RADIORECEPTOR ASSAY AND RADIOIMMUNOASSAY OF SELECTED BENZODIAZEPINES IN URINE SAMPLES FROM RACING GREYHOUNDS

Thesis submitted in accordance with the requirements of the University of Glasgow for the degree of Doctor of Philosophy by

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CHAPTER THREE

Analysis of Greyhound Samples for Selected Benzodiazepines by Radioreceptor Assay and Radioimmunoassay

Abbreviations

ATP	Adenosine Triphosphate
BZ	Benzodiazepine
BZ-R	Benzodiazepine Receptor
CNS	Central Nervous System
DMCM	Methyl 6,7-dimethoxy-4-ethyl-b-carboline-3-carboxylate
GABA	γ-Amino Butyric Acid
GABA-T	GABA-Transaminase
GC/MS	Gas Chromatography Mass Spectrometry
GLC	Gas Liquid Chromatography
FNZ	Flunitrazepam
NSB	Non Specific Binding
PBS	Peripheral Binding Sites
RIA	Radio Immunoassay
RRA	Radio Receptor Assay
SB	Specific Binding
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis.

B_{max}	Maximum number of binding sites
K_D	Dissociation Constant
IC_{50}	Concentration to inhibit 50% of ^{3}H-FNZ binding to BZ-R.
t_2	Half-Life

Glossary

Diazepam FNZ Triazolam	BZ-R agonists with anxiolytic, anticonvulsant, muscle relaxant and sedative effects.
Ro15-1788	Partial BZ-R agonist, competes with BZ agonists for the BZ-R and has weak BZ-like effects.
Ro15-4513	Inverse agonist at BZ-R, proconvulsant with some anxiogenic actions. Also a selective ethanol antagonist.
Ro5-4864	Selective PBS agonist, it is inactive at central BZ-R.
DMCM	Inverse agonist at BZ-R and induces convulsions.
Muscimol	GABA agonist
Bicuculline	GABA antagonist
Assay Buffer	25mM Sodium phosphate buffer, pH 7.4.

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ABSTRACT

Radioreceptor assay and radioimmunoassay of selected benzodiazepines in urine samples from racing greyhounds.

Radioreceptor assay is a quick and relatively simple analytical method which can discriminate between classes of drugs with a high degree of specificity. As it is receptors which interact and "detect" the drugs, the particular class of drugs detected is dependant on the radiolabelled ligand which is displaced from the receptor. This eliminates the need to obtain a pure preparation of one particular type of receptor which contrasts with radioimmunoassay. With this method the antibodies have to be raised against a specific drug and then purified in order that crossreactivity with drugs from other pharmacological classes does not occur. Also radioreceptor assay will only detect the pharmacologically active compounds in the biological sample. This is advantageous as only they will be acting in the body to cause an effect, so only they need to be measured.

Prior to the analysis of greyhound urine samples, various aspects of the receptor assay were determined to ensure optimum results were obtained under an established set of conditions. Rat brain tissue was centrifuged in order to isolate the synaptosomal fraction containing the benzodiazepine receptors. The protein content of this fraction was estimated together with determination of the

actual number of receptors and their affinity for benzodiazepines. As the brain tissue was collected in batches and stored until required, the length of time it could be kept at -20^{o}C was determined together with the length of time prepared synaptosomal fractions could be stored under similar conditions.

The limits for the incubation period which enabled enough time for an equilibrium to be established between the benzodiazepine/radiolabelled benzodiazepine and the receptor were determined. These also allowed for the separation of large numbers of samples before disruption occurs naturally. Separation of bound and free radioligand was achieved by filtration. The filters were washed with sufficient assay buffer to minimise the non-specific binding whilst maintaining an almost instantaneous separation time. Radioreceptor assay of a variety of benzodiazepines, and their pharmacologically active metabolites, was carried out to assess the affinity of each one for the benzodiazepine receptor and therefore enabling their potency to be calculated. The very specific nature of the benzodiazepine-radioreceptor assay for benzodiazepines was tested by assaying a wide variety of non-benzodiazepines. Relatively high concentrations of these other drugs were used, compared to the diazepam standards, to increase the possibility of cross reaction with the benzodiazepine receptor.

Once the experimental laboratory conditions had been established, a pilot study was carried out with the greyhounds to uncover any practical problems relating to oral dosing and sample collection. This study also allowed the laboratory procedures to be tested with actual greyhound urine samples containing diazepam. The major studies were carried out with triazolam and flunitrazepam, both short acting benzodiazepines. They both have half-lives of only 2 to 3 hours in dogs and consequently can only be detected in samples taken within a few hours of dosing. Also the concentration of drug present in each sample is less as triazolam and flunitrazepam are more potent than diazepam so lower doses are required to obtain a similar effect.

To increase the sample content of the amount of benzodiazepines and their metabolites which can be detected by radioreceptor assay, the urine underwent mild hydrolysis with ß-glucuronidase. This releases the metabolites from the glucuronide conjugate. By increasing the amount of pharmacologically active metabolites in the sample this serves to increase the sensitivity of the assay. In order to increase the sensitivity of the receptor assay even more, the samples were extracted with a variety of organic solvents. However the increase in assay time due to the inclusion of this extra step was not balanced by the increase in detection efficiency. Therefore the laboratory protocol to assay the samples included centrifugation of samples, to deposit any

particulate matter, rather than a full extraction procedure.

In order to confirm the presence of benzodiazepines in the urine samples, they were also analysed by radioimmunoassay, both before and after hydrolysis. The statistical comparison of the receptor assay and the radioimmunoassay results demonstrated the relative non-specificity of the benzodiazepine antibody used in RIA compared to the relative high specificity of the benzodiazepine receptor. The differing affinities of triazolam and flunitrazepam metabolites for the receptor had been previously determined and as the proportion in which they are excreted is known from the literature, it was possible to re-calculate the receptor assay results. Using only the increase in benzodiazepines detected after hydrolysis, accurate pharmacokinetic data could be obtained. As only a single oral dose was given with only serial urine samples collected, such data is limited to the calculation of the elimination rate constants and the half-lives of triazolam and flunitrazepam in greyhounds. Re-assay of triazolam urine samples after they had been stored at $-20^{o}C$ for 12 months gave results which were statistically compared to previous results from the assay of samples within two days of collection. This was to determine the stability of the samples under such conditions and has implications in cases where samples cannot be analysed on receipt and have to be stored for some time.

CHAPTER ONE

1:1 Radioreceptor Assays

Radioreceptor assays (RRA) were introduced in 1970 and are analogous to radioimmunoassay (RIA) as they are simple, quick and easy to carry out. Requiring only a basic scintillation counter they are a sensitive and relatively cheap way of screening samples for groups of drugs. Assays have been developed for most pharmacological classes of drugs, neurotransmitters and hormones. All follow the same principles usually requiring no more than an appropriate radioligand and range of standards to detect a wide variety of drugs [1, 2]. The receptor (R) source can be the same in all cases, homogenised brain tissue, as the majority of drug receptors can be found there. Specialized tissue is used in some cases such as insulin [3] where insulin free plasma is the receptor source and [^{125}I]insulin the radioligand. A variety of ligands for RRA of different classes of drugs and hormones are listed in table 1:1.

Table 1:1 Radioligands Required for the Radioreceptor Assay of Different Pharmacological Classes of Drugs.

