below the line of identity demonstrates that the two sample preparation methods yield similar results. There was good correlation of results from the two methods, r = 0.998 over the concentration range tested.

Sensitivity

The lower limit of detection of frusemide by both liquid-liquid extraction and solid phaes extraction in plasma and urine was 5 ng/ml.

Fig.2.8. Frusemide urinary concentrations: Correlation of two extraction methods, liquid-liquid vs. solid phase extraction



2.4. DISCUSSION

Our use of thin-layer chromatography was merely as an initial screening technique. The method used was modified from that of Roberts *et al.* (1978), which is the usual method for routine screening of post race urine samples in Kentucky. If the TLC plates are sprayed in sequence with sulphuric acid, sodium nitrite, ammonium sulphamate and N-(1-naphthyl)-ethylene diamine dihydrochloride (NED), a bright pink spot develops which corresponds to frusemide. This is based on the Bratton Marshall reaction, a colorimetric reaction used to detect sulphonamides; the free amino group couples with NED to produce the colour spot which is stable for several weeks. However, as we were not using this method for quantitative purposes a permanent record of the plates was not necessary. A problem encountered when colorimetric reactions are used, is high and variable blanks (Roberts *et al.* 1978; Mikkelsen and Andreasen, 1977). The variation in the blank values after fluorimetric analysis of urinary extracts has also been highlighted (Forrey *et al.* 1974). Though TLC is a laborious and time consuming technique it has been used for clinical pharmacokinetic studies. Mikkelsen and Andreasen (1977) have reported a quantitative fluorimetric TLC method which apparently has a higher specificity than the fluorimetric method without TLC, however, the sensitivity is lower.

In future it seems likely that the use of TLC even as a screening technique will be limited as more and more HPLC assays are developed for this purpose. These are considerably more reliable and quantitation is possible. Fullinfaw *et al.* (1987) have developed a HPLC method using UV detection at 271 nm, which is reliable for the detection of 12 of the 15 K^+-depleting diuretics available in Australia for up to 24 hours in urine.

Frusemide cannot be derivatized with pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA), or thionylchloride (Roberts *et al.* 1978). Using THAHS has been found to be satisfactory, but the technique is somewhat laborious, so that due to the need for derivatization of frusemide prior to GC we decided not to use this technique routinely for our pharmacokinetic studies.

The HPLC method described above using liquid extraction of frusemide was developed to allow the accurate and reproducible quantitation of frusemide in plasma and urine. This method was used in preference to previous methods for a number of reasons. Initially we used UV detection, but preliminary investigations showed that detection of frusemide in equine plasma and urine using HPLC with ultraviolet detection lacked the required sensitivity and specificity. Equine urine is renowned for the number of endogenous compounds present and, there was excessive interference from this endogenous material at 254 and 280 nm. However, the fluorescence properties associated with the ring structure make frusemide particularly amenable to this form of detection, permitting the development of a selective method of assay.

Another reason why certain of the previously used methods were not adopted was that they did not use an internal standard (Blair *et al.* 1975; Nation *et al.* 1979). A number of compounds were tested in the search for a suitable internal standard. Some of these have been used before, anthranilic acid, bumetanide and sulphanilamide were tried. All of these were unsuitable for one reason or another, for example the retention time of bumetanide was very long compared with frusemide making the time required for chromatography unacceptably long. The analogue N-benzyl-4-chloro-5-sulphamoylanthranilic acid fulfilled many of the criteria of an ideal internal standard; i.e. it is very similar in structure to frusemide thus its extraction from biological fluids is comparable, its retention time is not very different so the run time was not affected and there was still good resolution from the drug. Another benefit is that this method is relatively simple and thus suitable for detailed pharmacokinetic studies. We have eliminated some additional procedures found in some assays, for example a washing step (Fullinfaw *et al.* 1987) or a back extraction (Swezey *et al.* 1979).

The HPLC conditions were selected after preliminary investigations to improve the chromatography, for example mobile phase compositions were modified in order to get good peak separation and resolution. A number of organic solvents were tried as extracting solvents and the wavelengths (excitation and emission wavelengths) were changed slightly until optimum conditions were obtained.

Solid phase extraction is becoming increasing popular and is gaining widespread acceptance in analytical work. ToxElut and Extrelut in particular are widely used in doping analysis (Delbeke *et al.* 1988). Our primary aim in developing a solid phase extraction method was to make the sample preparation and chemical separation more efficient and cost-effective. The main practical advantage gained by using solid phase extraction over previous methods found in the literature is the time factor. Sample preparation time is very much faster than with liquid extraction. Using the VacElut system it is possible to prepare 24 samples simultaneously in approximately 45 min, whereas in the previous method it took this long just to evaporate off the ether phase. Thus this method facilitates the rapid analysis of hundreds of plasma and urine sample generated by pharmacokinetic and pharmacodynamic studies. Other benefits are that the method does not involve the handling of organic solvents, thus eliminating the hazard associated with this, it is also less expensive and requires limited technical expertise.

The calibration curves achieved by both methods were linear over the concentration range studied in plasma and urine as indicated by the high value of the correlation coefficient for the linear regression ($r > 0.990$). The replicability of the assays was acceptable over the entire concentration range investigated, and the data compares favourably with that reported earlier by other methods of analysis (Lindstrom and Molander, 1974; Blair *et al.* 1975; Carr *et al.* 1978; Nation *et al*. 1979). Recovery of frusemide and internal standard was high and comparable by the two methods. Similar data has been reported recently by Ameer and Mendoza (1990) who examined a solid phase extraction method for bumetanide, a loop diuretic analogous to frusemide.

Roberts *et al.* (1978) state that gas chromatography is the only method suitable for detection of frusemide in equine plasma and urine. They found that fluorimetric methods were totally incapacitated by the fluors present in equine urine. Forrey *et al.* (1974) also report that the main disadvantage in the use of fluorescence is the presence of extractable, spectrally interfering and quenching impurities. The presence of urinary fluorophores is a recognized limitation in solid phase extraction because more fluorophores elute from the solid phase column than would be extracted into an organic solvent. However, this obstacle can be overcome in a number of ways. Ameer and

Mendoza (1990) ameliorate the problem with the use of an acetonitrile column cleanup between injections. In our method the problem is reduced by having a number of sequential wash steps to remove as much matrix interference as possible, also the eluting solvent was the most dilute solution which could effectively elute frusemide and the internal standard. Additionally, the use of a protective guard cell increased the efficiency and life of the HPLC column.

Our results indicate that there is good correlation between the solid phase extraction method and the liquid extraction procedure. As such, both these methods were employed in pharmacokinetic studies throughout this thesis, the liquid extraction method for the initial studies (Chapters 4 and 5) and solid phase subsequently.

Chapter 3

THE PHARMACOKINETICS, METABOLISM AND DISPOSITION OF FRUSEMIDE IN THE HORSE

3.1. INTRODUCTION

Pharmacokinetics of Frusemide

Since the introduction of frusemide into clinical medicine by Kleinfelder in 1963, numerous investigations have been carried out with this agent. Due to its extensive therapeutic use, many of these studies have been on patients; however, Benet (1979) and Cutler and Blair (1979) reviewed most of the previously known pharmacokinetic data in normal subjects and revealed large variations in published results.

Calesnick *et al.* (1966) were the first to describe the pharmacokinetics of frusemide in man using [^{35}S]-frusemide. They reported that 51-94% of an intravenously administered dose was excreted in the urine and that much less appeared in the urine after oral administration. Only 2% of the label was found in the faeces so it is unlikely that the decreased elimination was due to a lack of absorption following oral absorption, but faeces were only collected for 24 hours. After intravenous administration, plasma frusemide concentration-time curves fitted a multi-compartment open kinetic model when a sensitive assay system, for example a radiometric method, was used (Beermann *et al.* 1975; Rupp 1974). Earlier studies using less sensitive assays found that data were adequately described by mono- and bi-exponential equations. It is likely that the slower terminal phase noted when radiometric assays are used may represent labelled metabolites with a slower elimination rate than that of frusemide itself.

Rupp and Hajdu (1970) described the pharmacokinetics following 40 mg of frusemide (i.v. and p.o.) to seven healthy male subjects. After i.v. dosing, the plasma level-time curves were described by three exponential segments, 5-30 min, 0.5-4 h, and 4-8 h. The mean half-lives for the segments were 16.6 min, 57.2 min and 4.48 h respectively.

There is wide variation in the terminal half-life of frusemide between reported studies, which could be due to both interindividual variation in the populations studied as well as differences in assay sensitivity and precision. However, the discrepancies

seem much greater than one would expect from study to study. Terminal half-lives of frusemide vary widely from 26 ± 10 to 105 ± 45 min. Several possibilities could account for these differences e.g. different assay methods, or different sampling times. As a rule, in studies where samples were collected for longer periods, longer half-lives were reported, a phenomenon observed with other drugs (Ishizaki *et al.* 1979). However, Chennavasin *et al.* (1981) evaluated the effect of a number of factors. They concluded that the discrepancies of the pharmacokinetics of frusemide may, in large part, be due to different methods of analysis of the data.

There are differences in other pharmacokinetic parameters of frusemide between the many studies, but the terminal elimination half-life appears to be subject to the greatest degree of variability. Renal clearance and the fraction of drug excreted unchanged (f_e) is also variable, which may possibly reflect the problems of accurately evaluating urinary metabolites of frusemide even when HPLC methods are used. In the studies reviewed by Benet (1979), f_e ranged from 0.34 to 0.92 and consequently nonrenal clearance values varied, being low when f_e is high.

AUC and plasma clearance values are more uniform, because the contribution of the final segment of the plasma concentration vs. time curve to total AUC is negligible for frusemide. Consequently plasma clearance calculated by different methods varies little.

Metabolism of Frusemide

Although frusemide is used in a range of clinical situations, data on its metabolism is rather controversial, and there is no clear consensus of opinion on its fate in man. During the 1970's a number of studies were carried out in different species on the metabolism and pharmacokinetics of frusemide but its metabolic disposition remains uncertain. Table 3.1. summarises the studies performed to determine the metabolism of frusemide in man (healthy volunteers and patients) as well as other species. Intact frusemide is the major urinary product, and a free amine metabolite and frusemide glucuronide have also been identified. Andreasen *et al.* (1978) also found anthranilic

Table 3.1. Summary of the metabolism of frusemide following studies in man and other species

Methods	Metabolites	Reference
Paper chromatography & spectrofluorimetry	2-Amino-4-chloro-5-sulphamoyl anthranilic acid (CSA)	Hajdu and Haussler, (1964)
Paper chromatography & spectrofluorimetry	2-Amino-4-chloro-5-sulphamoyl anthranilic acid (CSA)	Haussler and Wicha, (1965)
Paper chromatography, & radiometry	Only frusemide	Calesnick (1966)
Thin-layer chromatography, fluorimetry	Possible glucuronide	Kindt and Schmid, (1970)
Radiometric	Only frusemide	Kelly *et al.* (1974)
Radiometric & chromatography	2-Amino-4-chloro-5-sulphamoyl anthranilic acid (CSA)	Rupp (1974)

Table 3.1. continued.

Thin-layer chromatography, radiometry	Glucuronide, unidentified minor metabolite	Beermann (1975)
Fluorescence	2-Amino-4-chloro-5-sulphamoyl anthranilic acid (CSA)	Blair *et al.* (1975)
Thin-layer chromatography, & radiometry	Frusemide, 2-amino-4-chloro-5-sulphamoyl anthranilic acid (CSA), two unknown metabolites	Yakatan *et al.* (1976)
Thin-layer chromatography, β-glucuronidase	Glucuronide	Andreasen and Mikkelsen, (1977)
Thin-layer chromatography, fluorimetry	Anthranilic acid, 2-amino-4-chloro-5-sulphamoyl anthranilic acid (CSA)	Mikkelsen and Andreasen, (1977)
Thin-layer chromatography fluorimetry	2-Amino-4-chloro-5-sulphamoyl anthranilic acid (CSA),	Andreasen *et al.* (1978)

Table 3.1. continued.

HPLC β-glucuronidase	Glucuronide	Andreasen *et al.* (1978)
HPLC fluorescence detection	2-Amino-4-chloro-5- sulphamoyl anthranilic acid (CSA),	Carr *et al.* (1978)
Gas chromatography with acid extraction	2-Amino-4-chloro-5- sulphamoyl anthranilic acid (CSA), glucuronide	Perez *et al.* (1979)
HPLC fluorescence detection	Glucuronide	Smith *et al.* (1980a)

acid in the serum of healthy adults and patients with renal failure. The contradictions in the data may reflect problems with different assay procedures as reviewed by Benet (1979).

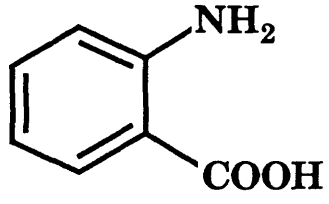
Hadju and Haussler (1964) and Haussler and Wicha (1965) reported that 2-amino-4-chloro-5-sulphamoylanthranilic acid (CSA) (Fig.3.1.) was the only metabolite of frusemide in man, dog and rat. Other investigators have also reported CSA as a metabolite of frusemide (Rupp 1974; Andreasen *et al.* 1978; Perez *et al.* 1979; Yakatan *et al.* 1976). Using radiochemical measurements and radio-chromatographic experiments, Rupp (1974) states that within the first 4 hours i.e. the period during which the diuretic effect is exerted, only unchanged frusemide is excreted. After this time, up to 24 hours, there is only one metabolite the free amine- CSA (see Discussion).

The presence of a glucuronic acid conjugate of frusemide has been reported, however, it is poorly quantitated in some studies and also found to vary with dosing history and renal function (Kindt and Schmid, 1970; Beermann *et al.* 1975; Andreasen and Mikkelsen, 1977, Andreasen *et al.* 1978; Perez *et al.* 1979; Smith *et al.* 1980a).

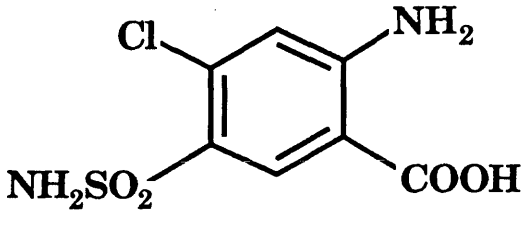
In their study on normal subjects and three groups of heart failure patients, Andreasen and Mikkelsen (1977) found that in patients not previously receiving frusemide, only 0.7-0.8 mg of the frusemide dose could be accounted for as the glucuronide. However, in patients who had been treated with frusemide chronically for at least the preceding 6 months, an average of 6.4 mg of frusemide was excreted as the glucuronide in the same 24 h period. The authors speculate that long-term use of the diuretic may be able to induce the glucuronidation process.

Smith *et al.* (1980a) imply that alkaline conditions cause degradation of frusemide glucuronide and can lead to erroneous results i.e. the extent of glucuronidation can be underestimated. This raises doubts over the results of Perez *et al.* (1979) who report that 3.3-40.4% of a frusemide dose in patients with pulmonary oedema is excreted as the glucuronide, as alkaline conditions are used in their analytical procedure. Smith *et al.* (1980a) found that glucuronidation accounted for approximately

Fig.3.1. Structures of possible metabolites of frusemide



Anthranilic acid



2-amino-4-chloro-5-sulphamoylanthranilic acid

(CSA)

14% of the available dose of frusemide whether given orally or by intravenous administration to healthy volunteers.

The enzymes responsible for frusemide metabolism in the liver are unknown. Herzberg *et al.* (1977) have illustrated that frusemide is one of many drugs which is capable of stimulating the activity of hepatic microsomal enzymes as measured by an increased urinary excretion of glucaric acid. Mitchell *et al.* (1974) have shown that large doses of frusemide produce massive hepatic necrosis in mice. The incidence and the severity of this decreases with prior treatment of mice with inhibitors of cytochrome P-450 drug oxidations (piperonyl butoxide, cobalt chloride or α-naphthylisothiocyanate) (Mitchell *et al.* 1976). In contrast, pretreatment with phenobarbitone, an inducer of several drug metabolizing enzymes in the liver increases the incidence and possibly the severity of necrosis. Mitchell *et al.* (1976) report that frusemide is metabolically activated to an arylating intermediate by a cytochrome P450 mixed function oxidase. It is believed that the furan ring of frusemide was the portion activated possibly by epoxidation and this combines covalently with hepatic macromolecules *in vitro* and *in vivo*.

Pharmacokinetics and Metabolism of Frusemide in the Horse

Following the approval of frusemide for the prophylaxis of epistaxis in horses there have been a few studies on its pharmacokinetics in this species. Before 1978 the diuretic effect of frusemide was not well characterized in the horse and the pharmacokinetics and disposition of this drug were essentially unknown. Tobin's group in Kentucky were the first to report in a series of papers, on the pharmacology of frusemide in the horse (Tobin *et al.* 1977; Roberts *et al.* 1976; Roberts *et al.* 1978; Shults *et al.* 1978).

Roberts *et al.* (1978) found that after frusemide (1 mg/kg, i.v.), plasma concentrations were well fitted by a 2-compartment open model, with an initial α phase half-life of about 5 min and an elimination phase half-life of 38 min. Frusemide was not detectable in plasma after 4 hours. Urinary concentrations of frusemide also fell rapidly from an initial value of about 25 μg/ml to less than 9 μg/ml at 45 min after dosing.

Roberts *et al.* (1978), Shults *et al.* (1978) and Tobin (1978) report that at least 60% of a dose of frusemide was excreted unchanged in equine urine, but they make no mention of the fate of the remaining 40% or mention any other metabolites.

Intramuscular administration of frusemide at the same dose level resulted in an enhanced diuretic affect (Roberts *et al.* 1978). Frusemide was rapidly absorbed, plasma levels peaked within 8 min, drug levels then declined monoexponentially with an apparent half-life of about 86 min. The relatively prolonged plasma half-life of frusemide after i.m. injection correlated well with the prolonged diuretic response seen after administration by this route.

Further work by Chay *et al.* (1983) investigating both a 1 mg/kg and a 0.5 mg/kg dose, used a modified GC method with improved sensitivity allowing the detection of frusemide for up to 8 h. Their findings were in agreement with those of Roberts *et al.* (1978). However, instead of a 2-compartment model to describe frusemide distribution, triexponential equations were found to fit the data better. The γ-phase represented less than 6% of the area under the plasma concentration-time curve, this is due to the very rapid decay in drug levels.

Todi and Fenwick (1983) studied the excretion of three diuretics in the horse. 200 mg frusemide was administered intravenously and intramuscularly. Using HPLC they were able to detect frusemide for up to 3-6 h in plasma and 20-24 h in urine. No metabolites were detected in urine or plasma.

Thus despite the extensive use of frusemide in the horse, its metabolism in this species has not been investigated in any detail. This study was designed to evaluate the diuretic effect of frusemide and to gain information on its metabolic disposition following administration of 1 mg/kg by three different routes, namely intravenously, intramuscularly and orally. There are no reports in the literature on the oral administration of frusemide in the horse.

3.2. METHODS

Experimental Protocol

In this study one horse received 3 treatments on successive dosing sessions, these were separated by 1 week.

Treatments

A. [^{14}C]-Frusemide 1 mg/kg (100 μCi) intravenously administered via the jugular vein.

B. [^{14}C]-Frusemide 1 mg/kg (100 μCi) intramuscularly.

C. [^{14}C]-Frusemide 1 mg/kg (100 μCi) orally.

Compounds

[7-^{14}C]-Frusemide specific activity 30.81 μCi/mg; radiochemical purity > 97% by TLC and HPLC (Hoechst, Frankfurt, F.R.G.). Frusemide (4-chloro-5-sulphamoyl N-furfurylanthranilic acid) (Lasix 5% solution; Hoechst, Milton Keynes, Bucks. U.K.).

Determining Radiochemical Purity

The radiolabelled frusemide was dissolved in methanol (2.5 ml) and a 20 μl injection (approx. 9000 dpm) made on the HPLC column. The HPLC system was that described in Chapter 2: briefly the conditions were a mobile phase of water: acetonitrile: acetic acid (67:28:5 by vol.). The column was a Lichrosorb RP C-18 (E. Merck, Darmstadt, F.R.G.), 125 x 4mm i.d. 5 μm particle size. The flow rate was 1.4 ml/min, and the excitation and emission wavelengths were set at 354 and 404 nm respectively.

Animal Information

One Thoroughbred male horse (body weight 465 kg) belonging to Horse Racing Forensic Laboratories, Newmarket, was used in this series of experiments.

Before each experiment, the horse was fasted overnight prior to dosing the following morning and for two hours after dosing. Water was available *ad libitum*.

Dose Preparation and Administration

[7-^{14}C]-Frusemide (16.8 mg; approx. 500 μCi) was dissolved in methanol (2.5 ml). For the intramuscular and intravenous administrations, 0.5 ml aliquots of this solution were then removed and added to a 10 ml Lasix injection vial (5% frusemide solution). The solution was well mixed and then withdrawn into a 10 ml syringe through a Millisort 0.22 μm filter. The excess dose was removed for dosimetry, leaving 9.75 ml, the volume required for a dose of 1 mg/kg. The i.m. dose was administered directly into the muscles in the neck, and the i.v. dose via the jugular vein over 30 sec. For the oral dose, 0.5 ml of [7-^{14}C]-frusemide was transferred to a 100 ml volumetric flask. 9.3 ml of the injection solution was added and the volume made up to 100 ml with water. After mixing 1 ml was removed for dosimetry and the remainder transferred to a plastic bottle for dosing. Water (600 ml) was used to flush the stomach tube following dosing.

Blood Collection

An indwelling cannula was inserted into a jugular vein prior to dosing.

(a) Whole blood samples (2 x 25 ml) were collected into tubes containing lithium heparin (Monovettes, Sarstedt) before drug administration and at various intervals during the course of the experiment. The blood was centrifuged at 2000 rpm for 5 min, and the plasma aspirated. Before storage of plasma at -20°C, duplicate 1 ml samples were counted for ^{14}C.

(b) Blood samples (2.5 ml) were also collected into EDTA tubes at each time point.

Urine Collection

Following cannulation, the horse was moved to a metabolism stall in which the movement of the horse is restricted, designed for the convenient collection of samples. Urine was collected using the method described by Marsh (1983). A harness is placed

over the horse's back holding in place a fabric reinforced latex funnel, adjusted so it surrounds the penis. The urine runs from the funnel, via flexible plastic tubing into collection bottles placed in a pit below the level of the animal. Urine samples were collected individually as far as possible, and during the night this was achieved by using a large fraction collector.

The time at which each urine sample was voided was noted and the volume measured. A small volume of each sample (ca. 120 ml) was taken for further analysis and the rest discarded. Each urine sample (15 ml) was centrifuged at 3500 rpm for 10 mins to obtain a clear supernatant, 0.1 ml aliquots were removed for scintillation counting. The excretion of radioactivity was followed and the collection of samples discontinued when the level of radioactivity in the urine had fallen to background (approx. 48 h). All urine samples were frozen and transported from Newmarket to London and stored at -20 °C prior to analysis.

Faeces Collection

Faeces were only collected following the oral administration of frusemide. In the first 24 hours pooled voidings were collected, i.e. a 0-12 h and a 12-24 h collection. Subsequently each individual faecal void was collected, this was generally in 3 h batches. After collection the samples (7-10 kg/12 h; 1.5-3 kg/3 h) were individually weighed and thoroughly mixed in an electric cement mixer without the addition of water. A sample of this crude homogenate (300 g) was frozen and transported to London for analysis.

The radioactivity in faeces was determined by weighing a small sample of faeces and adding a known volume of water. The mixture was then liquidised in a Moulinex food blender for 2 min. Aliquots of the homogenate (2 ml) were removed into a measuring cylinder and hydrogen peroxide (2 ml) and 5M sodium hydroxide (1 ml) added. To control the foaming caused by the hydrogen peroxide, iso-amyl alcohol (0.1 ml) was added. The measuring cylinders were covered in aluminium foil and left at room temperature until decolourized and the foaming had stopped (1 h). The solution was then neutralized with acetic acid (0.2 ml) and the volume made up to 10 ml

with the addition of ethanol (4.7 ml). After heating in a water bath at 60°C for 30 min, aliquots (3 x 500 µl) were taken into scintillation vials and Ecoscint scintillation fluid added (10 ml). The ^{14}C content was determined using the method below. All determinations were performed in triplicate.

Sample Analysis

Blood: Packed cell volume (PCV) was determined by the microhaematocrit method.

Urine: Immediately on collection the pH of the urine was measured and the specific gravity obtained using a refractometer. Urinary sodium levels were determined by flame photometry, and chloride levels were measured using a chloridometer. 0.1 ml aliquots of urine were used to determine creatinine concentration using the Jaffé method. These analyses were performed by the Animal Health Trust, Newmarket.

Treatment of Urine

In order to identify any possible metabolites of frusemide, urine was subject to different treatments.

β-Glucuronidase Incubation

Ester glucuronides were identified by adjusting urine (2 ml) to pH 5 with glacial acetic acid. 0.2M sodium acetate buffer pH 5 (1 ml) and bovine liver β-glucuronidase (1 ml Glucurase, 5000 units/ml, Sigma, Poole, Dorset, U.K.) were added, and after shaking, the mixture was incubated at 37°C for 20 h.

To ensure specificity of enzyme activity another incubation was prepared. This was identical to the above, with the exception that D-saccharic acid 1,4 lactone (0.8 mg, Sigma, Poole, Dorset, U.K.), a specific inhibitor of the enzyme, was added prior to incubation.

Alkaline Hydrolysis

Urine was adjusted to pH 9 with molar sodium hydroxide and left at room temperature for 12 h. The sample was adjusted to pH 7 with hydrochloric acid (1 M), before HPLC analysis.

Acid Hydrolysis

Urine was adjusted to pH 1 with hydrochloric acid (6M) in a screw capped vial. After mixing, the vial was sealed, and heated at 130°C, for 12 h. After cooling the mixture was neutralized before HPLC analysis.

Radiochemical Techniques

The ^{14}C content of plasma and urine was determined by scintillation spectrometry. Initial analysis was carried out at the Newmarket laboratories using a Betamatic ll liquid scintillation counter (Kontron Instruments, Watford, Herts, U.K.) with channels ratio quench correction, using Instagel scintillant (Canberra-Packard, Pangbourne, Berks, U.K.). Subsequent analysis was done using Ecoscint scintillation fluid (National Diagnostics, Reading, U.K.), with a Minaxi TriCarb 4000 Series, Model B4450 liquid scintillation spectrometer (Canberra-Packard, Pangbourne, Berks, U.K.). Quench correction was by the channels ratio method.

High Performance Liquid Chromatography

The HPLC system used was that described in Chapter 2 following solid phase sample preparation. Urine was centrifuged at 2500 rpm for 10 min, before injection of small volumes on to the HPLC cartridge.

Quantitation of frusemide and metabolites was achieved by collecting 0.3 ml fractions of eluent into scintillation vials, using a LKB RediRac fraction collector and counting for ^{14}C activity. The identity of peaks was assigned by comparison of retention times with those of authentic standards.

Pharmacokinetic Methods

Pharmacokinetic parameters were calculated using the Kinet Pharmacokinetic analysis package on an Apricot Xi 10 computer.

Peak plasma concentration, C_{max}, and the time to reach peak plasma concentration T_{max}, were obtained by inspection of graphical data. The terminal elimination rate constant β, was calculated from the lines of best fit which were obtained from least squares regression analysis of the terminal log-linear portion of the plasma concentration/time curves. The elimination half life, $T_{0.5\beta}$, is obtained from:

$$T_{0.5\beta} = \ln 2/\beta \qquad (1)$$

The area under the plasma concentration/time curve (AUC), was calculated by the trapezoidal method. The volume of distribution, V_d, was determined from equation (2):

$$V_d = \text{Dose}/\beta.\text{AUC} \qquad (2)$$

The apparent plasma clearance of radioactivity was calculated from equation (3):

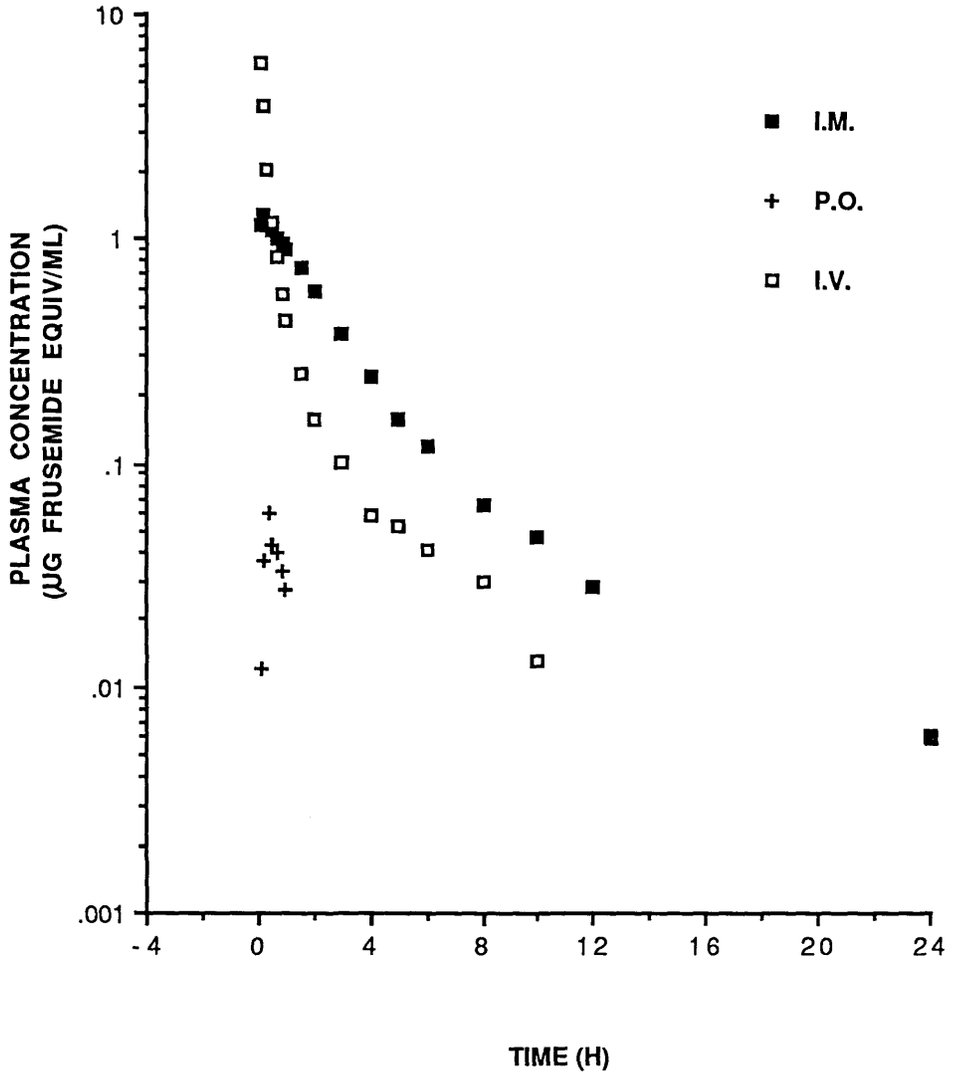
$$Cl = \text{Dose}/\text{AUC} \qquad (3)$$

3.3. RESULTS

Plasma Pharmacokinetics

The frusemide concentration data obtained from the HPLC analysis of plasma revealed that at all time points the major radioactive component (>90%) was unchanged frusemide, thus it is possible to equate plasma radioactivity directly with frusemide concentration. Plasma concentration-time curves for frusemide following intramuscular, intravenous and oral administration are presented in Fig.3.2.

Fig.3.2. Plasma concentration of frusemide in a horse given [14C]-frusemide (1 mg/kg) intravenously, intramuscularly and orally on three separate occasions



Intravenous Administration

Fig.3.2. shows that frusemide plasma concentrations decline biexponentially with time. The data were well fitted to a two compartment open model; a correlation coefficient of greater than 0.9 being achieved for both the distribution (α) and elimination (β) phases. Pharmacokinetic parameters describing the experimental data after i.v. administration of frusemide are given in Table 3.2. The peak plasma concentration (C_{max}) was 6.128 μg/ml, this declined very rapidly, the distribution phase ($t_{1/2\alpha}$) lasting 8 min. As the peak plasma concentration C_{max} was 6.128 μg/ml, the length of time for which plasma levels were 50% of this i.e. over 3.046 μg/ml was just 15.3 min. ^{14}C was not detectable in plasma by 10 h post-dosing, by this time the concentration had fallen to 0.013 μg equivs/ml. The shorter elimination half-life for frusemide of 1.6 h after i.v. injection compared to i.m. administration (2.8 h) was due to both a smaller volume of distribution (V_d) and a higher plasma clearance (Cl) ($t_{1/2\beta} = \ln 2\ V_d/\ Cl$).

Intramuscular Administration

The semi-log transformation shows that following intramuscular administration the plasma concentrations of frusemide declined biphasically with time after absorption was complete (Fig.3.2.). The experimental data were well fitted to a two compartment open model; the correlation coefficient for the distribution and elimination phase being greater than 0.990. The pharmacokinetic parameters describing this, corrected as appropriate for the weight of the horse, are listed in Table 3.3.

Frusemide was very rapidly absorbed, the peak plasma concentration (C_{max}) being 1.288 μg/ml, achieved within 0.17 h as indicated by the time to achieve maximum concentration (T_{max}).The length of time for which plasma levels were 50% of maximum i.e. above 0.644 μg/ml, was 2.1 h, this gives an indication of the duration of action of frusemide following intramuscular injection. ^{14}C activity was detectable in plasma up to 24 h post-dosing and resulted in a plasma elimination half-life of 2.8 h. The distribution phase, the volume of distribution and the AUC were all

Table 3.2. Pharmacokinetic parameters describing the plasma level-time curve of frusemide following the intravenous administration of [^{14}C]-frusemide (1 mg/kg) to one horse

Body Wt.	(kg)	465
C_{max}	(μg/ml)	6.128
t_{max}	(h)	0.080
α	(h^{-1})	5.141
$t_{1/2\alpha}$	(h)	0.135
β	(h^{-1})	0.431
$t_{1/2\beta}$	(h)	1.608
V_d	(l/kg)	0.803
Cl_p	(l/h/kg)	0.346
$AUC_{(trap)}$	(μg/ml.h)	2.890

Table 3.3. Pharmacokinetic parameters describing the plasma level-time curve of frusemide following the intramuscular administration of [^{14}C]-frusemide (1 mg/kg) to one horse

Body Wt.	(kg)	465
C_{max}	(μg/ml)	1.288
t_{max}	(h)	0.170
K_{abs}	(h^{-1})	3.926
α	(h^{-1})	0.706
$t_{1/2\alpha}$	(h)	0.978
β	(h^{-1})	0.244
$t_{1/2\beta}$	(h)	2.842
V_d	(l/kg)	1.202
Cl_p	(l/h/kg)	0.293
$AUC_{(trap)}$	(μg/ml.h)	3.410

greater following i.m. administration then after i.v. administration, however the total plasma clearance was reduced (0.293 vs. 0.346 l/h/kg). The systemic bioavailability (F) of frusemide after i.m. injection compared to that achieved following i.v. administration (absolute bioavailability) was determined by urinary excretion ratios and found to be 86%.

Oral Administration

Following oral administration, frusemide appears to be very poorly absorbed from the gastrointestinal tract. The peak plasma concentration (C_{max}) was only 60 ng/ml and this was achieved 21 min after administration. Frusemide was not detectable in plasma for more than 1 h after dosing (Fig.3.2.). The systemic bioavailability (F) of frusemide after oral administration compared to i.v. administration (absolute bioavailability) was determined from urinary excretion ratios and found to be 4.4%.

Diuretic Effect

Frusemide administration produced a rapid and short lasting diuretic effect after intravenous administration. Intramuscular injection also produced a marked diuretic effect, however, oral dosing of frusemide resulted in a very slight but nevertheless discernable diuresis. Fig.3.3a. shows the cumulative urine volume produced following frusemide administration by three different routes. The graph highlights the speed of onset and the intensity of the diuretic effect with about 10 litres of urine being voided after i.v. administration and about 13 litres after i.m. dosing. The transient nature of the diuresis is also evident.

The more prolonged action of frusemide after i.m. dosing is also evident from the sodium excretion data. Fig.3.3b. shows the cumulative excretion of sodium at certain time intervals up to 6 h after both i.v. and i.m. administration. It can be seen that i.v. frusemide has an immediate but short lasting effect, sodium excretion declined exponentially. However, after i.m. administration the peak natriuretic effect is only about 50 % of that after i.v. injection but this increased natriuresis lasts for

Fig.3.3a. Cumulative urine volumes produced in a horse following frusemide (1mg/kg) administration by different routes.

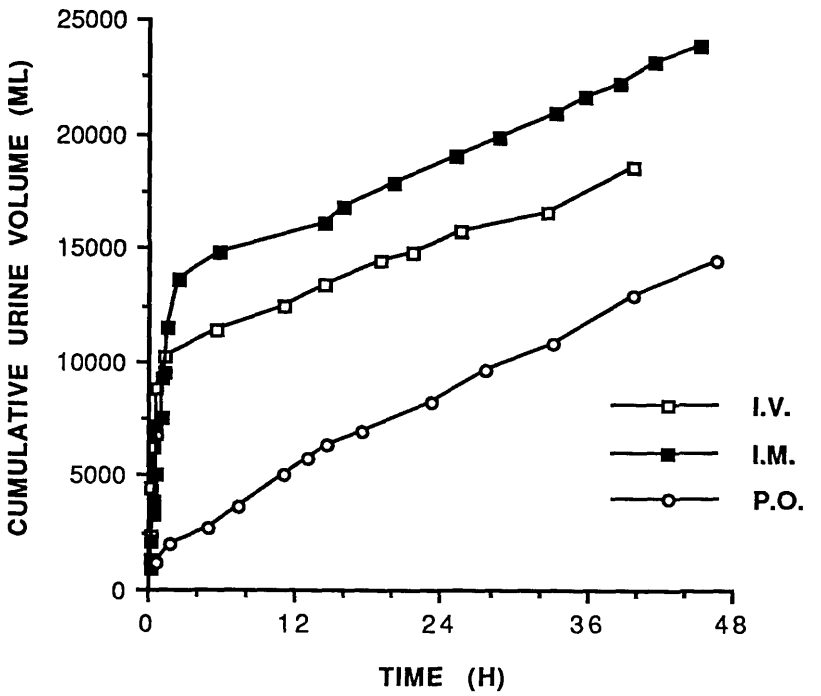
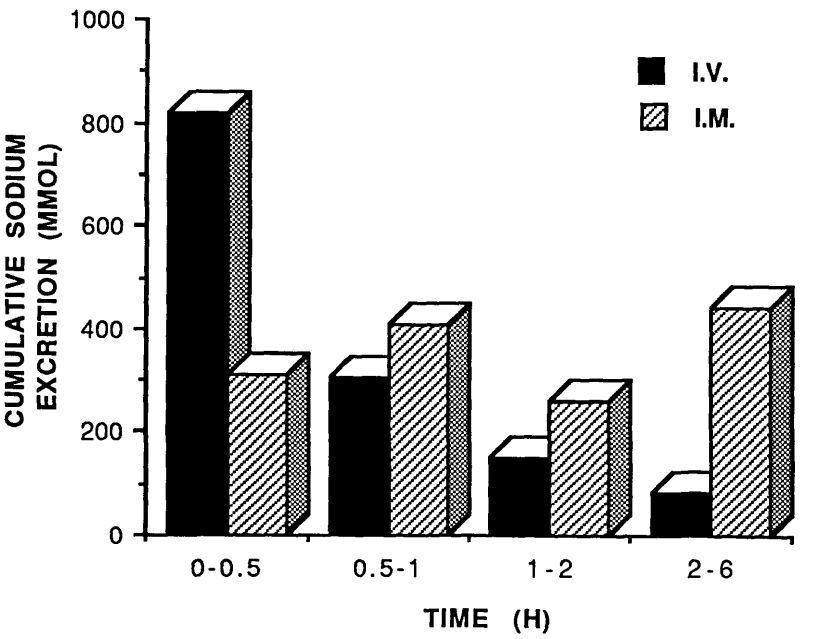


Fig.3.3b. Sodium excretion in pooled urine samples following the intravenous and intramuscular administration of [14C]-frusemide (1 mg/kg) to one horse on separate occasions



considerably longer, resulting in a slightly greater overall effect (1417 vs. 1359 mmol in 6 h).

Urine flow rates increased considerably during the period of diuresis. Within the first hour flow rates of about 300 ml/min were obtained after i.v. administration. Similar flow rates were sustained for longer (2.4 h) after i.m. administration resulting in a greater number of voidings; eleven within 2 h compared with six after i.v. dosing.

The increase in urine volume after each of the three treatments was mirrored by a rapid fall in specific gravity, Fig.3.4. shows that after i.m. injection specific gravity fell from 1.028 to 1.003 in 17 min. after which values gradually returned to normal. A urine sample voided at 5.9 h had a low specific gravity, but that of the subsequent sample (14.5 h) was similar to control levels. Due to the 8.6 h interval between these two urine samples it is difficult to say precisely when values return to normal, however it is about 10 - 12 h after treatment. Similar data were obtained after i.v. dosing. The specific gravity data after oral dosing provides evidence of the small diuretic effect, as two urine samples were voided in 1.7 h and the specific gravity of both samples was lower than normal (1.009 and 1.011); subsequent samples had higher values.

The pH of individual urine samples ranged from 7.0 - 8.5 during the course of the experiment. The more alkaline values were generally obtained for the urine samples which were voided during the night and as such remained in the fraction collector for a number of hours prior to being processed and the pH determined.

Urinary Excretion of ^{14}C

From the plot showing the urinary concentration of frusemide following i.v. and i.m. administration (Fig.3.5.) it can be seen that after i.v. injection frusemide is very rapidly eliminated in the urine. Drug concentration in the first urine sample voided 7 min after dosing was 53.5 μg/ml. 57.3% of the dose was excreted within 30 min. In contrast, after i.m. administration, urinary elimination of frusemide was much slower initially, frusemide concentrations in urine samples voided within the first 2.5 h were below 25 μg/ml and accounted for 52% of the dose. However from 5 h post-dosing urinary frusemide concentrations were always higher after i.m. than i.v. administration.

Fig.3.4. Specific gravity of urine in one horse following frusemide (1 mg/kg) administration by three different routes on separate occasions

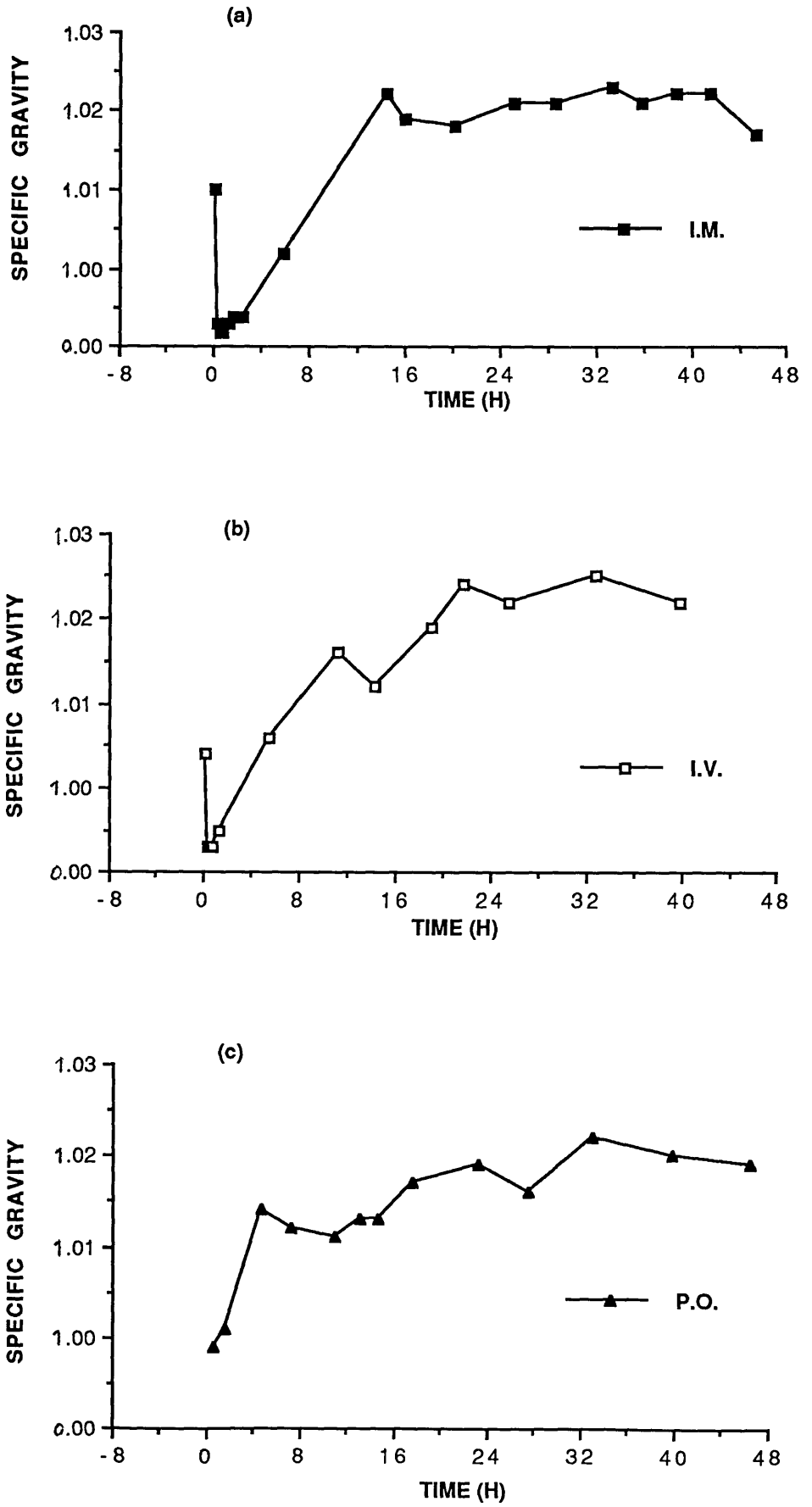
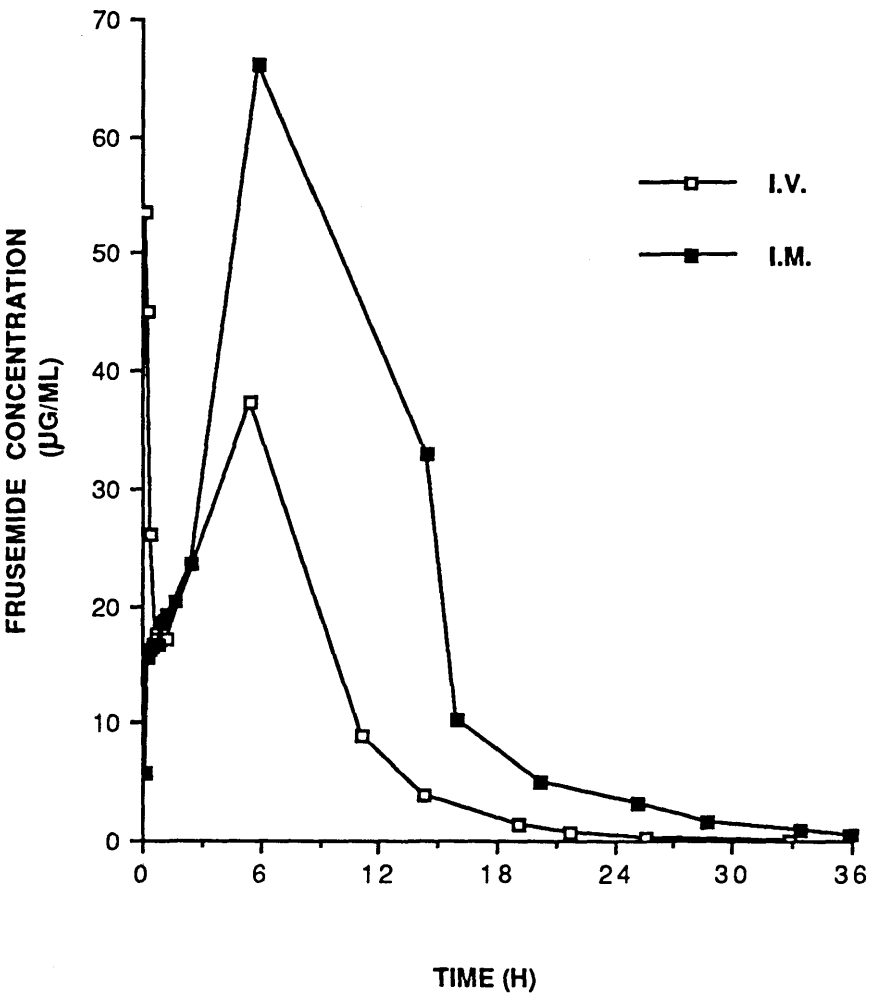


Fig.3.5. Urinary concentration of frusemide in one horse given [14C]-frusemide (1 mg/kg) intravenously and intramuscularly on separate occasions



Urine samples were collected for up to 48 hours after the administration of [^{14}C]-frusemide by the 3 different routes of administration. By this time the radioactivity levels had returned to background, the results obtained following each administration are presented in Fig.3.6. The urinary recovery of ^{14}C following the oral dosing of [^{14}C]-frusemide was very small, however urinary elimination was essentially complete within this time, and accounted for 78.9% (i.m.), 4.0% (p.o.) and 91.9% (i.v.), of the dose. The graph shows the very rapid urinary excretion of ^{14}C activity within 6 h, over 65% of the dose had been excreted following i.m. frusemide and almost 90% after i.v. injection. Fig.3.7. illustrates the urinary excretion of frusemide. From the terminal portion of the curve, it can be seen that after i.v. administration frusemide is eliminated more quickly with a urinary elimination half-life of 2.8 h, following i.m. administration the elimination half-life is 4.1 h.