Class of drug	Radioligand	Reference
BZ[a]	[^{3}H]diazepam	4,5,6,7,8
	[^{3}H]FNZ	9,10,11,12,13,14
Opioids	[^{3}H]naloxone	15,16,17,18,
	[^{3}H]fentanyl	19
Neuroleptics	[^{3}H]haloperidol	20,21,
ß-adrenergic agonists	[^{3}H]dihydro-alprenolol	22,23,24,25,26
anti-depressants	[^{3}H]QNB[b]	27,28
	[^{3}H]imipramine	29,30
anticons[c]	[^{3}H]phenytoin	31
hGH[d]	[^{3}H]hGH	32,33
Insulin	[^{125}I]insulin	3,34
ANP[e]	[^{125}I]ANP	35,36

a = Benzodiazepine

b = [^{3}H]quinuclidinyl benzilate

c = Anticonvulsants

d = Human growth hormone

e = Anti naturitic peptide

The value of the RRA is that it is specific for the pharmacological class of drugs under investigation. The BZ-RRA has been tested against over 100 non-BZ from 22 distinct pharmacological groups and 14 possible neurotransmitters. None had any affinity for BZ-R at levels below 0.1 mM [4], where as benzodiazepines could be detected at levels down to 20-50nM. This specificity together with the RRA ability to detect only pharmacologically active drugs and metabolites gives it an advantage over conventional RIA. RIA is less specific as antibodies can cross react with other drugs, and there is the time interval (months) required in order to raise and purify suitable antibodies. With RRA little time is wasted preparing a sample of homogenised brain tissue and finding an appropriate radioligand.

As with every new analytical method, its effectiveness has to be established and with RRA this was achieved by analysing the same samples by gas liquid chromatography (GLC) and RRA [9,10,12]. When the levels were quantified the correletion between the two sets was very good ($r = 0.96$) [11].

Now RRA has been established as a method it has been used in clinical trials to determine benzodiazepine levels in plasma, serum and cerebrospinal fluid [6,8,13].

Its use as an analytical tool to study BZ-R and their interaction with new BZ-type compounds has greatly increased over the last few years. It can be used with equal efficiency with in vitro [37,38,39] and in vivo [40,41,42,43,44] studies such as investigations into the way benzodiazepines and ethanol interact to depress the central nervous system. [45,46,47,48,49,50]. RRA of benzodiazepines can be used in combination with psychological tests for stress to assess the potential of a new BZ-type compound as an antianxiety agent [51,52] and anticonvulsant agent [46,53,54,55,56,57,58,59,60].

1:2 Benzodiazepines

Benzodiazepines come under the heading of tranquillisers as they are capable of calming a patient without producing hypnosis. As their main use is to treat anxiety, agitation and tension they are classified as minor tranquillisers [61,62]. These drugs are widely prescribed with twice as many women taking them as men [63]. Diazepam (Valium) and chlordiazepoxide (Librium) were first introduced in the 1960's to replace barbiturates as sleeping pills and sedatives. They had fewer side effects and there is less chance of overdosing at high levels [64]. These benzodiazepines are long acting and may cause a 'hangover effect' due to the relativly slow elimination from the blood [65,66,67,68].

Short acting benzodiazepines, such as triazolam (Halcion), and medium acting benzodiazepines, such as flunitrazepam (Rohypnol), have been developed which are eliminated from the blood within a few hours, thus fewer side effects are experienced [69,70,71,72].

Benzodiazepines are prescription only medicines and are controlled under class C of the Misuse of Drugs Act, 1971, thus it is illegal to supply them, but not illegal to possess them without a prescription. Due to their wide availability they are open to abuse and the possibility they may be used to dope sporting animals, such as greyhounds, has to be considered. Therefore ways of detecting the various benzodiazepines in urine samples at very low levels, ng/ml, have to be developed.

As with any accurate analytical method, the presence of an endogenous form of the drug under investigation has to be considered, for example, opioids and opiate peptides (ß-endorphins) [73,74,75]. Up to the last few years no evidence for an endogenous BZ-type compound was available, but recently information from electrophysical studies and those involving monoclonal antibodies have indicated the presence of such a peptide. When purified it was found to be a low molecular weight protein and preliminary results show it to have a similar molecular structure to N-desmethyl diazepam [76,77,78,79,80].

Monoclonal antibodies have detected BZ-like compounds, similar to N-desmethly diazepam, in mammalian brain tissue and cow's milk [81]. A dietary source appears to be the most likely possibility as synthesis of a benzodiazepaine-like structure, a heterocyclic ring with a chloride atom attatched, is highly unlikely. Some fungi such as Penicillium cyclopium are capable of synthesising such compounds and may prove to be the source by acting as contaminants of food stuffs. Lorazepam - a dichloro compound has been found in drug free plasma, again this was attributed to a dietary source [82]. Investigations into a possible dietary source - wheat and potatoes, revealed trace amounts of pharmacologically active benzodiazepines [83,84], with similar structures to diazepam.

Recently triazolam has become more widely available and as it is excreted from the body within a few hours via the urine, bile and faeces, sensitive methods are required to detect low concentrations. This is especially true for racetrack samples where doping may have taken place 3-4 hours prior to examination. The disposition of triazolam in dogs has been previously studied with [^{14}C]triazolam to establish its excretion profile and its metabolic products [85,86,87].

Triazolam plasma levels peak 0.5hr after oral dosing, decreasing by first order kinetics with a $t_{½}$ of 0.85 hours. Highest levels of parent compound are reached 1 hour after dosing followed by a rapid decrease in concentration. A plateau period occurs 4-5 hours after oral dosing where plasma levels of triazolam and its metabolites remain constant for a 6 hour period before continuing to decrease at a slower rate - $t_{½}$ 14.5 hours. This plateau period is possibly due to redistribution of the protein bound triazolam and/or enterohepatic circulation. The major metabolites in dog urine are 1'-hydroxymethyl triazolam, conjugated 1'-hydroxymethyl triazolam and conjugated 4-hydroxy triazolam. The conjugate in both cases being the glucuronide.

Triazolam has been found to affect the mammalian circadian clock [88], though information is scarce in this area. It is thought to act at receptor sites in the eyes and pineal gland, the latter being proposed as the organisation centre for circadian rhythm. Ro15-1788 was found to block the phase shifting effect of triazolam thus having the potential to be used as a tool to study such inbuilt biological rhythms.

The pharmacokinetics of flunitrazepam - a medium acting benzodiazepine, have also been studied in dog with urine and plasma samples [89,90,]. Following oral dosing it is rapidly metabolised, with a $t_{½}$ of 2 hours, and eliminated via the urine as the metabolites N-desmethyl flunitrazepam and 7-amino flunitrazepam. Both are excreted as the glucuronide [91].

1:3 Benzodiazepine Receptors

Papers relating to the observation and demonstration of BZ-R began to appear in 1977. Their presence in central nervous system (brain and spinal cord) can be demonstrated both by radioreceptor assay and autoradiography [92,93,94,96,97]. Preparations of synaptosomes from the cerebral cortex or whole brain minus the cerebellum, which has least benzodiazepine receptors (BZ-R), are used for benzodiazepine RRA. The BZ-R were shown to be highly stereospecific with (+) enantiomers having a higher affinity for the BZ-R than the (-) form. The (+) benzodiazepine enantiomers are 120x more potent than (-) at displacing [^{3}H]diazepam from the receptors.

The following criteria has been established in order to obtain good affinity binding of benzodiazepines to the BZ-R;

1 Binding must be specific with only one class of BZ-R involved.

2 Binding has to be saturable demonstrating a definite number of BZ-R sites in the tissue sample.

3 Labelled ligand must bind to the BZ-R with high affinity.

NB. Specific binding:- The amount of binding expected to occur at the receptors and is saturable.

Non-specific binding (NSB):- Remaining binding (background) - absorption to glassware etc which cannot be displaced by excess ligand. NSB increases linearly with increasing radioligand concentration.