Radiochromatographic analysis revealed the presence of two compounds which were not present in drug-free urine. A typical HPLC radiochromatogram is illustrated in Fig.3.8. By comparison of retention times with an authentic standard the major component was identified as unchanged frusemide. 62.2% (after i.m. administration) and 69.1% (after i.v. administration) of the dose was excreted as parent drug. The metabolite accounted for 13.2% (Fig.3.9a) and 17.4% respectively (Fig.3.9b.). The metabolite was identified as the free amine, 2-amino-4-chloro-5-sulphamoylanthranilic acid (CSA), as treatment of the urine with β-glucuronidase and alkaline hydrolysis did not alter the metabolic profile, however acid hydrolysis resulted in frusemide being converted to this metabolite. It is known that in acidic conditions frusemide is N-dealkylated to CSA.

Faecal Excretion of ^{14}C

Due to the very low urinary recovery of ^{14}C activity after the oral dose of [^{14}C]-frusemide, the faeces were collected after this treatment in order to obtain a quantitative recovery of radioactivity. Faecal elimination of ^{14}C was detectable up to 33 h post dosing and accounted for 82.6% of the dose (Fig.3.10.).

Fig.3.6. Cumulative urinary excretion of 14C-radioactivity in one horse, following [14C]-frusemide (1 mg/kg) by three different routes of administration on separate occasions

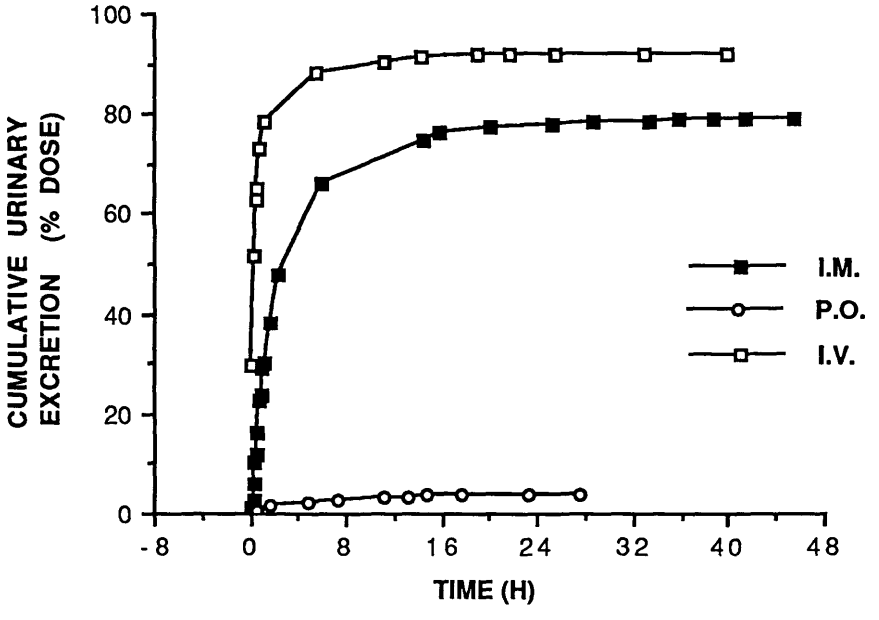


Fig.3.7. The urinary excretion rate of frusemide in one horse given [14C]-frusemide (1 mg/kg) intravenously and intramuscularly, expressed as percentage of dose excreted per hour

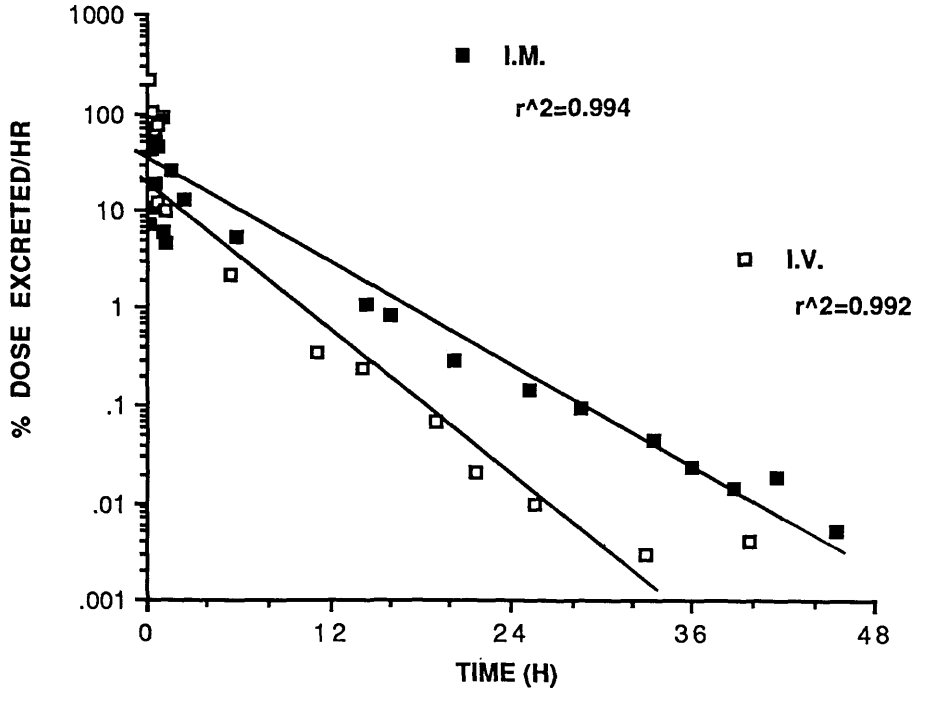


Fig.3.8. Radiochromatogram of urine from one horse following [14C]-frusemide (1 mg/kg; i.v.) showing the presence of parent drug and a single metabolite

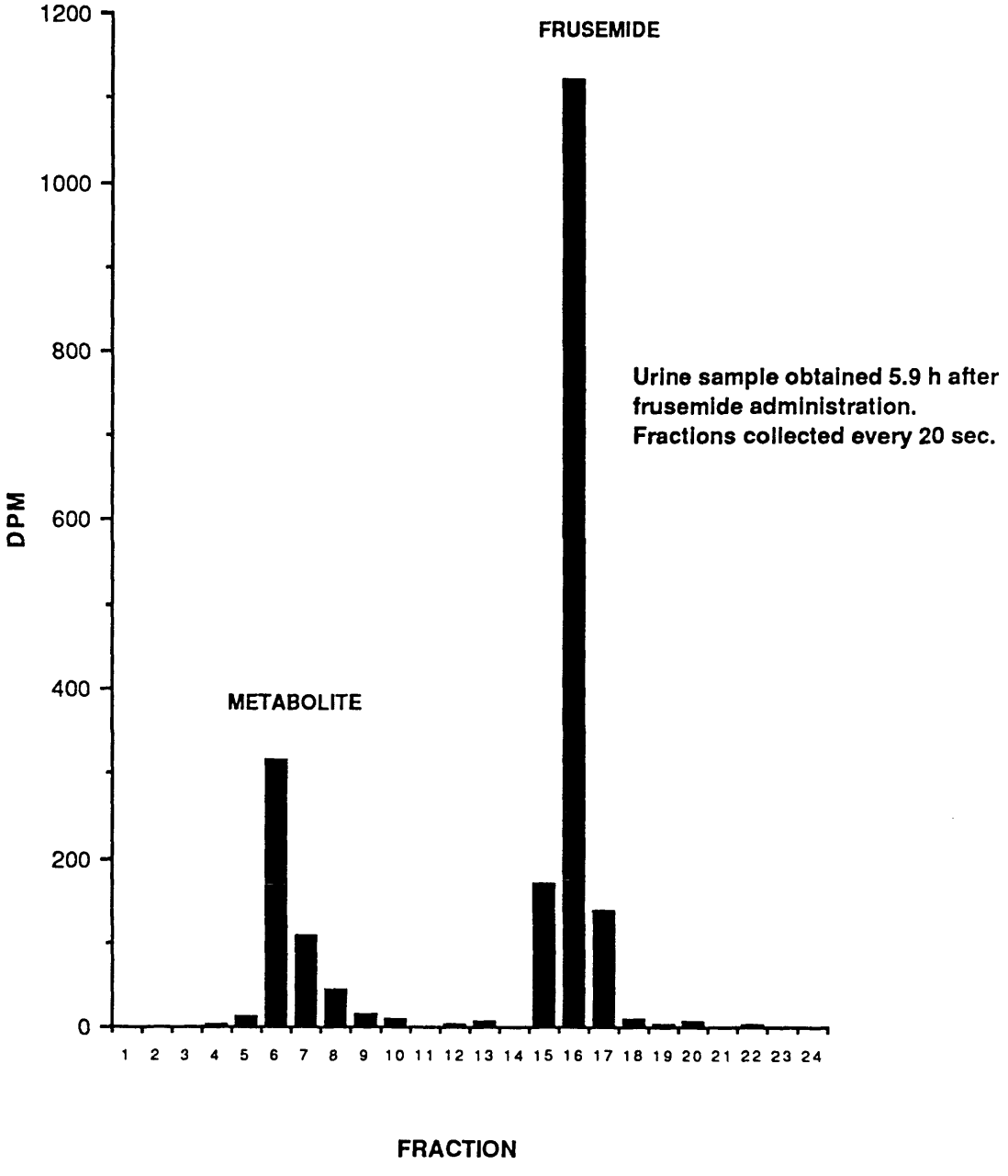
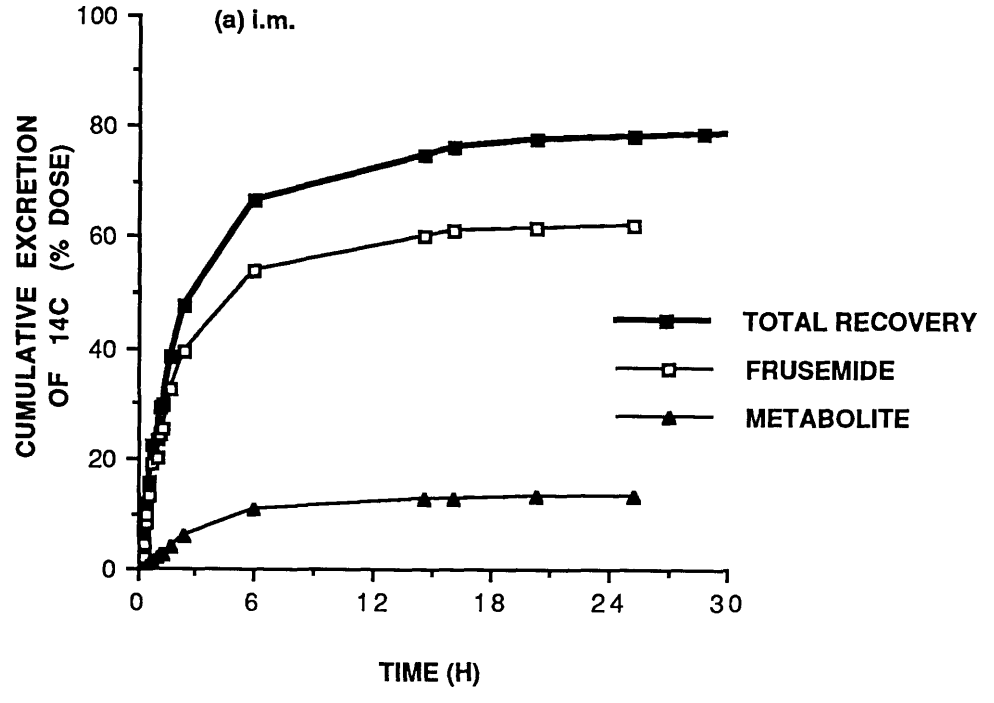


Fig.3.9. Cumulative urinary excretion of 14C-radioactivity in one horse given [14C]-frusemide (1 mg/kg), (a) intramuscularly and (b) intravenously on separate occasions



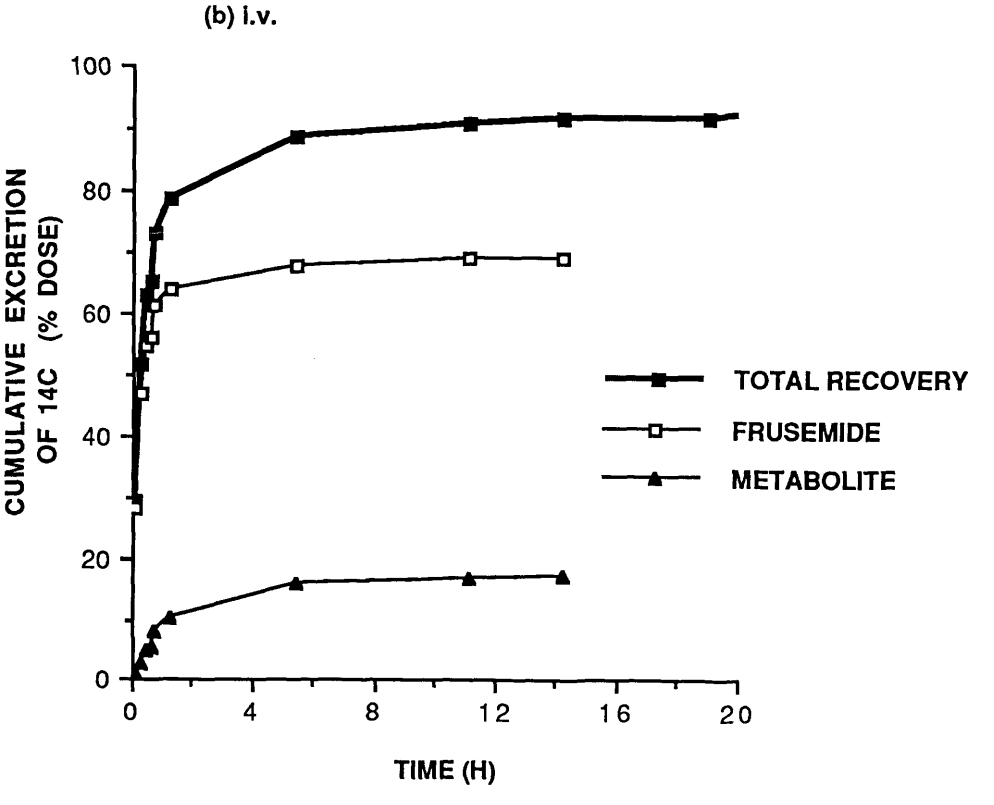
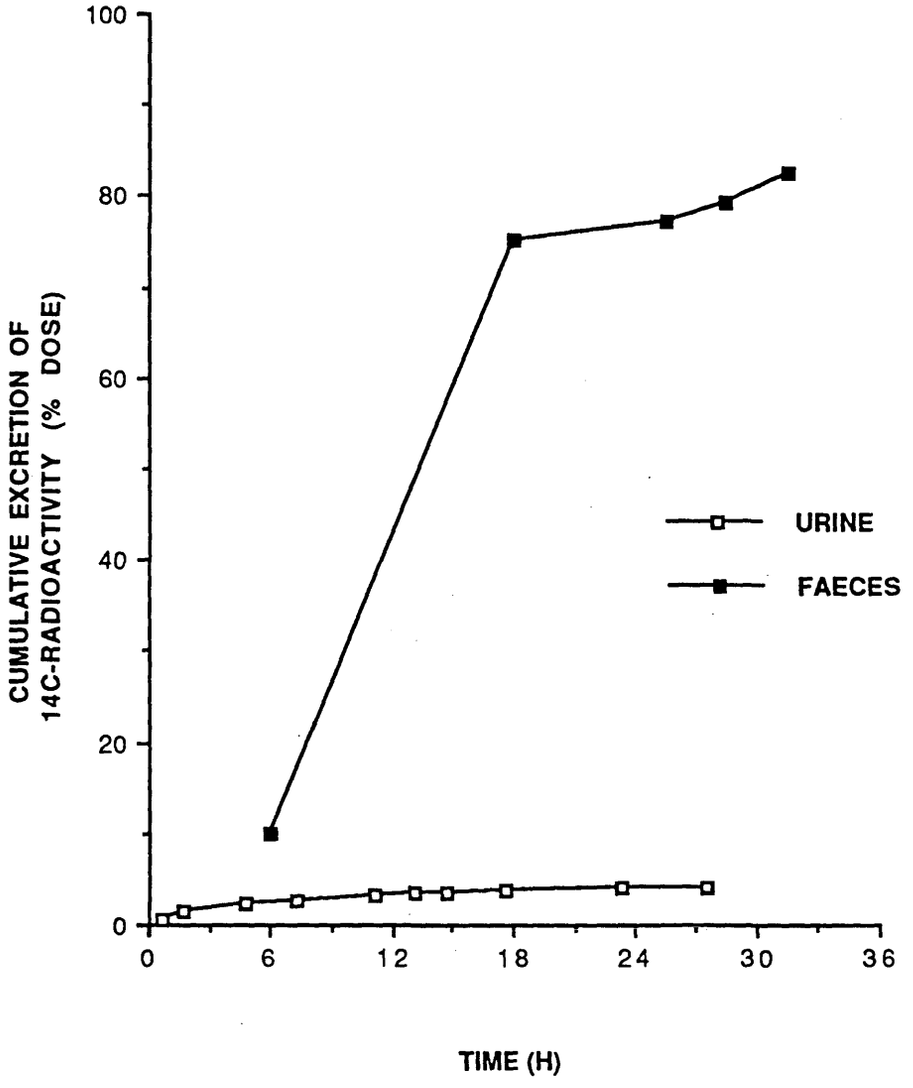


Fig.3.10. Cumulative urinary and faecal excretion of 14C-radioactivity by one horse given [14C]-frusemide (1mg/kg) orally



3.4. DISCUSSION

Administration of frusemide (1 mg/kg) by different routes either intravenously, intramuscularly or orally has marked effects on the pharmacokinetics and pharmacodynamics of the diuretic. A dose level of 1 mg/kg was chosen as this is the recommended therapeutic dose in man and other species. However, in America the lower equine dose of 0.5 mg/kg is most commonly used in jurisdictions where frusemide is permitted medication for the prophylaxis of epistaxis.

As there was only a single administration of frusemide by each route to one horse no account can be made about the statistical significance of these results. However, the disposition kinetics of frusemide following i.v. and i.m. administration was in fairly good agreement with previous reports on the horse.

The very fast distribution phase (8 min) following i.v. injection in the present study is similar to that reported by Roberts *et al.* (1978) and Chay *et al.* (1983). The elimination half-life of 1.6 h in our study was considerably longer than that reported by Roberts *et al.* (1978). As already mentioned (see Introduction above) the elimination half-life has been found to be the parameter with the greatest degree of variability. The discrepancy could be because we were able to detect frusemide in plasma for up to 10 h while in Roberts' study frusemide was not detectable in plasma after 4 h. It is generally accepted that the ability to detect drugs in plasma for longer will increase their "clearance" time. Thus it is likely that further enhancement of analytical methodology might demonstrate an even slower terminal plasma half-life for frusemide. However, Chennavasin *et al.* (1981) stress the importance of relating pharmacokinetic parameters to the pharmacodynamics of a drug, i.e. relate measured concentrations to response and calculate parameters based on biological relevance.

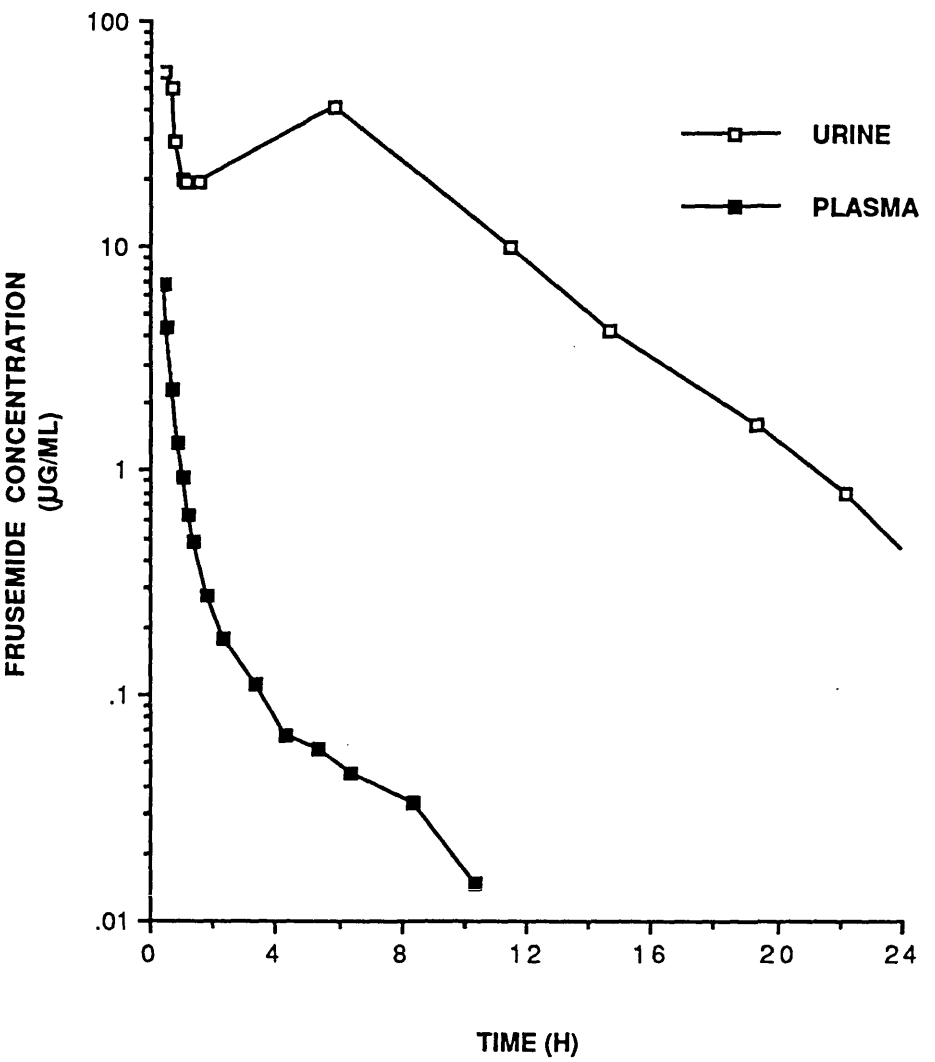
Chay *et al.* (1983) used triexponential equations to fit the data. From their results and Benet's (1979) analysis of the frusemide data of Rupp and Hajdu (1970) it has been shown that the fraction of the AUC, or the fraction of the clearance relating to the terminal exponential phase (γ-phase) was less than 10% and 20% respectively. This indicates that the terminal phase has little influence on the actual plasma concentration-

time curve, this is presumably due to the very rapid decay of drug levels. Thus most investigators recently have used a 2-compartment open model to fit the plasma concentration curve during the early part of the study, i.e. they lend less emphasis to those data points representing concentrations of drug having no pharmacological effect. Clinically this gives the "best" half-life as it provides the most meaningful information about the duration of the biological effect.

Our results show that frusemide plasma levels were above 3.0 μg/ml i.e. 50% of the plasma maximum (C_{max}) for just 15.3 min. Due to the distribution phase being very rapid, frusemide concentrations in plasma during this phase correlated with the period of spontaneous voiding elicited by the diuretic. This illustrates a correlation between pharmacokinetic data and pharmacodynamic findings similar to that reported by others (Rupp and Hajdu, 1970; Rupp 1974). This is of practical value in the clinical setting as it shows that the diuresis can be predicted from plasma concentrations. Control values for urine flow rate in the horse of up to 10 ml/min were reached approximately 1 h after dosing.

Urinary concentrations of frusemide after i.v. dosing also fell rapidly from an initial value of approximately 50 μg/ml to less than 20 μg/ml, 1 h after dosing. By comparing drug concentrations in plasma and urine (Fig.3.11.) it can be seen that urinary concentrations of frusemide are much higher than total plasma levels of the drug. This is likely due to the excretion of frusemide by the anion transport pathway. The involvement of this pathway in the excretion of frusemide was highlighted by Hirsch *et al.* (1975) who showed that renal tissue is capable of accumulating frusemide from an incubation medium. From repeated dosing (Tobin *et al.* 1978) it appears that the diuretic response to a single i.v. dose of frusemide at 1 mg/kg is limited primarily by rapid elimination of the drug and not due to limited fluid being available for excretion, as a second dose soon after the first immediately elicited a marked diuresis. The alkaline nature of equine urine also favours the rapid excretion of the acidic frusemide. In this study urinary concentrations initially were approximately 10 times that detected in plasma. However at later times i.e. after 6 h urinary concentrations were very much greater (1000 times) than plasma levels. Thus as frusemide is rapidly

Fig.3.11. Comparison of frusemide concentrations in plasma and urine following the intravenous administration of [14C]-frusemide (1 mg/kg) to one horse



pumped into urine mainly as unchanged drug it is quickly eliminated from the blood. This agrees well with the data reported by Cohen *et al.* (1976) who showed that renal tissue in the dog accumulates frusemide *in vivo* to concentrations about 10 times those observed in plasma. Roberts *et al.* (1978) working with the horse state that urinary concentrations of frusemide may be as much as 1000 times greater than total plasma levels of the drug.

69.1% of the intravenous frusemide dose was excreted unchanged a value similar to that reported by others in the horse (Roberts *et al.* 1978; Shults *et al.* 1978; Tobin 1978). In the present study, in addition to unchanged frusemide, 17.4% of the dose was excreted as 2-amino-4-chloro-5-sulphamoylanthranilic acid (CSA). This metabolite has been reported in a number of species (Hajdu and Haussler, 1964), however, there has been considerable debate concerning its existence.

Rupp (1974) suggests that the presence in the literature of metabolites other than CSA are artifactual, since moisture and heat can cause the degradation of frusemide. For instance, if urine extracts are applied to a TLC plate and dried with a fan, several artifacts are produced which may be wrongly identified as metabolites.

Yakatan *et al.* (1976) administered [^{35}S]-frusemide to dogs and monkeys. In both these species the label excreted in the urine was approximately 80% frusemide, 7% CSA, and 7% was attributable to two different unknown metabolites. Following oral and intravenous administration of [^{35}S]-frusemide to healthy subjects, Beermann *et al.* (1975) found unchanged frusemide, frusemide conjugated with glucuronic acid, and a polar metabolite which was not identified. A few studies have found no evidence of the amine CSA (Calesnick *et al.* 1966; Kindt and Schmid, 1970, Beermann *et al.* 1975).

In our study, preparation of samples for HPLC involved the addition of acid (HCl 6M). It has been suggested (Benet 1979) that acidification and extraction steps lead to formation of CSA during assay procedures. An *in vitro* study by Cruz *et al.* (1979) demonstrated the acid-labile hydrolysis of frusemide to CSA, and Smith *et al.* (1980a) indicate that the presence of CSA is an artifact due to errors inherent in analytical procedures. However, this metabolite was detected even when untreated

urine was injected on to the HPLC system. Thus CSA was a true metabolite and not just an artifact resulting from experimental procedures. This is substantiated by Carr *et al.* (1978) who used ether extraction of frusemide from acidified samples, however, their chromatograms do not indicate the formation of CSA. Similarly Blair *et al.* (1975) acidify their samples before analysis but this did not lead to the formation of CSA.

Frusemide was very rapidly absorbed following intramuscular administration. The peak plasma concentration (C_{max}) was 1288 ng/ml i.e. about a fifth of that after i.v. injection, but higher than the 800 ng/ml reported by Roberts *et al.* (1978) who administered the same i.m. dose. However, plasma levels declined more slowly, our data were best described by a 2-compartment open model. Roberts *et al.* (1978) found that frusemide drug levels declined monoexponentially in the horse after i.m. dosing. However our data gives a poor correlation coefficient of 0.740 if monoexponential decay is assumed. The considerably longer time (2.1 h vs. 15.3 min) that the plasma levels are maintained after i.m. administration than i.v. administration results in an enhanced diuretic effect. The prolonged plasma half-life of frusemide (2.8 h) correlated well with the diuretic response.

The volume of water eliminated after a 1 mg/kg i.v. dose (ca. 10 l) or i.m. dose (13 l) is approximately 3-4% of the total body water in a horse (Tobin 1981). If access to water is restricted, this deficit is probably distributed throughout total body water. In this experiment the horse had access to water two hours after dosing so this deficit is readily replaced and results in rapid reversal of any haemoconcentration.

Following i.m. injection of frusemide urinary concentrations rose during the first 6 h after dosing and then declined exponentially over the next 40 h, with a urinary half-life of about 4.1 h. Tobin (1981) reported a urinary half-life of 5.3 h for frusemide in the horse after i.m. administration. As with i.v. injection frusemide remains detectable in urine long after it is no longer detectable in plasma. These results are similar to those reported by Roberts *et al.* (1978). A similar proportion of the frusemide dose was excreted as unchanged drug and CSA after i.m. injection as after i.v. administration.

In this study there was no evidence for a glucuronide of frusemide after any of the different routes of administration. Throughout this study urinary pH was slightly alkaline, which may have resulted in the glucuronide not being detected as alkaline urine has been found to degrade this conjugate (Smith *et al.* 1980a). Data on the metabolism of frusemide in the horse is limited, most of the studies describing frusemide disposition have been performed in man and the dog (Benet 1979; Verbeeck *et al.* 1982).

The elimination of frusemide is predominantly by renal clearance of unchanged drug due to renal tubular secretion and partly by nonrenal routes. In the past it has been assumed that nonrenal clearance results as a consequence of metabolic degradation in the liver. However, a number of studies have shown that neither renal clearance (Cl_r) nor nonrenal clearance (Cl_{nr}) are affected in patients with hepatic disease (Fuller *et al.* 1981; Sawhney *et al.* 1981,;Verbeeck *et al.* 1982). In another study in dogs, it was demonstrated that these parameters were not altered in functionally hepatectomized dogs i.e. when the entire liver was removed, also the urinary recovery of frusemide glucuronide was unchanged (Verbeeck *et al.* 1981). This has led to suggestions that glucuronidation may occur in extrahepatic sites, possibly the kidney, and that the liver does not play a major role in the nonrenal component of elimination (Branch 1983).

As already mentioned there is a lack of information on the disposition of frusemide after oral administration in the horse. This is probably because the oral route is not recommended therapeutically in this species.

After p.o. administration, the bulk of the frusemide dose was eliminated in the faeces. A large proportion (65%) was excreted relatively quickly in the 12-24 h faecal sample. However, the recovery of frusemide (9.9%) in faeces within 0-12 h was unexpected as previous studies investigating gastrointestinal transit time in the horse have indicated a lag-time of up to 20 h before material is detected in the faeces (Vander Noot *et al.* 1967; Smith 1987). The faecal elimination rate was rapid and frusemide was not detectable 33 h after dosing. The horse had been fasted overnight prior to dosing, this may possibly have reduced gut transit time. Vander Noot *et al.* (1967) using chromic oxide as an indicator found that 4 days were needed for the total collection

period for a digestion trial with horses and suggested 5 days be used to reduce the influence of individual animal differences. This rapid faecal excretion of frusemide after p.o. administration, is very likely a consequence of incomplete absorption. This is substantiated by the fact that after i.v. injection urinary elimination accounts for over 90% of the dose, thus the faecal route is very minor.

In this study faeces were only collected after the oral administration, so a comparison of the amount of drug excreted in faeces by other routes cannot be made. In man, Rupp (1974) estimated biliary excretion of approximately 12% as he found that after i.v. administration 12 ± 3% of the dose was recovered in the faeces compared with 46 ± 16% after p.o. dosing. Similarly Beermann *et al.* (1975) found faecal recovery of 7-9% in man after an i.v. dose of frusemide. However, Beermann *et al.* (1975) found that drug concentrations in bile were insufficient to explain the faecal recovery after i.v. treatment. They suggest that there may be other routes of excretion such as intestinal secretion. Therefore nonrenal clearance of frusemide may involve active anion transport into the gut; transport mechanisms in the renal tubule may have parallels in intestinal mucosa (Branch 1983).

The very low bioavailability (4.4%) found in the horse was not unexpected in that frusemide shows low and very variable oral bioavailability in other species. In man the extent of frusemide absorption is usually between 40 and 60% (Kelly *et al.* 1974; Waller *et al.* 1982; Rupp 1974; Beermann *et al.* 1975), but absorption less than 40% (Smith *et al.* 1980a) and even as low as 10% has been found (Brater *et al.* 1982). The reason for this incomplete and variable bioavailability has not been fully evaluated.

As frusemide was given orally on just one occasion it is impossible to say whether the poor absorption and consequent weak diuretic effect is peculiar to this horse, a result of the route of administration, or whether some other factor is responsible. Potential factors which may contribute to the observed incomplete bioavailability of frusemide include (a) the horse being in a fasted state prior to dosing, (b) dose formulation and precipitation of frusemide from its solution, (c) chemical degradation of frusemide, (d) presystemic first-pass metabolism and (e) site specificity

in absorption. Each of these factors will be discussed briefly. However, all the data available are obtained from other species, mainly man and the rat.

In our study the horse had been fasted overnight prior to dosing even though it has been shown that food does not affect the extent of absorption of frusemide (Kelly *et al.* 1974). Though drug concentrations were very low in plasma, peak levels were reached within 21 min. This agrees with data in man showing that absorption in the fasting state is rapid with peak plasma levels occurring within an hour (Kelly *et al.* 1974) thus it is unlikely that this is responsible for poor absorption in the horse.

It is well recognised that formulation factors can affect the *in vivo* performance of a dosage form. Frusemide like many other drugs when improperly formulated may be poorly absorbed, this may be critical for this diuretic because of its potential site specificity in absorption (see below). In this experiment we decided to dose orally with the same Lasix Solution that was used for i.v. and i.m. injection. This was used in preference to Lasix Oral Solution which contains sorbitol and glycerol. These constituents delay frusemide absorption (Waller *et al.* 1985) and would also have added an additional variable into the present study. Although Lasix Solution has been used for oral dosing by other groups (Waller *et al.* 1985, 1988) and good bioavailability was obtained it is likely that this could be the main contender for the poor results we obtained. Soon after dosing the horse was very fidgety and was continuously pounding the floor. It is possible that precipitation of frusemide from its solution in the GI tract probably the stomach might occur as it is a poorly water soluble compound. Waller *et al.* (1982) have shown that frusemide is poorly soluble in water but rapidly absorbed from tablet form. The acidic nature of frusemide may have caused some gastric irritation which resulted in the horse's restless behaviour.

Another explanation for the very low bioavailability could be hydrolysis of frusemide to CSA in the stomach prior to absorption. There have been a number of *in vitro* studies on the hydrolysis of frusemide in gastrointestinal (GI) juices (Cruz *et al.* 1979; Beermann *et al.* 1975). A recent study by Andreasen *et al.* (1982) showed that at the same pH, frusemide is much more soluble (40-50%) in gastric fluids than in buffer solution. These results indicate that the contribution of chemical decomposition of

frusemide in GI fluids is negligible and cannot explain the observed low absorption of the drug found after oral intake.

As peak plasma levels after oral dosing occur very rapidly it has been suggested by Chungi *et al.* (1979) that limitation of favorable absorption site may be the reason for incomplete absorption of frusemide. Lee and Chiou (1983) showed that in the rat the stomach was the organ with the fastest absorption rate. Their data also indicates that, as the rate of disappearance of frusemide from the GI tract was very rapid, the stomach may also be the major site of metabolism. Similar results were obtained in the dog and rabbit, in view of this data in three animal species it is possible that the same may also occur in the horse and also humans.

Finally, due to there being only a single administration of frusemide to one horse, it is not possible to assess any degree of inter- or intrasubject variation in bioavailability. Large inter- and intravariability in the disposition of frusemide has been demonstrated after oral administration in man (Waller *et al.* 1982, 1985). A study by Grahnen *et al.* (1984) in man indicated extensive intraindividual variation from two oral dosage forms, the magnitude of which was similar to interindividual variation. This variation has been attributable to differences in the rate and extent of absorption. It has been proposed that frusemide is absorbed in the upper segment of the GI tract, thus variability in absorption kinetics may be influenced by physiological factors such as gastric emptying, gastric pH and transit time. Future work to evaluate the oral bioavailability of frusemide in the horse should be performed using a greater number of animals to diminish any influence of intrasubject variability.

Chapter 4

THE EFFECT OF FRUSEMIDE ON THE DISPOSITION OF SALICYLIC ACID

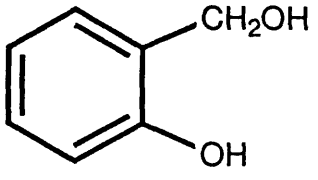
4.1. INTRODUCTION

The common belief that frusemide affects the detection of other drugs in horses has led to a series of studies. The diuresis following frusemide treatment can indeed dilute drugs and their metabolites in post-race urine samples, thereby rendering their detection more difficult (Roberts *et al.* 1976; Miller *et al.* 1977). The main purpose of the present study is to investigate the effect of frusemide on the metabolism pharmacokinetics and "detectability" of a model acidic drug, the non-steroidal anti-inflammatory drug (NSAID) salicylic acid.

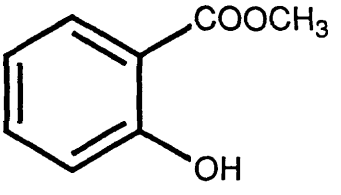
Among ancient folk remedies for pain and fever, extracts of the barks, leaves or fruits of a number of trees had a high reputation. It is now known that many of these herbal remedies owed their effectiveness to the salicylates they contained. The word salicylate is itself derived from *SALICACEAE*, the botanical name for the willow family. The medicinal properties of willow (Salix) bark were first described by the Rev. Edmund Stone in the mid-eighteenth century. Later the active ingredient of Salix bark was shown to be a bitter glycoside, salicin, which on hydrolysis yields glucose and salicyl alcohol (saligenin). Kolbe published methods for the synthesis of salicylic acid from phenol in 1860, and the first therapeutic use of salicylate reported for the treatment of rheumatic fever in man was described by Buss in 1875. It is still in common use today because of its anti-inflammatory, antipyretic and analgesic properties. It is now almost exclusively used in the form of the ester, acetylsalicylic acid. This was first synthesized by Hoffman in 1897, and introduced by Bayer under the tradename Aspirin in 1899, Fig.4.1. illustrates the structures of some of these salicylates.

The metabolism of salicylate has been examined in the horse. The drug is very rapidly eliminated, thus to use salicylate effectively large doses of the drug are needed and they must be given close to the time at which you want the drug to take effect. The plasma half-life in ponies was about 1 h following sodium salicylate (44 mg/kg; i.v.) (Davis and Westfall, 1972). Following a single oral dose of 35 mg/kg salicylic acid, the

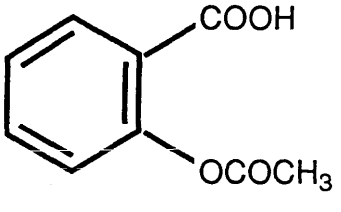
Fig.4.1. Structures of some salicylates



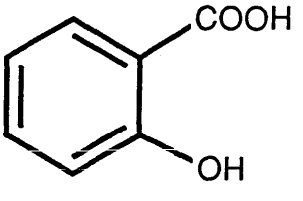
Salicyl alcohol



Methyl salicylate (oil of wintergreen)



Acetylsalicylic acid (aspirin)



Salicylic acid

drug was rapidly cleared with a plasma half-life of 2.8 h, and urinary elimination half-life of 3.6 h and 2.9 h (Marsh 1981,1983). Rapid clearance of this acidic drug is enhanced by the basic nature of horse urine. Over 97% of the dose is excreted unchanged, the drug being exclusively eliminated via the kidneys. Other studies have also shown that salicylic acid is the major drug-related urinary constituent and it is excreted unchanged (Davis *et al.* 1973; Schubert 1967).

NSAIDs are indicated for injury-related inflammation and many acute and chronic musculo-skeletal conditions in racehorses (Tobin 1979a, 1979b; Lees and Higgins, 1985). Whereas acetylsalicylic acid is not used as commonly as some other NSAIDs as it is less effective due to its pharmacokinetics in this species, methyl salicylate is present in a number of proprietary liniments e.g. Oil of Wintergreen, and is in common use. As mentioned in Chapter 1, an increasingly common type of doping is medication to "restore normal performance". NSAIDs are now the most frequently encountered drugs of this type.

There is a general opinion among users that NSAIDs are not drugs because they cannot affect a fit horse. This conviction is wrong and its implementation illegal, as to restore normal performance by a therapeutic agent is to administer a substance that can alter racing performance at the time of the race. This contravenes the Rules of Racing, particularly Rule 200 (Moss 1972). Thus salicylate is still a drug that concerns racing authorities as its multi-action properties and ready availability make it popular with horsemen. Salicylic acid and acetylsalicylic acid have been reported by members of the Association of Official Racing Chemists as being present illegally in the urine of racehorses on a number of occasions.

Salicylic acid presents a particular difficulty to the racing chemist being an endogenous component of horse urine, owing to the natural occurrence of salicylates in a number of edible plants, including legumes and grasses (Gross and Greenberg, 1948). As salicylates of dietary origin may contribute to the detection of salicylic acid in urine (Murdick *et al.* 1968; Moss *et al.* 1981), the detection of salicylic acid in equine urine does not necessarily imply that its presence is a consequence of drug administration. Normal values in horse urines quoted range from 3.97 mg/% ± 3.06

total salicylate (Hucklebridge 1979) to 30 mg/% (Murdick *et al.* 1968). In a recent study, Beaumier *et al.* (1987) determined levels of salicylic acid in horse urine and plasma following feeds containing different types of hay. Elevated levels were found in horses fed lucerne hay, elimination of salicylic acid exhibiting a diurnal pattern which was related to the type of feed and the feeding schedule. Hydrolysed lucerne hay was found to contain up to 485 μg salicylic acid/g, compared to timothy and clover hay which contained 2.4 and 32 μg salicylic acid/g respectively. This study emphasises the need of routine forensic laboratories to establish maximum tolerance levels of salicylic acid for plasma and urine (this will be discussed further later).

It is common practice in clinical medicine to find the concomitant use of diuretics and anti-inflammatory drugs. While there is abundant data on the role of aspirin in the diuresis induced by frusemide (see Chapter 5), the literature is sparse on the part played by frusemide in the pharmacokinetics of aspirin. There is every reason to speculate on an interference by frusemide due to its pharmacodynamics and it is the consequences of this interaction which is of particular interest to racing officials. Since its introduction into equine medicine frusemide has been used for the treatment of various forms of oedema and as discussed in Chapter 1 increasingly for the prophylaxis of epistaxis. Results by Trujillo *et al.* (1981) propose that in the horse haemostasis is affected negatively by aspirin. Thus if aspirin is administered for long periods of time, it may be a potential causative or predisposing factor of haemorrhagic problems in horses that suffer epistaxis.

The results of this study are discussed in terms of the pharmacokinetic drug interaction between frusemide and salicylic acid, and of how frusemide might affect other acidic drugs with which it may be administered.