In accordance with enzyme kinetics B_{max} refers to the number of BZ-R in the preparation, whilst K_D is the dissociation constant of the radioligand from the receptor; if K_D is small then the receptor has a great affinity for the ligand. B_{max} and K_D can be experimentally calculated by binding studies and Scatchard plots. Investigations into the effect of temperature on RRA with [^{3}H]diazepam gave optimal results after incubation at 0^oC, where as [^{3}H]FNZ RRA could be incubated at room temperature. As both ligands bind to the same receptors, the optimal incubation temperature difference is most likely due to a variation in K_D.

Comparison of *in vivo* and *in vitro* potencies of benzodiazepines is possible providing all the pharmacologically active metabolites involved are considered [95].

The evolution of the BZ-R has been studied as a means of determing its structure and is present in all mammals and birds studied, to date, though a species difference occurs relating to the proportion of binding sites per brain region [98,99,100]. Bony fish possess BZ-R with a high affinity for [^{3}H]benzodiazepine ligands, but not chondrichthyes (sharks) which indicates this receptor type was a late evolutionary development. Sharks do possess some BZ-R with low affinity for benzodiazepines which must have developed earlier in the evolutionary timescale.

Work is now being done to determine which specific protein sequences are necessary for benzodiazepine specific binding. [^{3}H]FNZ and [^{3}H]Ro15-4513 can be used to irreversibly photolable two proteins in brain tissue:- P_{51}-51kD and P_{55}-55kD. They are assumed to be associated with the central receptor as binding of [^{3}H]FNZ to each protein is inhibited by diazepam, but not by Ro5-4864 which acts only at peripheral receptor. As GABA - a CNS neurotransmitter is probably associated with the BZ-R it is possible P_{51} and P_{55} are different subunits of this complex [101]. Comparison of P_{51} and

P_{55} from different brain regions revealed a high degree of homology indicating a definite receptor area which will bind to a definite chemical structure such as a benzodiazepine [102,103].

The location of two BZ-R subtypes, I - a high affinity BZ-R and II - a low affinity BZ-R in human brain, was achieved via autoradiography. Quantification was also carried out by in vitro labelling of the BZ-R. As the two BZ-R subtypes were located in distinct areas of the brain:-

I predominantly in the ventral striatum matrix

II predominantly in the dorsal striatum matrix

it is possible they may be related to various functional effects. The proposal is that I is related to anti-anxiety effects of BZ and II is related to the mediation of muscle relaxation and ataxic effects of benzodiazepines [97,103,104].

Benzodiazepines bind to receptors on a variety of tissues outside the CNS including; alveolar type II cells, heart, lung, erythrocyte membranes and the pineal gland [105,106,107,108]. These peripheral binding sites (PBS) have been partially purified by gel filtration in an attempt to determine their structure [109]. Photolabelling with PK 14105, a nitrophenol derivative, followed by electrophoresis revealed a low molecular weight (18kD) acidic protein rather than the large and complex structure of the central BZ-R. Ro5-4864, Ro15-1788 and FNZ are used to study similarities and differences between the two types of receptors [110,111].

Diazepam and desmethyl diazepam can also be used in this sense as they have the same affinity for the central BZ-R but at the peripheral sites diazepam has more affinity for the receptors than its metabolite [112].

Surgery affected both central BZ-R and PBS. B_{max} had increased by ~30% 3 days after the operation and is thought to be related to stress and alterations of stress related hormones such as cortisol, growth hormone and ß-endorphins [113]. There is a correlation with healing processes as 7 days after surgery when the subcutaneous tissue and collagen had become re-established, the B_{max} was found to have returned to control levels.

As PBS have no known interaction with GABA, other hormones may be involved in the regulation of these receptors. An endogenous protein which inhibits Ro5-4864 binding to PBS has been isolated, purified and named antralin. It has no effect on the binding of [^{3}H]Ro15-1788 to central BZ-R. It is thought to regulate calcium ion channels via PBR and dihydropyridine binding sites [114].

1:4 The Benzodiazepine/GABA Receptor complex

ɣ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the vertebrate central nervous system. It is formed by the action of glutamate decarboxylase on glutamate produced by the Krebs cycle. By increasing permeability of neuronal membranes to chloride ions it causes hyperpolarisation and neuronal inhibition. GABA released from neurones is inactivated by re-uptake processes and by GABA-transaminase (GABA-T). Direct labelling of the receptor with [^{3}H]FNZ allows the degradation mechanism to be studied and preliminary conclusions are that the BZ/GABA-R complex is degraded via an energy dependant, non-lysosomal pathway [115]. Benzodiazepines exert their effects by acting on the GABA-R and increase GABAergic transmission.

Many projects and studies have since been carried out to establish the connection between BZ-R and GABA-R, the structure of such receptors, the effects exerted on one by the other under normal and stressed conditions and the effect of other drugs and chemicals on the proposed BZ-GABA complex. A comprehensive review of the electrophysiology of BZ-R ligands which incorporates work done on BZ-GABA-R complexes is available [116]. Such studies provide the most convincing results linking BZ-GABA systems, as it is possible to study subpopulations of benzodiazepines in the various brain regions. Thus

results from binding studies can be related to their physiological functions.

Incubation of ligand/receptor preparations in the presence of muscimol increased [^{3}H]diazepam binding, whereas incubation in the presence of bicuculline decreased [^{3}H]diazepam binding [117]. Binding to receptor preparations from 4 day old rats which contain 75% of adult BZ-R but only low levels of GABA-R was not affected by muscimol or bicuculline. This strengthens the case for a possible BZ-GABA complex with the receptor sites located either on the same membrane protein or on separate but closely associated membrane proteins.

Quantitative autoradiography has been used to study the reproducibility of benzodiazepine and GABA receptors and their binding parameters in different areas of cat brain tissue. Very high levels of BZ/GABA-R were found in the ventral area of the motor hypothalamus, which is the region thought to be associated with anxiety. Further evidence of a BZ/GABA-R complex came from autoradiographic studies combined with quantitative radiohistochemistry. The results showed an increase in [^{3}H]benzodiazepine binding in all brain regions following the addition of GABA [118,119].

<u>In vitro</u> study of BZ/GABA receptor development utilised mouse primary spinal cell cultures as their binding properties are similar to <u>in vivo</u> spinal cord cells [120]. During the first 14 days of development K_D remained the same whilst B_{max} increased. Addition of

GABA to the incubation medium led to a concentration dependant increase in [^{3}H]FNZ binding, thus BZ-R and GABA-R are coupled early in neuronal development. GABA gated chloride channels also develop early as bicuculline blockage of chloride ion transport was possible from day 3.

Cultured neurons were also used to investigate the effect of GABA has on the B_{max} value of [^{3}H]FNZ specific binding [121]. Chronic treatment with benzodiazepines caused tolerance to their sedative and anticonvulsant effects, but not to their anxiolytic effects. Prolonged treatment with diazepam decreased the ability of GABA to inhibit [^{3}H]DMCM binding. This is due to a decrease in BZ/GABA coupling rather than a change in the benzodiazepine recognition sites. Treatment of BZ-R <u>in vitro</u> with GABA agonists and antagonists, muscimol and (+)bicuculline, modulate the affinity of [^{3}H]diazepam binding increasing the evidence that benzodiazepine and GABA receptor sites are linked. Conversely, a study of BZ-R ligands on GABA-R revealed modulation of the receptors by the various ligands. Muscimol inhibits potassium stimulated release of GABA whereas clonazepam potentiates its release. This modulation of GABA-R by BZ-R may be related to the therapeutic actions of benzodiazepines [122,123,124].

As the BZ/GABA-R is sited in the cell membrane, membrane lipids can affect the conformation of the receptor protein. One such lipid is phosphotidylserine which specifically interacts with part of the BZ/GABA-R complex and facilitates [^{3}H]FNZ binding to the receptors [125]. It is possible that endogenous phosphotidylserine may be involved in the regulation of the BZ/GABA-R function.

The above papers describe the emergence of a single receptor protein which has a binding site for both benzodiazepine and GABA, a ligand-gated chloride channel sensitive to muscimol and bicuculline. This complex is refered to as the $GABA_A$ receptor. Its function can be modulated by a wide range of drugs including picrotoxin-like anticonvulsants, benzodiazepines and barbiturates, which bind directly to the GABA-R complex [126]. Photoaffinity labelling of crude brain homogenates with [^{3}H]FNZ has shown the benzodiazepine binding site to be a peptide of Mr = 50-53kD (α-subunit). Labelling with [^{3}H]muscimol identifies the GABA binding site as a peptide of Mr = 56kD (ß-subunit). These two peptides are thought to form an oligomer incorporating the modulatory ligands which form a membrane channel. Dose/response curves from electrophysical studies predict two or more binding sites for the GABA oligomer. The ß-subunit has 60% of the α-subunit's BZ binding capacity and as this binding can be prevented by excess cold ligand, it may function as a binding site along with the

∝-subunit. There may be a single oligomeric GABA receptor with benzodiazepine and GABA binding on both ∝ and ß subunits, which are similar. The differences between them probably results from variations in binding affinity and pharmacological specificity.

Following the emergence of the BZ/GABA-R complex as an integrated structure the search is now on to define the exact layout of the complex. As the complex shares the structural and functional properties with the nicotinic and glycine receptor-gated ion channels it is possible they evolved from a common gene pool. It has also enabled many of the techniques and approaches used to study the nicotinic acetylcholine receptor to be used to purify the BZ/GABA-R in sufficient quantities, thus enabling detailed studies of its molecular and structural properties [127,128].

The presence of an ∝-subunit of 50-53kD and a ß-subunit of 55-58kD have been confirmed by SDS-PAGE. Incubation of the ∝-subunit (BZ-R site) with glycosidases indicates the presence of glycosides in varying degrees depending on the brain region, which could relate to the BZ-R heterogenicity [128]. Using monoclonal antibodies to either the ∝ or ß subunit it is possible to show the $GABA_A$-R complex has both. The subunit stoichiometry has been found to be a tetramer - $\alpha_2\beta_2$, of Mr~230kD. Recombinant DNA cloning of ∝ and ß demonstrated a 35% homology in their amino acid sequences. Antibodies raised against a nonapeptide sequence of the BZ/GABA ∝-subunit

demonstrates the existence of heterologous regions of α-subunit in the cerebral cortex [129].

As benzodiazepines have sedative, muscle relaxant and anticonvulsant effects, projects to study modifications of these effects are often carried out using rat and mouse artificially induced seizure models [130,131,132,133]. An example of this is the interperitoneal (ip) administration of DMCM to mice to study its pharmacological effects. A series of benzodiazepines were given both ip and orally prior to DMCM dosing at concentrations which inhibited the seizures [130]. Theory indicates that the BZ/GABA-R complex has two conformations which are in equilibrium and benzodiazepines have a high affinity for the open chloride channel form. Any benzodiazepines binding to the receptor moves the equilibrium, increasing the number of open channels enabling chloride ions to enter the cell. DMCM is thought to have a high affinity for the closed channel conformation 'locking' the receptor in this state and decreasing benzodiazepine mediated GABA neurotransmission.

In addition to the study of *in vivo* and *in vitro* effects of drugs on the BZ/GABA-R complex, other factors such as age, ethanol and mental state of subject can also modulate the BZ/GABA-R complex.

Effects of ageing on ethanol and diazepam modulation of GABA binding sites have been demonstrated [134]. When taken together ethanol and diazepam act synergistically to depress the CNS, diazepam acts on the

BZ-R to increase chloride ion transport, whereas ethanol increases chloride ion transport in a non-specific way by increasing the fluidity of the cell membranes. Age has no effect on B_{max} but may affect K_D. In the absence of ethanol, diazepam influences the $GABA_A$ binding site in all age groups. When ethanol was present the influence of diazepam on the $GABA_A$ binding site decreased with age. This was due to age related changes in the phospholipid content and composition of the membranes. Chronic ethanol treatment is also thought to uncouple the BZ/GABA-R complexes in the brain [45].

GABA is also thought to be involved in depressive illnesses as low levels of GABA have been described in such patients prior to treatment [135].

CHAPTER TWO

To Establish the Radioreceptor Assay Protocol

2:1 Introduction

Before the radioreceptor assay could be used as a working method to analyse the greyhound urine samples, various parameters have to be experimentally defined. This involved a series of mini-experiments to look at the individual aspects of the RRA. Due to the nature of the assay, the first experiments were related to the preparation and evaluation of benzodiazepine receptors. Preparation involved separating the synaptosomal fraction, containing the receptors, from homogenised brain tissue by centrifugation. The resulting pellet was assayed to determine its protein concentration, which was used as a guide to receptor concentration. As the receptors are prepared in batches from rats of similar weights and ages, determination of the protein concentration of one vial can be used to estimate the protein concentration of the remaining vials.

Once the protein concentration of a particular vial has been established, a more specific estimation of the receptor number (B_{max}) can be made. This is done by carrying out a binding assay - a variation of the RRA requiring a series of radioligand standards rather than a

series of benzodiazepine standards. The Packard Combicept™ program translates the data into two graphs, calulating B_{max} from each. The dissociation constant (K_D) is also calculated in this way.

A group of three experiments relating to practical aspects of the RRA were carried out. The first was to establish the length of time the receptors could be stored at $-20^{o}C$, both as the whole brain tissue and as the prepared synaptosomal fraction. Once an assay has been set up, it has to incubate for a set period to allow the receptors and the ligand/radioligand to reach equilibrium. Determination of the optimum length of this period formed the basis of the second experiment. The reversal of this equilibrium by the addition of excess unlabelled ligand was also noted.

At the end of incubation the bound and free radioligand have to be separated. This can be achieved by either filtration or centrifugation [136], as the BZ-R is in the particulate form. Soluble receptors, such as those for hormones, require an alternative method such as precipitation or gel filtration chromatography [1]. Filtration is the most widely used separation method as it is quick and efficient. The total separation time - filtration and washing of the filters, is about 10-20 seconds/sample which requires K_D to be 10^{-8}nM or less. As values for benzodiazepines are in this range, filtration is applicable to RRA of urine samples for

benzodiazepines. The main disadvantage of filtration is the non-specific binding of the radioligand to the filters and filtration system. This can be minimised by washing the filters. The number of washes required to reduce NSB to a minimum, whilst retaining a total separation of 10 to 20 seconds formed the basis of the third experiment.

When the NSB cannot be reduced to manageable levels or when K_D is rapid, centrifugation is the alternative, though it is less efficient than filtration. Washing and re-centrifugation of the pellet increases separation time, thus K_D has to be small. Eliminating the wash period increases the NSB due to free radioligand trapped in the pellet.

Several studies were carried out to assess the potency of various benzodiazepines and non-benzodiazepines to displace [^{3}H]FNZ from the BZ-R. The potency of a compound in such circumstances is usually refered to as its K_i value. The smaller the K_i value, the lower the concentration of drug required to produce a biological or clinical effect. In this way K_i values are often used to predict the recommended daily doses of a drug. Experimentally it is the IC_{50} value - the amount of drug to displace 50% of radiolabel, which is measured and used to calculate the K_i value. IC_{50} and K_i values were obtained for several different benzodiazepines and a selection of their metabolites.

A variety of non-benzodiazopines, representing most pharmaceutical classes, were assayed with [^{3}H]FNZ as the radioligand. This was to establish their lack of cross reaction with the BZ-RRA and to demonstrate the low affinity of non-benzodiazepines for the BZ-R.