4.2. METHODS

Compounds

Frusemide (4-chloro-5-sulphamoyl N-furfurylanthranilic acid) (Lasix 5% solution; Hoechst, Milton Keynes, Bucks, U.K.). [carboxy-^{14}C]-Salicylic acid, specific activity 393 μCi/mg, radiochemical purity by HPLC >98% (Amersham International, Bucks, U.K.). Sources of salicylate and derivatives were as follows: salicylic acid (2-hydroxybenzoic acid) was supplied by the Pharmacy of St. Mary's Hospital (London, U.K.), gentisic acid (2,5-dihydroxybenzoic acid) was obtained from Sigma (Poole, Dorset, U.K.) and salicyluric acid (2-hydroxyhippuric acid) and 2-methoxybenzoic acid from Aldrich (Aldrich Chemical Company, Gillingham, Dorset, U.K.). HPLC grade solvents were purchased from Rathburn Chemicals Ltd., (Walkerburn, U.K.). Other materials were reagent-grade chemicals purchased from usual U.K. commercial sources.

Purification of [carboxy-^{14}C]-Salicylic acid

Thin layer chromatography (TLC)

Solutions of gentisic acid, salicylic acid and salicyluric acid were prepared by dissolving in ethanol. 10 μl of each solution was used for spotting on channelled TLC plates. TLC was carried out using silica gel F_{254} plates, layer thickness 0.2 mm, 20x20 cm on aluminium support (Catalogue No. 5554; E.Merck AG, Darmstadt, F.R.G.). The plates were run in two different solvent systems:-

(1) cyclohexane:chloroform:glacial acetic acid (20:80:10 by vol.) and

(2) ethyl acetate:methanol:ammonia (0.88 S.G.) (80:20:10 by vol.)

and the compounds located as dark quenching spots under UV light. The R_F value of salicylic acid was 0.86 and 0.52 in the two respective systems. Both gentisic acid and salicyluric acid did not move appreciably from the origin.

The [carboxy-^{14}C]-salicylic acid was supplied by Amersham as a crystalline solid from stock held at 2°C in borosilicate glass ampoules. Radiochemical purity by TLC was reported as being 97% (Nov.1982) and 95% (Aug.1985) in solvent system

(1). The crystalline solid was dissolved in ethanol (5 ml). Having obtained the R_F value for salicylic acid, 1 µl of [^{14}C]-salicylic acid was spotted on to the TLC plate, a small volume of unlabelled salicylic acid was also spotted so as to identify the compound. The plate was developed in solvent system (1).

UV light indicated the presence of a single major peak corresponding to salicylic acid (R_F = 0.85), no other spots were detectable. The plate was divided into 0.5 cm bands and then cut for ^{14}C determination, methanol (0.5 ml) was used to elute the compound. Ecoscint scintillation fluid (3 ml) was added prior to liquid scintillation counting which revealed the presence of a very minor band which had low chromatographic mobility. The radiochemical purity of [^{14}C]-salicylic acid by TLC before purification was found to be 96.9% (Fig.4.2.).

High Performance Liquid Chromatography (HPLC)

[^{14}C]-Salicylic acid (10 µl) was analysed by HPLC under the conditions described below. Fractions of eluent (0.5 ml) were collected and analysed by liquid scintillation counting. The radiochromatogram located two main bands of radioactivity, the peak corresponding to salicylic acid represented 84.1% of the total radioactivity and a second peak which eluted before the acid accounted for 14.2% (Fig.4.3.). Due to the detection of this second peak it was decided to purify the [^{14}C]-salicylic acid using HPLC.

The ethanolic solution (5 ml) was reduced in volume under a gentle stream of nitrogen to approximately 1 ml. A series of injections were then made on to the HPLC column. Fractions (0.5 ml) of eluent were collected and those corresponding to the salicylic acid peak were pooled and stored at 4°C. Fig.4.4. shows the single peak corresponding to salicylic acid, following injection of the purified [^{14}C]-salicylic acid on to the HPLC system. The total pooled volume (46 ml) was then divided so as to provide 6 individual doses. 3 doses containing approximately 20 µCi for the horses receiving salicylic acid alone and 3 doses of 35 µCi for the horses being dosed with both salicylic acid and frusemide. The aliquots were dried down individually by rotary

Fig.4.2. Radiochromatogram showing radiochemical purity of [14C]-salicylic acid (as purchased) following thin layer chromatography

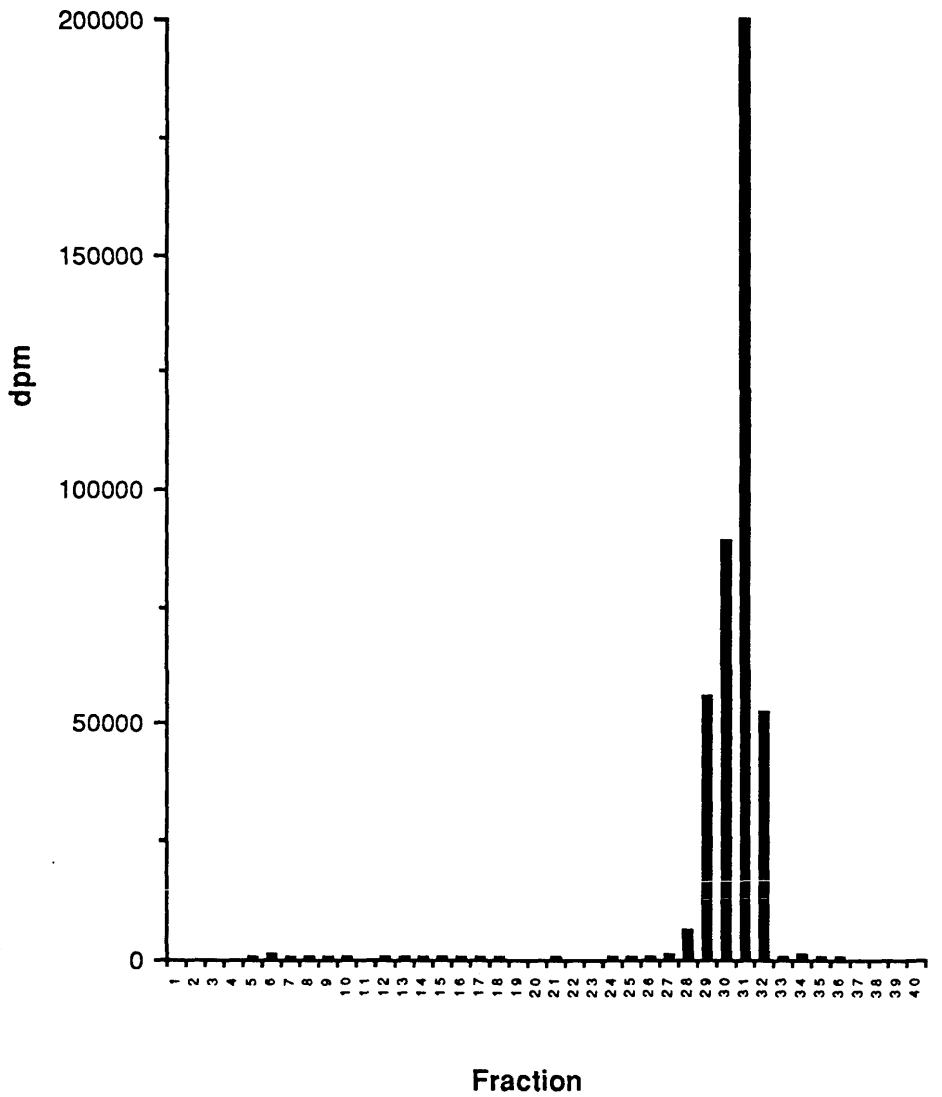


Fig.4.3. Radiochromatogram showing radiochemical purity of [14C]-salicylic acid (as purchased) following HPLC

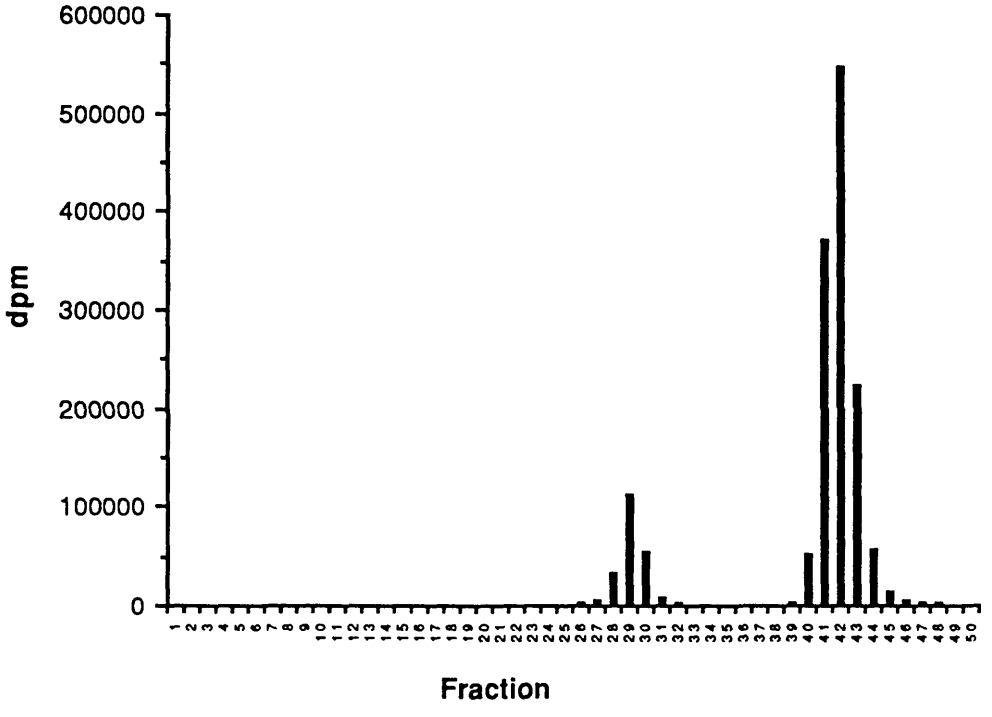
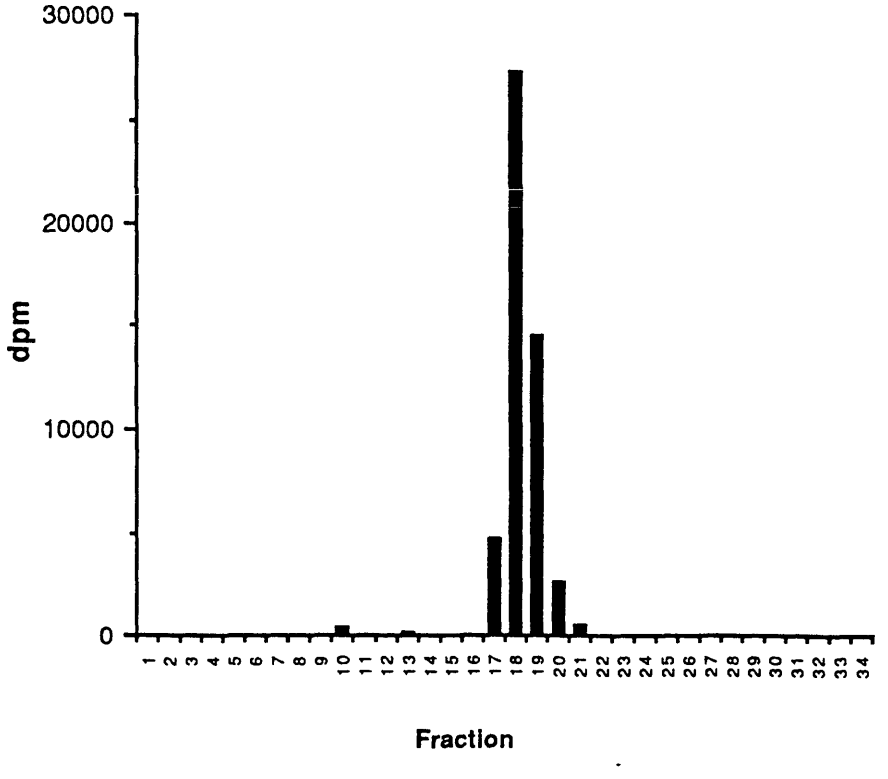


Fig.4.4. Radiochromatogram showing radiochemical purity of [14C]-salicylic acid following purification by HPLC



evaporation, ready for transporting to Newmarket where they were reconstituted before dosing.

Animals and Drug Administration

Drugs were administered at the Horseracing Forensic Laboratory to three thoroughbred horses (1 colt, 2 geldings; body weights 476-514 kg). Each horse received three different treatments at the same time of day in successive dosing sessions. Treatments were separated by a week to ensure that there was no interference.

Treatments

A. Frusemide (1 mg/kg) intravenously;

B. [^{14}C]-Salicylic acid (40 mg/kg; 20 μCi) orally;

C. [^{14}C]-Salicylic acid (40 mg/kg; 30 μCi) orally followed as soon as possible (within 8 min) by frusemide (1 mg/kg) intravenously).

[^{14}C]-Salicylic acid was administered by stomach tube as a solution of the sodium salt in 800 ml of water, after which the horses were moved to metabolism stalls where their movement is restricted, these are designed for the convenient collection of samples (Marsh 1983). Frusemide (9.52-10.30 ml) was then injected over 1 min into the jugular vein.

Food was withheld from the animals overnight before dosing, at 9.30 am, and for about 2 hours afterwards, but there was free access to water. Each morning and afternoon the horses were removed and exercised for 30 minutes before being returned to the metabolism stalls.

Blood Collection

Blood samples (50 ml) were collected from an indwelling jugular venous cannula into Monovettes (Sarstedt Ltd., Leicester, U.K.) containing lithium heparin crystals, and centrifuged at 2000 rpm for 5 min. Plasma was aspirated and duplicate 1 ml samples taken for ^{14}C measurements.

Urine Collection

Urine was collected as previously described (Marsh 1983). The time of voiding was recorded and the sample volume and pH immediately measured; specific gravity was obtained using a refractometer. A small volume of urine (15 ml) was centrifuged at 3500 rpm for 10 min and 0.1 ml aliquots of the clear supernatant were removed for ^{14}C determination and creatinine measurements.

Sample Analysis

Plasma and urinary concentrations of ^{14}C after [^{14}C]-salicylic acid were determined by liquid scintillation spectrometry. Electrolytes were determined, urinary sodium and potassium were measured by flame photometry, and chloride levels were measured using a chloridometer. Creatinine concentrations were measured, using the Jaffé method, by the Animal Health Trust, Newmarket.

Drug Analysis

Initially a colorimetric method was used to determine salicylic acid and its possible metabolites, subsequently a HPLC method was used as this was more suitable for quantification of metabolites in pharmacokinetic studies. However, having confirmed earlier findings of the lack of metabolism of salicylate in the horse (Marsh *et al.* 1981), it was possible to use radiochemical techniques and equate radioactivity directly with salicylate concentration.

(1) Colorimetric method

Trinder's reagent was prepared by dissolving mercuric chloride (40 g) in water (800 ml) with heating and stirring. 1N HCl (120 ml) was then added together with ferric nitrite (40 g). For the colorimetric reaction Trinder's reagent was added to urine (1 ml), the tubes were mixed and the solution transferred to a 1 ml glass cuvette, and the absorbance read at 530 nm (Trinder 1954). Calibration curves ranging from 0-1000 µg/ml for salicylic and salicyluric acid were prepared by spiking methanol with the

respective standard solutions, and then carrying out the above procedure. Correlation coefficients greater than 0.995 were obtained.

(2) HPLC method (Hutt *et al.* 1986)

To aliquots (1 or 2 ml) of plasma were added the internal standard, 2-methoxybenzoic acid (0.1 ml of a 0.5 mg/ml solution), and trichloroacetic acid (0.1 ml of a 50% solution). The mixture was centrifuged, the supernatant removed and 20 µl injections were made on to the column. To aliquots (1 or 2 ml) of urine were added the internal standard (1 ml of a 0.5 mg/ml solution), the pH of the mixture adjusted to 1 by the drop-wise addition of 6M HCl and the whole extracted with diethyl ether (2x4 ml). The ether extracts were pooled and evaporated to dryness, the residue was dissolved in mobile phase (2 ml) before HPLC injection (20 µl).

The HPLC system employed a Lichrosorb RP-18 analytical column (250x5mm; E.Merck, Darmstadt, F.R.G. Cat. No. 15539) and injections were made using a Rheodyne valve loop injector. A reciprocating pump (model M-45; Waters Associates, Harrow, Middlesex, U.K.) and a mobile phase of methanol-glacial acetic acid-water (25:4:71 by vol.) were used at a flow rate of 1.5 ml/min. A UV detector set at 280 nm and linked to a chart recorder were used to detect the peaks. Calibration curves for salicylic acid, salicyluric acid and gentisic acid were prepared and quantification was achieved by the peak height ratio method (Fig.4.5.). The retention times (min) of standard compounds in this system were as follows: gentisic acid 4.3; salicyluric acid 6.3; 2-methoxybenzoic acid 10.2; salicylic acid 15.0 (Fig.4.6.).

(3) Radiochemical techniques

Plasma and urinary concentrations of ^{14}C after [^{14}C]-salicylic acid were determined by liquid scintillation spectrometry in a Betamatic II liquid scintillation spectrometer (Kontron Instruments, Watford, Herts, U.K.) with channels ratio quench correction, using Instagel scintillant (Canberra-Parkard, Pangborne, Berks, U.K.).

Fig.4.5. Calibration curves for salicylic acid, salicyluric acid and gentisic acid extracted from horse urine using a HPLC method (Hutt et al. 1986)

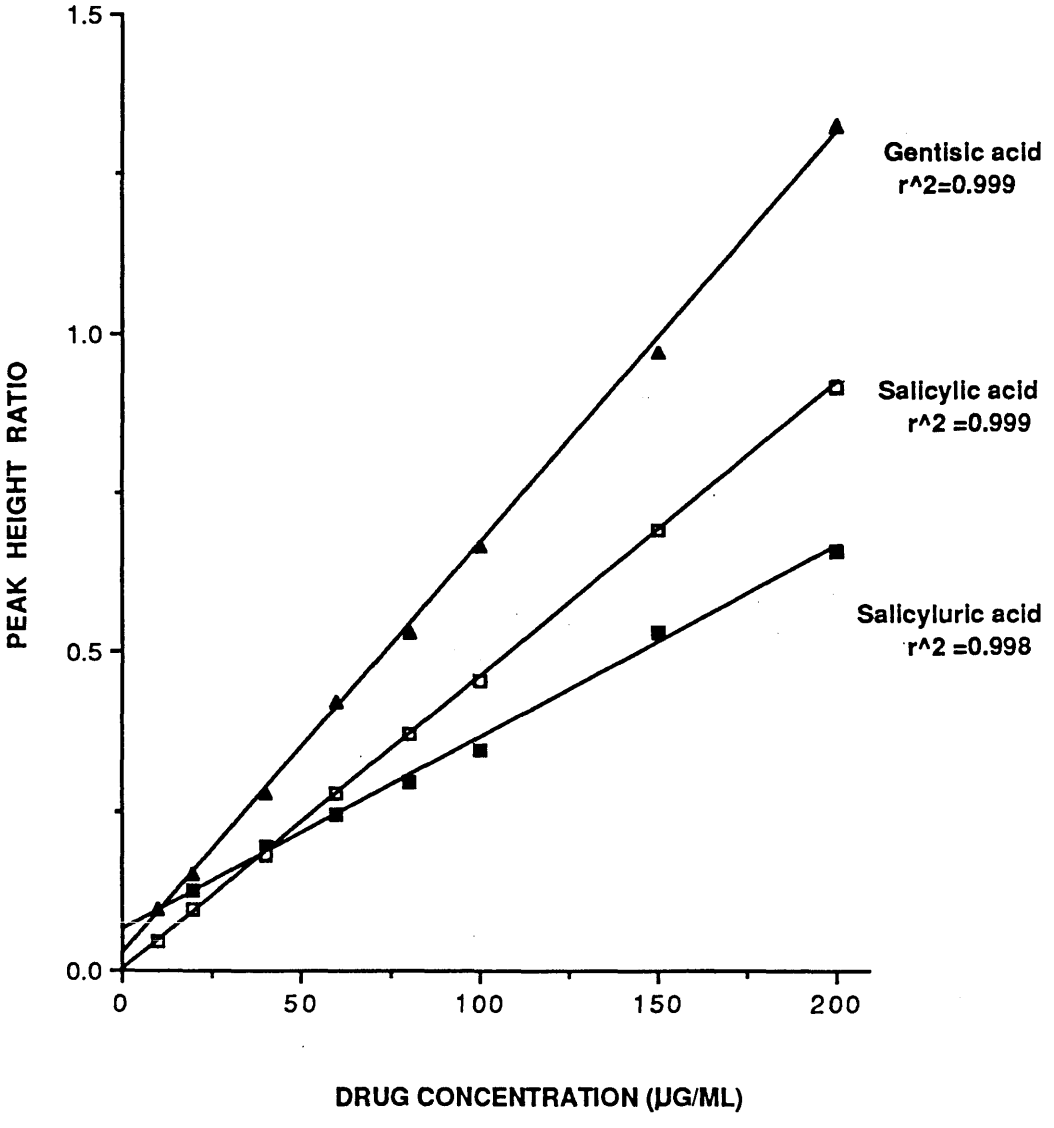
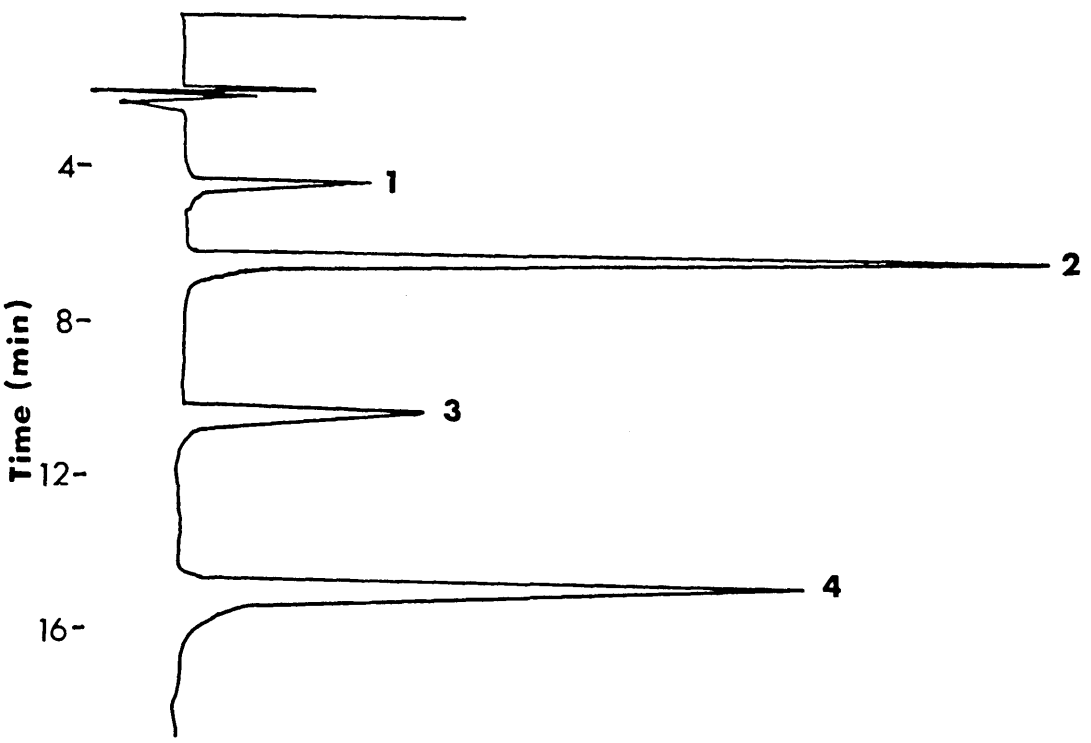


Fig.4.6. Typical HPLC chromatogram showing the retention times of salicylic acid, gentisic acid, salicyluric acid and the internal standard using a HPLC method (Hutt et al. 1986)



Peak	Compound	Retention time
1	Gentisic acid	4.3 min
2	Salicyluric acid	6.3 min
3	Internal standard	10.2 min
4	Salicylic acid	15.2 min

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated by least-squares regression analysis as described in Chapter 3.

4.3. RESULTS

Frusemide had an immediate but short-lasting diuretic effect. Between 7 and 9 litres of urine were produced in the first hour, this is more than the usual 24 h output of horses. The increase in urine volume was mirrored by a rapid fall in specific gravity (Fig.4.7.) after which values gradually returned to normal within 12 h after treatment. The effect of frusemide was the same with or without salicylate.

Plasma Pharmacokinetics

The graph of the log ^{14}C plasma concentrations (μg equivalents/ml) plotted against time (Fig.4.8.) illustrates that salicylate was rapidly absorbed. The effect of frusemide on subsequent salicylate disposition was striking; after frusemide, plasma salicylate concentrations were much higher in all three horses and peak concentrations were reached later. However, after the peak, elimination proceeded at the same rate, and the pairs of regression lines were parallel. Consequently for each horse the elimination half-life of salicylate was the same in the presence or absence of frusemide, so that the concentration differences persisted. Salicylic acid was not detectable in plasma 24 h after dosing, thus elimination of this dose (40 mg/kg; ca. 20 g) was essentially complete within this time.

The pharmacokinetic parameters presented in Table 4.1. for all three horses confirm that after frusemide the peak salicylate concentration (C_{max}) was significantly higher and was attained at slightly later times as shown by T_{max}; the elimination rate constant (K) and the half-life ($T_{0.5\beta}$) were unaltered. Co-administration of frusemide

Fig.4.7. Specific gravity of urine of a horse given [14C]-salicylate (40 mg/kg; p.o.), frusemide (1 mg/kg; i.v.) and [14C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

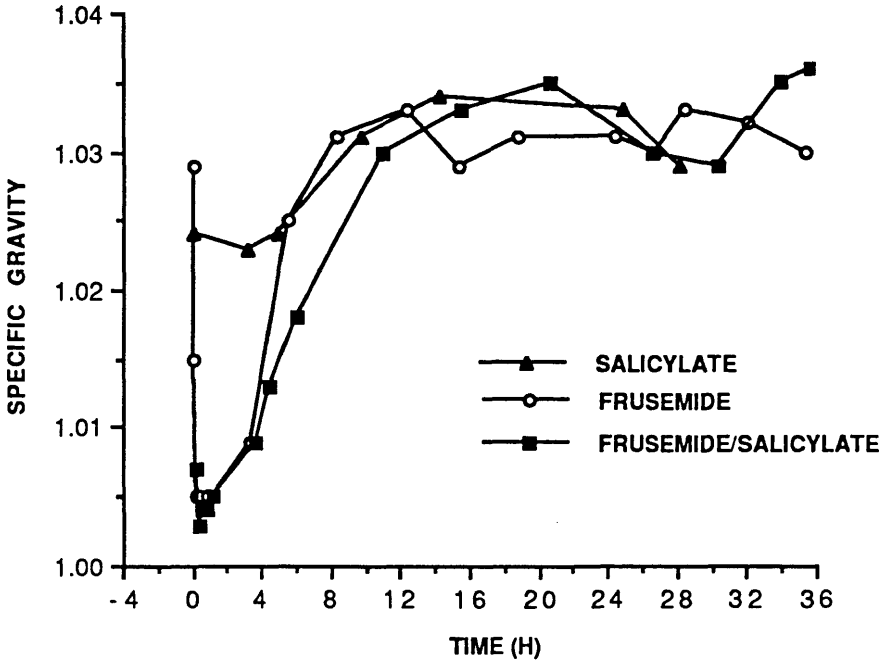


Fig.4.8. Plasma concentration of 14C-radioactivity in a horse given [14C]-salicylate (40 mg/kg; p.o.) and [14C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

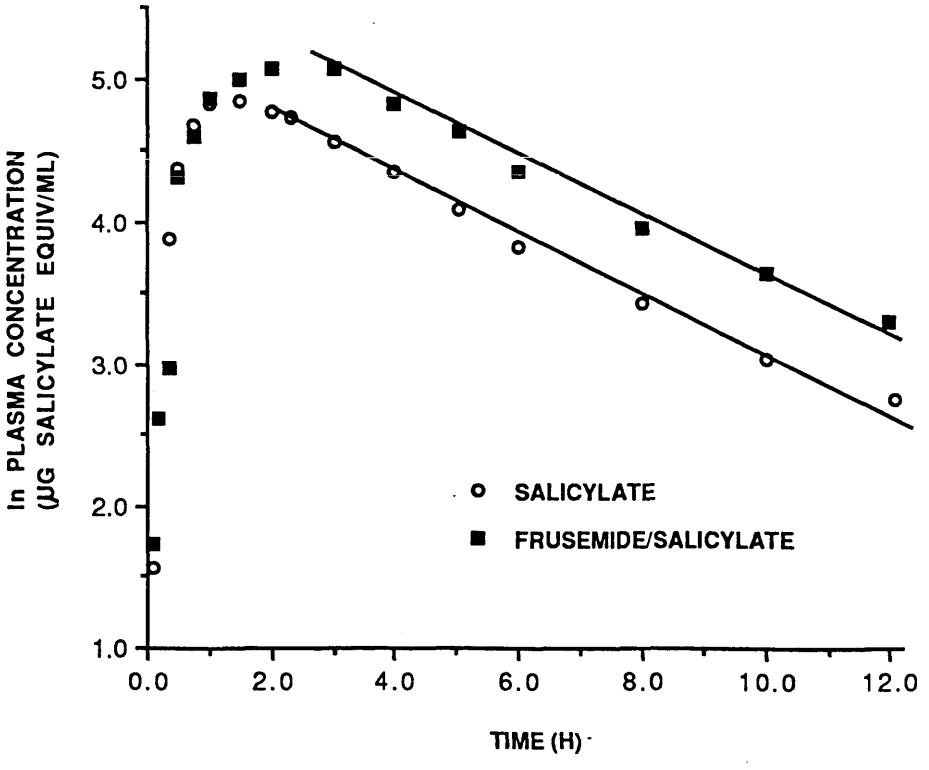


Table 4.1. Pharmacokinetic parameters describing the plasma levels of salicylate in the horse, following the administration of [^{14}C]-salicylate (40 mg/kg; p.o.) and [^{14}C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions (mean ± s.d., n = 3)

	Salicylate	Salicylate + Frusemide
C_{max} (µg/ml)	123 ± 5	160 ± 9 *
t_{max} (h)	1.67 ± 0.3	2.37 ± 0.7
$T_{0.5\beta}$ (h)	3.40 ± 0.6	3.61 ± 0.7
K (h^{-1})	0.208±0.04	0.197 ± 0.04
App V_d(l/kg)	0.22 ±0.02	0.15 ± 0.006 *
Cl (ml/h/kg)	53 ± 12	37 ± 10 *
AUC (µg/ml.h)	756 ± 137	1122 ±260 *

* $p < 0.05$, significantly different from salicylate alone

Table 4.2. Urinary excretion of salicylate within 4 h in the horse, following the administration of [^{14}C]-salicylate (40 mg/kg; p.o.) and [^{14}C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions (mean ± s.d., n = 3)

		Salicylate	Salicylate + Frusemide
Urine Volume	(l)	1.84 ± 0.67	10.08 ± 1.29 *
Amount of Salicylate	(g)	8.80 ± 2.87	5.87 ± 0.71
Concentration of Salicylate	(g/l)	5.10 ± 1.75	0.59 ± 0.10 *

* $p < 0.05$, significantly different from salicylate alone

also produced significant reductions in volume of distribution and clearance, and an increase in AUC.

Urinary Excretion

Plots of urine ^{14}C concentration against time are difficult to interpret as concentrations are much affected by variations in flow rate of urine (Fig.4.9.). Evidence that the excretion of salicylate is delayed after frusemide is given by plotting cumulative radioactivity excretion (Fig.4.10.) which eliminates the effect of urine volume. In the first 4 h after administration, frusemide significantly reduced the urinary excretion of salicylate (from 38% to 29%, n=3, $p<0.05$) after which it proceeded at the normal rate. There was a five-fold increase in urine volume during this time and an approximately 10-fold dilution in urinary salicylate concentration (Table 4.2.). However, total amounts excreted were the same, with percentage recoveries of 95.6-100.9% for all six administrations. 24 h after dosing salicylic acid levels had fallen to 262±105 μg/ml in the presence or absence of frusemide.

The pH of each urine sample was determined, but no differences were observed between the different treatments. The urine tended to be slightly alkaline (especially those urine samples collected in the fraction collector overnight) or neutral throughout this study, and frusemide administration caused no observable changes.

Creatinine Levels

There was an initial steep fall in creatinine levels after frusemide, then concentrations returned to normal by about 10 h (Fig.4.11a.). The small fall after salicylate alone might be due to the diluting effect of the water in the dose (800ml). However, cumulative excretion of creatinine was linear in all three experiments showing that its excretion rate was unaffected by drug treatment (Fig.4.11b.).

Fig.4.9. Urinary concentration of 14C-radioactivity in a horse given [14C]-salicylate (40 mg/kg; p.o.) and [14C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

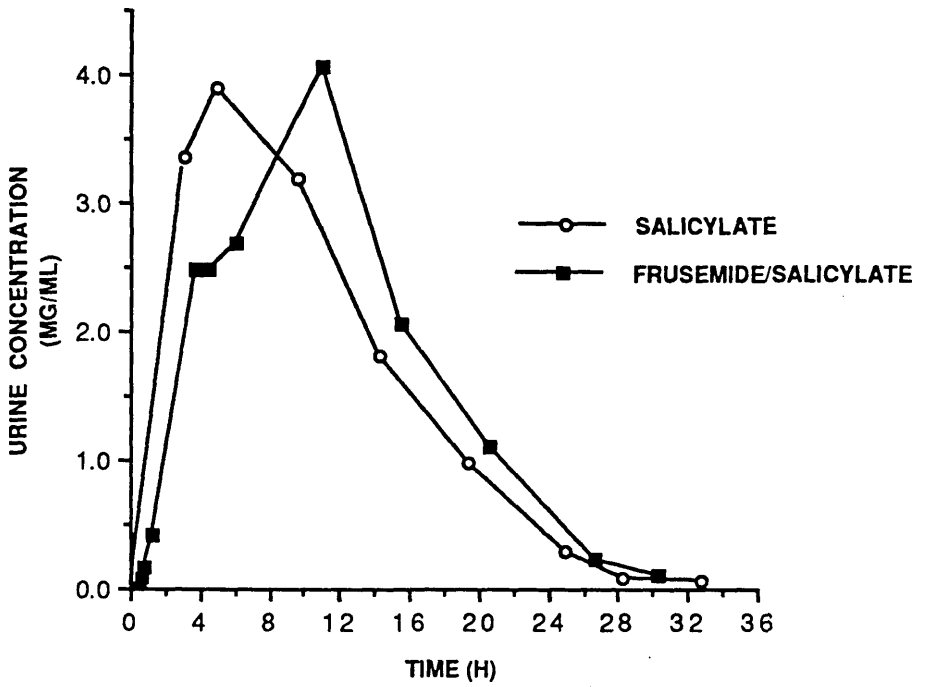


Fig.4.10. Cumulative urinary excretion of 14C-radioactivity by a horse given [14C]-salicylate (40 mg/kg; p.o.) and [14C]-salicylate (40 mg/kg;p.o.)+frusemide (1 mg/kg;i.v.) on separate occasions

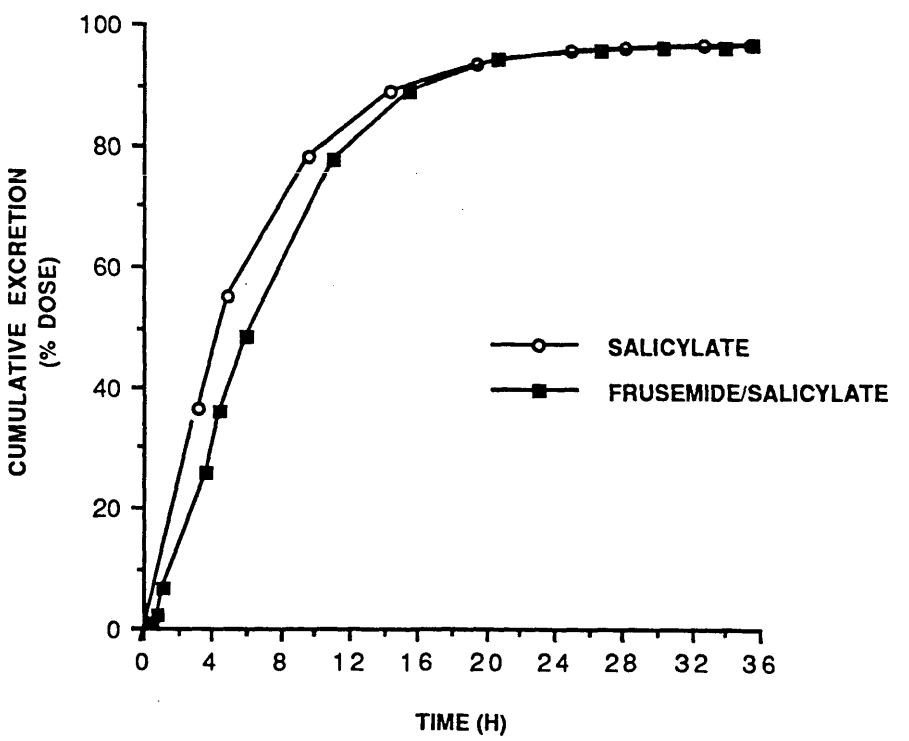
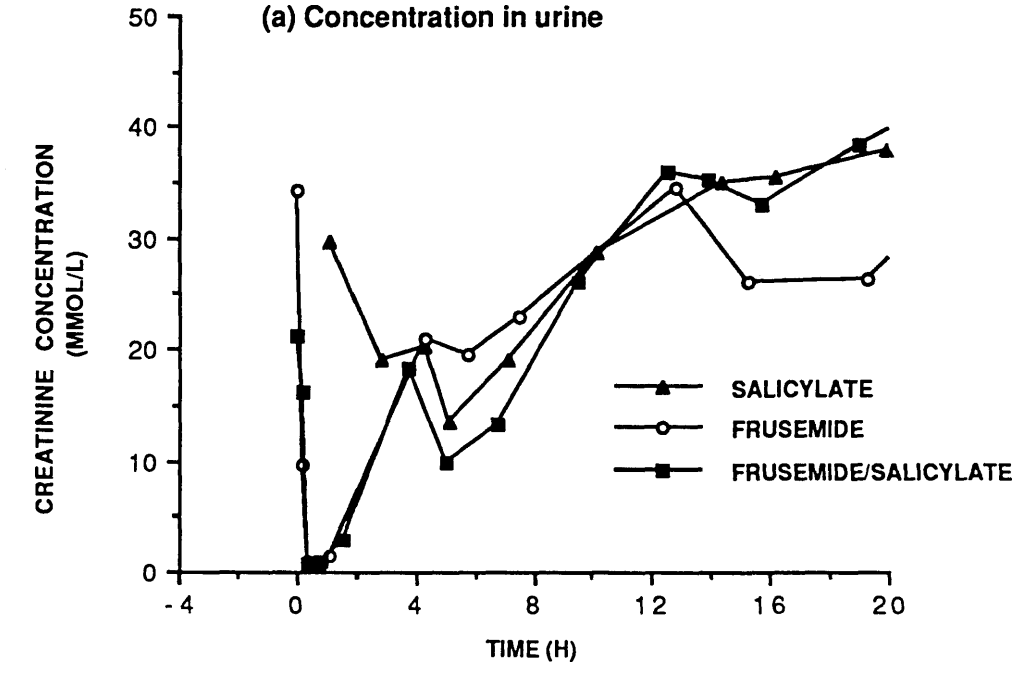
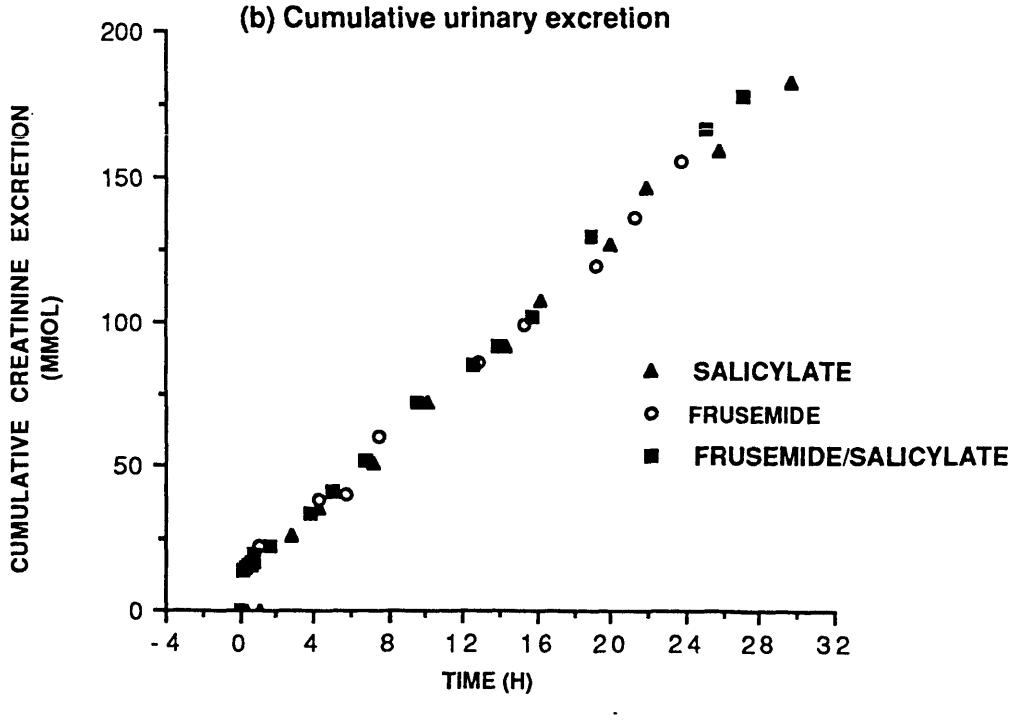


Fig.4.11. Creatinine excretion in a horse given [14C]-salicylate (40 mg/kg; p.o.), frusemide (1 mg/kg; i.v.) and [14C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions





Urinary Electrolyte Levels

Sodium

Changes in sodium excretion following frusemide administration resembled those in man. After frusemide, sodium concentrations in urine rose rapidly (Fig.4.12a.). Concentrations of approximately 120 mmol/l were reached within the first hour, these returned to starting levels, close to zero within 5 h. Fig.4.12a. also shows that with salicylate alone there was a small rise in sodium levels. This may possibly be due to the sodium in the dose i.e. 145 milliequivalents/500 kg horse.

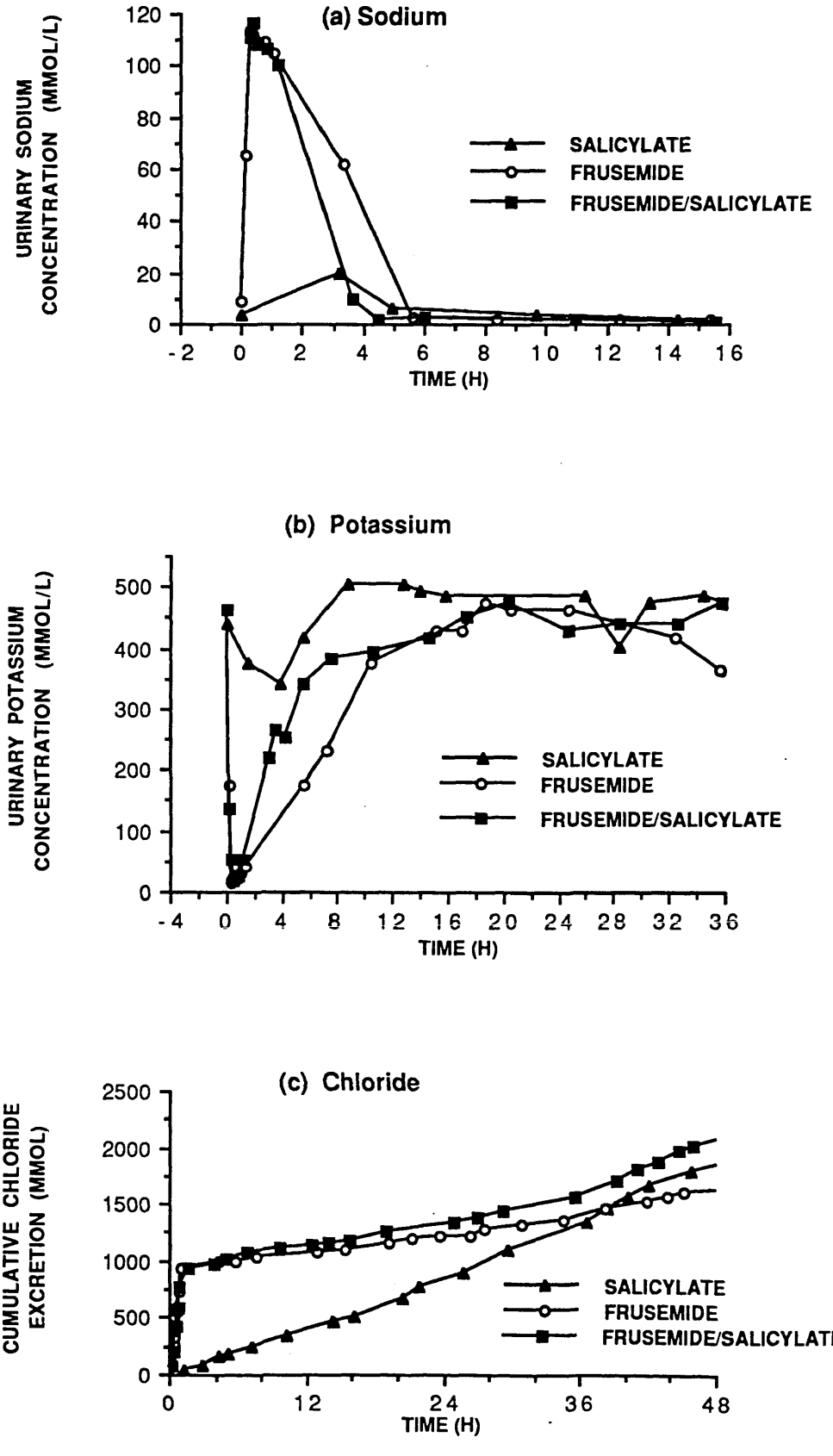
Potassium

Urine potassium concentrations fell sharply immediately after frusemide administration (Fig.4.12b.), but this was purely a dilution effect as the rate of excretion increased for a short period and cumulative excretion was linear following all three treatments. Values returned to normal after about 15 h.

Chloride

After frusemide, initial concentrations were in the normal range but total excretion is, of course, much increased because of the huge fluid output and body chloride is considerably depleted. Some chloride conservation was observed subsequently since the chloride concentrations in frusemide and salicylate/frusemide experiments were clearly lower than after salicylate alone. The increased excretion is clearly indicated in Fig.4.12c., which shows that frusemide causes a large initial increase in output which is slowly compensated for by a reduction in chloride excretion over the subsequent 2 days.

Fig.4.12. Electrolyte excretion in a horse given [14C]-salicylate (40 mg/kg; p.o.), frusemide (1 mg/kg; i.v.) and [14C]-salicylate (40 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions



4.4. DISCUSSION

The recommended therapeutic oral dose of aspirin for horses is 30 to 100 mg aspirin/kg bodyweight or 15-50 g for a 500 kg horse (Robinson 1983). The dose administered in this series of experiments was at the lower end of this range. It has been postulated that a threshold dose of approximately 5 g is required to elevate salicylic acid concentrations above background levels (Beaumier *et al.* 1987). As mentioned earlier salicylic acid is an endogenous component of horse urine.

This study confirms earlier findings that the elimination of salicylic acid is both rapid and complete, drug concentrations following administration were indistinguishable from levels quoted as being residual at 24 h. Studies have been carried out to define the maximum level of salicylic acid that may be expected in an untreated horse, in order to determine whether aspirin has been administered. Hucklebridge (1979) states that concentrations of up to 7 mg% (70 µg/ml) are acceptable as normal and a concentration exceeding this would indicate administration of the drug.

This value appears rather low in view of the findings of Beaumier *et al.* (1987). These authors found that a hay ration containing lucerne resulted in plasma and urine salicylic acid levels consistent with a daily intake of about 2.5 g of acetylsalicylic acid. From these results, Beaumier *et al.* (1987) established maximum tolerance levels of salicylic acid. They determined that plasma salicylic acid levels above 6.5 µg/ml or urine salicylic acid levels greater than 750 µg/ml may be credited to drug administration. In our study plasma concentrations of salicylate at 12 h when given alone were 16.9 ± 7.3 µg/ml. In the presence of frusemide the 12 h salicylate concentration was 26.0 ± 12.3 µg/ml. Urinary levels of salicylate were below the 750 µg/ml threshold, 20.0 ± 4.0 h after salicylate alone, and after 23.6 ± 4.9 h when given with frusemide. At the recommended therapeutic dose of 1 mg/kg intravenously, frusemide produced a marked diuresis in the horse, with an immediate increase in urine volume and a corresponding decrease in specific gravity and creatinine concentration. The diuretic effect was maximal during the first hour but ceased soon after, though

specific gravity and creatinine concentration took 8-10 h to return to pre-frusemide values.

When salicylate and frusemide were given together, plasma concentrations of salicylate always rose much higher than when salicylate was given alone. This is partly due to a haemoconcentration effect as a result of the considerable reduction in plasma volume which must always occur when there is such a large diuresis with no matching increase in fluid intake. After Muir *et al.* (1978) gave frusemide intravenously to horses at a dose of 1.1 mg/kg, plasma volumes decreased from an average of 62.8 ml/kg in resting horses to 55.6 ml/kg, an average reduction of 11.5%. In the same study a decrease in ECFV of 7.71% was reported.

Since urine is a filtrate of blood, this volume reduction increases the concentration of non-filterable components. Molecular size is an important factor that determines the degree of permeability, large molecules such as plasma proteins and erythrocytes, remain trapped in the circulation. The effect of protein binding is an extreme case of molecular size restriction, as the drug becomes part of a very large molecule. The concentration of any drug firmly bound to the "trapped" protein will therefore also be increased, while that of a non-bound drug would be unaffected. Salicylate, like many NSAIDs, is highly protein-bound (90%) (Horner 1976; Tobin 1981), so increased plasma concentrations are to be expected following frusemide administration. Frusemide administration causes increases in haemoglobin concentration, packed cell volume (PCV) and total protein concentration in the blood of resting or exercised horses, indicating a haemoconcentration and a possible reduction of plasma volume (Fregin *et al.* 1977; Gabel *et al.* 1977; Muir *et al.* 1978). However, this alone probably does not account for the observed differences in C_{max} (Table 1), other factors are also most likely involved.

Earlier work with other drugs in horses has not shown a reduction in their plasma concentrations as a result of frusemide diuresis (Gabel *et al.* 1977; Tobin 1981), and we have shown that salicylate plasma concentrations are in fact increased. There are therefore no grounds for the popular belief that frusemide flushes drugs out

of horses' blood, thus preventing its use for detection of illegal drug administration; detection would actually be made easier in the case of salicylate.

The urinary data indicates that frusemide delays salicylate excretion. Since these drugs are extensively protein bound, glomerular filtration is limited, and the renal organic anion transport system plays a predominant role in their excretion.

The involvement of the organic anion transport pathway in the excretion of frusemide was indicated by Hirsch *et al.* (1975) who showed that renal tissue is capable of accumulating frusemide from an incubation medium. The data indicates that frusemide accumulation by renal cortical slices involves a metabolically dependent active transport mechanism. Tubular secretion of frusemide was first demonstrated by a micropuncture study in the rat kidney by Deetjen (1966), who also suggested that there was competition for this secretion between frusemide and other acids. The delay in salicylate excretion could well be the result of competition between salicylic acid and frusemide for the transport system.

In another study, Oyekan *et al.* (1984) found that total amount of salicylate excretion increased (though not statistically significant) when aspirin was ingested before frusemide or vice versa. The mean percentage change from control was 10.8%. However, there was a significant decrease (30.4%) in the total amount of salicylate excreted when aspirin and frusemide were ingested simultaneously. Oyekan *et al.* (1984) also proposed that this was due to the fact there is an inhibition of salicylate proximal tubular secretion by frusemide when the diuretic occupies the renal receptors at a concentration that is proportionately higher than that of salicylate.

A striking feature of organic anion transport is that compounds with very different chemical structures are secreted, since organic anion substrates exhibit several interactions with the hydrogen receptor site on the carrier. These include binding of an aromatic group, hydrogen bonding via substrate carbonyl groups, electrostatic interactions, and unspecific hydrophobic interactions.

A speculative model for the substrate-receptor interaction has been proposed by Möller and Sheikh (1983) who stated that for substrates a three-point attachment of the substrate to a receptor site can be identified in addition to electrostatic interactions; they

concluded that a three-point attachment is the minimum required to obtain high affinity substrate binding. Application of this theory predicts that frusemide has the potential for high affinity interaction with the carrier, but salicylate can achieve only low affinity binding. This preferential binding of frusemide could explain the delay in salicylate excretion, when the compounds are co-administered.

The competitive blockade of salicylate excretion would be short-lived, as the elimination half-life of frusemide is only 35 min (Roberts *et al.* 1978). Meanwhile, absorption from the gut would have continued relatively unopposed by elimination, so that plasma concentrations would continue to rise until absorption was complete. The delay in salicylate excretion would therefore also contribute to the observed increase in C_{max} (Table 4.1.).

Another factor which might have some bearing is that frusemide can slow or even reverse the absorption of water from the gut. After an i.v. dose of 0.3 mg/kg in man the rate of water absorption from the jejunum was reduced by about 60%, while a dose of 0.6 mg/kg caused a net inflow of water (MacKenzie *et al.* 1974); however the relevance of this cannot be assessed because the volumes involved were very small and the species and anatomical region were different.

An effect of frusemide on absorption from the gut in man was also suggested by Brooks *et al.* (1974) who gave indomethacin (another carboxylic acid NSAID) orally together with oral frusemide. In contrast to the salicylate results, plasma concentrations of indomethacin were initially considerably less than in the absence of frusemide. Competition between the two drugs was thought responsible in this study too, either for plasma protein binding sites or for absorption sites in the gut. The latter seems more likely because the oral route of frusemide administration is the most significant difference between Brooks' work and our own, which gave the opposite result.

Oyekan *et al.* (1984) suggested a number of factors which may account for the frusemide-aspirin interference, including protein binding displacement and pH-dependent absorption of salicylates from the gastric mucosa. They suggested that increased salicylate excretion when frusemide is co-administered may be due to a

frusemide-induced alkalosis effect. Under these alkaline conditions salicylate is highly ionized and its clearance is greater than glomerular filtration rate, so that there is little reabsorption. Also, the high rate of urine flow produced by frusemide would be expected to decrease tubular reabsorption.

In this study the elimination of salicylic acid could not be correlated with urinary pH. It has been suggested that there is a link between equine urinary pH after exercise and the elimination of acidic drugs such as salicylates (Davis and Westfall, 1972; Tobin 1979a). The alkaline pH explains the rapid elimination of salicylate but pH would probably have few other effects as the pK_a values of frusemide and salicylate are similar (3.9 and 3.0 respectively).

The principal effect of frusemide on urinary levels of salicylate appears to be a diluting effect, and in this experiment the increase in urinary volume during the first 4 h after frusemide was sufficient to account for the lower salicylate concentration. This was confirmed by changes in creatinine concentrations, which are believed to be an effective measure of urine dilution because the rate of its excretion is essentially constant. Our results support this, since although creatinine concentrations initially fell sharply and were later rather variable, these were again purely dilution effects. The excretion rate for each horse was constant and was unaffected by the presence of frusemide or salicylate; rates for all three horses were very similar. This agrees with another study which looked at the effect of diuresis on urinary excretion and creatinine clearance in the horse (Gronwall 1985). The mean control value for urinary creatinine excretion was 22.5 µg/min/kg compared with 24.2 µg/min/kg following frusemide treatment.

A previous report (Tasker 1967) on electrolyte levels in normal horses emphasized the need for very strict sodium economy in the horse. This is because the sodium concentration of hay is very low, and careful regulation is needed when no supplementary sodium is provided. This is reflected in the fairly rapid return to normal levels. During the course of this experiment the horses were in metabolism stalls where they do not have a salt lick to supplement their diet. Rebound conservation of sodium was not observed, probably due to the baseline being so low (1-2 mmol/l). Tasker

estimated that sodium intake from a hay diet resulted in 329 mequivalents/day/500kg horse. Thus the 145 mequivalents of sodium in the dose i.e. an increase in sodium intake of 44% is likely to lead to an increased urinary output. The horse ingests extremely large quantities of potassium estimated at approximately 3930 meq/day (Tasker 1967). Thus the kidney has a very important function in these animals to rid the body of the potentially toxic quantities of potassium consumed.

The effect of frusemide on the disposition and urinary elimination of a number of drugs has been studied, with particular regard to how routine forensic screening may be affected (Chapter 1). The present results show that frusemide treatment can potentially reduce urinary concentrations of salicylate for a period of hours after its administration, this could seriously impair the detection of salicylate in routine screening tests. This phenomenon was highlighted when routine screening of race-track samples from frusemide-treated horses showed phenylbutazone consistently present in plasma samples but only occasionally in urine (Roberts *et al.* 1976).

As described in Chapter 1, diuretics are one of the five categories of drugs screened for by the IOC. As testing relies on urine samples, frusemide is often illegally used in an attempt to "mask" by dilution the presence of another banned substance in the urine. The belief that the detectability of drugs in urine can be reduced by the administration of diuretics is also widespread in horse racing. It is therefore important to test the truth of the belief in studies such as this. The effect of frusemide on other categories of drugs will be reported in the following chapters.

Chapter 5

THE EFFECT OF SALICYLIC ACID ON THE PHARMACOKINETICS AND DIURETIC RESPONSE OF FRUSEMIDE

5.1. INTRODUCTION

As already mentioned, it is not uncommon in clinical practice to administer diuretics and non-steroidal anti-inflammatory drugs (NSAIDs) concomitantly. The NSAID-diuretic interaction is important in the clinical situation. Congestive heart failure and conditions where NSAID therapy is required is particularly frequent in the elderly (Baker 1988). There is considerable literature on the interaction between diuretics, especially frusemide and NSAIDs (Rawles 1982; Favre *et al.* 1983). The interaction between indomethacin and frusemide has been known since the mid-1970's when Patak *et al.* (1975) showed that indomethacin therapy significantly reduced the diuretic and hypotensive effects of frusemide in normal subjects and in hypertensive patients. Since then similar interactions have been observed with sulindac, naproxen, flurbiprofen, piroxicam, diflunisal and ibuprofen (Ryan *et al.* 1984; Holford 1985; Laiwah and Mactier, 1981; Tempero *et al.* 1977; Baker 1988; Passmore *et al.* 1989).

Williamson *et al.* (1975) were the first to suggest that prostaglandins (PGs) may be mediators of the effects of loop diuretics. They reported that inhibition of cyclooxygenase by indomethacin in dogs, blocked the increase in renal blood flow caused by frusemide. An inter-relationship between renal PGs and frusemide administration is now firmly established. Inhibition of prostaglandins by NSAIDs has been found to have a distinct effect on the physiological actions of frusemide. For example, there is considerable evidence that cyclooxygenase inhibitors block the ability of frusemide to increase renal blood flow. This implies that the dilation of the renal vasculature is mediated by PGs (Data *et al.* 1978; Gerber and Nies, 1980; Williamson *et al.* 1975). The ability of frusemide to elevate plasma renin activity is also blocked by NSAIDs (Weber *et al.* 1977; Favre *et al.* 1983).

Collated data suggests that the systemic venous effects of frusemide are dependent on renal PG generation. Indomethacin in normal volunteers, or nephrectomy in dogs inhibits the ability of frusemide to increase venous capacitance which results in a prompt decrease in left atrial pressure and reduced cardiac pre-load (Gerber *et al.* 1981; Bourland *et al.* 1977).

The role of renal PGs in the tubular response to frusemide is uncertain. Possible mechanisms by which NSAIDs could alter the diuretic response to frusemide include a direct effect at the thick ascending limb of the loop of Henlé or by blocking the vascular effects of frusemide. Renal vasodilation *per se* causes diuresis by diminishing proximal tubular reabsorption of salt and water. Thus indomethacin, by inhibiting vasodilation, blocks frusemide's indirect effect on the proximal tubule, and could alter overall natriuresis.

In the last decade numerous studies have attempted to define the role of renal PGs as possible mediators of the effects of diuretics. Three criteria must be met in order to demonstrate that frusemide's action is mediated by PGs. Firstly, frusemide administration should enhance PG synthesis or decrease PG degradation, secondly infusing PGs or the PG precursor arachdonic acid must mimic the effect of the diuretic, and finally inhibition of PG synthesis should alter the response to the drug. Data from the many studies have often been difficult to interpret and contradictory. NSAIDs have been shown to reduce the diuretic and natriuretic effects of loop diuretics in several (Frolich *et al.* 1976; Brater 1979; Nies *et al.* 1983; Kirchner 1985) but not all studies. In a number of experiments in both animals and man, NSAIDs like aspirin and indomethacin have failed to impair the diuretic effects of frusemide, ethacrynic acid or the novel potent high ceiling diuretic torasemide (Berg 1977; Weber *et al.* 1977; Data *et al.* 1978; Van Ganse *et al.*1986). The disparity in the results may be due to species differences, use of different experimental preparations and variations in experimental design or conditions.

As far as we know there are no reports in the literature on the effects of NSAIDs on frusemide in the horse, the protocol for the present experiment was not specifically designed to study this particular interaction, but, as described in the previous chapter, to look at how the diuretic influences the disposition and detectability of salicylic acid. However, analysis of the data provides the opportunity of looking at the results from a different viewpoint. Although the results are limited and not conclusive, this does provide a useful pointer to further work on the NSAID-diuretic interaction. Information of this kind is of practical value in the light of the widespread

use of these types of drugs, especially phenylbutazone and frusemide. Both drugs are commonly used in the horse and concurrent administration is therefore not unusual. Thus in this chapter the data following frusemide's intravenous administration in the horse and salicylate's influence on it when the two drugs are given together will be discussed.

5.2. METHODS

Details of the compounds used and the experimental procedures are described in detail in the previous chapter. Briefly, three horses received three different treatments in successive dosing sessions. The treatments were:-

Treatments

A. Frusemide (1 mg/kg) intravenously;

B. [^{14}C]-Salicylic acid (40 mg/kg; 20 μCi) orally;

C. [^{14}C]-Salicylic acid (40 mg/kg; 30 μCi) orally followed as soon as possible (within 8 min) by frusemide (1 mg/kg) intravenously.

This chapter deals specifically with the consequences of frusemide administration and with salicylate's effect on this i.e. treatments A. and C.

Frusemide in plasma and urine was determined as described in Chapter 2. Initially the liquid-liquid extraction method was used prior to HPLC, but subsequently the solid phase extraction method was employed. Data in Chapter 2 illustrates that the two sample preparation methods yield similar results ($r^2 = 0.998$).

5.3. RESULTS

As already described in Chapter 4, frusemide administration resulted in a strong but short-lasting diuretic effect. Fig.4.7. illustrates the rapid fall in the specific gravity of urine elicited by the tremendous volume of urine produced after frusemide treatment. The effect of frusemide was the same with or without salicylate.

Plasma Pharmacokinetics

The graph of plasma concentration plotted against time (Fig.5.1.) illustrates that frusemide plasma concentrations decline as a biexponential function. The data represents the mean ± s.d. from three horses and indicates that frusemide plasma concentrations are not significantly affected by salicylate co-administration. During the first 4 h the plasma-level time curves with and without salicylate are very similar, however after this time it appears that frusemide is present in plasma for longer when salicylate is given. Frusemide levels were slightly higher and detectable for longer i.e. up to 10 h in one horse.

Table 5.1. shows the pharmacokinetic parameters for frusemide in the presence and absence of salicylate, and indicates that there are no significant differences between the two treatments. The elimination rate constant (K) is slightly smaller when salicylate is given, resulting in a mean half-life of 66 min compared with 54 min when frusemide is given alone, but this does not reach statistical significance. A number of parameters, namely the elimination rate constant (K), the elimination half-life ($t_{1/2\beta}$), and volume of distribution (V_d) all show a greater degree of variability when salicylate is co-administered. Due to the variability between the three horses, averaging the data results in very little difference between the plasma clearance and AUC values following frusemide or frusemide + salicylate.

Urinary Excretion

Plots of frusemide urinary concentration against time for each of the three horses are presented in Fig.5.2. The urinary excretion pattern is very similar for the

Fig.5.1. Plasma concentration of frusemide in the horse, following frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + salicylate (40 mg/kg; p.o.) on separate occasions

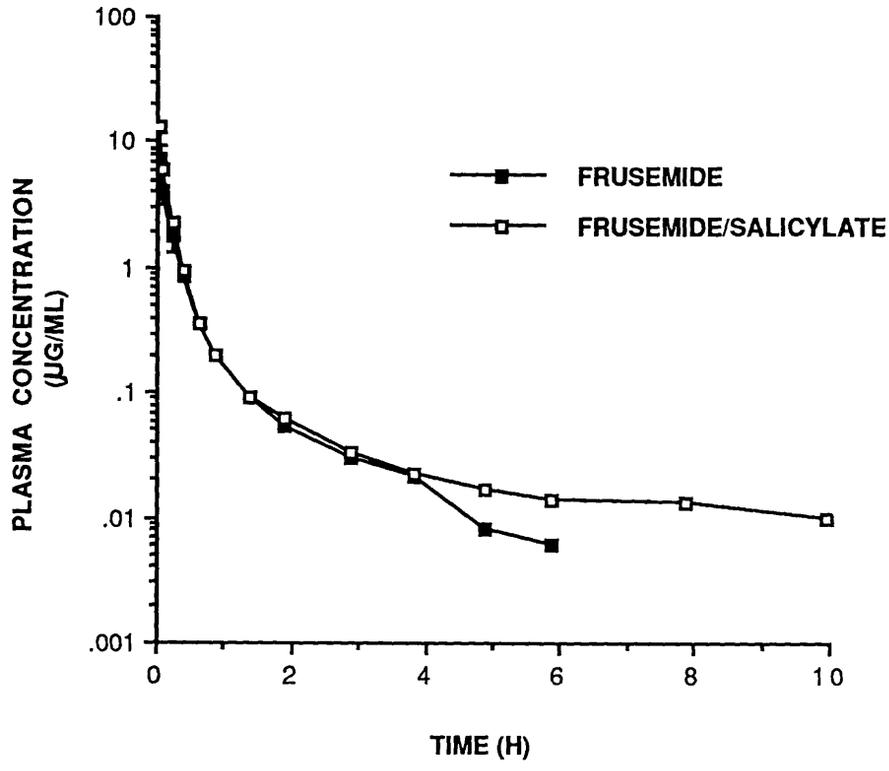
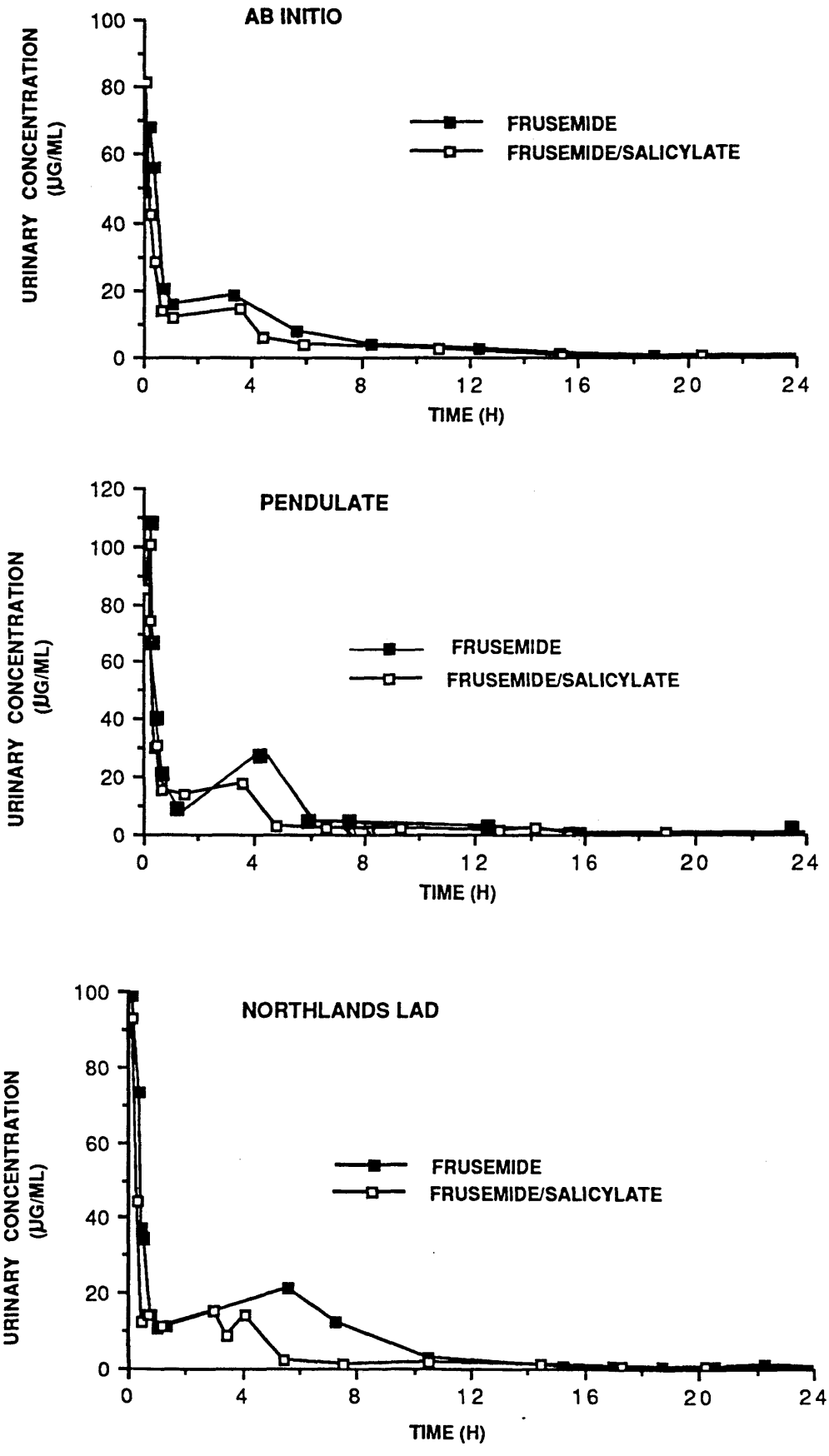


Table 5.1. Pharmacokinetic parameters describing the plasma levels of frusemide in the horse, following the administration of frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + [^{14}C]-salicylate (40 mg/kg; p.o.) on separate occasions (mean ± s.d., n=3)

		Frusemide	Frusemide + Salicylate
K	(h^{-1})	0.795 ± 0.164	0.760 ± 0.372
$t_{1/2\beta}$	(h)	0.897 ± 0.183	1.101 ± 0.636
V_d	(l/kg)	0.548 ± 0.185	0.718 ± 0.37
Cl	(l/h/kg)	0.415 ± 0.070	0.462 ± 0.025
AUC	(µg/ml.h)	2.46 ± 0.453	2.17 ± 0.123

Fig.5.2. Urinary concentration of frusemide in each of three horses given frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + salicylate (40 mg/kg; p.o.) on separate occasions



three horses following both treatments. Elimination of frusemide is very rapid, urinary drug concentrations within 7-10 min of dosing being as high as 90 μg/ml; however, after 1 h drug concentrations had fallen to approximately 15 μg/ml. The graphs show that after the initial marked diuresis there is a lag period before another sample is voided, allowing frusemide to accumulate in this sample, and resulting in a small peak in the curve. There are indications from the data that, in the presence of salicylate, frusemide is excreted more slowly. During the diuresis there is very little difference in the urinary concentrations of frusemide between the two treatments but from about 1 h onwards urinary levels of the diuretic are higher when it is given alone. Evidence that the excretion of frusemide is reduced after salicylate co-administration is given by plotting cumulative urinary excretion (Fig.5.3.). The percentage recovery of frusemide when given alone was 84.3 $\pm$ 9.4% and 75.9 $\pm$ 6.9% in the presence of salicylate. The graphs show that up to 30 min in two horses Pendulate (PE) and Northlands Lad (NL), and 45 min in the third horse Ab Initio (AI), the cumulative recovery is the same following both treatments accounting for approximately 60% in NL and AI and 70% in PE. Subsequently the urinary recovery in all 3 horses is greater when frusemide is given alone, this difference is maintained for the rest of the experiment and is most marked for NL (91.8% vs.74.1%).

Diuretic Effect

Intravenous administration of frusemide had a prompt diuretic effect which was not altered by salicylate. Fig.5.4. illustrates the cumulative urine volume data for each of the three horses following frusemide treament with and without salicylate. In two of the horses (AI and NL) the total volume voided is greater in the presence of salicylate. However, the disparity in urine volume is not apparent during the period of spontaneous voiding elicited by the diuretic which lasts for approximately 1 h. During this time the difference in urine volumes after the two separate treatments is negligible, but subsequently, i.e. from 3.5 h after frusemide the urine volume when salicylate is also given is greater such that at about 15 h post-dosing the difference in urine volume for AI is 2.3 l. This difference is maintained as the slopes of the two lines

Fig.5.3. Cumulative urinary excretion (0-6 h) of frusemide in each of three horses given frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + salicylate (40 mg/kg; p.o.) on separate occasions

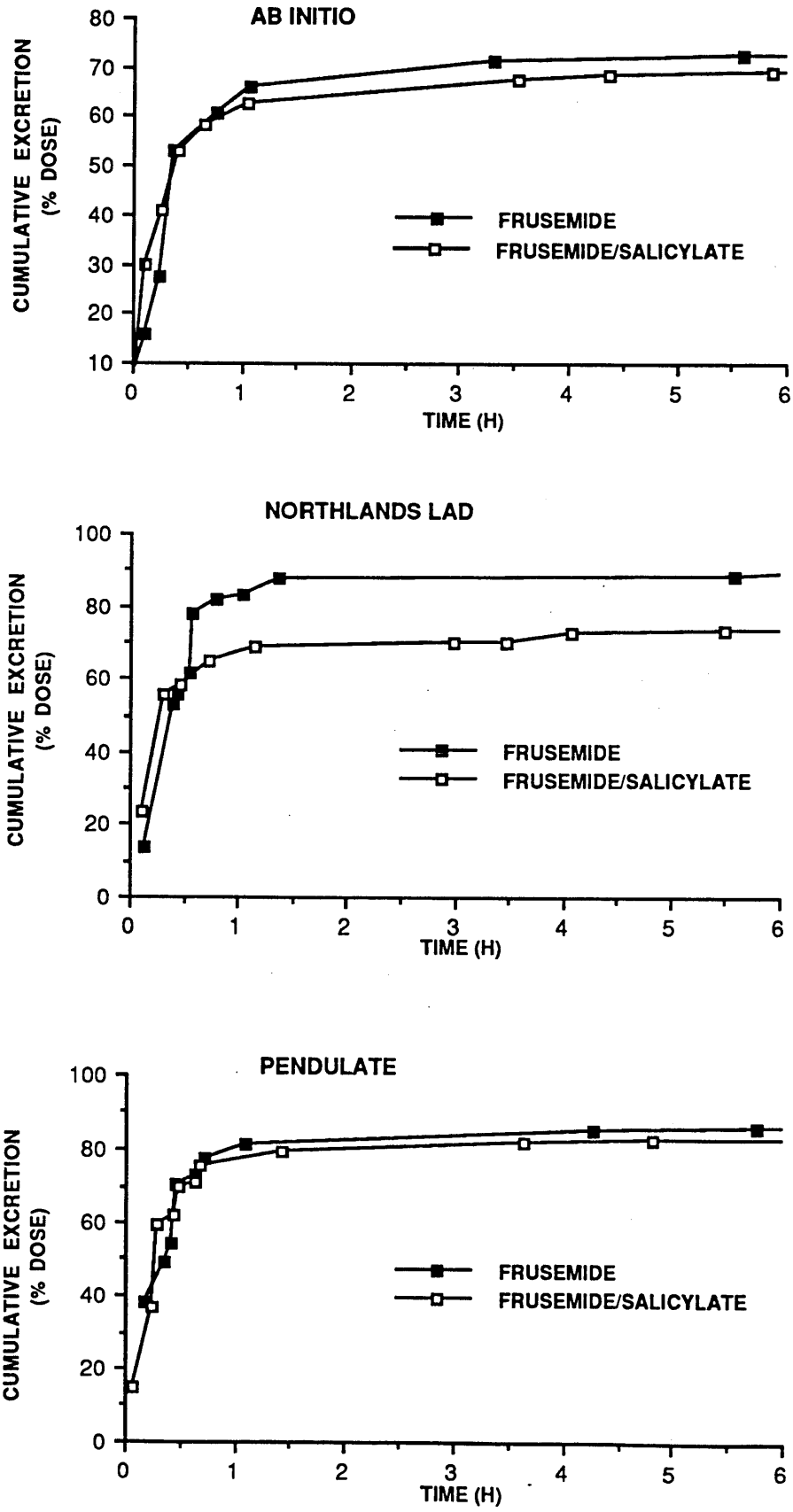
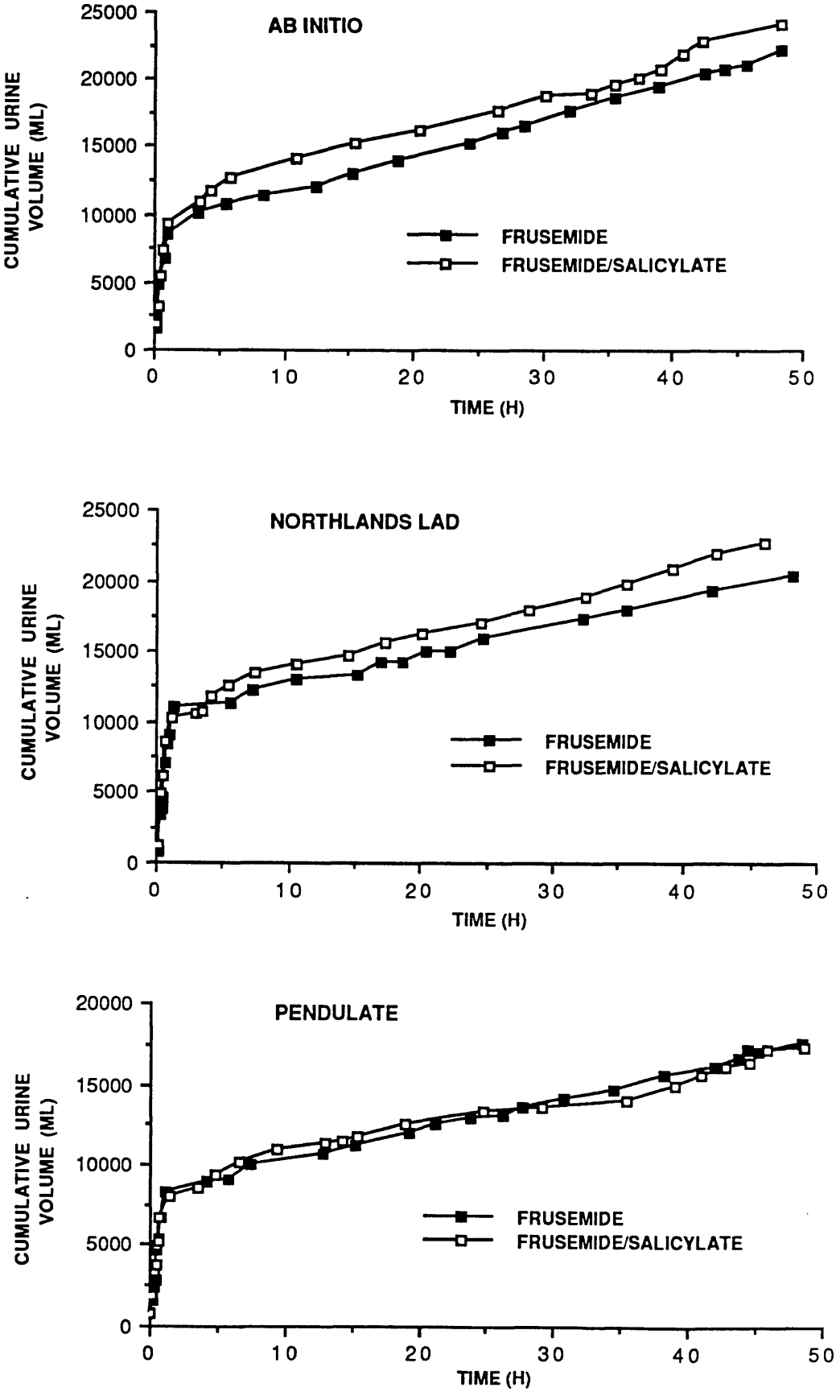


Fig.5.4. Cumulative urine volumes in each of three horses given frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + salicylate (40 mg/kg; p.o.) on separate occasions



corresponding to cumulative urine volume are essentially parallel. In the third horse (PE), the cumulative urine volume is very similar at all time points following either treatment.

The urine produced within 6 h following each of the three treatments is presented in Fig.5.5. This analysis of sequential 1 h or 2 h pooled urine samples from the three horses (mean ± s.d; n=3) after either salicylate, frusemide or salicylate + frusemide, shows that in the first hour frusemide induces a very potent diuresis with 7258 ± 980 ml of urine voided. Salicylate does not blunt this response at all, in fact there is a small increase in the volume of urine produced i.e. 7478 ± 975 ml. Between 1-2 h there is fractionally more urine produced by frusemide in the absence of salicylate. In the 2-3 h period, no urine is voided by any of the horses following frusemide or frusemide + salicylate. This is presumably an attempt by the horses to conserve fluid loss following the initial potent diuresis. Subsequently the volume of urine produced following the diuretic is similar to control, that is the amount voided when salicylate is given alone.

As expected, urinary flow rates increased dramatically and were very variable immediately after frusemide administration. Urinary elimination of frusemide was closely related to urine flow rate during these early time points (r^2=0.833) (Fig.5.6.). "Control" values of up to 10 ml/min for urine flow in the horse were obtained approximately 4 h after dosing.

Fig.5.5. Urine volume in sequentially pooled urine samples from horses given salicylate, frusemide and frusemide + salicylate on separate occasions (mean ± s.d; n=3)

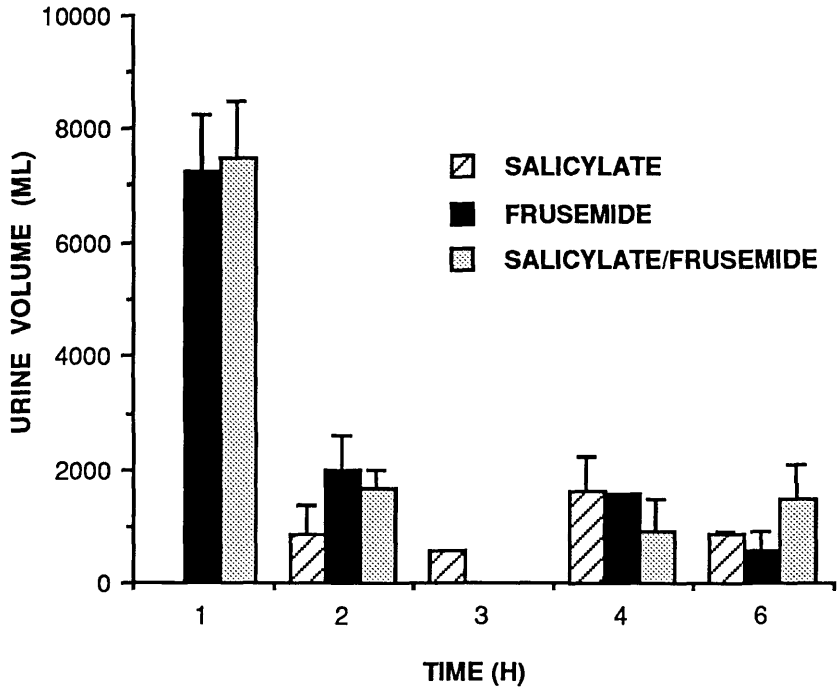
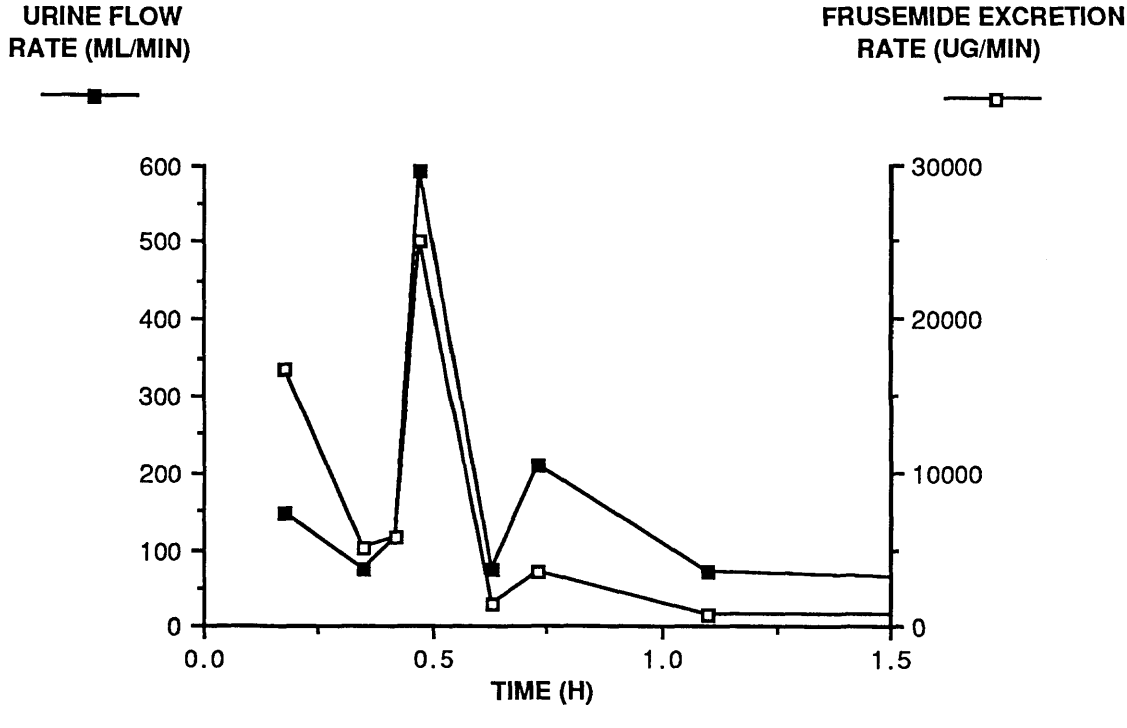


Fig.5.6. Correlation between urine flow rate and frusemide excretion rate in a horse given frusemide (1 mg/kg; i.v.)



5.4. DISCUSSION

Salicylic acid administration did not cause marked changes in the pharmacokinetic parameters of frusemide and the results also show that salicylate does not impair frusemide-induced diuresis and natriuresis in the horse. However, as already mentioned the primary purpose of this experimental protocol was not to define this interaction.

The majority of experiments looking at the effects of NSAIDs on loop diuretics have studied indomethacin, a potent prostaglandin synthetase inhibitor. These are concerned with the diuretic, natriuretic and renal haemodynamic effects of the NSAID on frusemide. There are only a few references in the literature which refer to the salicylate-frusemide interaction in animals and man (Berg 1977; Berg and Loew, 1977; Henry 1980; Bartoli *et al.* 1980; Planas *et al.* 1983).

A possible mechanism to explain how NSAIDs alter the diuretic and natriuretic response to frusemide may be a pharmacokinetic drug interaction. Frusemide, being a highly protein bound organic acid, gains access to its active site via the organic acid transport system (Deetjen 1966; Burg *et al.* 1973; Hirsch *et al.* 1975). Indomethacin and salicylic acid are similarly eliminated via this system and may competitively block this secretory pathway and thereby alter the diuretic response to frusemide. A pharmacokinetic interaction would affect two factors which determine overall response to frusemide, firstly the amount of drug delivered to the active site and secondly the time course of the delivery.

The present results on the pharmacokinetics of frusemide in the horse after concurrent salicylate administration indicate that salicylate has no effect on either frusemide plasma levels or pharmacokinetic parameters. The very similar plasma concentration time-curve for frusemide after salicylate differs from the results obtained by others investigating the effect of NSAIDs on diuretics who found elevated plasma concentrations in the presence of the NSAID (Chennavasin *et al.* 1980; Tempero *et al.* 1977). In this experiment it is only at later time points i.e. after 4 h that the salicylate appears to make a difference in that frusemide concentrations are slightly higher.

However, these results do not achieve statistical significance and also at these later time points frusemide concentrations are below 20 ng/ml.

A likely reason for not observing a change in the plasma level-time curve is that the two drugs were administered within 10 min of each other in this experiment, the salicylate orally and frusemide intravenously. Table 4.1. reveals that the time to reach peak plasma concentration (T_{max}) for salicylate is 1.67 ± 0.3 h and slightly longer, 2.37 ± 0.7 h in the presence of the diuretic. Consequently it is unlikely in these circumstances that salicylate will influence frusemide plasma concentrations as after intravenous administration frusemide is very rapidly cleared from the plasma, such that its pharmacological actions are over while salicylate is still being absorbed from the gut.

In contrast, in the study by Chennavasin *et al.* (1980) eight normal volunteers ingested 100 mg of indomethacin the night before and 100 mg on arising the morning of the study (30-60 min before frusemide 20 or 40 mg; i.v.). Indomethacin significantly increased the serum concentration of frusemide after 60 min resulting in marked changes in a number of pharmacokinetic parameters including a prolonged elimination half-life for frusemide. However, the effect of indomethacin on the kinetics of frusemide did not change the total amount of drug or the time course of its delivery to the active site. This indicates that the lag period between the two drugs is an important factor, more critical than the actual dose size. In our study the dose of salicylate was considerably higher (40 mg/kg; p.o.) than that of Chennavasin *et al.* (1980) and the frusemide dose was approximately double (1 mg/kg; i.v.). Brater (1979) using 20 or 40 mg doses of frusemide demonstrated that the effect of indomethacin differed with dose of frusemide and implied a dose-dependent interaction, this emphasises the need for care in choosing the diuretic dose in further studies of this nature.

A third factor which determines overall response to the diuretic is the dose-response relationship of frusemide. Chennavasin *et al.* (1980) found that the inhibitory action of indomethacin on the effect of frusemide was caused primarily by a change in the dose-response relationship, i.e. a pharmacodynamic rather than a pharmacokinetic interaction. The curve of serum concentration vs. response was significantly shifted to

the right, in contrast, the urinary frusemide-response curves did not shift. This confirms that the pharmacokinetic interaction by blocking the renal secretion of frusemide disrupts the relationship between the amount of drug in serum and urine.

The current study shows that immediately after frusemide administration a strong diuresis ensues and this is not impaired by salicylic acid. As already mentioned this is presumably due to salicylate still being absorbed from the gut during this time and so it does not exert an inhibitory effect on frusemide excretion. Also MacKenzie *et al.* (1974) have shown that frusemide can slow or even reverse the absorption of water from the gut so this may further delay salicylate absorption. As discussed in Chapter 4, frusemide has the potential for high affinity binding with the receptor site on the carrier of the organic anion transport system, while salicylate can achieve only low affinity binding (Möller and Sheikh, 1983). Therefore frusemide itself can impede salicylate excretion, but this competitive blockade is short-lived due to frusemide's own rapid elimination.

In spite of this both the frusemide urinary concentration and the cumulative excretion data show that at later time points salicylate does appear to exert an effect on frusemide excretion. As this is at approximately 1 h post-frusemide dosing and salicylate was given before, it is feasible that some salicylate is being eliminated via the transport system and this is slowing frusemide elimination. However, by this time the majority of the frusemide (ca. 70%) has already been eliminated. Thus salicylate is acting on a very small proportion of frusemide molecules such that no blunting of the diuretic or natriuretic effect was observed.

In this study it is not possible to say what role sodium levels played in the NSAID and loop diuretic interaction as the horses received their usual diet, they were neither sodium loaded nor sodium restricted during the course of the experiment. However it is known that the sodium concentration of hay is very low and so it is strictly regulated by the horse, consequently normal excretion is low (1-2 mmol/l) (Tasker 1967). It has been suggested that sodium balance significantly influences this interaction. Nies *et al.* (1983) showed in dogs that indomethacin impaired the natriuretic effects of frusemide in salt-depleted but not in salt-loaded dogs. Similarly Berg (1977)

found that acetylsalicylic acid exerted the most pronounced effects on urinary sodium excretion when the sodium excretion on the preceding control day was low. In a recent study Herchuelz *et al.* (1989) reported that a modest degree of sodium restriction was required to detect the existence of an interaction between torasemide and indomethacin in young healthy volunteers. However, there are studies where an interaction has been observed in volunteers on a normal sodium diet (Patak *et al.* 1975; Frolich *et al.* 1976; Brater 1979; Bartoli *et al.* 1980). It is conceivable that sodium restriction enhances the expression of the interaction and that its detection or extent is dependent on the actual sodium balance of each individual subject.