When all these experiments had been carried out, the collective results were assessed and a protocol for radioreceptor assay of benzodiazepines drawn up. This formed the basis for the analysis of the greyhound urine samples for selected benzodiazepines.

2:2 Equipment and Materials

Centrifuge	Beckman JA 2-21 with JA 20 rotor.
Scintillation Counter	Packard 2200 CA Tri-Carb® Liquid Scintillation Analyser.
Data processing	Binding Assay - Packard Combicept™ program for IBM®-PCAT. RIA - Packard SecuriaT program for IBM®-PCAT.
Radioligand	N-Methyl [^{3}H]Flunitrazepam Amersham International.
Filters	Whatman® GF/B glass microfibre filters.
Scintillant	Ecoscint A®, National Diagnostics.
Pico 'Hang-In' vials	Packard®.
LP3 tubes	Luckham Ltd., Disposable Laboratory Plastics.

Drugs

Alprazolam	Upjohn Ltd.
Bromazepam	Roche Products Ltd.
Clonazepam	Roche Products Ltd.
Diazepam & metabolites	Roche Products Ltd.
Flunitrazepam & metabolites	Roche Products Ltd.
Lorazepam	Wyeth Laboratories.
Nitrazepam	Roche Products Ltd.
Oxazepam	M.A. Steinhard Ltd.
Triazolam & metabolites	Upjohn Ltd.
Amphetamine	Sigma® Chemical Company Ltd.
Aspirin	Sigma® Chemical Company Ltd.
Amytriptyline	Berk Pharmaceuticals Ltd.
Amylobarbitone	Eli Lilly & Company.
Caffeine	Sigma® Chemical Company Ltd.
Chloroquin	I.C.I. Pharmaceuticals (UK).
Chlorpromazine	May and Baker Ltd.
Morphine	Sigma® Chemical Company Ltd.
Paracetamol	Winthrop Laboratories.
Propranolol	Berk Pharmaceuticals Ltd.
Quinine	Sigma® Chemical Company Ltd.

All other chemicals were of analytical grade.

2:3 Experimental Methodology

Note: All [^{3}H]FNZ dilutions were made with 25mM sodium phosphate assay buffer, pH 7.4. The receptor preparation was diluted where necessary with 25mM sodium phosphate assay buffer, pH 7.4. Benzodiazepine standards were made up either in 25mM sodium phosphate assay buffer, pH 7.4, or drug free greyhound urine centrifuged to remove any particulate matter. Throughout all the experiments "assay buffer" refers to 25mM sodium phosphate buffer, pH 7.4.

2:3:1 Preparation of Benzodiazepine Receptors

Male Sprague-Dawley rats were stunned and decapitated to allow easy removal of the brain tissue. The whole brain minus the cerebellum was removed, immediately frozen and stored at -20^{o}C until required. The mitochondrial fraction containing the synaptosomes in which the BZ-R are located was prepared by hand homogenisation of 1g of brain tissue in 5 volumes of ice cold 0.32M sucrose solution. The homogenate was centrifuged for 10 minutes, spin speed 3500g, at 4^{o}C and the pellet discarded. The remaining supernatant was centrifuged for 20 minutes, spin speed 15000g, at 4^{o}C, this time the supernatent was discarded and the weight of the pellet noted. Resuspension of the pellet in 5 ml of assay buffer enabled it to be transferred to a suitable vial and stored at -20^{o}C until required.

2:3:2 Protein Assay

The protein assay, a colourimetric method [137], is carried out using bovine serum albumin to prepare the standard curve. The coloured complex which is measured in a spectrophotometer, and directly relates to the protein concentration, is due to tyrosine and tryptophan residues in the protein complexing with the alkaline copper-phenol reagent.

Reagents

1 Sodium carbonate, 2% $^{W}/v$ in 0.1M NaOH.

2 Copper sulphate (hydrated), 1% $^{W}/v$ in water.

3 Sodium potassium tartrate, 2% $^{W}/v$ in water.

4 Bovine serum albumin 100 ug/ml in 0.5M NaOH.

Prepare fresh copper reagent by mixing reagents 1:2:3 in the proportions 100:1:1.

The brain preparation is assayed at 2 dilution levels, 1/10 and 1/50, in order to obtain a more accurate determination of its concentration. 0.5M NaOH was the diluent in both cases.

Method

Reagent	Volume added (ml)					
Sample/Std	0.0	0.2	0.4	0.6	0.8	1.0
0.5M NaOH	1.0	0.8	0.6	0.4	0.2	0.0
Cu reagent	5.0	5.0	5.0	5.0	5.0	5.0
Vortex, stand for 10 minutes at 25°C						
1N Folin reagent	0.5	0.5	0.5	0.5	0.5	0.5

Vortex, stand for 30 minutes at 25°C.
Read absorbance at 540nm using one 0.0mg.ml sample, as blank.

Plot absorbance against bovine serum albumin (ug) per assay to generate a standard curve from which the protein concentration of the samples can be read directly. The standard curve is linear over the range of standards used.

2:3:3 Binding Study

This was carried out to determine the dissociation constant, K_D, and the number of receptors available for binding in a preparation of brain tissue, B_{max}. The data generated can be plotted in a variety of ways which are discussed in section 2:4.

Standards, made up in drug free greyhound urine:

Diazepam: 0, 10, 25, 50, 100, 200ng/ml.

Triazolam: 0, 1, 5, 10, 15, 20, 25, 35, 50ng/ml.

FNZ: 0, 10, 25, 50, 100, 200ng/ml.

[^{3}H]FNZ, made up in assay buffer:

10, 25, 50, 100, 200, 400, 500, 600, 800, 1000nCi,.

Method - Total Binding

10 pairs of LP3 tubes were set up to enable each [^{3}H]FNZ concentration to be assayed in duplo. Each tube contained an incubation mix consisting of 400ul receptor preparation, 1mg/ml, 100ul of the appropriate [^{3}H]FNZ concentration and 50ul assay buffer. After each tube was vortexed, to ensure thorough mixing, they were incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

Method - Non-Specific Binding

10 pairs of LP3 tubes were set up to enable each [^{3}H]FNZ concentration to be assayed in duplo. Each tube contained an incubation mix consisting of 400ul receptor preparation, 1mg/ml, 100ul of the appropriate [^{3}H]FNZ concentration and 50ul triazolam 1ug/ml. After each tube was vortexed, to ensure thorough mixing, they were incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

The DPM of total added label was determined by addition of 100ul of each radioligand concentration directly to 4ml of scintillant which was counted for 1 minute.

2:3:4 Rat Brains and Receptors - Storage Study

The length of time rat brains and receptors can be stored at -20^{o}C was determined by preparing sets of receptors and assaying them over a period of six weeks. The triazolam standards were made up in blank greyhound urine. 10 pairs of LP3 tubes were set up enabling duplicates of each standard to be assayed.

Std. Curve

Triazolam		[^{3}H]FNZ	R preparation
(ng/ml)	volume (ul)		volume (ul)
0	50	100	400
1	50	100	400
5	50	100	400
10	50	100	400
15	50	100	400
20	50	100	400
25	50	100	400
35	50	100	400
50	50	100	400
NSB			
1000	50	100	400

Each tube was vortexed, to ensure thorough mixing, and incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

Non-specific binding (NSB) includes any free radioligand not washed away, that bound to the non-receptor fraction of the brain preparation, and that bound to the separation

materials - filters, assay tubes etc. Following assay of the standards, C_o/C_x is calculated for each standard and plotted against the triazolam concentration to create a standard curve. An example of a set of results for such a standard curve is shown in table 2:1, with the appropriate graph shown in figure 2:1.