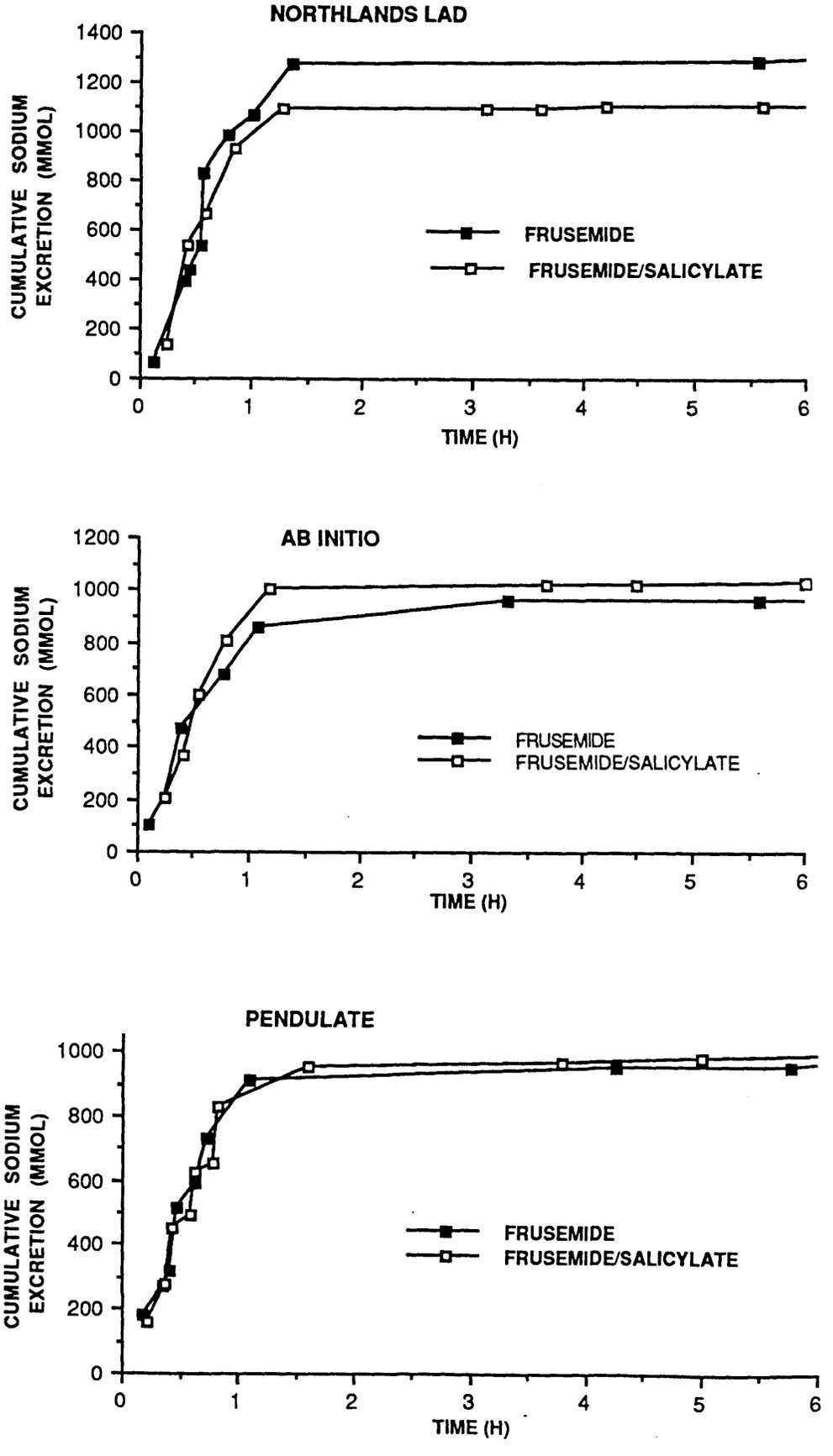
A few studies in man have shown marked effects of salicylate on frusemide diuresis. Henry (1980) found that urine output following aspirin + frusemide was significantly less after 2 h than either diflunisal + frusemide or placebo + frusemide. He stated that the mechanism may be related to competition of tubular excretion or to inhibition of synthesis of intrarenal prostaglandins. In another study Bartoli *et al.* (1980) found that changes in frusemide clearance as a result of aspirin administration were correlated with simultaneous changes induced in the diuretic response to frusemide. They concluded that the blunting of the diuretic effect of frusemide by aspirin is due to inhibition of tubular secretion rather than inhibition of PG synthesis. Their evidence against PGs playing a role was that the blunting of the diuretic action should have been greater with increased doses of aspirin, but the opposite effect was observed. The NSAID-diuretic interaction is important in the clinical situation (Patak *et al.* 1975), it is also of particular interest in racehorses due to the increased use of these drugs in recent years. In the present study it is difficult to draw any definitive conclusions on the salicylate-frusemide interaction, as data was obtained from only three horses. It was not practical to repeat the experiment under optimal conditions due to the species used. Obviously the horse is not an ideal experimental animal for metabolism studies mainly as a result of its size. They also require considerable manpower during experimental periods, as well as special facilities such as metabolism stalls and equipment for the collection of biological samples. Consequently it was not possible to use them in large numbers, however, each horse was used as its own

control so that intra-animal variation was eliminated, but there was considerable inter-horse variability in this experiment. For example cumulative sodium excretion data indicated that in one horse (NL) sodium excretion was reduced in the presence of salicylate, in another (AI) excretion was slightly greater in the presence of salicylate and there was very little difference between the two treatments in the third horse (PE) (Fig.5.7.). It was not possible to do a statistical analysis on the urinary data, so it is difficult to comment upon the validity of the differences observed between the two treatments.

The results presented in this preliminary study provide a basis for the design of a more detailed experiment to determine whether the NSAID-diuretic interaction is simply an acid-acid interaction, or whether inhibitors of PG synthesis affect the response to diuretics in the horse. Our data indicates that a number of factors are likely to be critical in determining an effect of NSAID treatment on diuretic response. In a future protocol the dose of aspirin can safely be increased as the recommended therapeutic dose for horses is 30-100 mg/kg bodyweight (Robinson 1983). Thus the dose used in the present study (40 mg) was at the lower end of this range. Ideally aspirin should be administered orally and intravenously at a lower dose, on separate occasions.This will help to determine the role of absorption in the interaction between the two drugs. Another important factor is the time of administration of the two drugs. From previous results it appears that a lag period is essential. Concomitant administration may give rise to false negative results, due to frusemide's short-lasting pharmacological effect and its very rapid elimination. To have the best chance of observing an interaction the NSAID should be administered as a "loading dose" some hours before frusemide and again approximately 1 h before the diuretic in a manner similar to that of Chenavasin *et al.* (1980).

Studies of this kind will lead to an increased understanding of the NSAID-diuretic interaction in racehorses, which is of particular interest due to the advent of controlled medication programmes and the widespread use of these classes of drugs in recent years.

Fig.5.7. Cumulative urinary excretion (0-6h) of sodium in each of three horses following frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + salicylate (40 mg/kg; p.o.) on separate occasions



Chapter 6

THE FRUSEMIDE-PROBENECID INTERACTION IN THE HORSE

6.1. INTRODUCTION

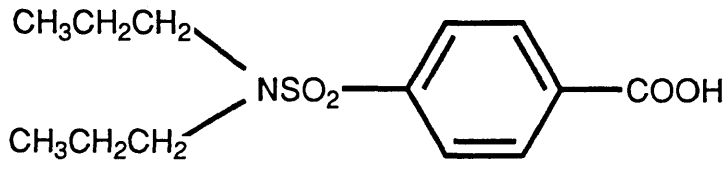
The kidney is responsible for the removal of endogenous and exogenous compounds from the body. Many compounds of very different chemical structure are substrates of the organic anion transport system of the kidney, since substrates exhibit several interactions with the hydrogen receptor site on the carrier. These include binding of an aromatic group, hydrogen bonding via substrate carbonyl groups, electrostatic interactions and unspecific hydrophobic interactions (Möller and Sheikh, 1983). Substances secreted include hippurates, benzoates, sulphonamides and heterocyclic carboxylates. Pharmacologically active compounds include diuretics, salicylate, probenecid, phenylbutazone and antibiotics with a penicillic acid nucleus; these compounds are often excreted after conjugation with glycine, sulphate or glucuronic acid. Tubular secretion often potentiates the pharmacological effect of drugs with an intratubular site of action e.g. diuretics and uricosurics.

The secretion of organic anions is completely separate from that of organic cations which have an independent transport mechanism within the kidney (Peters 1960; Rennick 1981). These transport systems are mutually exclusive, i.e. inhibition or stimulation of one system does not affect the other. Renal secretion of organic anions is confined to the proximal tubule, where the anion system is thought to be composed of several different subsystems which have a spectrum of overlapping specificities, thus a substrate may be transported by more than one subsystem.

Much of our present knowledge about the renal secretory system is based on studies with p-aminohippurate. This has been used as a model to study the properties of the organic anion transport mechanism because it is efficiently secreted, undergoes limited reabsorption in most species, and also because it is only weakly bound by plasma proteins, and is easy to determine chemically. Probenecid is another representive substrate of the system (Weiner 1960), as a selective competitor which has a central place in the detection of active transport of anions due to its inhibitory potency.

Probenecid ((p-[dipropylsulphamoyl]benzoic acid); Fig.6.1.) is a highly lipid-soluble benzoic acid derivative (pK_a 3.4). It was first synthesized in 1951 as a result of

Fig.6.1. Structure of probenecid



PROBENECID

(p-[dipropylsulphamoyl]benzoic acid)

a project directed towards the production of non-toxic compounds that would inhibit the secretion of penicillin by the kidney tubules. When penicillin was first introduced it was very much in demand and in extremely short supply, thus the rapid excretion of the antibiotic was of practical significance. Probenecid was found to depress the tubular secretion of penicillin, thus reducing its concentration in urine and increasing plasma concentrations sometimes up to fourfold. It is therefore used as an adjunct to penicillin therapy in conditions where very high or prolonged concentrations of penicillin are needed. Hence, competitive inhibition may be regarded as the basis of probenecid's action on renal secretory transport.

Probenecid is also a potent uricosuric agent; it promotes excretion of urates by inhibiting tubular reabsorption, which results in a lowering of the elevated concentrations of uric acid in plasma. In the past it has been routinely used in the treatment of chronic gout and to prevent hyperuricaemia during treatment with diuretics of the thiazide or similar type (Freis and Sappington, 1966; Smilo *et al.* 1962).

Frusemide is extensively bound to plasma proteins, so glomerular filtration of this diuretic is limited and tubular secretion, as opposed to filtration is the principal determinant of frusemide's access to the lumen and its subsequent excretion. Tubular secretion of frusemide was first demonstrated in a micropuncture study in the rat kidney by Deetjen (1966) when investigating the site and mode of its diuretic action. Others showed by clearance methods that frusemide was secreted by the kidney of the intact animal (Calesnick *et al.* 1966; Gayer 1964). Preliminary results by Deetjen (1966) also suggested that there was competitive inhibition of frusemide transport by other acids. The involvement of the organic anion transport pathway in the excretion of frusemide was indicated by Hirsch *et al.* (1975) while working on kidney slices from rabbits, dogs, mice and rats. They showed that renal cortical slices are capable of accumulating frusemide from the incubation medium, by a metabolically dependent active transport mechanism.

Experiments on the renal secretion of [^{35}S]-frusemide by Bowman (1975) show that tubular secretion accounts for essentially all frusemide clearance by the isolated perfused rat kidney and this secretion is inhibited by probenecid. The binding

of frusemide to proteins and specifically to albumin has been reported to be high (> 95%) in all the species so far studied including the horse (Roberts *et al.* 1978). Bowman (1975) has shown that, in the absence of albumin, tubular clearance of frusemide is considerably increased, so that plasma protein binding seems to reduce frusemide secretion. At physiological albumin concentrations, binding of frusemide appears to play a significant role in determining the rate at which frusemide is secreted, the rate being dependent on the availability of unbound drug.

Any drug or chemical substance that competes for the renal organic anion transport system may prevent frusemide from reaching its site of action and thereby attenuate its diuretic response. Frusemide is actively secreted by the renal tubules in the hen. This secretion probably involves an anion transport system since a diminution of frusemide's effect has been demonstrated by three structurally different inhibitors of this system, namely novobiocin, iodipamide, and o-iodohippurate (Odlind 1979). Probenecid pretreatment has also been shown to alter the diuretic response of frusemide. Honari *et al.* (1977) were the first to examine whether probenecid interferes with frusemide elimination kinetics and diuretic action in man. The effect of other diuretics with probenecid has been studied namely, the chlorothiazide-probenecid and bumetanide-probenecid interactions (Brater 1978a; Friedman and Roch-Ramel, 1977).

It is predictable that probenecid will reduce frusemide delivery to the luminal side of renal tubules thereby reducing the natriuretic action of frusemide. This effect has been confirmed in the dog (Hook and Williamson, 1965), cat (Friedman and Roch-Ramel, 1977) and rat (Senft 1965). However studies in man on the effect of probenecid on diuresis and natriuresis have resulted in contradictory results (Homeida *et al.* 1977; Honari *et al.* 1977; Brater 1978b; Chennavasin *et al.* 1979; Smith *et al.* 1980; Andreasen *et al.* 1980). All these studies were carried out in healthy volunteers, while Hsieh *et al.* (1987) have evaluated the frusemide-probenecid interaction in hospital patients.

Frusemide and Doping

As described in Chapter 1, the IOC have categorized a number of doping classes and doping methods. These include blood doping and pharmacological, chemical and physical manipulation. The IOC Medical Commission has banned the use of substances and techniques which alter the integrity and validity of urine samples used in doping controls. Examples of banned methods are catherisation, urine substitution and/or tampering and inhibition of renal excretion, e.g. with probenecid or related compounds. Due to probenecid's ability to inhibit the renal excretion of other drugs and so hinder their detection in urine, probenecid has come to be regarded as a "masking agent", and its use among sportsmen has increased in recent years. In 1987-88 probenecid was identified in urine samples on 43 occasions by IOC-accredited laboratories, having not been reported in previous years. In 1987 five athletes were found to have very low levels of androsterone (Geyer 1989). It was shown that probenecid administration had decreased the excretion rate of this steroid. Four hours after probenecid the urinary concentration decreased from 3000 ng/ml to 100 ng/ml, and the very weak diuretic effect of probenecid resulted in further dilution of steroid in the urine.

There is limited information on probenecid in the horse; Gronwall and Brown (1988) have studied probenecid's effect on *p*-aminohippurate clearance and Jouany *et al.* (1988) have looked at the interference by probenecid on nandrolone urinary excretion in this species. They reported that probenecid significantly increased the urinary elimination of the estranediol metabolite of the anabolic steroid. Their findings are contradictory to the so-called "masking effect" of probenecid.

To the best of our knowledge, there are no reports in the literature on a possible frusemide-probenecid interaction in the horse. As such this study was undertaken to elucidate the mechanism of action of the potential pharmacokinetic and pharmacodynamic interaction of these two drugs.

6.2. METHODS

Experimental Protocol

Details of the experimental procedures are described in Chapter 3. Briefly one thoroughbred horse received two different treatments. Blood and urine were collected at various times after dosing and the ^{14}C content determined. Any additional information is described below.

In this study one horse received 2 treatments in successive dosing sessions, separated by 1 week.

Treatments

1. [^{14}C]-Frusemide (1 mg/kg; 100 μCi) intravenously administered via the jugular vein.
2. Probenecid (15 mg/kg) orally followed 1 hour later by [^{14}C]-frusemide (1 mg/kg; 100 μCi) intravenously.

Compounds

[7-^{14}C]-Frusemide, specific activity 30.81 μCi/mg; radiochemical purity >97% by TLC and HPLC (Hoechst, Frankfurt, F.R.G.). Frusemide (4-chloro-5-sulphamoyl N-furfurylanthranilic acid) (Lasix 5% solution; Hoechst, Milton Keynes, Bucks. U.K.). Probenecid (p-(dipropylsulphamoylbenzoic acid) (Sigma, Poole, Dorset, U.K.).

Dose Preparation and Administration

Probenecid (7 g) was added to water and then sodium hydroxide added dropwise until it dissolved. The final dose volume was 500 ml (pH 9.5), and was administered via a stomach tube.

Pharmacokinetic Methods

Pharmacokinetic parameters were calculated by least-squares regression analysis on an Apricot Xi 10 computer.

Peak plasma concentration, C_{max}, was obtained by determination of graphical data. The terminal elimination rate constant β, was calculated from the least squares regression line of the terminal log-linear portion of the plasma concentration/ time curves. The elimination half life, $T_{0.5\beta}$, is given by equation (1):

$$T_{0.5\beta} = \ln 2/\beta \qquad (1)$$

The area under the plasma concentration/time curve (AUC), was calculated by the trapezoidal method. The volume of distribution, (V_d), was determined from equation (2):

$$V_d = \text{Dose}/\beta.\text{AUC} \qquad (2)$$

The apparent plasma clearance (Cl_p) of radioactivity was calculated from equation (3):

$$Cl_p = \text{Dose}/\text{AUC} \qquad (3)$$

Renal clearance (Cl_r) of frusemide was determined by the equation:

$$Cl_r = U_{6h}/\text{AUC} \qquad (4)$$

where U_{6h} is the frusemide excreted unchanged in urine and AUC is the area under the plasma concentration-time curve to infinity ($\mu g\ h\ ml^{-1}$).

Nonrenal clearance (Cl_{nr}), was determined by the equation:

$$Cl_{nr} = Cl_p - Cl_r \qquad (5)$$

The contribution of glomerular filtration to the renal clearance of frusemide in horses can be calculated from equation (6):

$$Cl_r' = Cl_{GFR}(1-p) \qquad (6)$$

where Cl_r' = renal clearance due to glomerular filtration,

Cl_{GF} = glomerular filtration rate in horses. This is estimated from creatinine clearance which is approximately 1.48 ml/min/kg (Gronwall 1985).

p = fraction of drug bound to plasma proteins, assumed to be 95% (Roberts *et al.* 1978).

6.3. RESULTS

The results obtained after intravenous administration of frusemide have been described in Chapter 3. This study will refer to them only to compare the effects of probenecid on diuresis and natriuresis and the possible pharmacokinetic interaction of probenecid and frusemide. As there was only one administration of each treatment to a single horse, no account can be made of the statistical significance of these observations.

Frusemide Pharmacokinetics

Following probenecid pretreatment, frusemide plasma concentration declined as a biexponential function (Fig.6.2.). Plasma concentrations of frusemide with probenecid were higher at all time points, the C_{max} being 6128 ng/ml for frusemide alone and 7299 ng/ml for frusemide + probenecid. Determination of ^{14}C-activity (almost all unchanged frusemide) was not possible later than 10 h after frusemide dosing but with probenecid, ^{14}C was detectable in the 12 and 24 h plasma samples. Table 6.1. shows the pharmacokinetic parameters for frusemide in the presence and absence of probenecid. There is a decrease in the plasma clearance of frusemide after the oral administration of probenecid together with an increase in the volume of distribution. These differences are reflected in the considerably increased elimination half-life of frusemide (1.61 h vs. 3.48 h). The increase in AUC after probenecid is in agreement with the observation of higher plasma concentrations of frusemide.

The amount of frusemide excreted per minute in urine was well correlated with the plasma frusemide concentration when the diuretic was given alone (r^2=0.949; Fig.6.3a.). The solid line represents the line of best fit through the data points. However when probenecid was coadministered, a very poor correlation coefficient was obtained (r^2=0.112) due to the variable and massive excretion rate in the presence of the competitive inhibitor (Fig.6.3b.), the straight line is the line of best fit.

Table 6.2. shows the differences in clearance values (expressed as ml/min) in the presence of probenecid. The change in total plasma clearance was due to a reduction

Fig. 6.2. Plasma concentration of 14C radioactivity following [14C]-frusemide (1 mg/kg; i.v.) and [14C]-frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) administration to one horse

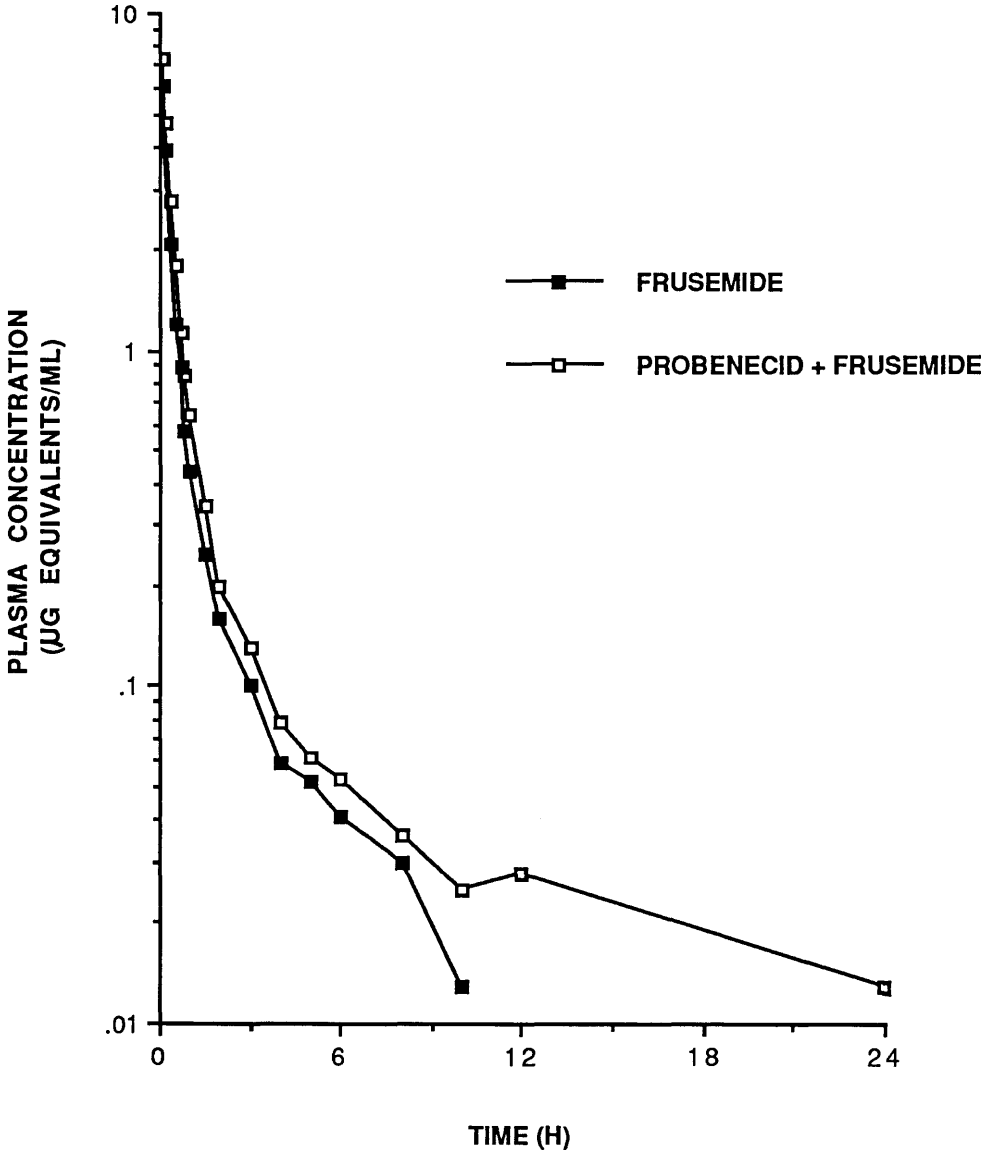


Table 6.1. Pharmacokinetic parameters describing the plasma levels of frusemide following the administration of [^{14}C]-frusemide (1 mg/kg; i.v.) and [^{14}C]-frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) to one horse on separate occasions

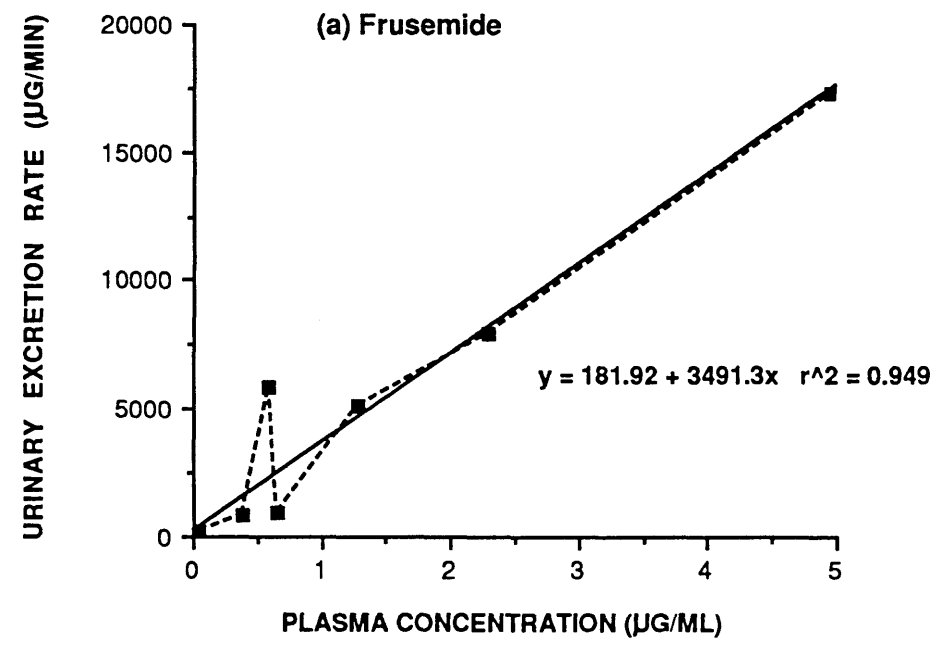
	F	F + P
C_{max} (μg/ml)	6.128	7.299
β (h^{-1})	0.431	0.199
$t_{0.5\beta}$ (h)	1.608	3.476
V_d (l/kg)	0.803	1.259
Cl (l/h/kg)	0.346	0.251
$AUC_{(trap)}$(μg/ml.h)	2.89	3.99

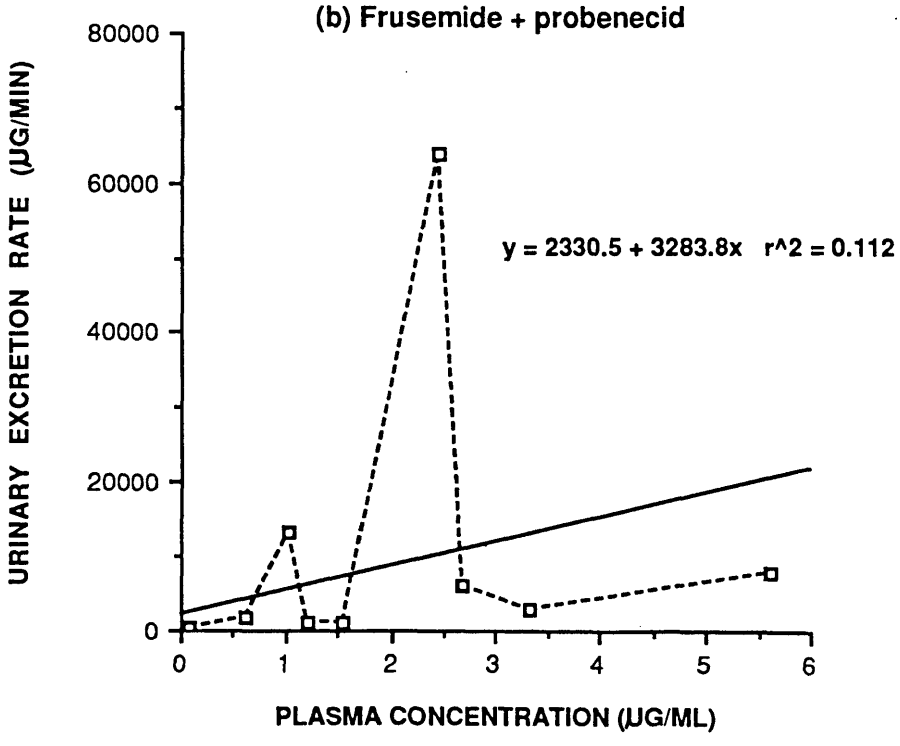
Table 6.2. Clearance (ml/min) values for frusemide following the administration of [^{14}C]-frusemide (1 mg/kg; i.v.) and [^{14}C]-frusemide (1 mg/kg;i.v.) + probenecid (15 mg/kg; p.o.) to one horse on separate occasions

		F	F+P	% change
Plasma clearance	(Cl_p)	2679	1944	27
Renal clearance	(Cl_r)	1816	1352	25
Nonrenal clearance	(Cl_{nr})	863	592	31

Cl_p = Dose/AUC $Cl_r = U_{6h} / AUC$ $Cl_{nr} = Cl_p - Cl_r$

FIG.6.3. **Relationship of the urinary rate of frusemide excretion and plasma concentration following (a) frusemide (1 mg/kg; i.v.) and (b) frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) in one horse**





in both the renal and nonrenal clearances of frusemide in the presence of probenecid. From the equations above, the glomerular filtration rate of frusemide in horses is approximately 34 ml/min which accounts for only 1.89% and 2.55% of the total renal clearance for frusemide alone and frusemide with probenecid respectively. However, the fraction of frusemide excreted unchanged in the urine was very similar after the two treatments, 69.13% (frusemide alone) and 70.46% (frusemide + probenecid).

Diuretic Effect

Frusemide had an immediate and short lasting diuretic effect, which was not altered by the administration of probenecid. However probenecid's presence resulted in a greater number of urine samples being produced due to small volumes being voided (8 samples within 1 h compared with 5 samples with frusemide alone). This resulted in the urinary flow rate also being very much more variable in the presence of probenecid. A huge urinary flow rate of 1214 ml/min was obtained 0.4 h after frusemide (Fig.6.4.), subsequent flow rates after the diuresis i.e. after 1 h were 2-9 ml/min. During the period of spontaneous voiding elicited by the diuretic 10222 ml of urine was produced after frusemide alone compared to 8280 ml with probenecid. This difference is maintained as later the slopes of the two lines corresponding to cumulative urine volume are essentially parallel (Fig.6.5.). Table 6.3. indicates that the greatest difference in urine volume is at 0.5 h i.e. there is a 32% decrease in the volume of urine produced when probenecid is given. At subsequent time points the % change in urine volume between the two treatments is very much less.

The intense diuresis due to frusemide administration resulted in a rapid fall in specific gravity and creatinine concentrations. Urinary pH values were alkaline during the course of both treatments, slightly more so after probenecid.

Plots of urine concentration against time (Fig.6.6.) indicate that after the initial marked diuretic response there is a lag period of 3-4 h before another sample is produced. Frusemide consequently becomes concentrated in the urine, resulting in a high concentration of the drug in the first urine sample produced after the initial diuresis. Thus 9.5% of the dose was eliminated in the sample produced at 5.50 h after

Fig. 6.4. Variation in urine flow rate following frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) in one horse

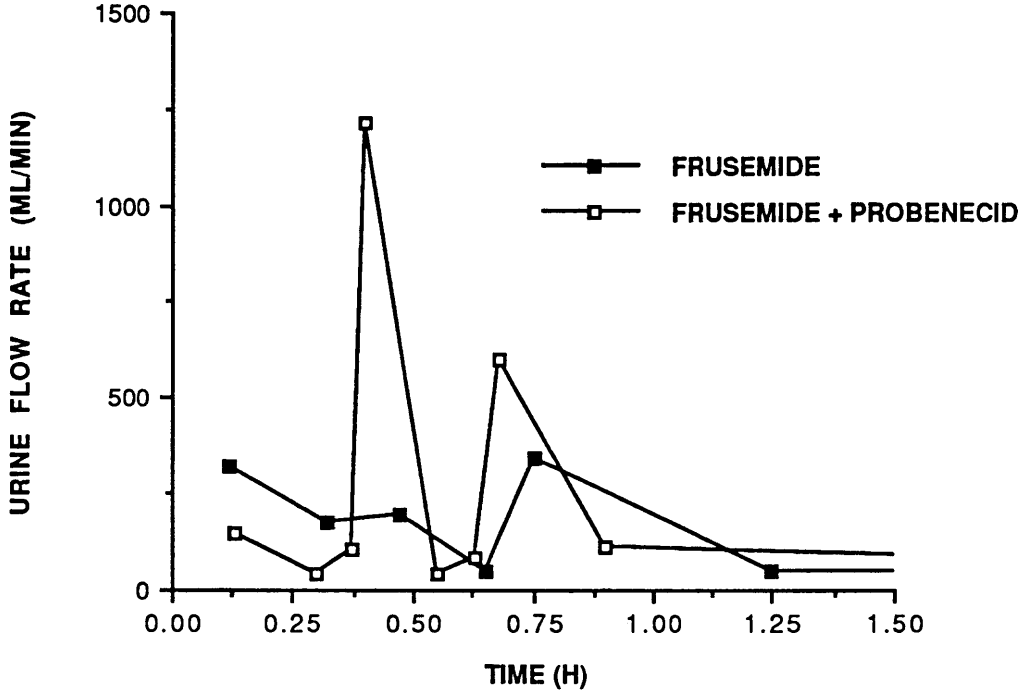


Fig.6.5. Cumulative urine volume following frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) to one horse

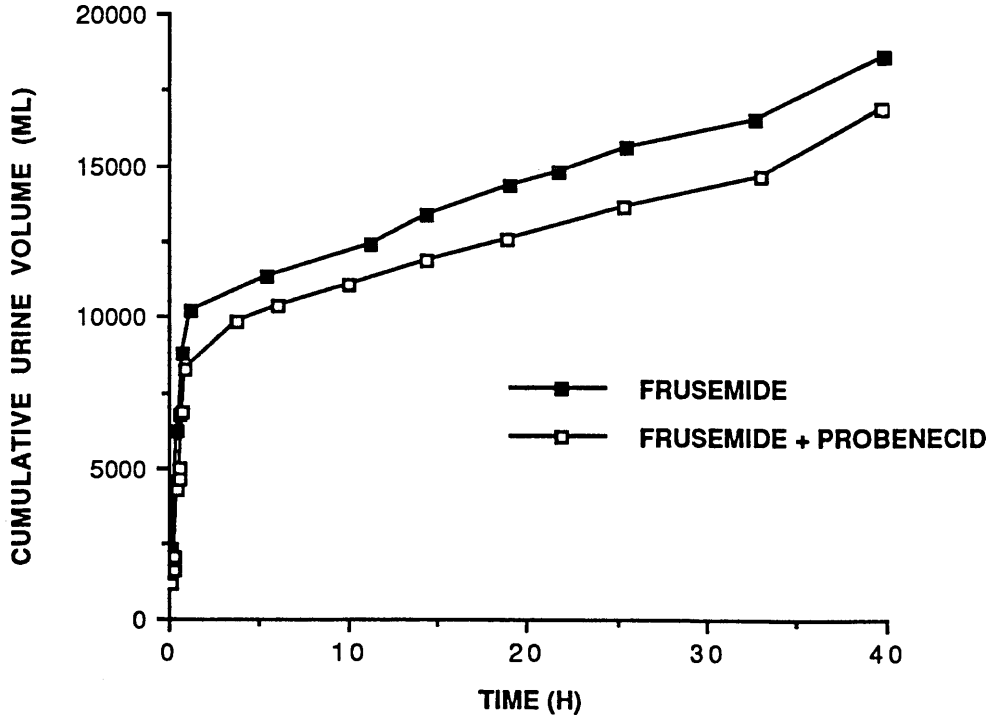


Table 6.3. 6 h Cumulative urine volume and sodium excretion following the administration of [^{14}C]-frusemide (1 mg/kg; i.v.) and [^{14}C]-frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) to one horse on separate occasions

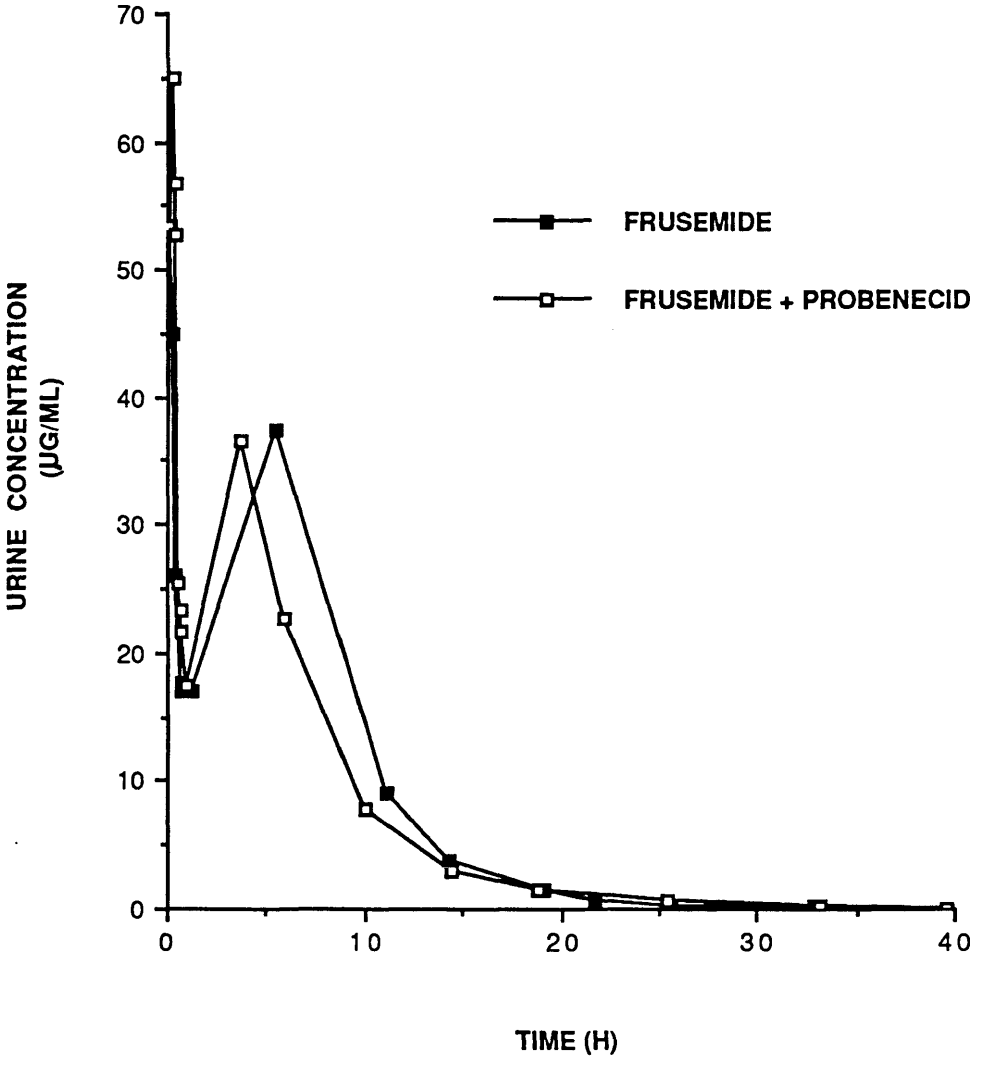
Urine Volume

	F	F + P	
Time after Frusemide	Urine vol. (ml)	Urine vol. (ml)	% Decrease
0.5	6225	4225	32.1
1.0	8842	8280	6.3
2.0	10222	9860	3.5
6.0	11482	10425	2.9

Sodium Excretion

	F	F + P	
Time after Frusemide	Sodium excretion (mmol)	Sodium excretion (mmol)	% Decrease
0.5	819	478	41.6
1.0	1127	888	21.2
2.0	1276	1021	19.9
6.0	1359	1047	22.9

Fig.6.6. Urinary concentrations of 14C following [14C]-frusemide (1mg/kg; i.v.) and [14C]-frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) administration to one horse



frusemide alone, and 12.5% in the urine sample voided at 3.72 h with probenecid. This concentration effect results in the distinct peak seen in the urine curve.

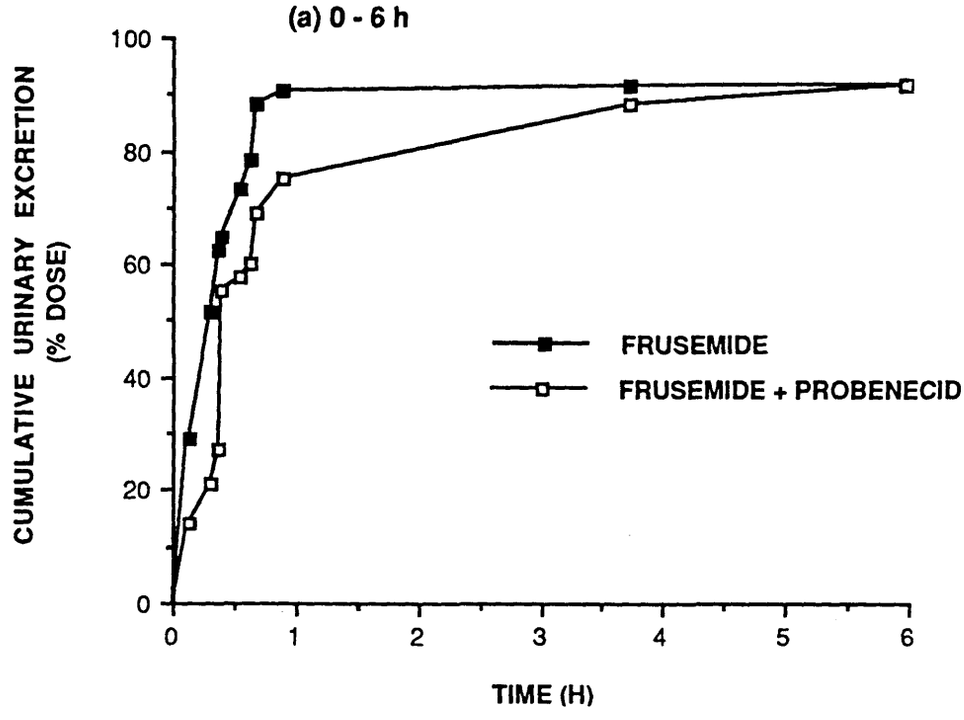
Plotting cumulative urinary excretion eliminates the effect of sample size and illustrates the delay in excretion of ^{14}C-radioactivity when probenecid is administered. Initially, the urinary excretion of total ^{14}C activity with probenecid was considerably lower than when [^{14}C]-frusemide was administered alone (Fig.6.7a.). However, at approximately 6 h the two curves intersect and subsequently the excretion of frusemide is slightly higher with probenecid, the cumulative recovery being 91.91% for frusemide and 93.76% for frusemide with probenecid (Fig.6.7b.). For the first few samples the ^{14}C-activity is almost all unchanged frusemide, the metabolite being detectable from 0.4 h onwards.

Natriuretic Effect

The effect of probenecid on frusemide-induced natriuresis is illustrated in Fig.6.8. The excretion of sodium closely mirrored urine volume and plotting cumulative data eliminates the influence of sample size. The initial natriuretic response to frusemide after probenecid pretreatment was reduced compared to that of frusemide administered alone. Table 6.3. shows the differences in sodium excretion at various time points and the % change when frusemide is given. Initially there is a large decrease (42%) in cumulative sodium recovery with probenecid. Subsequently this diference is reduced to approximately 21% and this is maintained for the rest of the experiment.