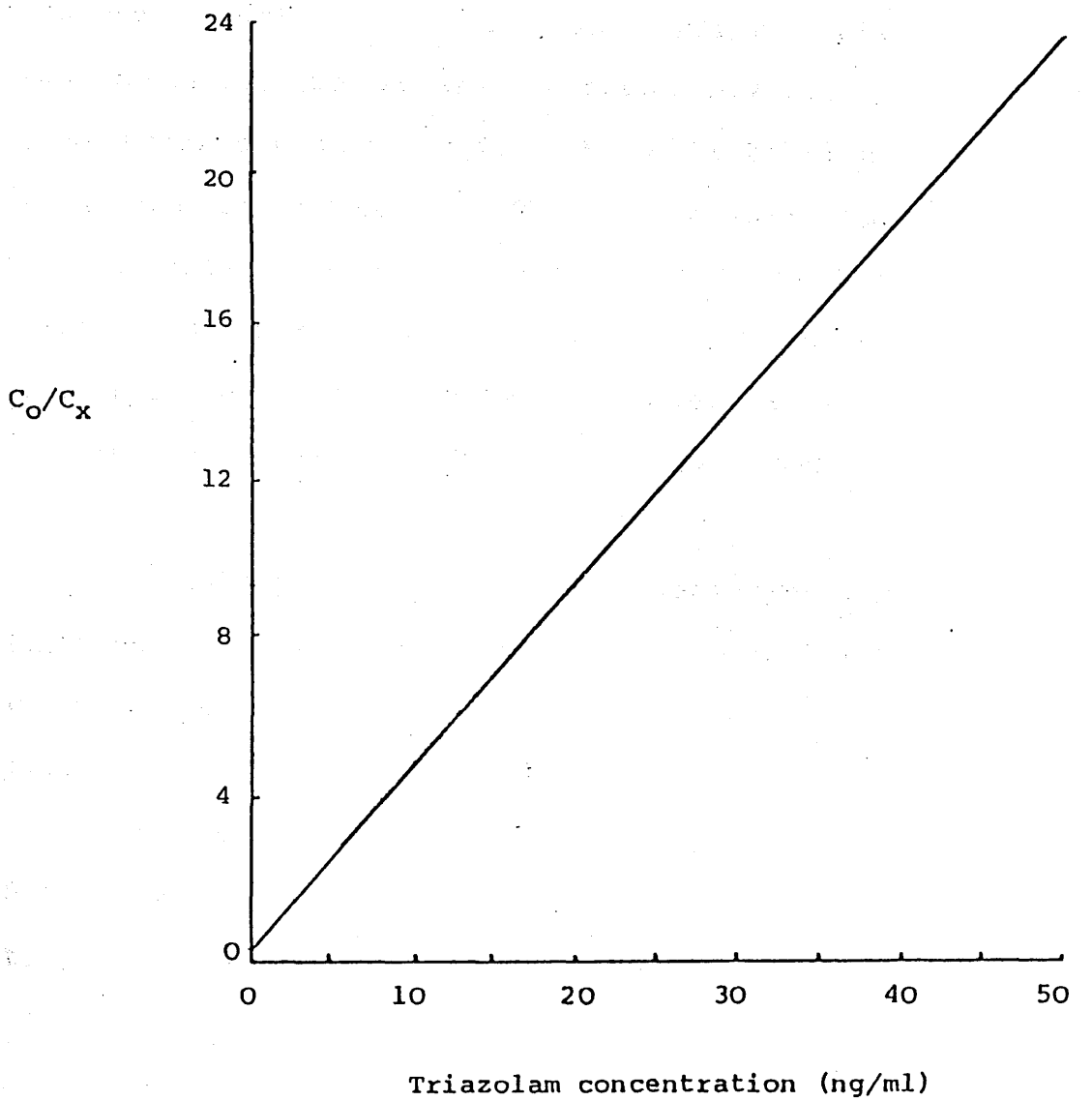
C_o = CPM for the 0 standard (50ul AB) minus CPM for the NSB standard.

C_x = CPM for 50ul standard/sample minus CPM for the NSB standard.

Table 2:1 Set of Experimental Data to Illustrate a Radioreceptor Assay Standard Curve.

Triazolam (ng/ml)	$\bar{x}$ DPM	C_x	C_o/C_x
0	824.4	786.6	
1	693.3	655.5	1.2
5	599.6	561.8	1.4
10	198.3	160.5	4.9
15	122.3	84.5	9.3
20	136.1	98.3	8.0
25	100.7	62.9	12.5
35	77.1	39.3	20.0
50	70.0	32.2	24.4
NSB	37.8		

Figure 2:1 An example of a radioreceptor assay standard curve for triazolam.



2:3:5 Specific Binding v. Time

Firstly, to determine the stable period of the receptor/ligand equilibrium. 42 LP3 tubes were set up in pairs, enabling duplicates to be filtered every 15 minutes from t_0 to t_{300}. Each tube contained an incubation mix consisting of 400ul receptor preparation, 1mg/ml, 100ul of [^{3}H]FNZ ,50nCi, and 50ul assay buffer. After each tube was vortexed, to ensure thorough mixing, the bound and free fractions were separated by filtration under vacuum at 15 minute intervals. The filters were washed with 5 x 1ml of assay buffer and placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand. DPM were plotted against time.

Secondly, to obtain the association curve where the BZ-receptors and [^{3}H]FNZ reach equilibrium and to demonstrate its reversal by the addition of excess triazolam.

50 LP3 tubes were set up in pairs, enabling duplicates to be filtered every 5 minutes from t_0 to t_{120}.

Incubation Mix - Association Curve

Each of the first 13 pairs of tubes contained an incubation mix made of 400ul receptor preparation, 1mg/ml, 100ul of [^{3}H]FNZ, 50nCi, and 50ul assay buffer. After each tube was vortexed, to ensure thorough mixing, they were incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum at 5 minute intervals. The filters were washed with 5 x 1ml of assay buffer and placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand. DPM were plotted against time.

Incubation Mix - Dissociation Curve

The remaining 12 pairs of LP3 tubes had 400ul of 1mg/ml receptor preparation and 100ul of 50nCi [^{3}H]FNZ added to them and were vortexed. After a 60 minute incubation period 50 ul of 1ug/ml triazolam was added to the tubes followed by thorough mixing. Filtration was begun immediatley at 5 minute intervals. The filters were washed with 5 x 1ml of assay buffer and placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand. DPM were plotted against time.

2:3:6 Separation of Bound and Free Radioligand

RRA, as described below, were carried out with several sets of triazolam standards, each made up in drug free greyhound urine. At the filtration stage each set of filters were washed with different volumes of assay buffer. Following several repetitions, the data was assessed to determine the least number of washes required to minimise the NSB. Four different volumes of assay buffer were used: 1ml, 3ml, 5ml & 10ml.

As before 10 pairs of LP3 tubes were set up, one for each of the triazolam concentrations in duplicate.

Std. Curve

Triazolam		[^{3}H]FNZ	R preparation
(ng/ml)	volume (ul)		volume (ul)
0	50	100	400
1	50	100	400
5	50	100	400
10	50	100	400
15	50	100	400
20	50	100	400
25	50	100	400
35	50	100	400
50	50	100	400
NSB			
1000	50	100	400

Each tube was vortexed, to ensure thorough mixing, and incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

2:3:7 Inhibition of Radioligand Binding to Receptors

RRA, as described below, were carried out with sets of benzodiazepine standards covering a range of 0 to 200ng/ml, each made up in drug free greyhound urine. Nine different benzodiazepine and six metabolites were used as below:

Benzodiazepine	Metabolite
Alprazolam	
Bromazepam	
Clonazepam	
Diazepam	3-hydroxy diazepam
	Desmethyl diazepam
Flunitrazepam	Desmethyl flunitrazepam
	7-amino flunitrazepam
Lorazepam	
Nitrazepam	
Oxazepam	
Triazolam	1'-hydroxy triazolam
	4-hydroxy triazolam

Standard ranges:

0, 1, 5, 10, 25, 50, 75, 100, 200, 1000ng/ml

for alprazolam, bromazepam, clobazam, clonazepam, diazepam, N-desmethyl diazepam, 3-hydroxy diazepam, flunitrazepam, desmethyl flunitrazepan, 7-amino flunitrazepam, lorazepam, nitrazepam, oxazepam, triazolam & 1'-hydroxy triazolam.

and 0.1, 0.5, 1, 2, 4, 5, 6, 8, 10ug/ml

for 4-hydroxy triazolam.

As before 10 pairs of LP3 tubes were set up, one for each of the benzodiazepine concentrations in duplicate.

Std. Curve

Benzodiazepine		[^{3}H]FNZ	R preparation
(ng/ml)	volume (ul)	volume (ul)	
0	50	100	400
1	50	100	400
5	50	100	400
10	50	100	400
15	50	100	400
20	50	100	400
25	50	100	400
35	50	100	400
50	50	100	400
NSB			
1000	50	100	400

Each tube was vortexed, to ensure thorough mixing, and incubated for 30 minutes at 25°C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

2:3:8 BZ-RRA and Non-Benzodiazepines

50ug/ml stock solutions of each of the non-BZ were made up in and diluted with assay buffer to give a working standard range as follows:

0, 10, 20, 30, 40, 50ug/ml.

To confirm the presence of the drugs, each standard was subjected to a U.V. scan against blank assay buffer. Where necessary the pH of the buffer was changed. Table 2:2 lists the compounds used together with their U.V. absorbance maxima at pH 7.4, (pH changes indicated where made).

Table 2:2 List of Non-benzodiazepines and Their U.V. Absorbance Maxima.

COMPOUND	U.V. ABSORBANCE MAXIMA nm
Amphetamine	258 265
Aspirin	230 280 (pH 2)
Amytriptyline	242
Amylobarbitone	256 (pH 13)
Caffeine	275
Chloroquin	261 334 347
Chlorpromazine	264
Morphine	287
Paracetamol	245
Propanol	288 306 319
Quinine	253 320 (pH 2)

6 pairs of LP3 tubes were set up, one for each of the non-BZ concentrations in duplicate and 10 pairs of LP3 tubes were set up, one for each of the diazepam standards in duplicate.

Std. Curve

Diazepam		[^{3}H]FNZ	R preparation
(ng/ml)	volume (ul)	volume (ul)	
0	50	100	400
10	50	100	400
25	50	100	400
50	50	100	400
100	50	100	400
200	50	100	400
NSB			
1000	50	100	400

Each tube was vortexed, to ensure thorough mixing, and incubated for 30 minutes at 25^{o}C. The bound and free fractions were separated by filtration under vacuum and the filters washed with 5 x 1ml of assay buffer. The filters were placed in to Pico vials, 4ml of scintillant was added and the vials shaken vigerously. Counting was for 1 minute and gave the DPM of the bound radioligand.

The non-BZ standards were deliberately made 10^3x that of the diazepam standards to enable any cross reaction with the BZ-R to be seen.

2:4 Results and Discussion

The previous pages have described the experiments carried out to assess the various aspects of the RRA. Preparation of the receptors was a straight forward method whereby the homogenised rat brain tissue was centrifuged twice. This produced a pellet containing sufficient viable receptors per mg of protein to set up a RRA with appropriate volumes. Table 2:3 contains data to construct a protein calibration curve which is shown in figure 2:2.

Table 2:3 Experimental Data from a Protein Assay.

Standard Curve	
ug protein/ml	mean absorbance
0.0	0.07
20.0	0.1
40.0	0.12
60.0	0.14
80.0	0.16
100.0	0.19

Table 2:4 lists the data from the two dilutions of the receptor preparation used. When all the dilutions have been taken into account it is possible to calculate the protein concentration of the pellet.

Figure 2:2 An example of a protein calibration curve.

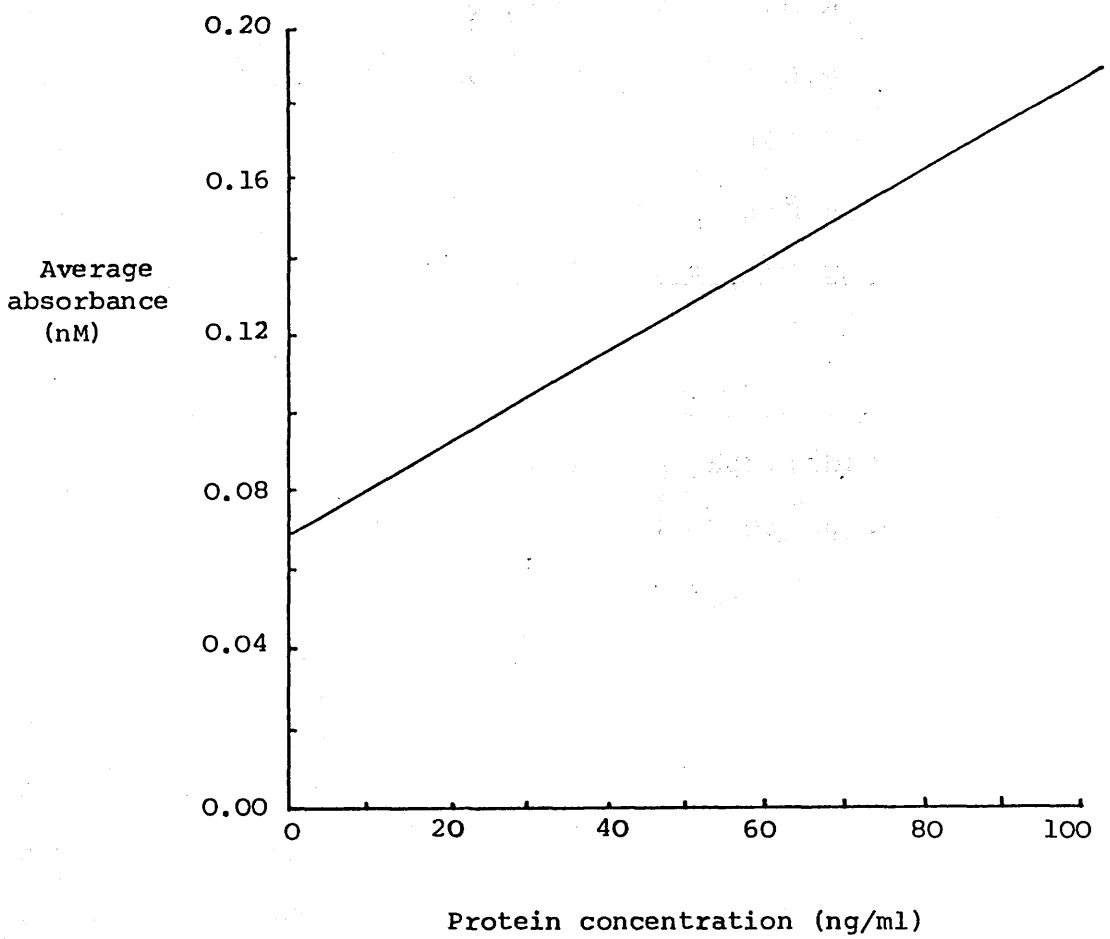


Table 2:4 U.V. Absorbtion Data from the Two Dilutions of the Receptor Preparation Pellet.