During the intense diuresis after frusemide administration the rate of urinary excretion of sodium was very variable. However, after this short period, the rate of excretion declined in parallel with frusemide plasma concentration and the rate of urinary frusemide excretion. Plotting plasma frusemide concentration vs. rate of urinary sodium excretion, and fitting a simple curve shows a linear relationship even though the points cluster towards higher plasma concentrations (Fig.6.9a.). The graph shows that probenecid pretreatment is associated with a shift to the right in the relationship between the plasma concentration of frusemide and its natriuretic effect. In contrast, the

Fig.6.7. Cumulative urinary excretion of 14C following [14C]-frusemide (1 mg/kg; i.v.) and [14C]-frusemide (1 mg/kg; i.v.) +probenecid (15 mg/kg; p.o.) administration to one horse (a) 0 - 6 h and (b) 0 - 36 h



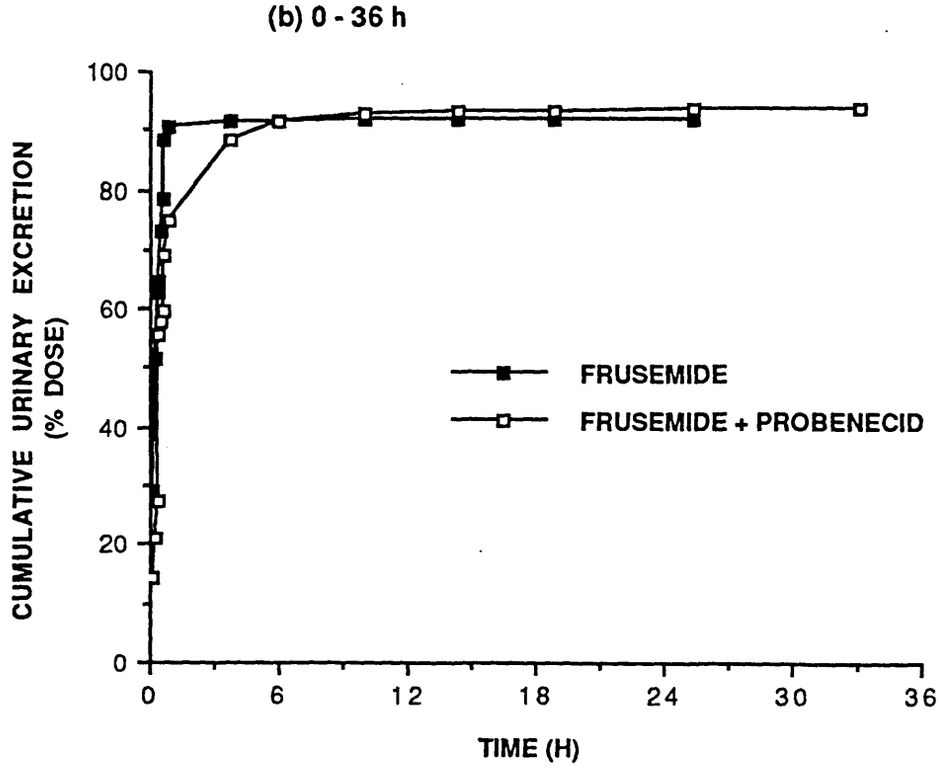


Fig.6.8. Cumulative sodium excretion following frusemide (1 mg/kg; i.v.) and frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) administration to one horse

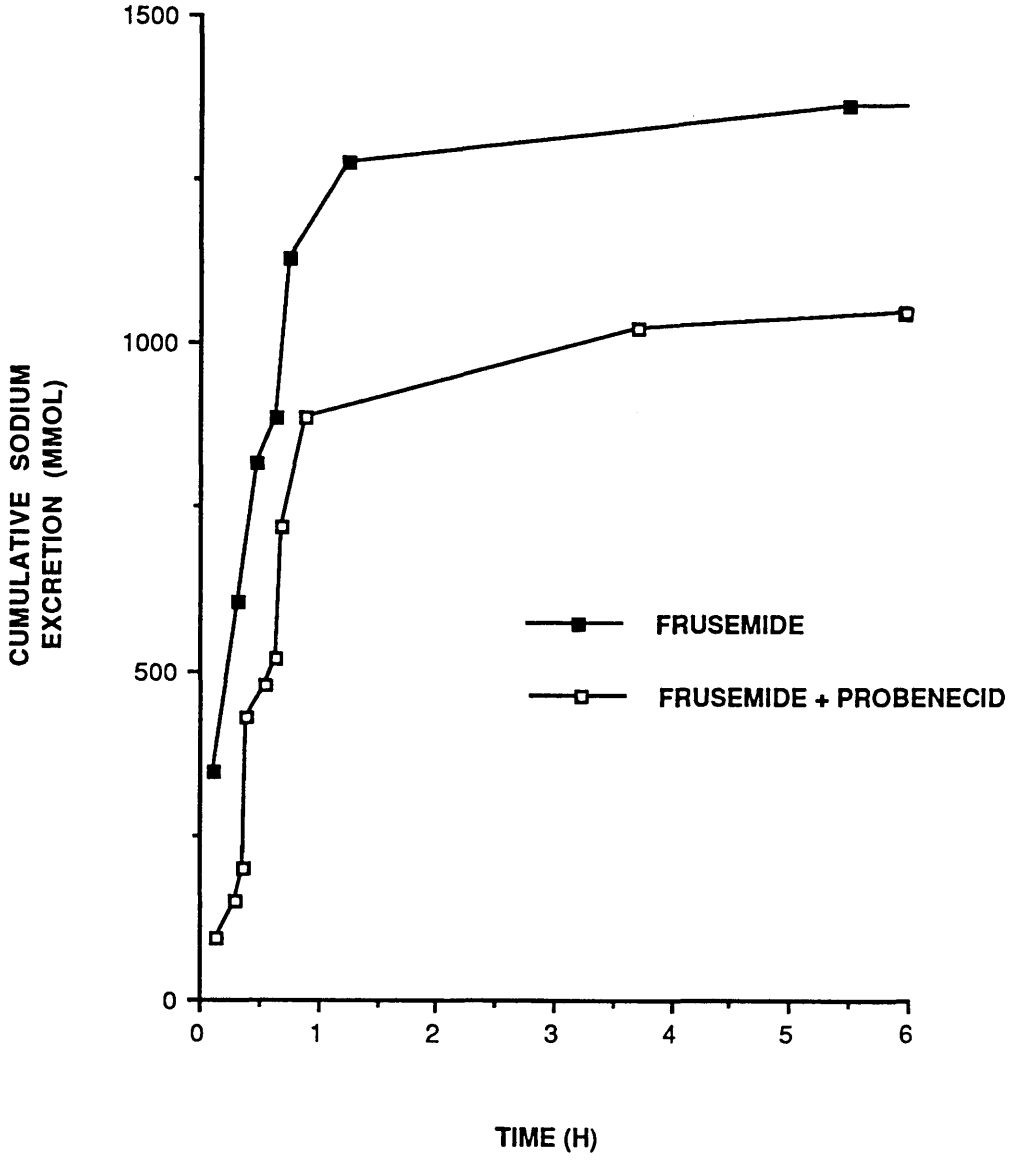
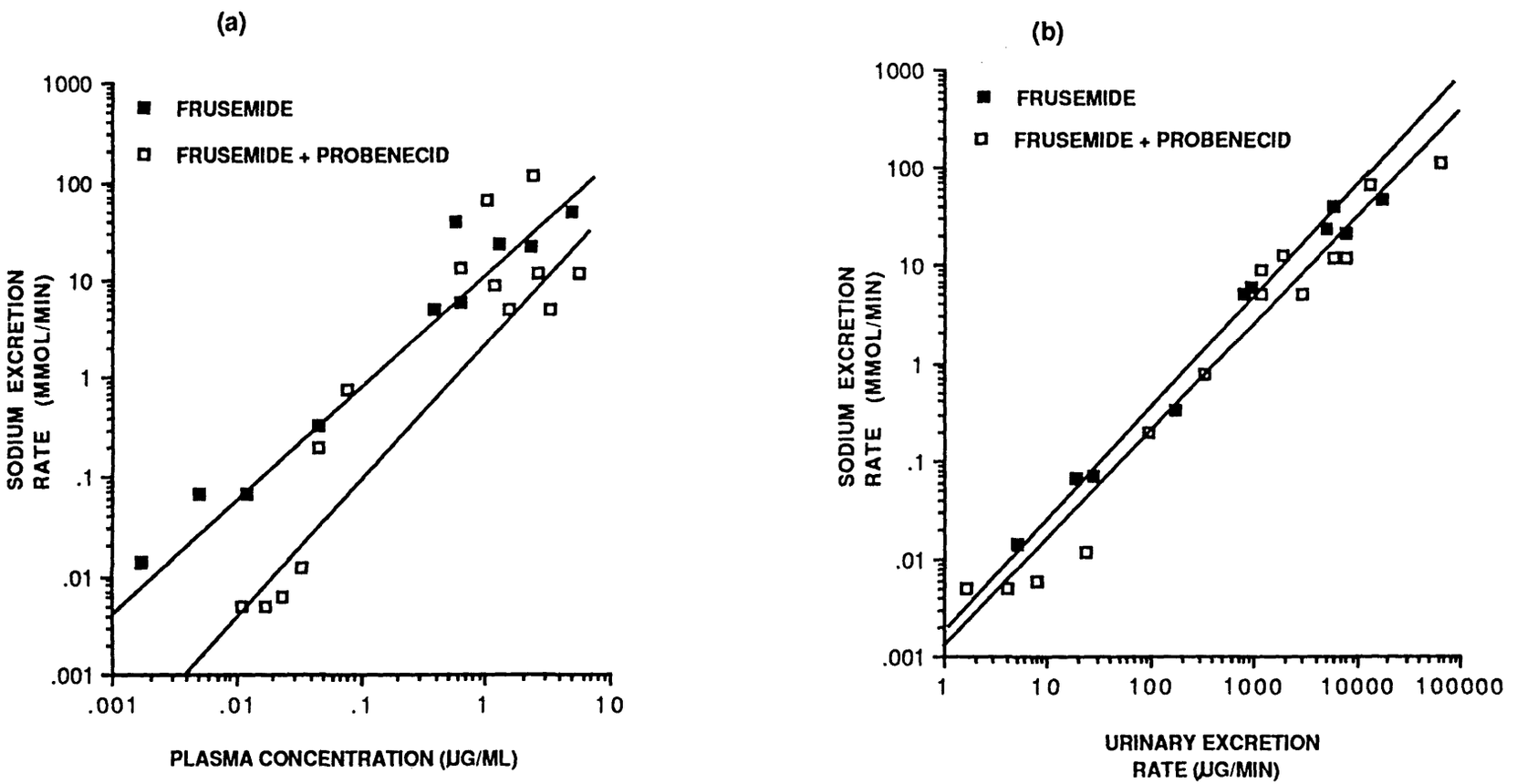


Fig.6.9. Concentration-response curves for frusemide, (a) the relationship between frusemide in plasma and response expressed as sodium excretion rate and (b) the relationship between the urinary excretion rate and response



relationship between urinary concentration of frusemide and sodium excretion rate was not affected by probenecid pretreatment (Fig.6.9b.).

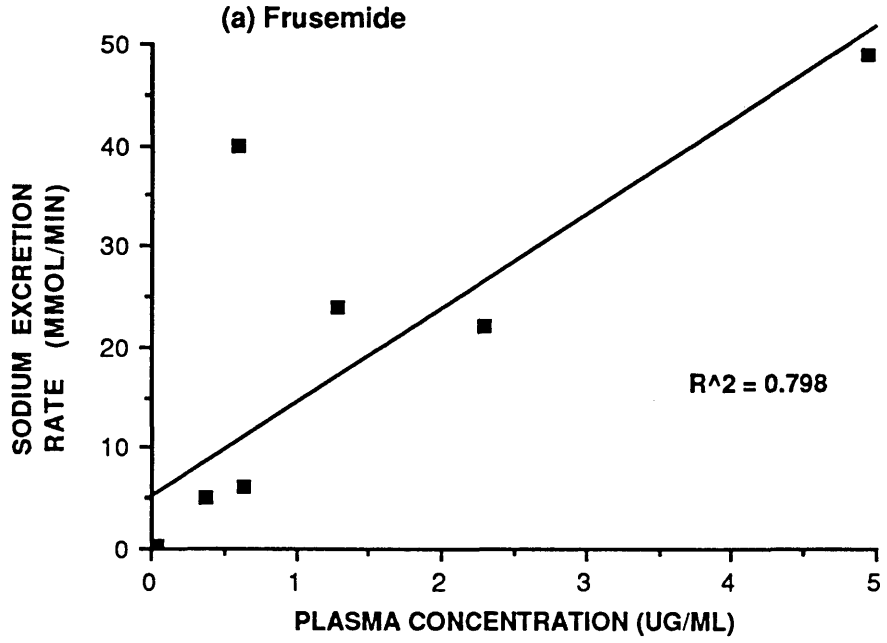
Concentration/effect relationships for frusemide are much better correlated with urine than plasma. The correlation between the natriuretic response and plasma concentration of frusemide was poor (r^2=0.798) (Fig.6.10a.). The administration of probenecid made it impossible to predict the natriuretic response to a given change in frusemide plasma concentration (r^2=0.244) (Fig.6.10b.). On the other hand, the correlation coefficient between natriuretic response and urinary excretion rate with and without probenecid, was greater than 0.900 (Fig.6.11.).

6.4. DISCUSSION

Probenecid caused marked changes in the pharmacokinetic parameters of frusemide, and the results also show that probenecid inhibits frusemide-induced natriuresis in the horse. This is in agreement with earlier findings in other animal species. Intravenous administration of probenecid (50 mg/kg) to dogs over 5 min completely attenuated the response to intraarterial infusion of 1 mg/kg/min of frusemide (Hook and Williamson, 1965). Similarly Friedman and Roch-Ramel (1977) who studied this drug interaction in the cat found that probenecid abolished the natriuretic action of frusemide when infused into the renal artery.

Results from human studies (normal volunteers) on the natriuretic effect do not always corroborate these findings in animals; probenecid was variously found to cause no change (Smith *et al.* 1980; Homeida *et al.* 1977) a small decrease (Andreasen *et al.* 1980) or even a significant increase in the natriuretic response (Brater 1978b; Chennavasin *et al.* 1979). In these studies where an increase in response was reported, the time course of the effect is important. This showed that the initial response to the diuretic was decreased by probenecid, but after about 2 hours, probenecid caused an increased response to frusemide; so that the overall effect was a higher total amount of

Fig.6.10. Relationship between the plasma frusemide concentration and the sodium excretion rate after (a) frusemide (1 mg/kg; i.v.) and (b) frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) administration to one horse



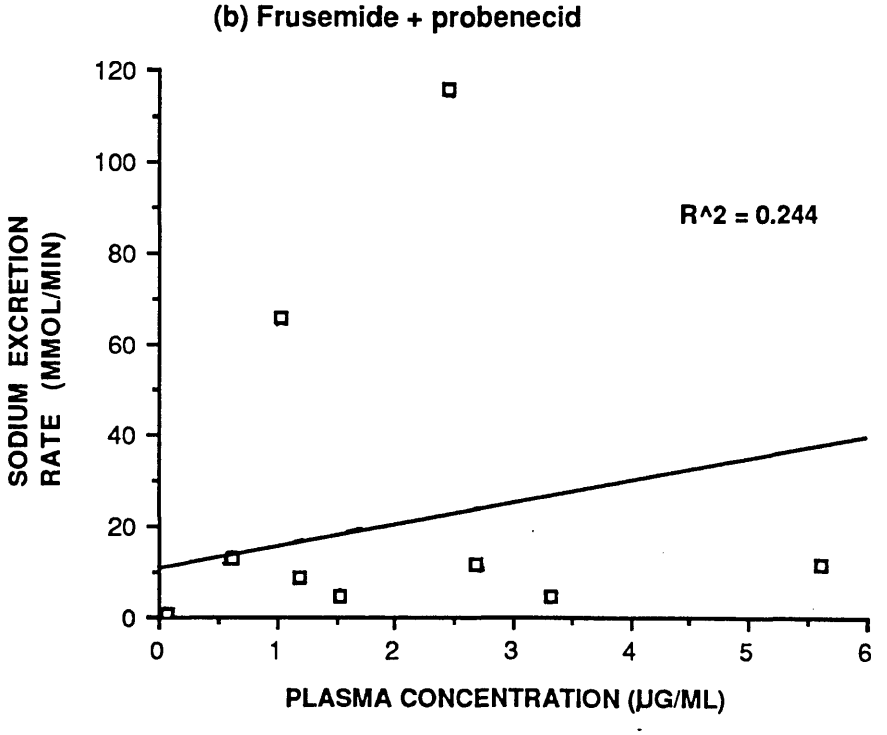
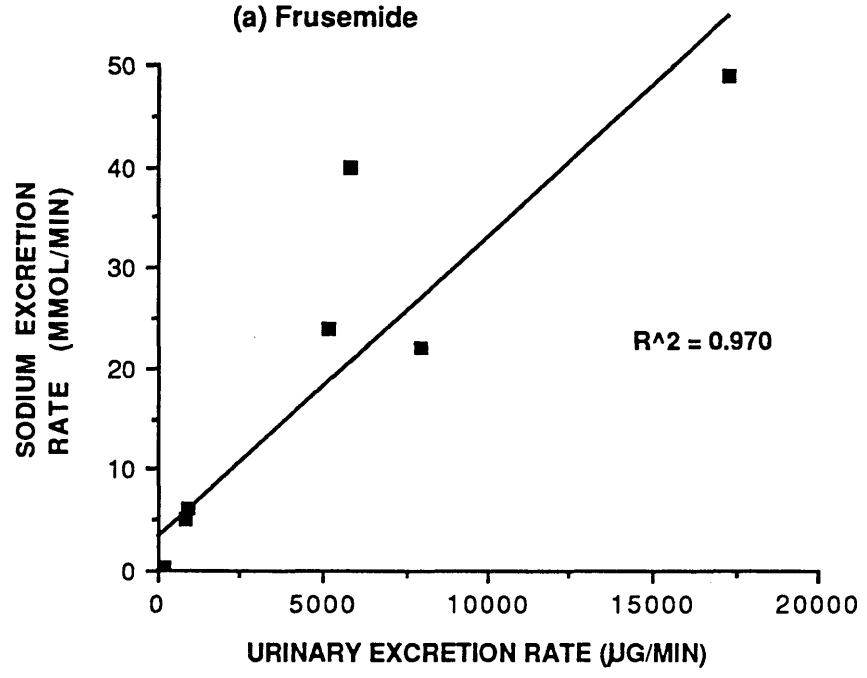
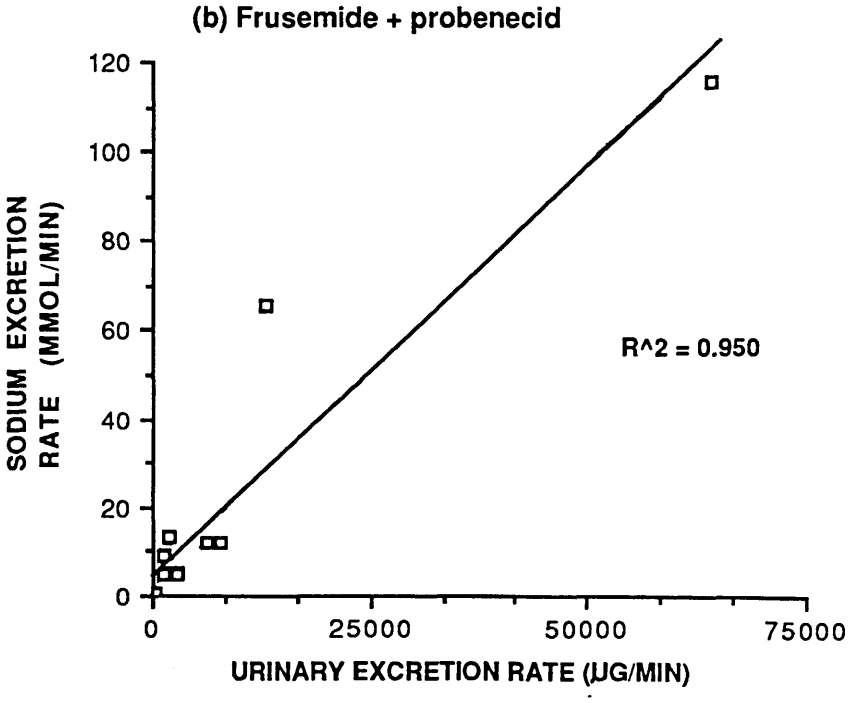


Fig.6.11. Relationship between the urinary frusemide excretion rate and the sodium excretion rate after (a) frusemide (1 mg/kg; i.v.) and (b) frusemide (1 mg/kg; i.v.) + probenecid (15 mg/kg; p.o.) administration to one horse





sodium excreted over 8 hours. Honari *et al.* (1977) observed no diminished natriuretic effect after a single intravenous dose of frusemide: however they did find significant attenuation of both the natriuretic and diuretic effect when probenecid was given during a continuous infusion of frusemide. In hospital patients receiving long-term frusemide therapy, the natriuretic action of frusemide was attenuated, when steady state was reached before probenecid was given, this is similar to a continuous infusion study (Hsieh *et al.* 1987). These conflicting results suggest a more complex interaction in man between probenecid and frusemide than has been described in experimental animals.

The present results on the kinetics of frusemide in the horse after concurrent probenecid administration agree quite well with the pharmacokinetic studies in man. The elevated plasma concentrations of frusemide after probenecid found at all time points have also been reported by Smith *et al.* (1980), who studied four healthy subjects and were able to show that the differences achieved statistical significance at all time points except at 5 minutes. Chennavasin *et al.* (1979) and Andreasen *et al*. (1980) have also shown considerably increased plasma concentrations of frusemide during probenecid treatment.

In the horse the prolongation of the elimination half-life of frusemide from 96 min to 208 min in the presence of probenecid represents a very large (117%) increase, which is similar in magnitude to that reported by Smith *et al.* (1980) i.e. 82 ± 5 min vs. 175 ± 17 min a 113 % increase, and 105 ± 16 min to 169 ±15 min (Chennavasin *et al.* (1979). An approximate doubling of the half-life of frusemide in the presence of probenecid has been reported by others (Honari *et al*. 1977; Homeida *et al.* 1977). The range in the reported half-lives of frusemide in different species is quite large, our values for the elimination half-lives in the horse are longer than those previously reported in this species (1.6 h). This is most likely because we followed concentrations of frusemide for a longer period of time, enabling the elimination phase to be more clearly defined. The ability to analyse small concentrations of drug allows the detection of a slower and slower decay of drug concentration which could result in the elimination half-life being longer.

The longer half-life and the slightly higher plasma concentrations of frusemide after probenecid enabled us to detect the diuretic in plasma up to 24 h post dosing, and there was a 38% increase in the AUC. An increase in AUC of more than 300% has been reported after probenecid in man (Smith *et al.* 1980), as a consequence of the considerably elevated plasma concentrations of frusemide. In Smith's study probenecid (15 mg/kg) was given on two occasions, this was twice the dose that we administered and may possibly have resulted in greater inhibition of frusemide excretion via the organic acid transport system.

The decrease in the plasma clearance of frusemide after probenecid from 2679 to 1944 ml/min in the horse is consistent with the findings of other groups in man (Honari *et al.* 1977; Homeida *et al.* 1977; Chennavasin *et al.* 1979; Andreasen *et al.* 1980; Smith *et al.* 1980). As probenecid was administered to only one horse in this experiment we cannot say for certain that the 27% (735 ml/min) decrease in plasma clearance is significant. However, this horse (Pendulate) has been given frusemide (1 mg/kg; i.v.) on three separate occasions, i.e. when looking at frusemide disposition following different routes of administration, and in the frusemide/salicylate and frusemide/theophylline studies (Chapter's 3, 4 and 8). Collating the plasma clearance data from these experiments results in a mean value of 2680 ± 115 ml/min; thus it is likely that the 735 ml/min fall after probenecid is statistically significant.

The fall in plasma clearance is primarily due to a decrease in renal clearance presumably as a result of a reduction in tubular secretion of frusemide. An alternative explanation for the decrease in renal clearance would be an increase in reabsorption of the secreted frusemide. However, this is unlikely as we found that urinary pH was unaffected by probenecid. At near neutral or slightly alkaline pH, frusemide is almost completely ionized so there is an insignificant amount of reabsorption. Previous human studies have all demonstrated a significant and marked reduction in renal clearance of frusemide by probenecid (Honari *et al.* 1977; Homeida *et al.* 1977; Chennavasin *et al.* 1979; Odlind and Beermann, 1980, Smith *et al.* 1980).

The importance of the tubular mechanism for the excretion of frusemide is highlighted by the fact that in the horse, glomerular filtration accounts for only about

2% of the renal clearance. A more detailed study of the renal mechanisms involved in the probenecid-frusemide interaction has been done by Andreasen *et al.* (1980). The glomerular filtration rate was measured by determining inulin clearance, and the influence of probenecid on frusemide binding to serum proteins was investigated. These workers also found that about 2% of frusemide was excreted by glomerular filtration and 98% was excreted by tubular secretion. This calculation assumed that there was no reabsorption. Probenecid was found to cause a small but significant decrease in binding of frusemide to plasma proteins, and secondly, tubular secretion of frusemide was impeded. Frusemide was displaced from its protein binding sites, so the amount filtered by the glomerulus was increased to 8%. This is contradictory to the findings of Chennavasin *et al.* (1979) and Odlind and Beermann (1980) who found that probenecid did not affect the extent of binding of frusemide. The increased glomerular filtration of frusemide is confirmed by the fact that inulin clearance increased, the significance of this being that inulin is excreted exclusively by filtration.

In our study we found that glomerular filtration accounts for a little more of the renal clearance when probenecid is given, but as we did not determine glomerular filtration rate and frusemide protein binding in the horse during the experimental period, our calculations are based on previously reported values for these parameters, and were assumed to be the same for both treatments.

Our data indicates that the decrease in plasma clearance was also augmented by a small fall in nonrenal clearance this has been observed in man (Homeida *et al.* 1977; Chennavasin *et al.* 1979), but not in animal studies as in these frusemide was administered intraarterially and probenecid's effect on nonrenal clearance could not be observed. In the above human studies the probenecid dose was 2 g in 24 h (30 mg/kg); when the dose is 1 g in 24 h a fall in nonrenal clearance is not always found (Honari *et al.* 1977). A likely explanation for this is that the renal clearance of frusemide is more sensitive to the inhibitory action of probenecid than the nonrenal clearance. In our study the significance of the suppression of nonrenal clearance is difficult to evaluate due to the limited data, but it seems that inhibition of nonrenal clearance, most likely hepatic, allows accumulation of frusemide in plasma. At later times these elevated plasma levels

result in greater amounts of frusemide being delivered into the urine despite the reduced renal clearance. Thus probenecid's associated inhibition of nonrenal clearance may explain the greater overall natriuretic effect found by Brater (1978b) and Chennavasin *et al.* (1979).

In our study, the attenuation of response to frusemide within the first hour after probenecid pretreatment is not compensated for later, most likely since some 75% of the frusemide dose is excreted during this time. The difference in the diuretic effect established between the two treatments is maintained during the rest of the experiment. The effect of probenecid on the pharmacokinetics and pharmacodynamics of frusemide in the horse might have been more striking if we had administered two 15 mg/kg doses of probenecid, one the night before as well as 1 h before the diuretic. This was not done as intubating the horse twice in close succession was undesirable. In future experiments it may be better to give probenecid in tablet form the night before the study, and then stomach tube a dose prior to the frusemide.

Reports in man which cite a similar initial attenuation but a subsequent increased response do not give any indication of the fraction of frusemide excreted during the period of diuresis (Brater 1978b; Chennavasin *et al.* 1979). It is likely that in these studies the amount of frusemide excreted is less as inhibition of tubular secretion is greater due to the increased probenecid dose also the plasma levels of frusemide are substantially higher due to reduced nonrenal clearance. The delayed response found by Brater (1978b) highlights the importance of following the time-course of the frusemide-probenecid interaction, and he suggests that differences in his data from that of Honari *et al.* (1977) and Homeida *et al.* (1977) may be due to the latter's shorter observation period (6 and 5 h respectively).

A number of factors determine the response to a diuretic, these include the total amount of drug delivered into the urine, the delivery of solute to the site of action, the dynamics of interaction of drug with its receptor and whether or not solute is reabsorbed distal to the site of action. Kaojarern *et al.* (1982) have shown that the time course of delivery of frusemide to the active site is an important independent determinant of overall response.

Our results show that in the presence of probenecid there are a greater number of voidings even though the total volume of urine produced is reduced. The effect of this can be visualized as "small packets" of frusemide passing intermittently through the renal organic anion transport system, their movement being restricted by blockade with the competitive inhibitor probenecid. Thus probenecid reduces the delivery rate of frusemide to its site of action. This secretion of frusemide takes place in the proximal portions of the nephron, resulting in an elevated concentration in the tubule fluid, which then flows to the thick ascending limb of Henlé's loop, the main site of diuretic action (Seldin *et al*; 1966, Burg *et al*. 1973). Using tubular segments dissected from rabbit kidneys and perfused *in vitro,* Burg *et al.* (1973) showed that low concentrations of frusemide in the tubular lumen (10^{-6}M) are much more inhibitory of sodium chloride transport than higher concentrations (10^{-4}M) bathing the peritubular surface.

In all the studies mentioned above in man and animals the effects of probenecid on urinary frusemide excretion and urinary sodium excretion rate are easy to follow as urine was collected by spontaneous voiding at set time points in man and by cannulation of the ureters in experimental animals. However, in our experiments in the horse this was not possible, so variation in urinary flow rate at early times is an important factor that complicates the interpretation of data.

The effect of variations in flow rate in the horse can be seen when looking at the relationship between urinary excretion rate of frusemide and serum concentration of frusemide (Fig.6.3.). The correlation in the presence of probenecid is poor due to the very high excretion rates at 0.40 h and 0.68 h i.e 64012 µg/min and 13102 µg/min, with corresponding flow rates of 1214 ml/min and 603 ml/min respectively. This is likely due to the variability in the delivery rate of frusemide to the luminal side of the renal tubules in the presence of probenecid, as a good correlation coefficient (0.949) was obtained when the diuretic was given alone.

Fig.6.7b. also shows that this variability results in delay in the excretion of frusemide during the intense diuresis. The two curves intersect at about 6 h and recovery of frusemide is slightly greater with probenecid. This could be because probenecid is eliminated faster. In this study it is difficult to ascertain whether

frusemide exerts an effect after inhibition by probenecid is over as at 6 h the diuretic response is over and excretion of frusemide is virtually complete, we are dealing with just 3% of the dose after this time. However, Chennavasin *et al.* (1979) state that the effects of probenecid on organic acid transport were long-lasting, they observed a constant degree of inhibition for up to 6 h.

"Concentration-response" curves for concentrations of frusemide in plasma and urine relating to the natriuretic effect are depicted in Fig.6.9. The shift to the right after probenecid pretreatment shows that the plasma concentration required to achieve a urinary rate of sodium excretion of 1 mmol/min after probenecid is greater than that required in the control period before probenecid. There was no change in the relationship between concentration of frusemide in urine and response.

The concentration/effect relationships shown in Figs.6.10. and 6.11. demonstrates that the urinary excretion rate of frusemide is a better indicator of natriuresis than is its plasma concentration. It is of interest that frusemide excretion rate, rather than urinary frusemide concentration was correlated with natriuretic effect. Thus our data are consistent with the principle that it is the amount of frusemide present on the luminal side of the tubular epithelium that predicts the natriuretic response rather than the plasma concentration. This agrees with the *in vitro* findings of Burg *et al.* (1973) as well as with other studies in humans (Homeida *et al.* 1977; Chennavasin *et al.* 1979; Andreasen *et al.* 1980; Smith *et al.* 1980).

In summary, probenecid alters the pharmacokinetics of frusemide, the results are consistent with the findings in man. Knowledge about this interaction is specifically valuable for racing authorities as both frusemide and probenecid are drugs whose use has been increasing. Frusemide due to its "diluting" effects and probenecid because of its "masking" effect. This study has shown that probenecid delays the excretion of frusemide, as such the detectability of frusemide in urine may be affected. Probenecid has the potential to reduce renal clearance and thus the urinary concentrations of other acidic drugs excreted via the organic anion transport system e.g. salicylate, and so hinder the forensic screening of these compounds. Information on probenecid and its interaction with other drugs in the horse is useful as data are very limited in this species.

Chapter 7

THE METABOLISM AND PHARMACOKINETICS OF THEOPHYLLINE IN THE HORSE

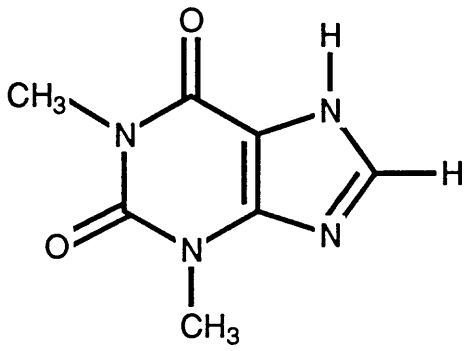
7.1. INTRODUCTION

The naturally occurring alkaloids theophylline (1,3-dimethylxanthine), caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) (Fig.7.1.) are derivatives of xanthine and are among the many drugs which have been used as stimulants in racehorses (Green *et al.* 1983; Lambert and Evans, 1983). Drug-positive samples from racehorses originate both from deliberate doping and accidental or inadvertent doping, especially from compounds present in the diet (Kelly and Lambert, 1978). In the early 1970's there was a spate of positives for caffeine and theobromine, which were eventually traced to the inclusion of cocoa husks in horse rations by a feedstuff manufacturer. Recently a trainer was fined when a post-race urine sample revealed the presence of caffeine, theobromine and theophylline in his horse. The source was found to be a feed, mixed by a local supplier (Daily Mail, 7th June 1989). In 1987 the feeding of a seemingly innocuous Mars bar to a race horse made front page news "Mars bars De Rigeur from £10,000 win" (The Guardian, 7th January 1987). The horse was disqualified when theobromine was detected in a post-race urine sample and it was traced to the chocolate.

Theophylline, a methylxanthine found in tea and coffee, has been used for many years as a bronchodilator and respiratory stimulant for the treatment of acute and chronic bronchial asthma, apnoea in premature infants and as an adjunct in the treatment of congestive heart failure and acute pulmonary oedema (Hendeles *et al.* 1983; Svedmyr and Simonsson, 1978; Webb-Johnson *et al.* 1977; Shannon *et al.* 1975).

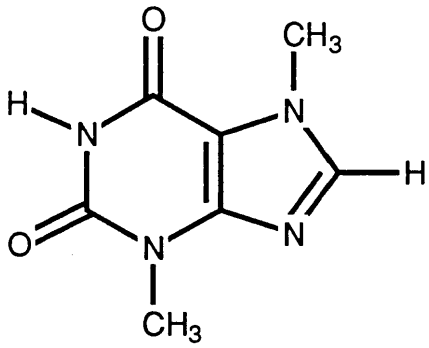
Theophylline and the more soluble form aminophylline (theophylline ethylene-diamine) are routinely used in veterinary medicine to treat respiratory conditions where bronchospasm is believed to play a role in the disease process (Davis 1980; Derksen 1983). The effects of these drugs are only of 2 to 4 h duration, thus they are useful in the initial management of acute clinical cases when administered intravenously or by inhalation, but do not bring about complete recovery. The pharmacokinetics of

Fig.7.1. Structures of the methylxanthines



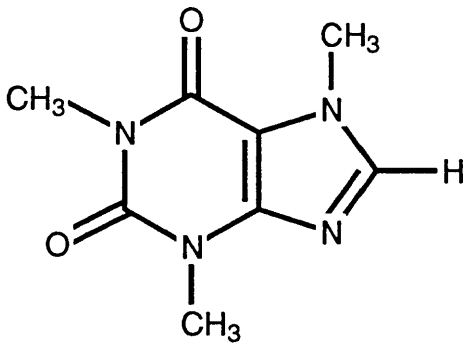
THEOPHYLLINE

(1,3-dimethylxanthine)



THEOBROMINE

(3,7-dimethylxanthine)



CAFFEINE

(1,3,7-trimethylxanthine)

theophylline have been reported in a number of species of veterinary importance; the ox (Davis 1978), the dog (McKiernan *et al.* 1981), the cat (McKiernan *et al.* 1983) and the pig (Koritz *et al.* 1981).

There have been a small number of studies on the pharmacokinetics of theophylline in horses following intravenous and intragastric administration. A review paper by Davis (1978) reported the half-life to be 3 h, but no supporting data were included. The results reported by others indicate rapid absorption after oral administration with a mean elimination half-life of between 9.7 and 19.3 h, and a volume of distribution of 0.85 to 1.02 l/kg (Kowalczyk *et al.* 1984; Errecalde *et al.* 1984; Ingvast-Larsson *et al.* 1985; Ayres *et al.* 1985). However, the literature apparently contains no detailed study of the metabolic fate of theophylline in the horse. Following intravenous administration of aminophylline, Sams and Liu (1983) reported that approximately 35% of the dose was eliminated in the urine as 1,3-dimethyluric acid, with 27% eliminated as theophylline. No other metabolites were detected and the fate of the remaining 38% is unknown. In another study, after [^{14}C]-theophylline was administered orally, 80% of the radioactive dose was recovered in the urine, 26% as theophylline and the remainder presumably as 1,3-dimethyluric acid (Moss and Horner, unpublished data).

Thus, despite the obvious therapeutic potential of theophylline in horses and its occasional occurrence as a drug of abuse, there is a lack of information of its metabolic fate. Chapter 8 details the results of a study investigating the effects of frusemide on theophylline. In that experiment three horses were used and each served as its own control; i.e. each horse received theophylline alone, frusemide alone and frusemide with theophylline. This chapter describes the pharmacokinetics and metabolism of theophylline following oral administration in the horse.

7.2. METHODS

Compounds

[8-^{14}C]-Theophylline, specific activity 317 μCi/mg, radiochemical purity >98% by HPLC, was purchased from Amersham International (Aylesbury, U.K.). Sources of xanthine and uric acid derivatives were as follows: theophylline (1,3-dimethylxanthine, 1,3-DMX), and 1,3-dimethyluric acid (1,3-DMU) from Sigma (Poole, U.K.); 1-methyluric acid (1-MU) and 3-methyluric acid (3-MU) from Fluka (via Fluorochem, Glossop, U.K.). Other materials purchased were: sodium acetate trihydrate (Analar grade) from BDH (Poole, U.K.). HPLC grade acetonitrile from Rathburn Chemicals Ltd. (Walkerburn, U.K.).

Animals and Drug Administration

Three thoroughbred male horses (body weights 474-530 kg) of Horseracing Forensic Laboratory Ltd. were used in this experiment. Each horse received [^{14}C]-theophylline 1 mg/kg; 100 μCi orally. [^{14}C]-Theophylline (1 mCi) was dissolved in methanol (10 ml). Prior to administration, [^{14}C]-theophylline (1 ml of the methanolic solution) was added to water (100 ml) and heated to 40°C with occasional stirring for 1 minute. Unlabelled theophylline (523-585 mg) was added slowly with continuous stirring, and when dissolved the solution was well mixed and transferred to a plastic bottle, cooled and made up to 400ml with water. The [^{14}C]-theophylline was administered by a stomach tube, with a total volume of 1 litre of water. Following administration of theophylline, the horses were moved to metabolism stalls in which the movement of the horse is restricted, to permit the convenient collection of samples (Marsh 1983). Water was available before dosing but food was withheld overnight prior to dosing, which was at approximately 8.30 am. Following the theophylline dose, both food and water were withheld until approximately 2.00 pm. Each morning and afternoon during the course of the experiment the horses were exercised for 30 minutes before being returned to the metabolism stalls.

Blood Collection

Blood samples (50 ml) were obtained at various times after dosing, from an indwelling jugular venous cannula. The blood was collected into Monovettes (Sarstedt Ltd. Leicester, U.K.) containing lithium heparin crystals and was centrifuged at 2000 rpm for 5 min. The plasma was aspirated, and before storage of plasma at -20°C, duplicate 1ml samples were removed and counted for ^{14}C.

Urine Collection

Urine was collected as previously described (Marsh 1983) for 96 hours. The time at which each urine sample was voided was recorded and the volume measured. A small volume of each urine specimen (15 ml) was centrifuged at 3500 rpm for 10 minutes to obtain a clear supernatant, and 0.1 ml aliquots were removed in duplicate for scintillation counting. An aliquot of each sample (ca. 120 ml) was kept for drug analysis and the rest discarded. All urine samples were frozen and transported to London for storage at -20°C prior to analysis.

Radiochemical Techniques

The ^{14}C content of plasma and urine was determined by liquid scintillation spectrometry, using Ecoscint scintillation fluid (National Diagnostics; Reading, U.K.) with a Minaxi Tricarb 4000 Series, Model B4450 liquid scintillation spectrometer (Canberra Packard; Bucks. U.K.). Quench correction was by the channels ratio method.

High-Performance Liquid Chromatography

A highly selective HPLC method was used for the assay of theophylline and its major metabolites in urine (Hotchkiss and Caldwell, 1987). The HPLC system consisted of two LC-6A solvent delivery systems, a SCL-6A system controller, a SIL-6A autoinjector and a SPD-6A UV spectrophotometric detector set at 280 nm, linked to a C-R6A Chromatopac integrator (all from Shimadzu Corp., supplied by Dyson Instruments; Houghton-le-Spring, Tyne and Wear, U.K.).

The analytical HPLC column (250 x 5mm i.d.) was a LiChrosorb C18 cartridge (Cat. No. E.Merck, Darmstadt, Germany) and a gradient system was used. Mobile phase A consisted of 1.28 g/l sodium acetate trihydrate containing 0.4% (v/v) glacial acetic acid, pH 4, and mobile phase B consisted of 1.28 g/l sodium acetate trihydrate containing 20% (w/v) acetonitrile and 0.5% (v/v) glacial acetic acid. Both solutions were filtered and degassed with a Millipore HA (0.45 μm) solvent filtering system prior to use.

Compounds were eluted from the column using a linear gradient from 0 to 80% solvent B over 22 min with a hold of 5 min at the final conditions. The flow rate was 1 ml/min throughout. Between each urine injection the column was washed with methanol for 15 min, and then re-equilibrated for 15 min with mobile phase A.

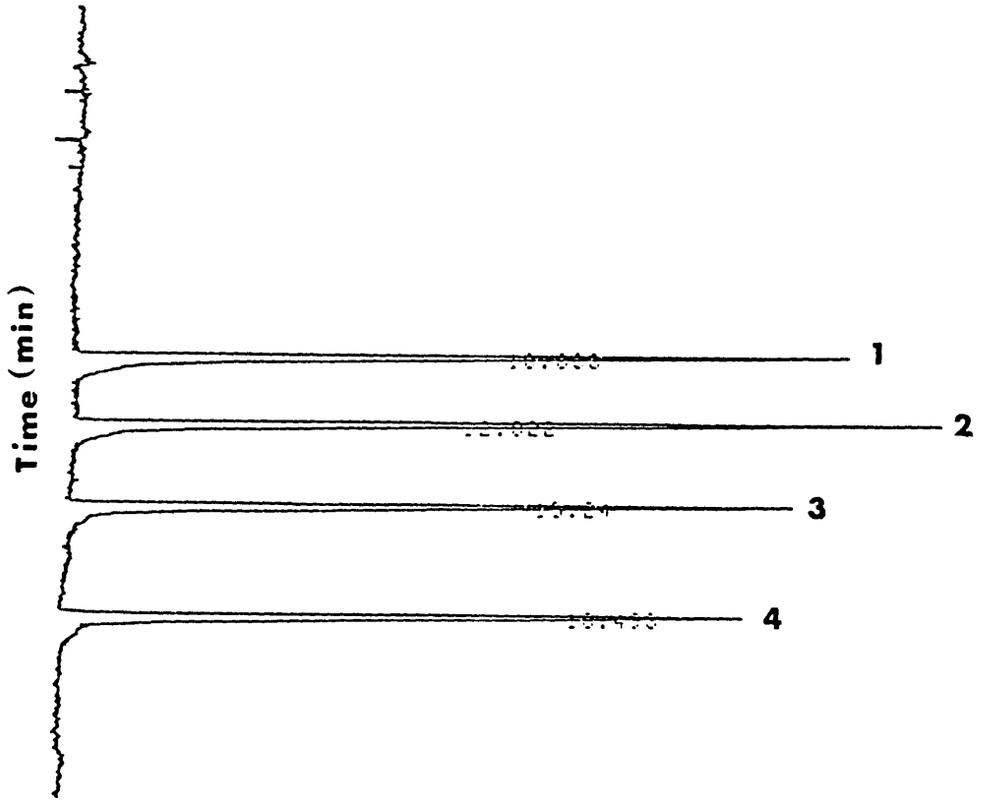
Urine was centrifuged at 2500 rpm for 10 minutes, prior to injection directly on to the HPLC column. Quantitation of theophylline and its metabolites was achieved by collecting 0.5 ml fractions of eluent and counting for ^{14}C activity.

The identity of peaks were assigned by comparison of retention times with those of authentic standards. Under the conditions described the elution times for 1,3-DMX, 1,3-DMU, 1-MU and 3-MU were 18.4, 15.2, 12.8 and 10.8 minutes respectively (Fig.7.2.).

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated by least-squares regression analysis on an Apricot Xi 10 computer as described in Chapter 3.

Fig.7.2. Chromatogram showing the retention times of theophylline and its metabolites following the injection of authentic standards and HPLC separation by the method of Hotchkiss and Caldwell (1987)



Peak	Compound	Retention time
1	3-Methyluric acid	10.8 min
2	1-Methyluric acid	12.8 min
3	1,3-Dimethyluric acid	15.2 min
4	Theophylline	18.4 min

7.3. RESULTS

Excretion of ^{14}C

Urine samples were collected for 96 h after the oral administration of [8-^{14}C]-theophylline. Equating radioactivity directly with theophylline concentration indicated that by 96 h after dosing urinary concentration of ^{14}C activity was 1.16 ± 0.2 μg equivalents/ml. An aliquot of the 96-120 h urine sample was counted to ensure that the radioactivity levels had returned to within background, the concentration of this was found to be 0.02 ± 0.04 μg equivalents/ml. This data indicates that the urinary elimination of ^{14}C was essentially complete within 120 h. Fig.7.3. illustrates the cumulative excretion against time up to 96 h. This graph implies that excretion is not over but after 120 h, ^{14}C activity was not detectable in individual urine samples so the curves for each horse appear to stop abruptly but excretion at this time is very low. Thus the urinary elimination of ^{14}C was essentially over by 96 h and accounted for 64.0 - 75.1% of the dose (n=3).

Plasma Pharmacokinetics

The log ^{14}C levels (μg equivalents) in plasma were plotted against time (Fig.7.4. mean ± s.d.). Peak plasma levels were obtained within 45 minutes (t_{max}) indicating rapid absorption. The plasma theophylline concentrations declined biphasically with time and the experimental data were adequately described by a 2-compartment open model for the three horses.

Individual and mean pharmacokinetic parameters describing the plasma data are listed in Table 7.1. corrected as appropriate for the weight of the horse.

Following oral administration, theophylline was very rapidly absorbed. In horse 3 the drug was essentially completely absorbed within 20 minutes, and since only two blood samples were taken during this phase, a rate constant for absorption (K_{abs}) could not be obtained due to an inadequate number of concentration vs. time points. The half-life of absorption (t_{abs}) in the other two horses was 8 minutes. The mean peak theophylline plasma concentration (C_{max}) was 1.81 μg/ml which was achieved within

Fig 7.3. Cumulative urinary excretion of 14C in three horses given [14C]-theophylline (1mg/kg;p.o.). 40.8±5.5% of the dose excreted in 0-24 h, 14.4±2.4% in 24-48 h, 6.1±0.4% in 48-72 h and 2.7±0.8% in 72-96 h

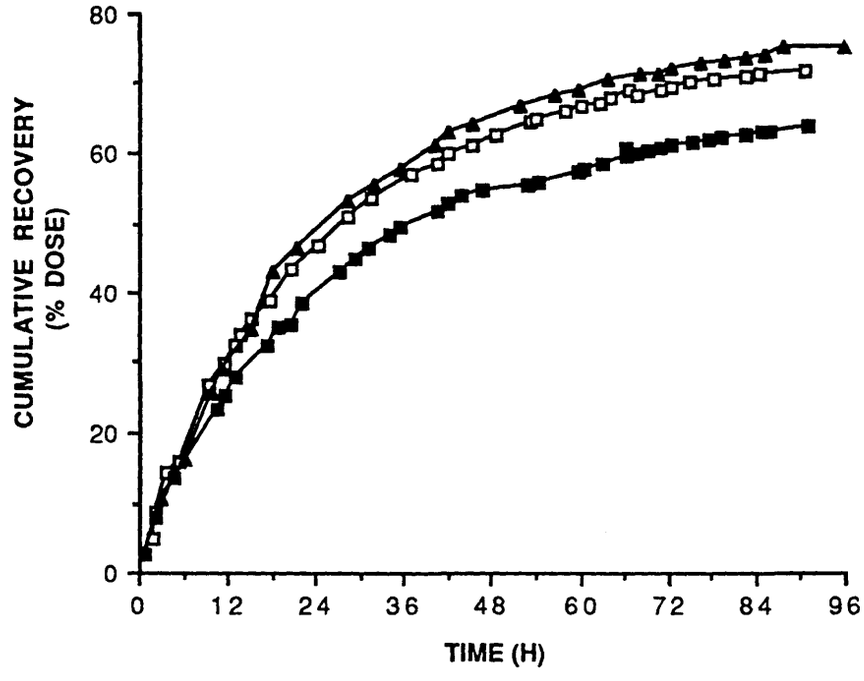


Fig.7.4. Plasma concentration of 14C-radioactivity in three horses given [14C]-theophylline (1 mg/kg; p.o.) (mean ± s.d; n=3)

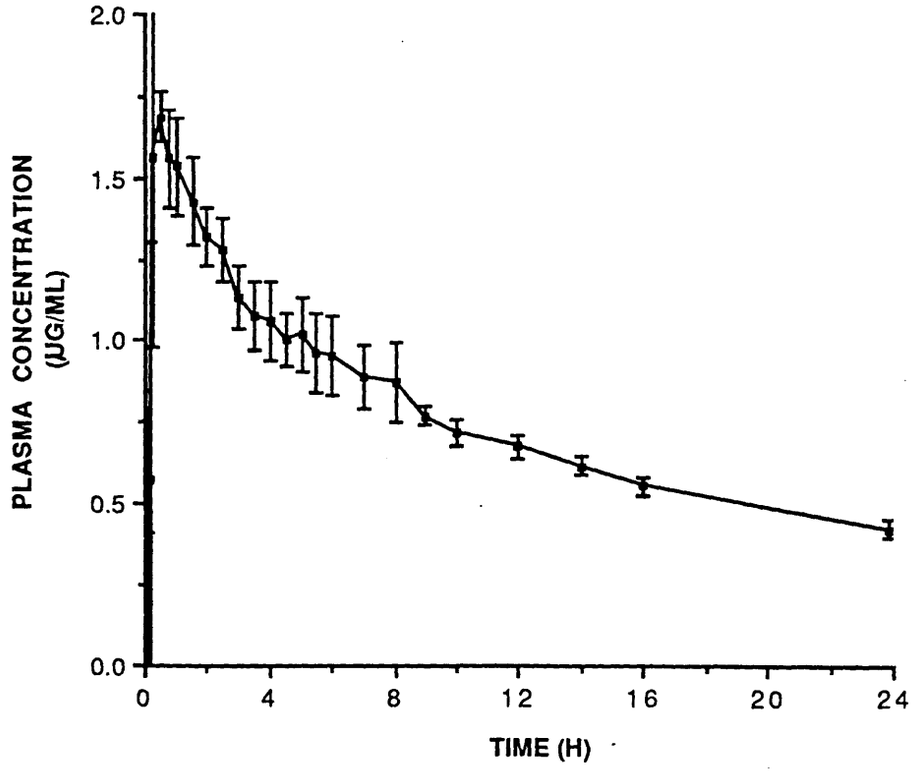


Table 7.1. **Pharmacokinetic parameters describing the plasma levels of theophylline in the horse, following the oral administration of [^{14}C]-theophylline (1 mg/kg) (mean ± s.d., n=3)**

	HORSE			
Parameter	1	2	3	Mean±s.d.
Body Wt.(kg)	474	530	516	507 ±29
C_{max} (μg/ml)	1.74	1.7	2.00	1.81 ±0.16
t_{max} (h)	0.50	0.75	0.33	0.53 ±0.21
$K_{abs}(h^{-1})$	7.62	4.077	-	5.849±2.51
$t_{1/2abs}$(h)	0.091	0.170	-	0.131±0.05
α (h^{-1})	0.144	0.986	0.521	0.550±0.42
$t_{1/2\alpha}$ (h)	4.187	0.703	1.331	2.284±2.216
β (h^{-1})	0.034	0.044	0.035	0.038±0.006
$t_{1/2\beta}$ (h)	20.5	15.6	19.9	18.7 ±2.67
V_d (l/kg)	0.928	0.874	0.890	0.897±0.028
Cl (l/h/kg)	0.031	0.038	0.031	0.033±0.004
AUC (μg/ml.h)	31.7	26.0	32.1	29.9 ±3.4

Table 7.2. **Urinary metabolites of theophylline in the horse, following the oral administration of [^{14}C]-theophylline (1mg/kg) (mean ± s.d., n=3)**

	% Dose in 96 h
1,3-MU	42.8 ± 1.3
THP	15.9 ± 6.1
1-MU	8.9 ± 2.6
3-MU	1.6 ± 0.4

45 minutes in all cases, and declined to a mean concentration of 0.425 μg/ml at 24 h.

The distribution phase (α) was significant in that it was relatively long compared to that found in other species, lasting for between 42-250 minutes ($t_{1/2\alpha}$), thus high plasma concentrations were maintained for longer than that found in cats and dogs (see Discussion). The elimination half-life ($t_{0.5\beta}$) of theophylline showed a degree of variability (15.6-20.5 h). The mean volume of distribution (V_d) of theophylline was 0.897 l/kg, and was very consistent in the three horses. The total plasma clearance (Cl) of theophylline in the horse was low (0.033 l/h/kg), and similarly showed little variability.

Identification of Urinary Metabolites

HPLC analysis of the urine by the method described showed the presence of 4 discrete radioactive components (Fig.7.5.), corresponding to theophylline and three metabolites namely 1,3-dimethyluric acid (1,3-DMU), 1-methyluric acid (1-MU), and 3-methyluric acid (3-MU) (Table 7.2.). Following [^{14}C]-theophylline (1 mg/kg p.o.) a mean of 40.8±5.5% of the dose was excreted in the urine in 24 hours and a total of 70.3±5.7% by 96 hours after administration. 1-Methyluric acid and 3-methyluric acid have not previously been detected in the horse. Fig.7.6. illustrates the proposed pathways of theophylline metabolism in the horse. The mean recovery of unchanged theophylline in the urine determined in this study (15.9%) is similar to that reported by others in man (Monks *et al.* 1979). Analysis of sequential 24 hour pooled urine samples showed that the quantitative pattern of metabolites did not change over the study period (Fig.7.7.).

[^{14}C]-Theophylline administration induced a transient diuretic effect. In one of the horses the urine flow rate increased to 86 ml/min, 2.2 h after drug administration. The data indicates that there was considerable variation in urine flow, this varied from approximately 2-20 ml/min over 24 hours. In the horse control values for urine flow rate average 10 ml/min. Elimination of unchanged theophylline was closely related to urine flow rate at all time points (Fig.7.8.).

Fig.7.5. Radiochromatogram showing theophylline and its metabolites in urine following the administration of [14C]-theophylline (1 mg/kg; p.o.) in the horse

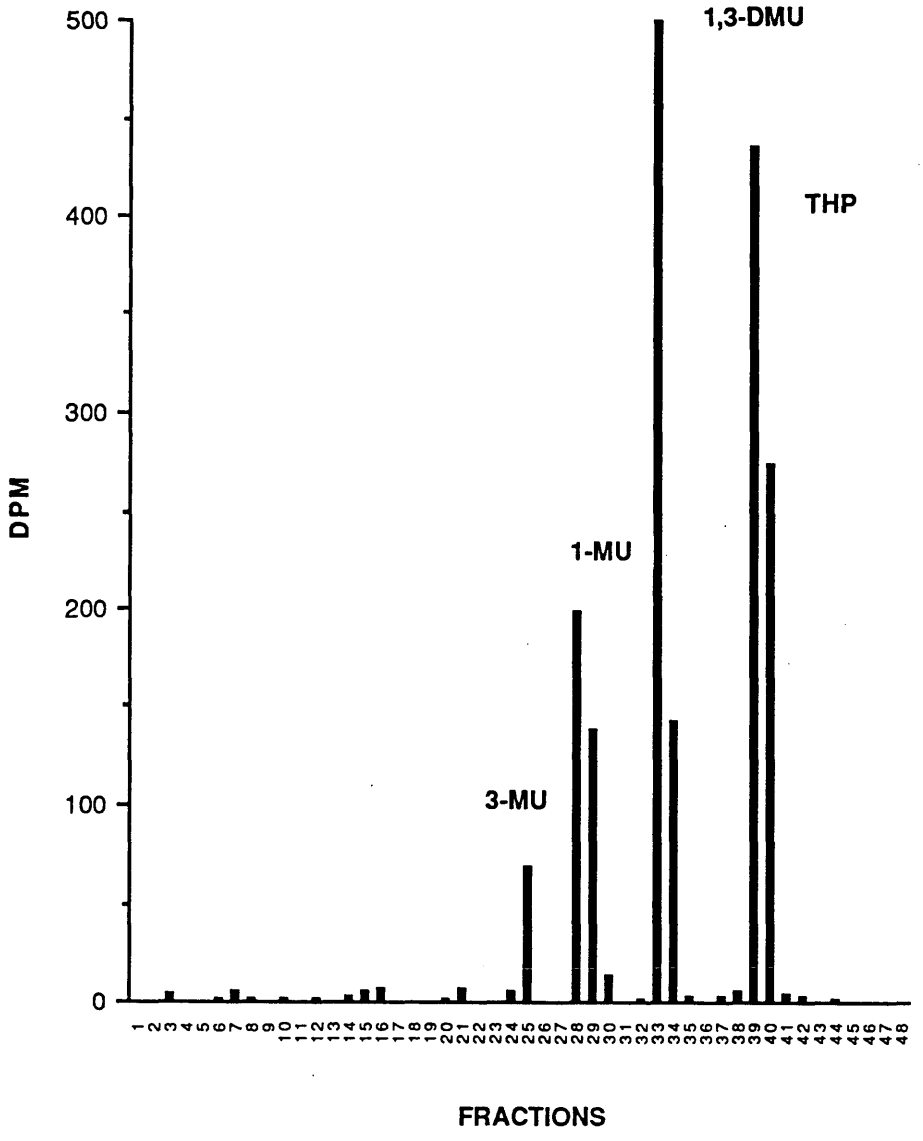


Fig.7.6. Pathways of theophylline metabolism in the horse

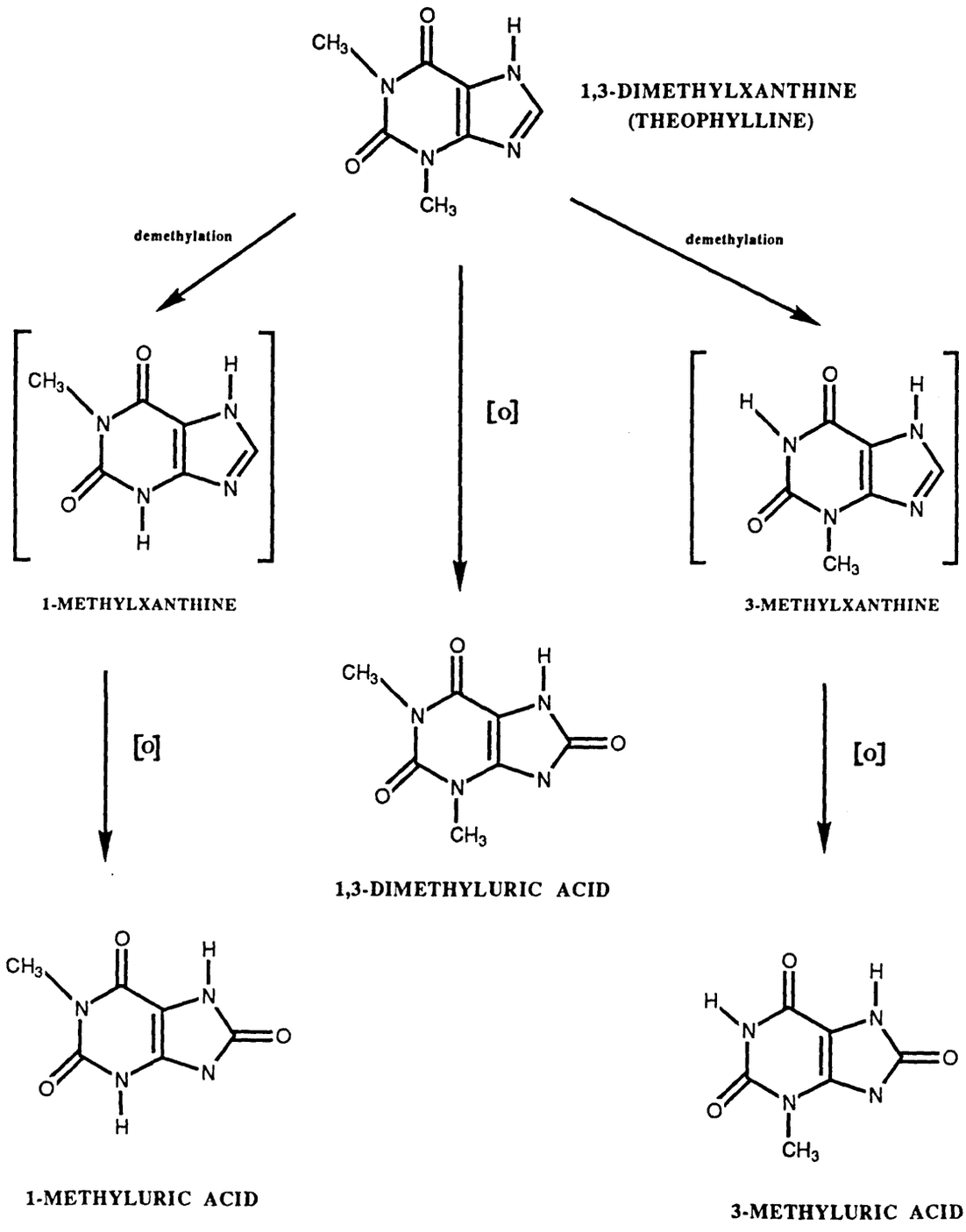


Fig.7.7. The urinary metabolite profile of 24h poded urine samples following the administration of [14C]-theophylline (1mg/kg; p.o.) in the horse. Results expressed as a % of urinary recovery (mean±s.d; n=3)

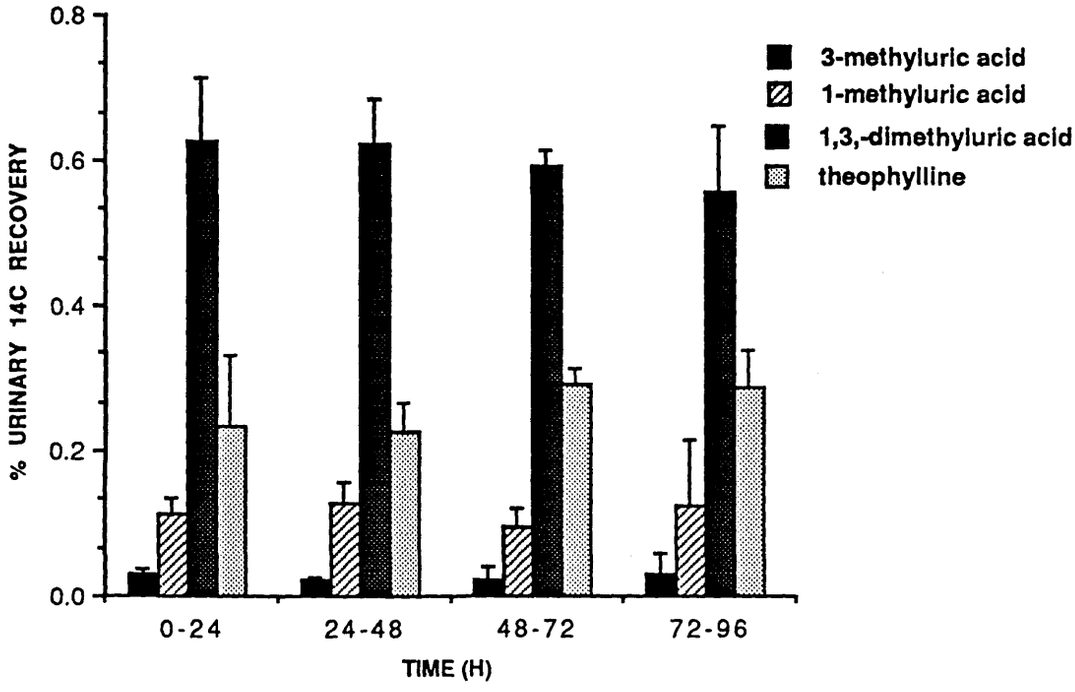
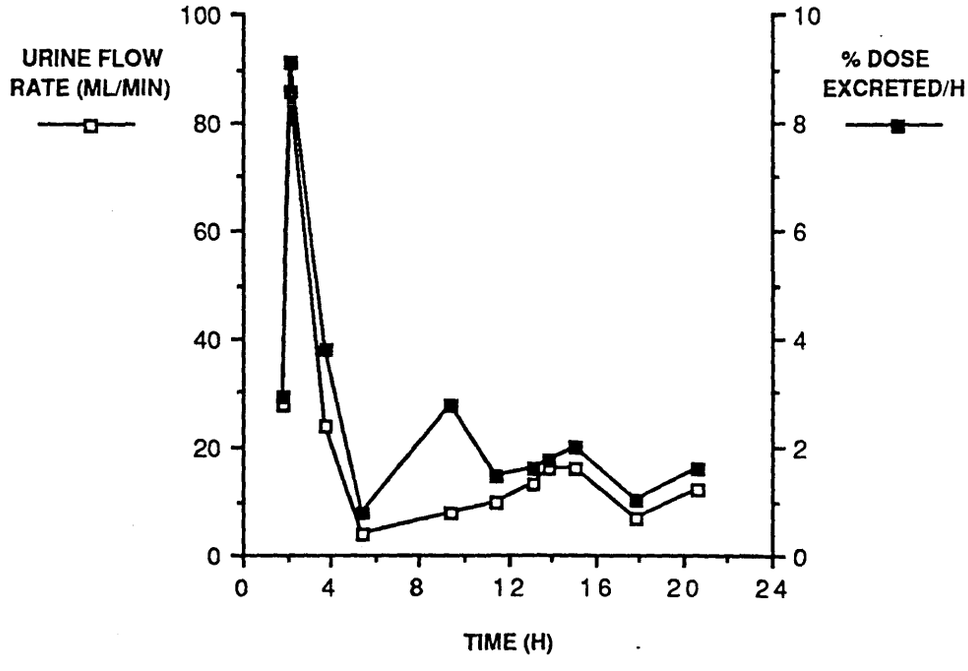


Fig. 7.8. Relationship between urine flow rate and theophylline excretion (expressed as % dose excreted/h) in a horse following [14C]-theophylline (1 mg/kg; p.o.) administration



7.4. DISCUSSION

The present results indicate that the plasma theophylline concentrations were best described by a 2-compartment open model. However there have been a number of studies in the horse where a triexponential equation was found to offer the best fit (Ayres *et al.* 1985; Ingvast-Larsson *et al.* 1985). In this study the distribution of theophylline from the central compartment was slow ($t_{1/2\alpha}$ 2.284 $\pm$ 2.216 h), and showed considerable animal variation. It is similar to that reported by Kowalczyk *et al.* (1984) following intravenous administration of theophylline and for caffeine in the horse (Green *et al.* 1983). Theophylline is rapidly absorbed (t_{max} 0.53 $\pm$ 0.21h), thus the slow distribution phase could be a problem in veterinary practice when using high doses of theophylline in that the very rapid absorption of the drug could lead to toxic concentrations due to plasma concentrations not falling as rapidly as predicted from other species. Errecalde *et al.* (1985) found that theophylline associated side-effects were found at all doses administered intravenously (5,10 and 15 mg/kg) during the distributive phase. It is relevant to note that, in contrast, the distribution phase in cats and dogs is extremely fast (15 min in both species) (McKiernan *et al.* 1983; McKiernan *et al.* 1981).

The mean elimination half-life of 18.7 h is consistent with the findings of other investigators (Kowalczyk *et al.* 1984; Errecalde *et al.* 1984; Ingvast-Larsson *et al.* 1985; Ayres *et al.* 1985). Davis (1978) reported a half-life of 3 h, this discrepancy cannot be evaluated as neither plasma concentrations nor the kinetic analysis were detailed in the paper. Our $T_{1/2\beta}$ value is very similar to that given for caffeine (18.2 h) in the horse, which is perhaps to be expected, in that theophylline differs only by the absence of a methyl group (Green *et al.* 1983). However in man the half-life for theophylline is nearly three times that of caffeine. The elimination rate in the horse is slower than that documented for man, dogs, cats and swine (Rall 1980; McKiernan *et al.* 1981; McKiernan *et al.* 1983; Koritz *et al.* 1981). It has been shown in all these species that $T_{1/2\beta}$ is the pharmacokinetic parameter with the greatest degree of

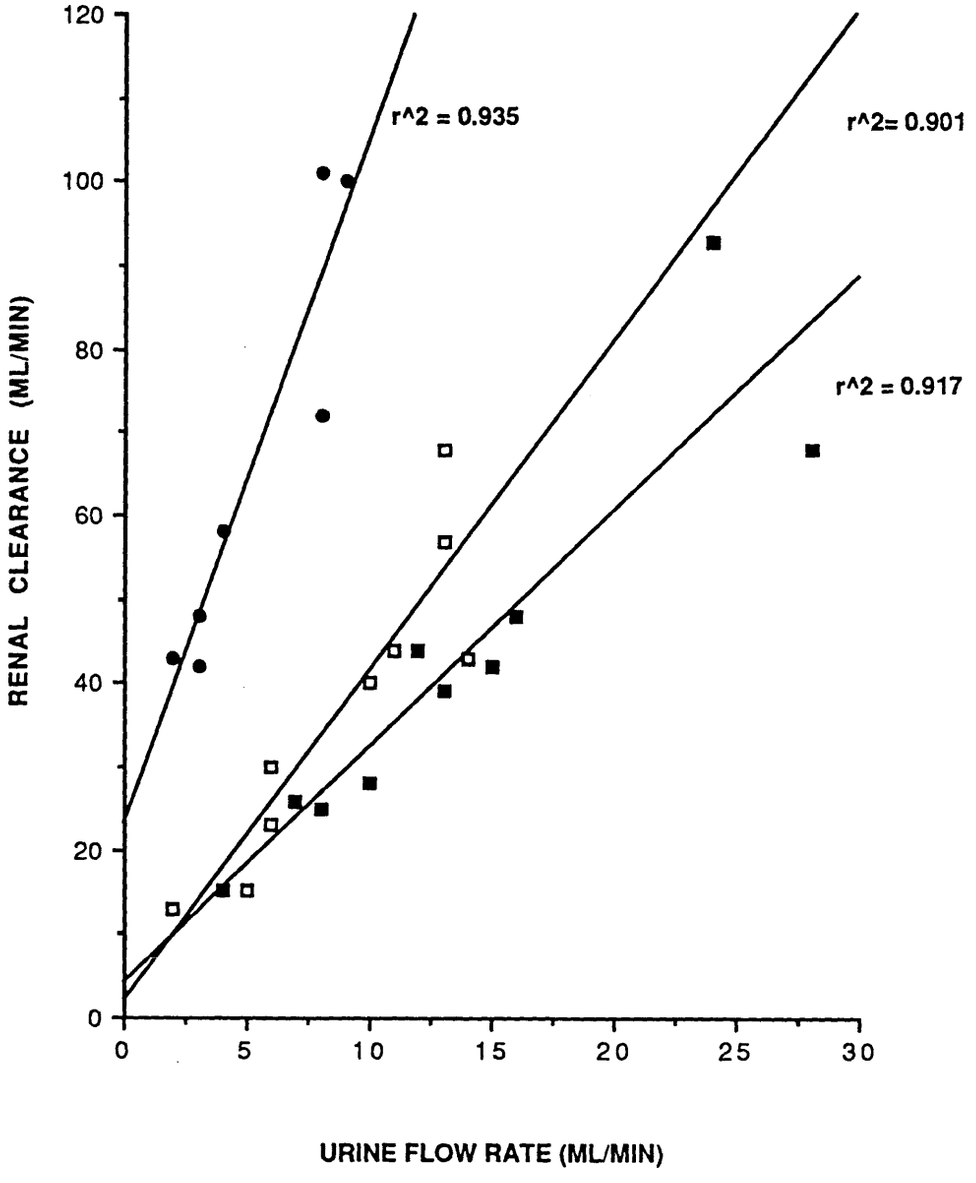
variability, presumably due to theophylline being extensively metabolised in the liver, and the rate of metabolism being the critical factor.

The volume of distribution determined in this study is very similar to that found previously in the horse, a value of 0.897 l/kg indicating fairly extensive distribution to the tissues. The V_d is almost twice that reported in man, the average being between 0.4 to 0.5 l/kg (Mitenko and Ogilvie, 1973; Piafsky and Ogilvie, 1975). In man once theophylline enters the systemic circulation, approximately 60% becomes bound to plasma proteins (Simons *et al.* 1979; Lesko *et al.* 1981). The larger V_d in the horse could be due to less extensive plasma protein binding. This is supported by Kelly and Lambert (1978), who state that only 20% of the total plasma theophylline concentration is bound to plasma protein in the horse. The total plasma clearance of theophylline found in this study is low (0.033 l/h/kg) and agrees with the findings of others (Ingvast-Larsson *et al.* 1985; Kowalczyk *et al.* 1984).

Truitt *et al.* (1950) found that administration of a single dose of theophylline as aminophylline caused an increase in urine flow rate within 1 hour after drug administration. He states that a blood level of theophylline of 5 µg/ml is necessary for a diuretic effect. However, in this study the same initial increase in urine flow rate was observed a little later with lower blood levels. Since fluid intake was restricted for 6 hours after theophylline administration, this indicates that the increased flow rate was due to a brief diuretic effect rather than a change in the rate of fluid intake by and hydration of the horses with time. As has been found in other species (Levy and Koysooko, 1976), the renal clearance of theophylline in the horse is urine flow-dependent (Chapter 8). Thus the increased urine output leads to increased elimination of unchanged theophylline: when the urine flow rate was 28 ml/min the renal clearance was 68 ml/min compared to 15 ml/min at a flow rate of 4 ml/min. Fitting simple curves to the data shows that the correlation coefficients between urine flow rate and renal clearance for the 3 horses over 24 h were greater than 0.9 (Fig.7.9.).

Oxidation at C-8 giving 1,3-dimethyluric acid is the major route of metabolism accounting for 42.8% of the dose. Theophylline is a good substrate for the microsomal cytochrome P450 enzyme system and it has been shown in man that the C-8 oxidation

Fig.7.9. Correlation between renal clearance of theophylline and urine flow rate in three horses following [14C]-theophylline (1 mg/kg; p.o.) administration



to 1,3-dimethyluric acid is mediated by this system (Grygiel *et al.* 1979). It is likely that this same enzyme is responsible for the oxidation in the horse. 1-Methyluric acid and 3-methyluric acid are most probably formed by N-demethylation of theophylline followed by oxidation, since it has been shown in man that the dimethyluric acids are not demethylated to produce monomethylurates (Birkett *et al.* 1983). N-demethylation of theophylline to the monomethylxanthines has been shown to be mediated by a single isozyme of the P-450 system, which has a distinct substrate specificity from that responsible for C-8 oxidation (Grygiel and Birkett, 1980, 1981). This is supported by the ability of cimetidine to inhibit the N-demethylation, but not C-8 oxidation, of theophylline (Grygiel *et al.* 1984).

The intermediate metabolite 1-methylxanthine is rapidly converted to 1-methyluric acid (Grygiel *et al.* 1979). The rate of formation of 1-methylxanthine is the rate limiting step, this being slower than its conversion to 1-methyluric acid, thus it is not often possible to detect the xanthine in the urine. 3-Methylxanthine was not detected at all in the horse, presumably due to its rapid conversion to 3-methyluric acid. This is in stark contrast with the data found in man, 3-methylxanthine being a quantitatively important urinary metabolite (Arnaud and Welsch, 1981). The monomethylxanthines are not substrates for the mixed function oxidase (cytochrome P_{450}) system at all (Birkett *et al.* 1981). It has been reported that 1-methylxanthine is a good substrate for the cytosolic enzyme xanthine oxidase, whereas the dimethylxanthines and 3-methylxanthine are not oxidised by this enzyme at all in man (Birkett *et al.* 1981, 1983). From our results it appears that 3-methylxanthine is a substrate for xanthine oxidase in the horse. This indicates an important species difference in the hepatic biotransformation of theophylline.

A time-dependent change in the pattern of urinary metabolites will result in the ratio of metabolites changing, and may enable analysts to determine the time of drug administration much more precisely (Smith 1987). This technique has important forensic implications especially when enforcing controlled medication programmes and "time rules" (Houghton *et al.* 1986). However, the absence of a time dependent pattern in the urinary metabolic profile for theophylline, does not enable one to measure the

change in the ratios of different metabolites. Thus for this compound it is not possible to determine the time of drug administration from a single urine sample.

This study is of potential benefit, as an increased knowledge of the pharmacokinetics and metabolic disposition of theophylline is useful in assisting racing chemists in their efforts to control illicit drug use, the methylxanthines being the second most commonly encountered class of compounds after the non-steroidal anti-inflammatories.

Chapter 8

THE EFFECT OF FRUSEMIDE ON THE DISPOSITION OF THEOPHYLLINE

8.1. INTRODUCTION

Frusemide's extensive use in racing and in sport in general, has been highlighted by the increased incidence of its detection in recent years. Frusemide was the sixth most commonly encountered drug, by members of the Association of Official Racing Chemists (AORC) in 1987-1988; and at the Seoul Olympics in 1988, four of the ten positives in athletes were for frusemide. As already discussed, the increasing popularity of frusemide treatment has led to widespread concern over its effect on the detection of other drugs. A likely consequence is that the diuresis following frusemide treatment may result in the dilution of drugs and drug metabolites in post-race urine samples thereby rendering their detection more difficult (Roberts *et al.* 1976; Tobin *et al.* 1977; Miller *et al.* 1977; Combie *et al.* 1981; Soma *et al.* 1984). Having investigated the effect of frusemide on the fate of an acidic drug, salicylic acid (Chapter 4) it was decided to study a basic drug. Theophylline was chosen in view of its therapeutic use and intermittent occurrence as a drug of abuse in the horse.

The methylxanthines are among the many drugs which are sometimes used as stimulants by sportsmen (Delbeke and Debackere, 1988) and in racehorses (Green *et al.* 1983; Lambert and Evans, 1983). Drug-positives for methylxanthines originate both from deliberate doping and accidental or inadvertent doping, especially from compounds present in the diet. Data reported by the AORC for the period 1947-1973 show that the methylxanthines accounted for 18.9% of all drugs detected (Tobin 1981; Smith 1987). In 1987-1988, though their use was reduced, they still represented some 10% of all positives.

The therapeutic uses of theophylline have been outlined in the previous chapter. Theophylline has also been indicated to treat respiratory conditions where bronchospasm plays a role in the disease process (Davis 1980; Dersken 1983). It is potentially useful in the treatment of chronic obstructive pulmonary disease (COPD) and congestive heart failure in the horse (Gerber 1973; Davis 1978; Thomson and McPherson, 1983; Kowalczyk *et al.* 1984). Theophylline is one of the most extensively studied drugs and there is an abundance of information on its disposition, efficacy and

toxicity. However, though theophylline has been reported to interact with other drugs of clinical importance (Jacobs *et al.* 1976), data on drug interactions involving theophylline are more limited. Many of the interacting drugs influence the metabolic clearance of theophylline (Jonkman and Upton, 1984) e.g. inhibition of cytochrome P450 by erythromycin and cimetidine increases serum theophylline concentrations and phenobarbitone and phenytoin decrease serum concentrations by enhancing hepatic metabolism of theophylline (Cummings *et al.* 1977; Landay *et al.* 1978; Weinberger *et al.* 1981).

The concurrent administration of theophylline and diuretics is common in clinical practice, for the treatment of patients with chronic obstructive pulmonary disease accompanied by congestive heart disease. Since it has been shown in man that the renal clearance of theophylline is dependent on the urinary flow rate (Levy and Koysooko, 1976; Tang-Liu *et al.* 1982), concomitant treatment with a potent diuretic such as frusemide could theoretically lead to a significant interaction by increasing theophylline clearance, and so cause a fall in plasma concentrations. However, reports on this possible interaction are limited and conflicting, an increase, a decrease and no change in the plasma concentration of theophylline having been reported (Grambau *et al.* 1978; Vrhovac 1978; Carpentiere *et al.* 1985; Janicke *et al.* 1987).

It has been suggested that frusemide consistently increases plasma theophylline concentrations (Grambau *et al.* 1978; Vrhovac *et al.* 1978). However, Toback and Gilman (1983) found that, in newborns, plasma theophylline concentrations decreased when frusemide was given simultaneously. Reductions in plasma concentrations were also found by Carpentiere *et al.* (1985), this being attributed to increased clearance of the drug. Finally, Janicke *et al.* (1987) found that frusemide did not influence the steady-state plasma concentrations of theophylline in any way. The possibility of a theophylline-frusemide interaction has important clinical implications, pharmacokinetic dosing regimens need to be applied cautiously to patients receiving both drugs due to theophylline's narrow therapeutic range (Conlon *et al.* 1981).

This experiment was designed to investigate the effect of frusemide on the metabolism, pharmacokinetics and detectability of a model basic drug. The results are

discussed in terms of the pharmacokinetic drug interaction between frusemide and theophylline and of how frusemide might affect other basic drugs with which it may be administered.

8.2. METHODS

Compounds

Frusemide (4-chloro-5-sulphamoyl-N-furfurylanthranilic acid; Lasix 5% solution;) was purchased from Hoechst (Milton Keynes, Bucks, U.K.). [8-^{14}C]-Theophylline specific activity 317 μCi/mg, radiochemical purity >98% by HPLC was purchased from Amersham (Amersham International, Aylesbury, U.K.). Sources of xanthine and uric acid derivatives were as follows: theophylline (1,3-dimethylxanthine, 1,3-DMX), and 1,3-dimethyluric acid (1,3-DMU) from Sigma (Poole, U.K.); 1-methyluric acid (1-MU) and 3-methyluric acid (3-MU) from Fluka (via Fluorochem, Glossop, U.K.). Other materials purchased were: sodium acetate trihydrate (Analar grade) from BDH (Poole, U.K.); HPLC grade acetonitrile from Rathburn Chemicals Ltd. (Walkerburn, U.K.).

Animals and Drug Administration

Drugs were administered at the Horseracing Forensic Laboratory to three thoroughbred horses (body weights 474-530 kg). Each horse received three different treatments at the same time of day in successive dosing sessions. A wash-out period of one week was allowed between treatments to ensure that there was no interference.

Treatments

A. Frusemide (1 mg/kg) intravenously;

B. [8-^{14}C]-Theophylline (1 mg/kg; 100 μCi/horse) orally;

C. [8-^{14}C]-Theophylline (1 mg/kg; 200 μCi/horse) orally followed 4 hours later by frusemide (1 mg/kg) intravenously.

[^{14}C]-Theophylline (1 mCi) was dissolved in methanol (10 ml). Prior to administration [^{14}C]-theophylline (1 ml of the methanolic solution) was added to water (100 ml) and heated to 40°C with occasional stirring for 1 min. Unlabelled theophylline (523-585 mg) was added slowly with continuous stirring, and when dissolved the solution was well mixed and transferred to a plastic bottle, cooled and made up to 400ml with water. The [^{14}C]-theophylline was administered by a stomach tube, with a total volume of 1 litre of water. Following administration of theophylline, the horses were moved to metabolism stalls in which the movement of the horse is restricted, this is designed for the convenient collection of samples (Marsh 1983).

Frusemide (9.5-10.6 ml of a 5% solution) was administered by intravenous infusion over 30 sec via the jugular vein.

Water was available before dosing but food was withheld overnight prior to dosing which started at approximately 8.30 am. The frusemide dose was given at approximately midday for both treatment A. and C. Both food and water were withheld until approximately 2.00 pm; i.e. two hours after the frusemide dose or six hours if theophylline was administered alone. Each morning and afternoon during the course of the experiment the horses were exercised for 30 minutes before being returned to the metabolism stalls.

Blood Collection

Blood samples (50 ml) were obtained at various times after dosing, from an indwelling jugular venous cannula. The blood was collected into Monovettes containing lithium heparin crystals (Sarstedt Ltd. Leicester, U.K.). 0.5 ml of the blood was removed into glass tubes for packed cell volume determination by the microhaematocrit

method. The rest was centrifuged at 2000 rpm for 5 minutes and the plasma aspirated. Before storage of plasma at -20°C, duplicate 1ml samples were removed and counted for ^{14}C. Total plasma proteins were determined by the method of Bradford (1976).

Urine Collection

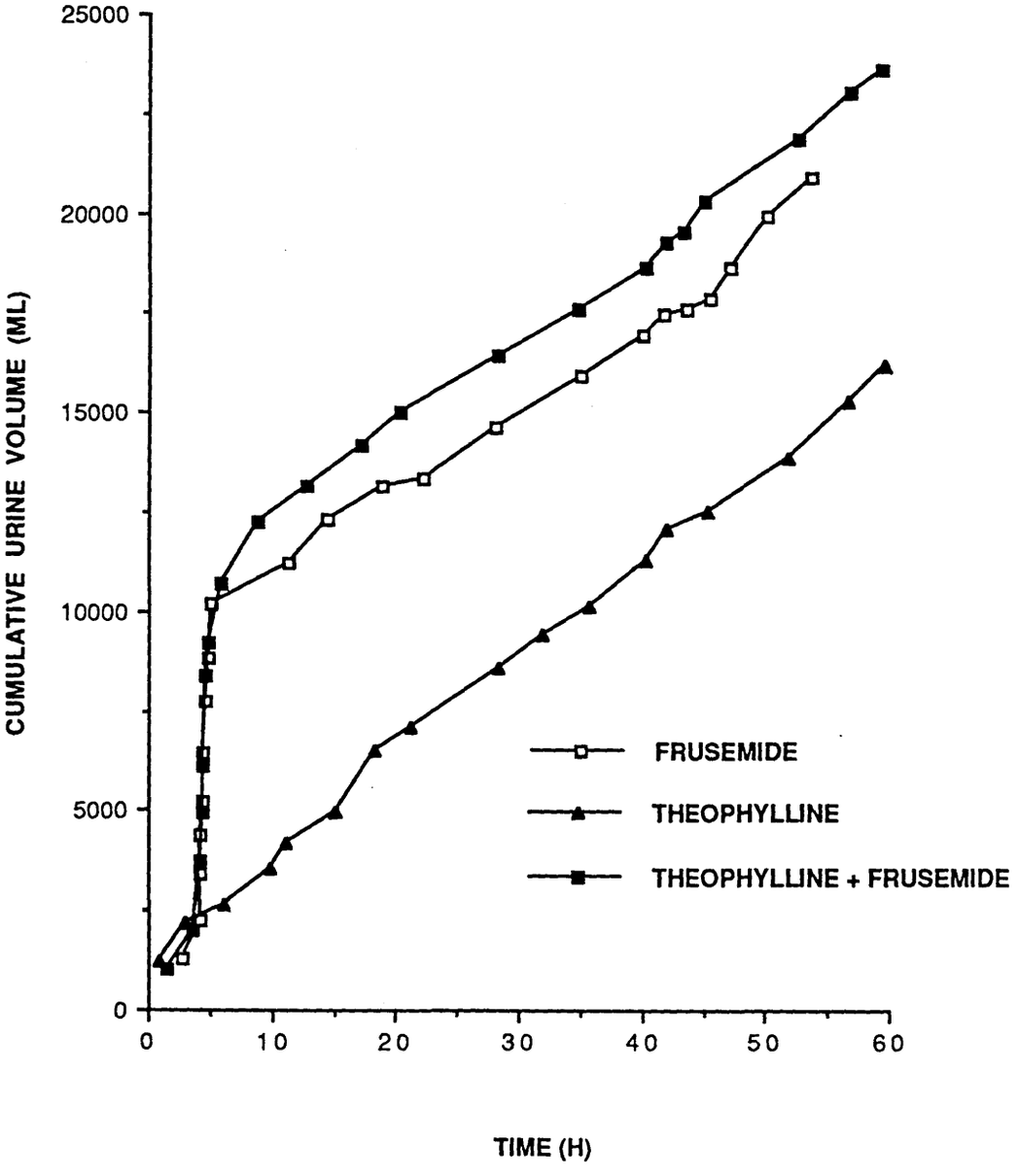
Urine was collected as previously described (Marsh 1983) for approximately 100 h. The time at which each urine sample was voided was recorded and the volume measured. Immediately on collection the pH was measured and the specific gravity obtained using a refractometer. A small volume of urine (15 ml) was centrifuged at 3500 rpm for 10 min to obtain a clear supernatant and, 0.1 ml aliquots were removed in duplicate for scintillation counting when theophylline was administered. An aliquot of each sample (ca. 120 ml) was kept for creatinine (Jaffé method) and drug analysis and the rest discarded. All urine samples were frozen and transported to London for storage at -20°C prior to analysis.

Radiochemical techniques, high-performance liquid chromatography for theophylline and its metabolites and pharmacokinetic methods were performed as described in Chapter 7.

8.3. RESULTS

Frusemide produced a rapid and short-lasting diuretic effect. Urine flow rates increased to 200 ml/min and approximately 8 litres of urine were produced within one hour of administration. This increase is illustrated in Fig.8.1. which shows cumulative urine volumes in one horse, following the three treatments. The transient nature of the diuresis is also evident, the urine flow rate returning to normal quite rapidly, the slopes of the three lines corresponding to cumulative urine volume being parallel after the

Fig 8.1. Cumulative urine volumes in a horse given [14C]-theophylline (1 mg/kg; p.o.), frusemide (1 mg/kg; i.v.) and [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions



potent diuresis. The data also indicate that theophylline itself may exert a diuretic effect. The cumulative urine volume was slightly higher when theophylline was administered with frusemide compared with frusemide given alone. 36 h after frusemide the cumulative urine volume was 15.9 l compared to 17.6 l when theophylline was also given. This difference was maintained over the whole collection period.

Plasma Pharmacokinetics

The ^{14}C levels (μg equivalents) in plasma plotted against time are presented in Fig.8.2. which shows plasma radioactivity concentrations in the same horse with and without administration of frusemide. Frusemide does not appear to have any effect on plasma concentration of theophylline. Plasma theophylline concentrations declined biphasically with time and the experimental data were well described by a 2-compartment open model for all six administrations. Fig.8.3. illustrates plasma level-time curves for both theophylline and frusemide when they are administered concomitantly. It can be seen that theophylline plasma concentrations decline very slowly in comparison with frusemide. The concentration of both drugs is almost the same i.e. 1.17 μg/ml for theophylline and 1.09 μg/ml for frusemide, 0.5 h after frusemide administration, however frusemide concentrations fall to below 15 ng/ml within 6 h while theophylline decays much more slowly (510 ng/ml at 12 h). The graph also shows that there is no change in the plasma theophylline curve as a result of the diuretic.

The pharmacokinetic parameters describing the plasma concentrations with and without frusemide are listed in Table 8.1. corrected where appropriate for the weight of the horse.

Frusemide was administered 4 h after theophylline, thus the absorption phase was complete and frusemide's effect on the elimination of theophylline could be assessed. Peak theophylline concentrations (C_{max}) were just under 2 μg/ml and were obtained within 45 min following each administration, indicating rapid absorption. The elimination half life ($t_{0.5\beta}$) of theophylline showed a degree of inter-animal variability (14.9-20.5 h) but this was not attributable to the diuretic. Only in one horse was the

Fig 8.2. Plasma concentration of 14C-radioactivity in a horse given [14C]-theophylline (1 mg/kg; p.o.) and [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

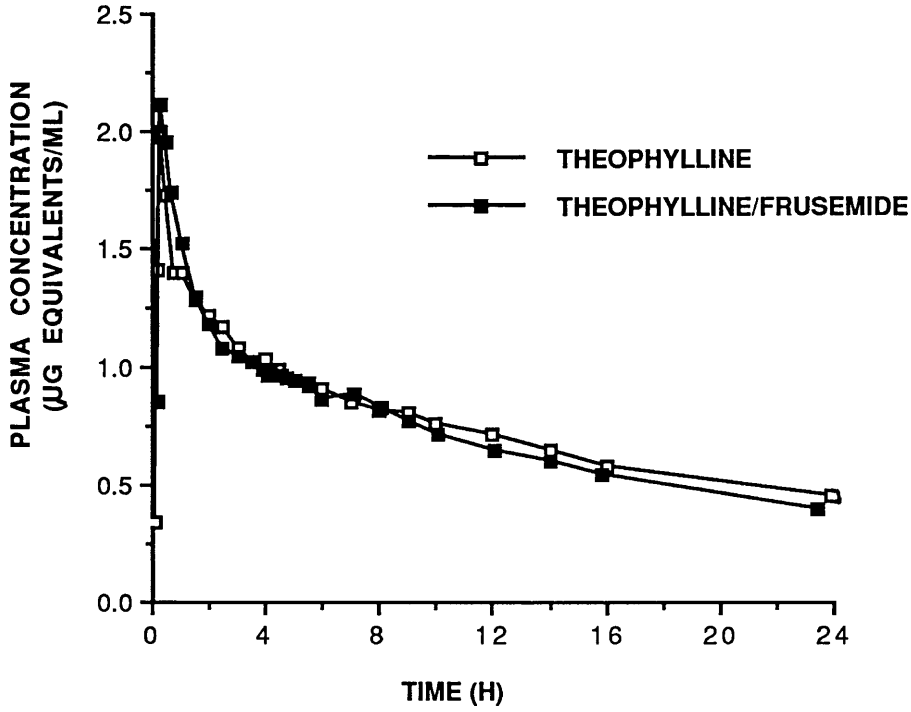


Fig.8.3. Plasma level-time curves for theophylline and frusemide when [14C]-theophylline (1 mg/kg; p.o.) and frusemide (1 mg/kg; i.v.) were administered together in the same horse

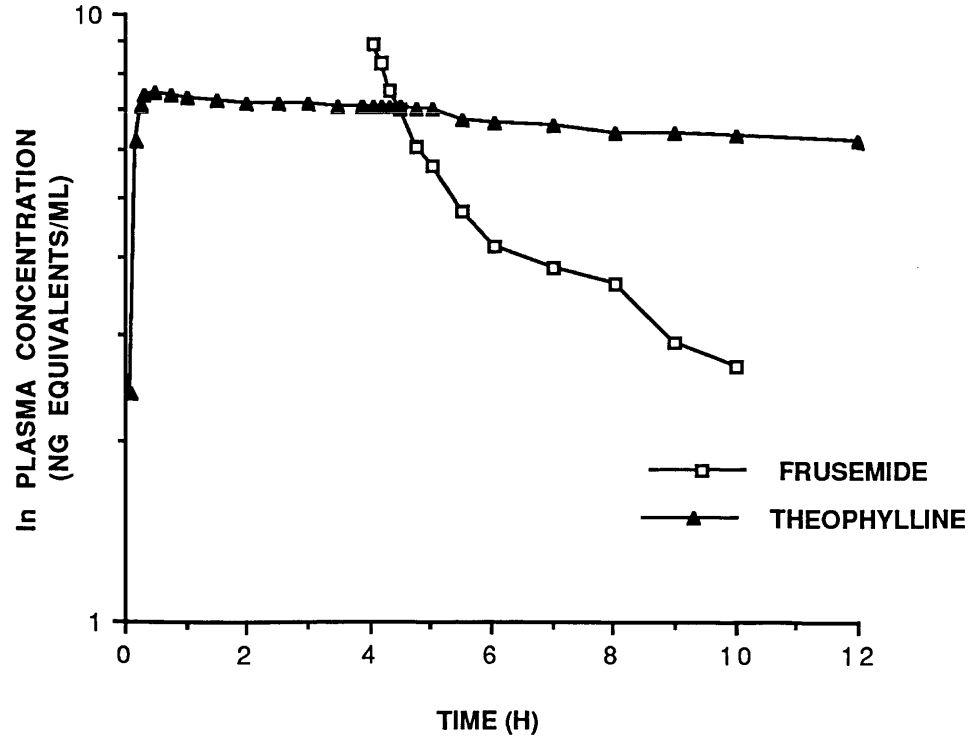


Table 8.1. Pharmacokinetic parameters describing the plasma levels of theophylline in the horse, following the administration of [^{14}C]-theophylline (1 mg/kg; p.o.) and [^{14}C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions (mean ± s.d., n=3)

		Theophylline	Theophylline + Frusemide
C_{max}	(μg/ml)	1.81 ± 0.16	1.74 ± 0.34
K	(h^{-1})	0.038 ± 0.006	0.042 ± 0.007
$T_{1/2\beta}$	(h)	18.7 ± 2.67	16.9 ± 2.82
V_d	(l/kg)	0.897 ± 0.028	0.965 ± 0.144
Cl	(l/h/kg)	0.033 ± 0.004	0.040 ± 0.002*
AUC	(μg/ml.h)	29.9 ± 3.4	25.3 ± 1.4

* $p < 0.05$, significantly different from theophylline alone

half life decreased in the presence of frusemide. The mean volume of distribution of theophylline was very consistent and was unaltered by frusemide, but there occurred a significant (0.033 l/h/kg vs. 0.040 l/h/kg; n=3, $p < 0.05$) increase in the clearance of theophylline. The AUC was also reduced, but this did not achieve statistical significance.

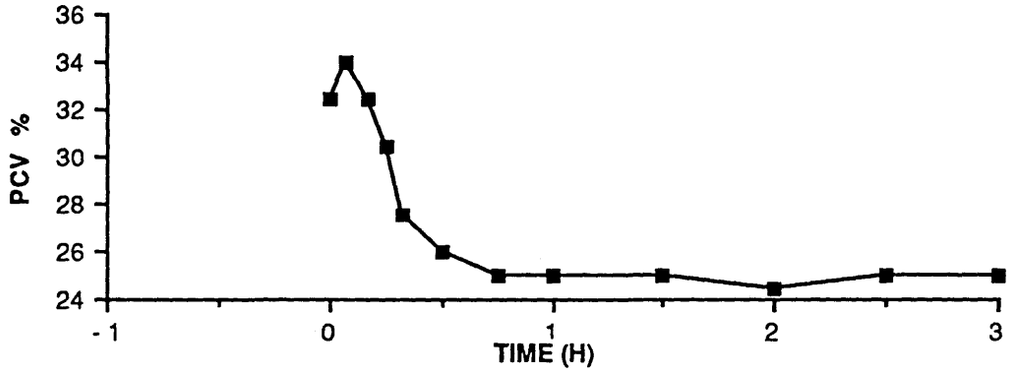
Packed Cell Volume Determinations

Packed cell volume (PCV) and total plasma proteins (TPP) were determined in order to obtain a measure of fluid withdrawal from the plasma during the course of the experiment. Fig.8.4. shows the effect of cannulation, frusemide administration and feeding on PCV.

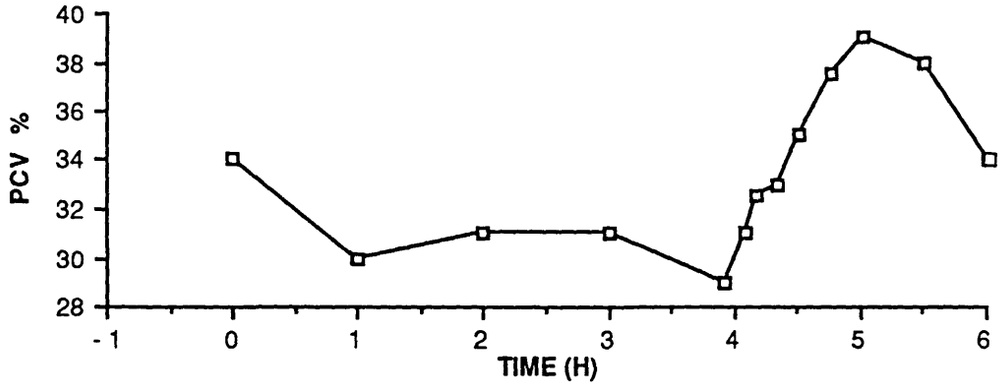
One horse (Theeb) used in these experiments was very excitable when cannulae were inserted and when the nasogastric tube was passed and the effect of these procedures on PCV are very evident (Fig.8.4a.). PCV declined by as much as 10% after this, back to the presumed resting level. Fig.8.4b. shows the marked increase seen in one horse after frusemide. Similar results were obtained for all the horses. The increase in PCV for this horse from 29% just before frusemide administration to 39% one hour later was due to an estimated loss of 32% in plasma volume, equivalent to about 8 litres of fluid over the period of diuresis. This is in keeping with the increased urine output at the time. Even larger increases in PCV occurred when the horses were fed, which was after they had been fasting for 20 h. These changes were seen in all nine experiments, but were more clear when no frusemide had been given (Fig.8.4c.). An increase in PCV from 23% to 34% which was detected over a period of 2 h represents a reduction in plasma volume equivalent to the abstraction of some 11 litres of fluid from the systemic circulation, greater than was seen after frusemide. Fig.8.4c. also illustrates that after an animal has been fasted, when the gut presumably contains relatively little fluid, the PCV is appreciably lower i.e. 23-24%. After feeding the PCV is almost constant, being about 28% up to 24 h, about 5% higher than before feeding.

Fig.8.4. The effects of cannulation, frusemide administration (1 mg/kg; i.v.) and feeding on packed cell volume (PCV) in the horse

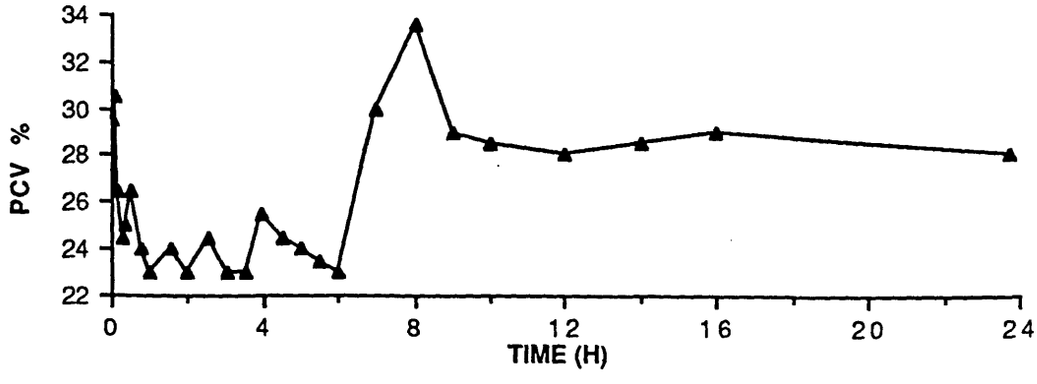
(a) Effect of the cannulation procedure (Theeb)



(b) Effect of frusemide (1 mg/kg; i.v.) administration (Pendulate)



(c) Effect of feeding after a 20 h fast (Pendulate)



Total Plasma Protein Determinations

Measuring total plasma proteins (TPP) revealed similar results to PCV determinations, reflecting fluid shifts in plasma volume. Fig.8.5. illustrates the data from a single horse following each of the three treatments. When theophylline was given there was little change in TPP concentrations, however, after feeding protein concentrations rose by 18% to a peak of 79.6 mg/ml over 2 h (Fig.8.5a.).

Following frusemide TPP concentrations increased from 70.5 mg/ml to 79.8 mg/ml, i.e. 13% within 20 min (Fig.8.5b.). Protein concentrations then fell gradually, possibly due to the horses having access to water during this time, so that plasma volume may be replenished. However, following feeding, shortly after the 6 h blood sample was taken TPP rose again to a peak of 87.6 mg/ml, causing a larger increase than after frusemide, but over a longer period of time.

With concurrent administration of theophylline and frusemide (Fig.8.5c.), it can be seen that the diuretic causes a large increase in protein concentration, from 67.9 mg/ml to 88.4 mg/ml, in 0.5 h. Feeding at approximately 6 h does not increase TPP, and the concentration remains fairly constant at about 80 mg/ml, similar to the PCV data following feeding (Fig.8.4c.).

Urinary Excretion

The excretion of theophylline in the horse was slow. Urine samples were collected for 96 h after the oral administration of [^{14}C]-theophylline, by which time urinary excretion was essentially complete. Total radioactivity recovery was 64.0-76.5% (n=6) of the dose.

Plots of urinary concentration against time show the influence of the large variations in flow rate of urine and highlight the considerable dilution of radioactivity caused by the increased fluid output following frusemide administration at 4 h (Fig.8.6.). Due to an approximate 5-fold increase in urine volume there was a decrease in urinary theophylline concentration (Table 8.2.). However, concentrations and urine volumes returned to pre-frusemide levels quite slowly i.e. 7-8 h after frusemide administration. Plotting the cumulative excretion of radioactivity (Fig.8.7.) eliminates

Fig.8.5. Effect of [14C]-theophylline (1 mg/kg; p.o.), frusemide (1mg/kg; i.v.) and [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) administration on total plasma proteins (TPP) in the horse

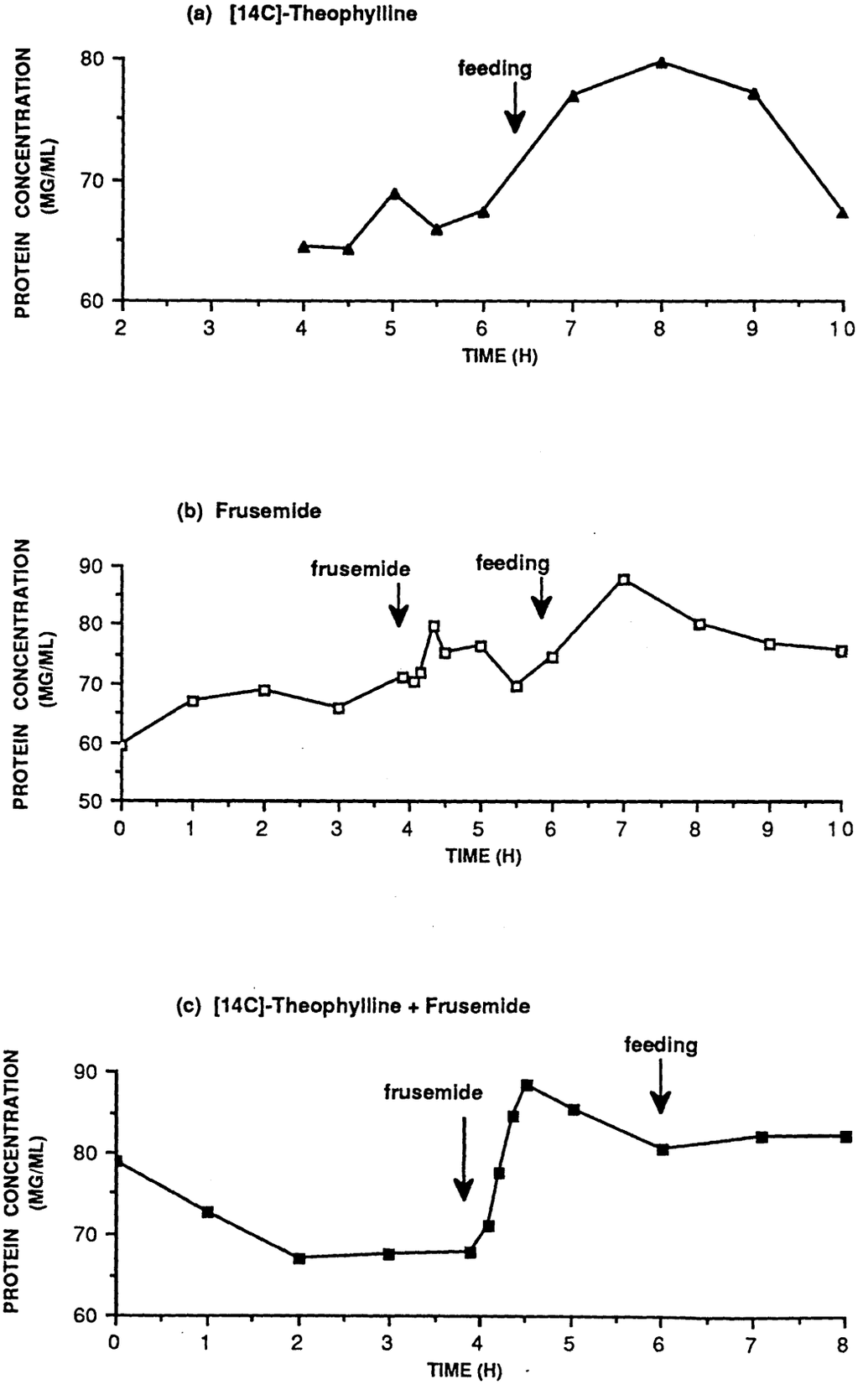


Fig.8.6. Urinary concentration of 14C-radioactivity in a horse given [14C]-theophylline (1 mg/kg; p.o.) and [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

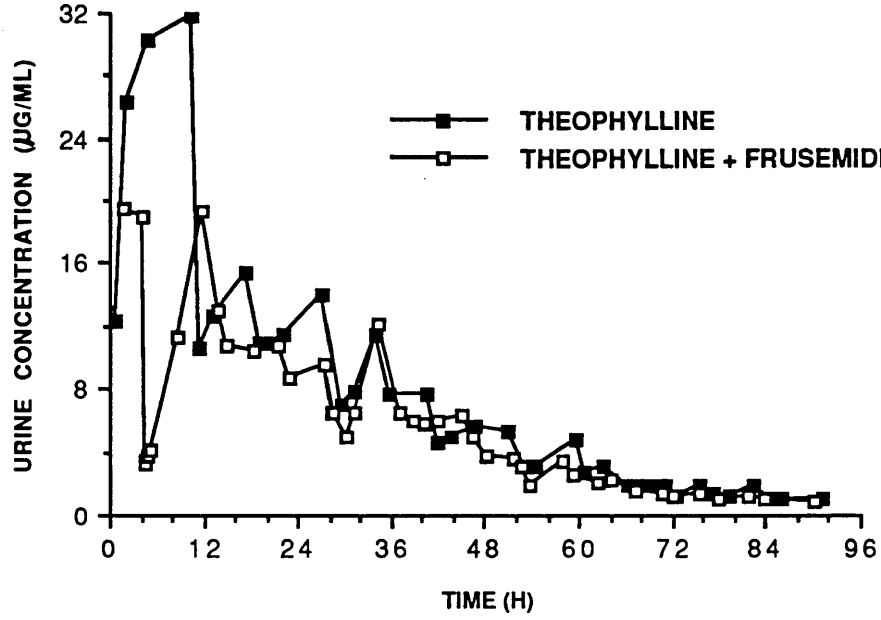


Fig.8.7. Cumulative urinary excretion of 14C-radioactivity by a horse given [14C]-theophylline (1 mg/kg; p.o.) and [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

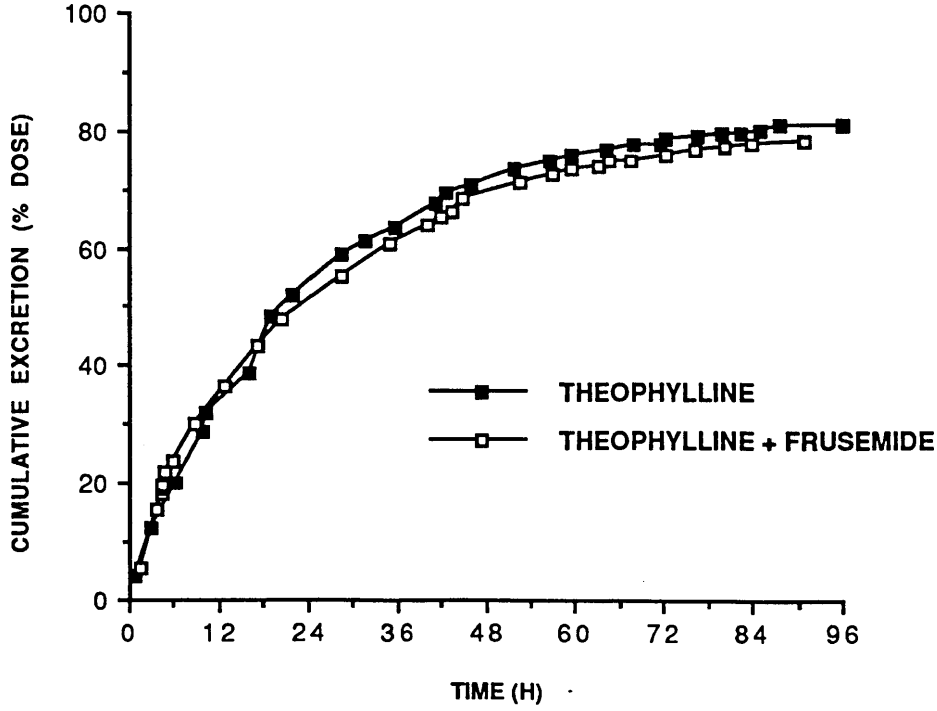


Table 8.2. Urinary excretion of theophylline within 6 h in the horse, following the administration of [^{14}C]-theophylline (1mg/kg; p.o.) and [^{14}C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions

	Theophylline	Theophylline + Frusemide
Urine Volume (ml)	2,647	10,695
Amount of Theophylline (mg)	94.5	112.2
Concentration of Theophylline (mg/ml)	35.70	10.49

Table 8.3. Urinary metabolites of theophylline in the horse, following [^{14}C]-theophylline (1 mg/kg; p.o.) and [^{14}C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) on separate occasions (mean ± s.d., n=3)

	% Dose in 96 h	
	Theophylline	Theophylline + Frusemide
Theophylline	15.9 ± 6.1	17.0 ± 5.0
1,3-Dimethyluric acid	42.8 ± 1.3	44.5 ± 5.5
1-Methyluric acid	8.9 ± 2.6	8.3 ± 1.2
3-Methyluric acid	1.6 ± 0.4	1.8 ± 0.2

the effect of urine volume and shows that the rate of excretion was unaffected, showing that frusemide did not affect the renal handling of theophylline. Fig.8.8. illustrates the pH of each urine sample following the three treatments to one horse (Pendulate). It can be seen that initially urinary pH was alkaline (8-8.5). Frusemide administration appears to reduce this alkalinity slightly as the pH falls to between 7.2-7.7, during 4-20 h post-dosing. A marked drop in pH was found after each treatment at 28 h post-dosing. This was at approximately 1 pm and was in the first urine sample voided after a 6 h period. This may be due to a cyclic variation dependent possibly on the time lag, feeding or exercise.

HPLC analysis of the urine by the method described showed the presence of 4 discrete radioactive components corresponding to theophylline and three metabolites, 1,3-dimethyluric acid, 1-methyluric acid and 3-methyluric acid. The quantitative recovery of urinary metabolites was very similar after frusemide administration (Table 8.3.). Plots of urinary excretion rate of theophylline and its metabolites show that immediately after frusemide administration at 4 hours there was a marked increase in the excretion of unchanged theophylline (Fig.8.9a.). This effect is short lasting and elimination returns to normal after the massive diuresis. Plotting cumulative recovery of unchanged theophylline illustrates this very clearly, and indicates the transient nature of the increased elimination (Fig.8.9b.).

Fig.8.8. Variation in urinary pH in one horse following theophylline (1 mg/kg; p.o.), frusemide (1 mg/kg; i.v.) and theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.) administration on separate occasions

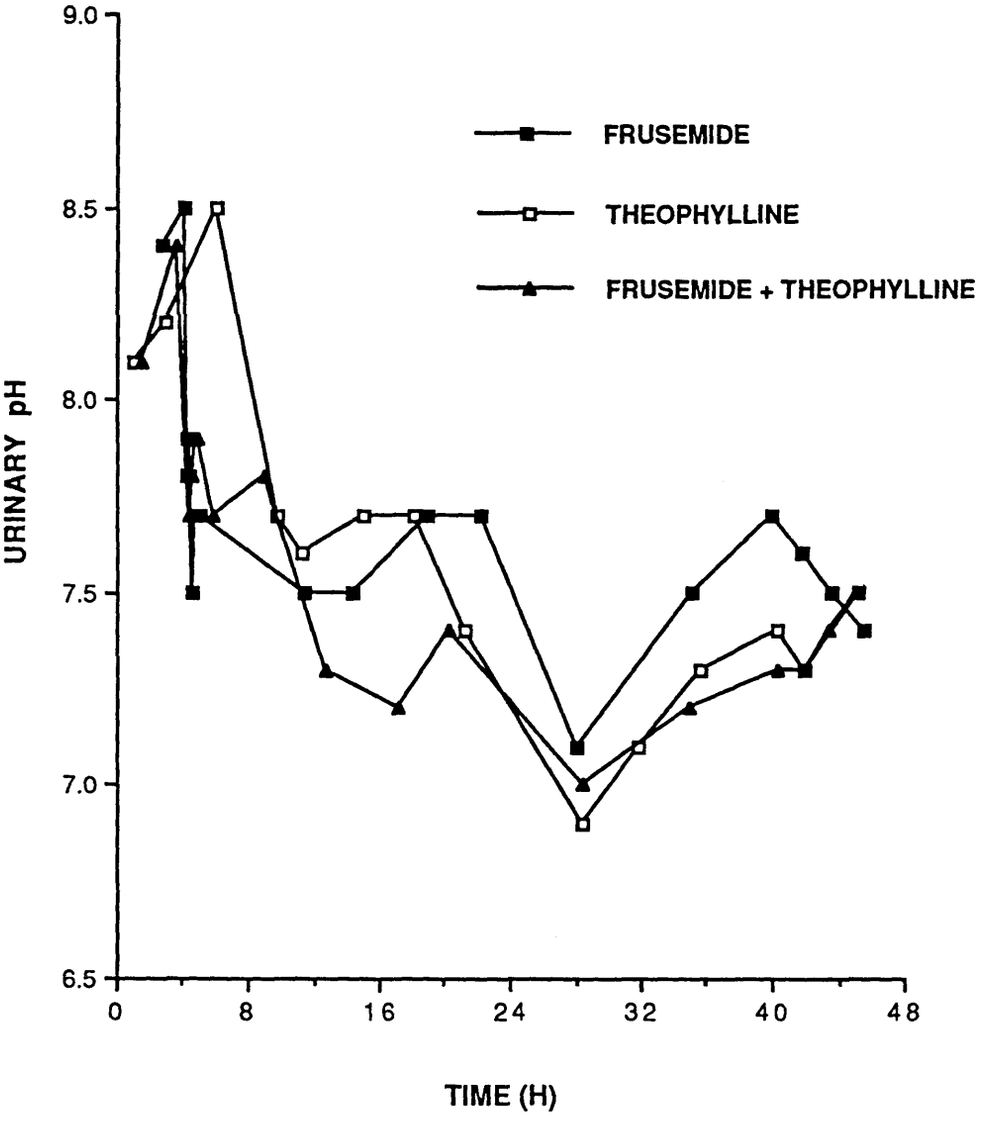
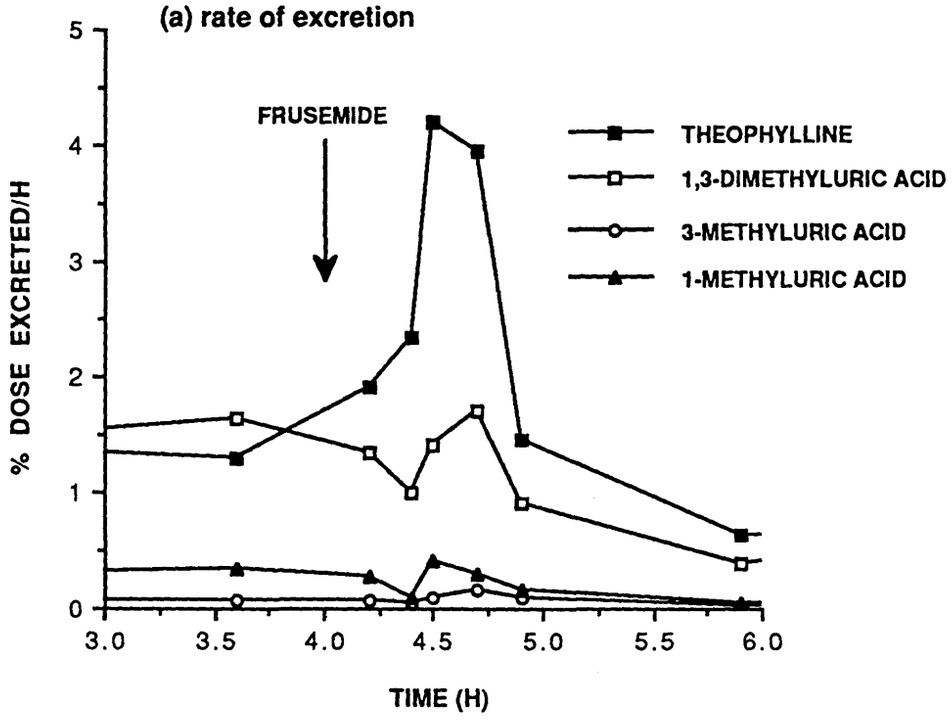
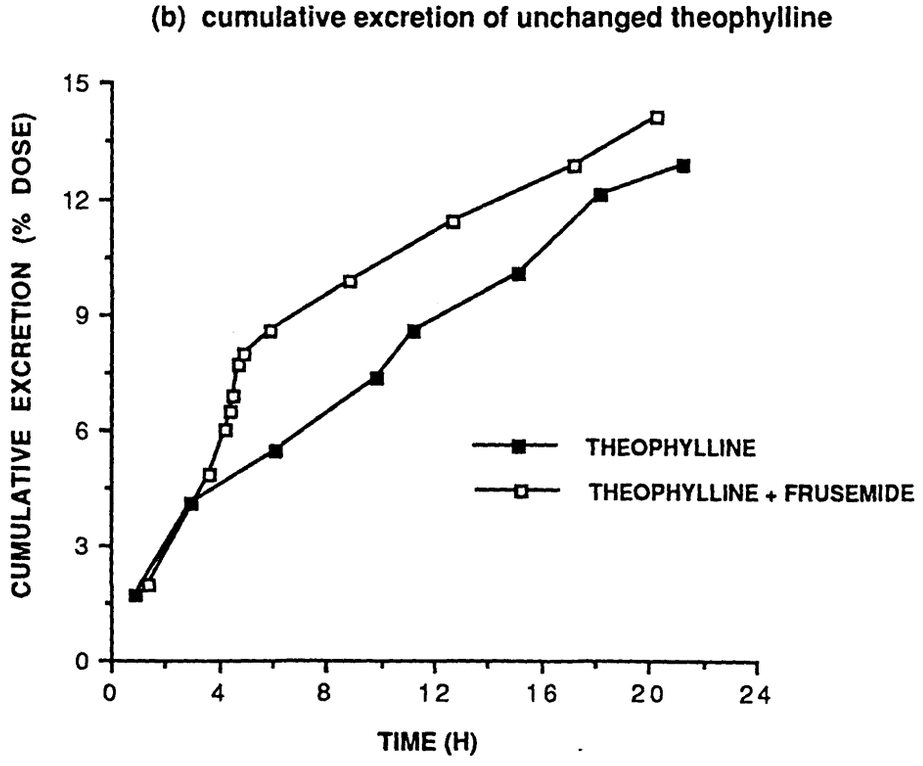


Fig.8.9. The urinary excretion of theophylline and its metabolites in a horse given [14C]-theophylline (1 mg/kg; p.o.) with frusemide (1 mg/kg; i.v.) 4 hours later





8.4. DISCUSSION

At the recommended therapeutic dose of 1 mg/kg intravenously, frusemide produced a marked diuresis in the horse. In this study, frusemide alone caused a striking increase in urine flow rate, resulting in a large increase in urine volume and a corresponding decrease in its specific gravity.

In this study the plasma concentration of theophylline was not affected by frusemide administration. This has also been shown recently by Stevenson *et al.* (1990), in the only report of this interaction in the horse. There have been a number of studies in man which have looked at the influence of frusemide on theophylline pharmacokinetics, particularly plasma concentrations. Our findings are in agreement with those of Janicke *et al.* (1987) who studied this interaction in healthy volunteers. A number of groups have reported a consistent increase in plasma theophylline concentration (Grambau *et al.* 1978; Vrhovac, *et al.* 1978; Conlon, *et al.* 1981), which was proposed to be due to a reduction in volume of drug distribution but this was only shown in one study (Vrhovac *et al.* 1978). A possible mechanism to explain this interaction may be that the marked diuresis induced by frusemide constricts the volume of distribution (V_d) of theophylline through haemoconcentration and reduction of the extravascular volume. Our findings indicated that frusemide did not reduce the V_d.

In the present experiment plasma clearance of theophylline increased significantly, this was the only pharmacokinetic parameter which was altered after frusemide. Janicke *et al.* (1987) also found an increase in renal clearance of theophylline, which was in parallel with the urinary flow rate after a single 20 mg dose of frusemide. However, after a second administration of frusemide (20 mg) the overall renal clearance calculated over 8 h was reduced by frusemide treatment despite a diuresis. The authors suggest that this may be due to increased reabsorption of water and theophylline in the distal region of the renal tubule following the intense diuresis soon after frusemide, resulting in a fall in the renal clearance of theophylline. Support for the increased clearance leading to lower plasma concentrations hypothesis, comes from a study which found concentrations of theophylline at 1 to 6 hours were

significantly lower when frusemide was given with theophylline than when theophylline was administered with placebo. The mean peak plasma theophylline concentration C_{max}, was approximately 40% lower, no other pharmacokinetic parameters were described (Carpentiere *et al.* 1985).

The elimination half life ($T_{1/2\beta}$) is a secondary parameter dependent on volume of distribution and clearance ($T_{1/2\beta} = \ln 2V_d/Cl$). In two of the horses the $T_{1/2\beta}$ was unaltered by frusemide, due to the increase in clearance being compensated for by a small increase in V_d. However, in one horse it was decreased from 19.9 h to 14.9 h. This was due to the increase in Cl as well as a small decrease in the V_d in this horse.

Parameters used to estimate and monitor the degree of water and/or electrolyte deficit include packed cell volume (PCV) and total plasma protein. It has been shown that frusemide causes increases in haemoglobin concentration, PCV and total protein concentration in the blood of resting or exercised horses, indicating haemoconcentration and a reduction of plasma volume (Fregin *et al.* 1977). Our results indicate that PCV is influenced by a number of factors. In this experiment the excitement caused by cannulating the horses led to an increase in PCV. As there was no fluid loss from the body this is probably due to the mobilization of erythrocytes from splenic stores into the circulation. It is well known that just trotting a horse causes a distinct increase in red cell count (Archer 1968): indeed increases of some $2x10^6$ erythrocytes/ml after 5 min of muscular exercise has been demonstrated (Archer and Clabby, 1965).

PCV is also altered by fluid shifts from the vascular compartment. Frusemide administration leads to pure water loss i.e. loss of solvent in excess of solute. This results in true dehydration, characterised by rising concentration of solutes producing a linear increase in haemotocrit. The fall in plasma volume is similar to the increased urine output elicited by frusemide, indicating that the fluid loss was not being made up from the tissues. If the horse is deprived of water and fluid loss is increased then water is drawn from the cells into the extracellular fluid, maintaining circulating blood volume despite the severe deficits of total body water (Brownlow and Hutchins, 1982).

The largest increase in PCV occurred when the horses were fed after a 20 h fast. During feeding there was a greater loss of water from the plasma than after

frusemide administration. Our results are similar to the findings of Kerr and Snow (1982) who looked at alterations in haematocrit, plasma proteins and electrolytes in horses following the feeding of hay. It is probable that salivary secretion is responsible for the changes in blood composition. Large amounts of saliva must be produced to masticate the hay, as the horse lacks the grinding reticulum and omasum of the ruminant, so coarse feed has to be masticated to a consistency suitable for passage through the small intestine. Alexander (1966) has shown that horses while masticating only secrete parotid saliva and as such large volumes are produced. In an experiment performed at the Horseracing Forensic Laboratory in Newmarket, a pony was found to produce saliva at a rate of 90 ml/min from a single explanted parotid gland (Horner 1989). If the horse did the same and this rate was maintained, it would amount to 11 l/h. This matches the PCV changes in that feeding led to an increase in PCV from 23% to 34% (Fig.8.4c.) which represents a reduction in plasma volume equivalent to the loss of some 11 litres of fluid.

These large volume changes following frusemide administration and feeding did not affect plasma theophylline concentrations. This was to be expected, since studies on drug transfer have shown almost identical concentrations of theophylline in plasma and saliva i.e. the saliva/plasma concentration ratio (S/P) is close to 1 (Horner 1976). Thus no matter how much fluid is transferred there is no change in plasma theophylline levels. Protein binding is an important factor affecting the movement of drugs into saliva. Compounds which are highly protein bound are unable to pass into saliva, they become trapped in the circulation, so that reduction of plasma volume to produce large volumes of saliva would cause their plasma concentrations to rise considerably. However, only 20% of the total plasma theophylline concentration is bound to plasma protein in the horse (Kelly and Lambert, 1978).

The effect of frusemide on urinary levels of theophylline was principally a diluting one. In the post-frusemide urine samples the urinary concentrations of theophylline were approximately 4 μg/ml compared to about 30 μg/ml without frusemide (Fig.8.6.). These were short-lasting dilution effects as cumulative excretion of radioactivity and creatinine data show that renal excretion was unaffected. Frusemide

treatment therefore, can potentially reduce urinary concentrations of theophylline for a number of hours (7-8 h) after its administration. Our results are very similar to those of Stevenson *et al.* (1990) who found that 2 doses of frusemide (150 and 250 mg; i.v.) significantly reduced the urinary concentrations of theophylline from 1-7 h after diuretic treatment. These workers also found that the effect of frusemide on urinary theophylline concentrations was prolonged compared to its effects on other drugs (Chapter 1), where a reduction was noted within 1-4 h of frusemide dosing.

Obtaining post-race urine samples for doping analysis is often difficult, it may take up to several hours before urine is voided spontaneously since horses are often in a negative water balance due to long water deprivation and excessive sweating. It has been suggested that the diuretic bumetanide is useful for obtaining rapid urinary samples for dope testing from horses (Frey *et al.* 1976). This group injected bumetanide intravenously in doses of 10 and 20 μg/kg, 5 to 10 minutes after a race. They reported that the strong increase in urine flow provoked by bumetanide reduces renal reabsorption of acidic and basic drugs and created favourable conditions for the detection of drugs used in doping. Frey *et al.* (1976) also state that the extraction of dilute urine after bumetanide gave fewer problems than did the normally highly concentrated mucous horse urine, and the advantages of this more than outweighs the lower drug concentrations in the urinary samples.

However, previously reported data (Roberts *et al.* 1976; Miller *et al.* 1977; Tobin *et al.* 1977; Combie *et al.* 1981) and the present results with theophylline do not support these conclusions. Frusemide and bumetanide are both acidic, high-ceiling diuretics which act in the same way to produce a strong diuresis. It is therefore probable that these drugs will have similar effects on the urinary concentrations of drug metabolites. In a routine dope testing situation a single urine sample is taken and this sample size is limited, thus changes in urinary concentrations of the magnitude which have been reported may be critical. Thus the rationale for the method of Frey *et al.* (1976) is debatable and their findings should be treated with caution.

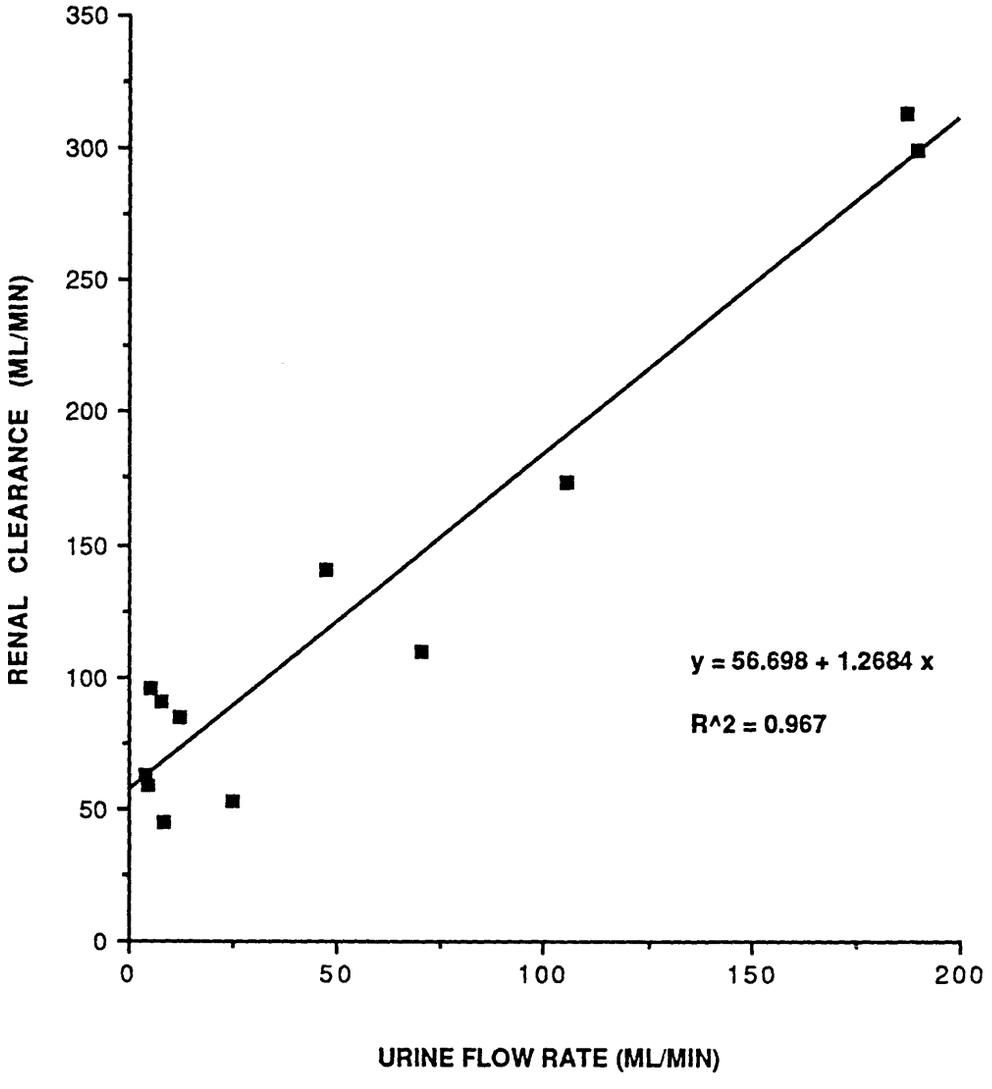
Based on normal urine flow values of 1.2 to 7.6 ml/min in the horse (Rumbaugh *et al.* 1982; Gronwall and Price, 1985) we found that theophylline itself

exerts a diuretic effect, the mechanism of the diuresis is unclear, but tubular solute reabsorption as well as a haemodynamic effect is thought to play a role (Brater *et al.* 1983). Diuresis caused by theophylline and frusemide can result in an increase in renal clearance. The renal clearance of unchanged theophylline in the horse is urine-flow dependent so that theophylline can accelerate its own urinary elimination by virtue of its diuretic action. Obviously, the influence of a potent diuretic able to increase flow rate to almost 200 ml/min is far more striking, the correlation coefficient between urine flow rate and renal clearance being 0.967 after frusemide administration (Fig.8.10.). However, frusemide does not alter the quantitative pattern of metabolites of theophylline as its duration of action is only one hour. This short action is due to the drug being rapidly eliminated in the urine via the organic acid transport system (Roberts *et al.* 1978).

At present the problem of the dilution caused by frusemide is overcome for forensic purposes by the use of a detention barn in jurisdictions where its use is permitted. In this system the horses are sequestered and supervised for 4 h before the race. Frusemide is administered at the approved dose and time pre-race under close and constant supervision. It is important that the rules regarding frusemide administration are strictly enforced, as unregulated use can hinder forensic detection of illegal medication. Recent unpublished work has shown that for at least the first 90 min after administration of frusemide, a recent administration of buprenorphine cannot be detected. Compliance with the "4 hour" rule should not affect urinary concentrations of other drugs as it has been shown in this study with theophylline and with other drugs that urinary levels return to pre-frusemide levels quite rapidly (Soma *et al.* 1984).

The present results indicate that after frusemide administration, a basic lipid-soluble drug such as theophylline will enter urine simply by diffusing across the walls of the renal tubules. The drug can also just as easily move from urine back into the kidney. For such drugs the final concentration in equine urine depends to a large extent on urine volume and pH.

Fig.8.10. Correlation between renal clearance of theophylline and urine flow rate in one horse given [14C]-theophylline (1 mg/kg; p.o.) + frusemide (1 mg/kg; i.v.)



Theophylline appears to diffuse across the renal tubule and equilibrate with urine at concentrations dependent on the pH of urine. The slightly alkaline urine found during this experiment may result in some reabsorption as an acidic pH favours the excretion of basic drugs, but the amount of drug eliminated in the urine will be temporarily increased due to the much increased urine volume. This contrasts with frusemide's effect on acidic drugs whose elimination is likely to be delayed due to competition for the organic acid transport system, even though the alkaline urine will favour the excretion of acidic drugs.

SUMMARY

This thesis has involved an investigation of the disposition and pharmacokinetics of frusemide in the horse, and its effects on the detectability of co-administered drugs. The results presented are summarized briefly below:-

1. Using a new HPLC method and fluorescence detection we were able to obtain accurate and reproducible quantitation of frusemide in biological fluids. Data on the plasma pharmacokinetics, the urinary and faecal excretion and the diuretic effect of frusemide following different routes of administration have been described. Frusemide causes a rapid intense but short-lasting diuretic effect. The 1.6 h elimination half-life after i.v. injection was considerably shorter than that after i.m. administration (2.8 h). This prolonged plasma half-life correlated well with the enhanced diuretic response following i.m. dosing. A similar proportion of the frusemide dose was excreted as unchanged drug (70%) in the urine after i.v. and i.m. administration. Only one metabolite was detectable in urine, 2-amino-4-chloro-5-sulphamoylanthranilic acid. Following oral administration frusemide was very poorly absorbed, faecal elimination accounted for 82.6% of the dose.

2. When salicylic acid and frusemide were given together both plasma and urinary data indicated that the diuretic delays the elimination of salicylic acid. The higher levels of salicylate in plasma are likely due to haemoconcentration as a consequence of the diuresis. Competition between the two acidic drugs for the organic acid transport system leads to the delayed appearance of salicylate in urine. The principal effect of frusemide on urinary levels of salicylate was a diluting one. The increase in urine volume following frusemide treatment can potentially reduce urinary concentrations of salicylate for a number of hours (4 h), this could seriously impair salicylate detection in routine screening tests when urine is analysed. Paradoxically the increased salicylate concentrations in plasma enhance the detection of the drug in blood samples and this should provide a reliable index of drug levels during or following a period of diuresis.

3. Salicylic acid administration did not cause significant changes in the pharmacokinetic parameters of frusemide, nor did it impair the frusemide-induced diuresis and natriuresis. However, there was evidence that the NSAID did exert an effect on frusemide excretion. This was a preliminary study and provides a useful starting point for future work to elucidate the role of prostaglandins as mediators of the effects of high-ceiling diuretics.

4. Probenecid caused marked changes in the pharmacokinetic parameters of frusemide. In the presence of probenecid the elimination half-life of the diuretic increased from 1.61 h to 3.48 h. Our data indicates that probenecid induced-inhibition affects the disposition of frusemide by altering both renal and nonrenal clearance. The decrease in diuresis (32.1%) and natriuresis (41.6%) (within 0.5 h) is due mainly to the reduced rate of delivery of frusemide to its site of action. Our results are compatible with the tenet that frusemide acts at the luminal side on the nephron and we have shown that urinary frusemide should be used in evaluating response rather than plasma concentrations.

5. A study on the pharmacokinetics and metabolic disposition of theophylline in the horse revealed that urinary elimination of the compound is slow in the horse. Plasma theophylline concentrations were best described by a 2-compartment open model. The elimination half-life following oral administration was 18.7 ± 2.7 h. The major metabolite was the C-8 oxidation product 1,3-dimethyluric acid (42.8%), unchanged theophylline (15.9%) and two metabolites not previously identified in the horse 1-methyluric acid (8.9%) and 3-methyluric acid (1.6%) were also detected. 3-Methylxanthine appears to be a substrate for xanthine oxidase in the horse, this highlights a species difference in the hepatic biotransformation of theophylline.

6. When theophylline and frusemide were given together, the diuretic did not alter the plasma concentration of theophylline. Frusemide did not affect the renal handling of theophylline as the rate of excretion was unchanged. However, urinary theophylline

concentrations showed a considerable dilution due to the 5-fold increase in urine volume. Drug concentrations returned to normal quite slowly i.e. 8 h after frusemide administration. The data presented shows that the renal clearance of unchanged theophylline in the horse is urine flow dependent following frusemide treatment, however, the quantitative recovery of urinary metabolites was not altered by frusemide. The results indicate that frusemide, in contrast to its effects on salicylic acid, does not hinder the detection of theophylline.

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