Samples			
1/10			
Dilution	Average absorbance	ug pt/ml	Equilibration of ug pt/ml to 1ml dilution
0.2	0.09	15.0	75.0
0.4	0.11	32.0	80.0
0.6	0.15	66.0	110.0
0.8	0.17	83.0	103.0
1.0	0.20	118.0	118.0
		Average	97.0
1/50			
Dilution	Average absorbance	ug pt/ml	Equilibration of ug pt/ml to 1ml dilution
0.2	0.07	0.0	0.0
0.4	0.07	0.0	0.0
0.6	0.08	7.0	11.6
0.8	0.09	15.0	18.7
1.0	0.09	15.0	15.0
		Average	15.1

Estimation of the protein concentration of each pellet by photospectrometry revealed the average protein concentration of 1g of brain tissue to be between 1 and 2mg, after resuspension in 5ml of assay buffer. A selection of prepared pellets, their weights and protein concentrations are shown in table 2:5. Also shown are the volumes of assay buffer required to give a final protein concentration of 1mg/ml.

Table 2:5 Results from the Assay of Prepared Pellets to show the Concentration of Protein in each Preparation.

Weight of pellet (mg)	Protein Conc. mg/ml	Vol made up to 1mg/ml with AB	Final protein conc. (mg/ml)
44.4	1.8	0.55	1.0
32.7	1.4	0.71	1.0
51.7	2.1	0.47	1.0
45.3	1.8	0.55	1.0
42.1	1.8	0.55	1.0
30.7	1.2	0.83	1.0
36.2	1.5	0.66	1.0
36.1	1.5	0.66	1.0

Accordingly a protein concentration of 1mg/ml was used as a working concentration for the benzodiazepine radiorecptor assay (BZ-RRA).

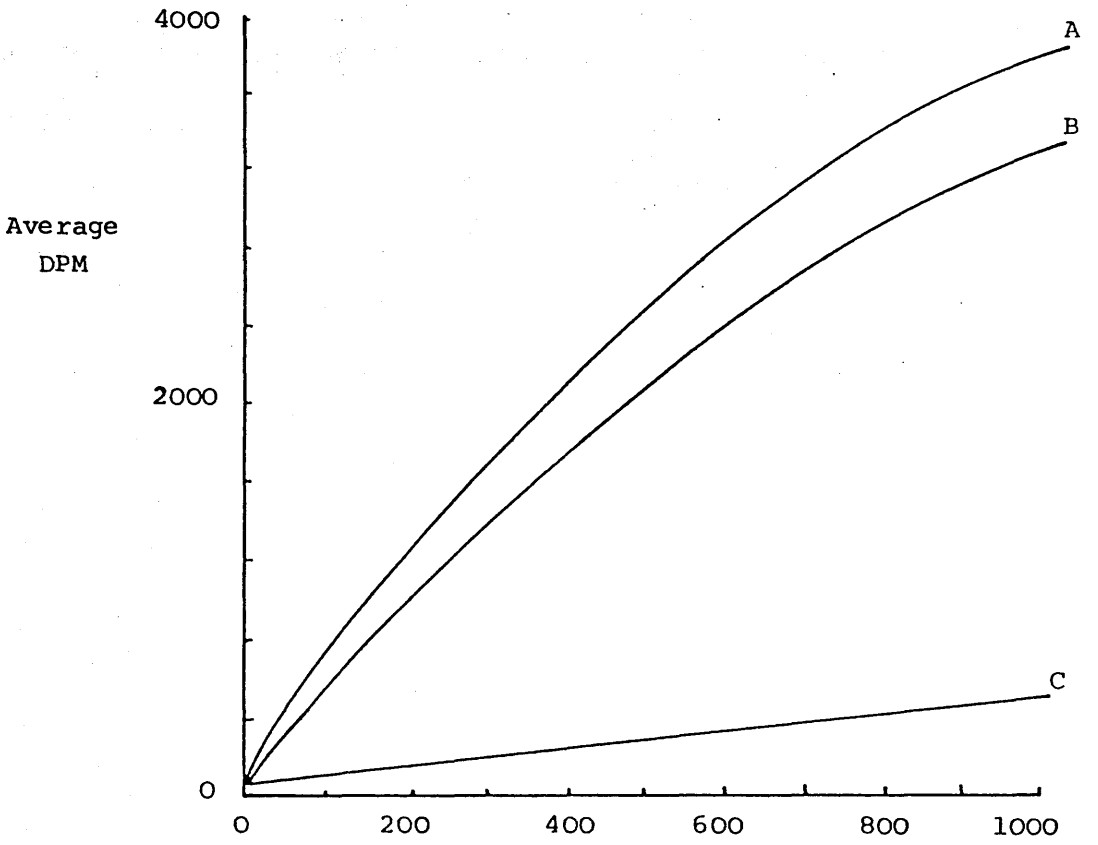
As the binding study and the experiments to assess the storage time of prepared receptors and whole rat brains, were carried out prior to those assessing the incubation period and washing of the filters, literature values were used where necessary. The incubation period was taken as between 30 and 60 minutes and the filters washed with 10ml of assay buffer [9,10,11,12,13,14]. When the appropriate experimental data had been established both studies were repeated with an incubation period of 30 minutes and with the filters being washed with 5 x 1ml assay buffer. Once the protein concentration for each pellet has been estimated it is possible to make a more accurate determination of the receptor number in each pellet. The binding assay forms the experimental part with the data obtained being transformed by a variety of calculations and graphs to determine K_D and B_{max}. Subtraction of the NSB DPM results from the total bound DPM results produces the DPM for the specifically bound [^{3}H]FNZ. A set of experimental results to highlight this part are shown in table 2:6.

Table 2:6 Experimental Results from a Binding Assay for Total, NSB and Specific Binding of [^{3}H]FNZ to the Benzodiazepine Receptors.

	Average DPM		
[^{3}H]FNZ (nCi)	Total Bound	NSB	Specifically Bound
10	99.75	104.5	-4.75
25	208.05	81.7	126.35
50	304.95	89.3	215.65
100	712.5	98.8	613.7
200	1411.7	168.15	1243.5
400	2489.0	281.9	2207.1
600	2874.7	395.4	2479.3
800	3459.9	411.3	3048.6
1000	3887.4	507.3	3380.1

Figure 2:3 is a plot of the data from figure 2:6 whereby total (A), specific (B) and non-specific binding (C) of [^{3}H]FNZ are plotted against increasing [^{3}H]FNZ concentration (nCi). The specific binding of the radioligand is saturable, whereas the NSB increases linearly with increasing radioligand concentration, both necessary requirements for a workable RRA. These results, together with the DPM of the total radioligand added, are transformed by the Combicept™ program [138], to determine K_D and B_{max} via the Scatchard plot,

Figure 2:3 Total, non-specific and specific binding of tritiated flunitrazepam.



A - Total binding.
B - Specific binding.
C - Non-specific binding.

[1,94,113,139,140,141,142,143]. This graphical method remains popular despite publications relating to the errors in the correction and interpretation of such plots, [144,145,146,147]. Often, as with the Combicept™ program, the data is used to draw a Scatchard plot and a saturation curve with a K_D value obtainable from each by extrapolation of the data. Theoretically these values will be the same from both plots, but often slight differences occur due to the different way the graphs, and therefore the values, are calculated. The same applies to the B_{max} value which is also obtainable from both graphs. Below is a comparison of Scatchard and saturation plots which emphasises the errors associated with plotting a Scatchard curve:

1 The points at the top of the Scatchard plot relate to the low concentrations of radioligand thus are the source of greatest error, but they are often used to determine where the line is drawn.

The same points on a saturation plot are near the origin and do not have as much bearing on the shape of the graph.

2 As the data points forming the Scatchard plot are often clustered in the top half of the graph, extrapolation to obtain B_{max} can result in a large error as there are fewer or no points near the intersection with the X axis.

With the saturation plot the data points are spaced more evenly with those more likely to be accurate near the top of the graph, the part used to determine K_D.

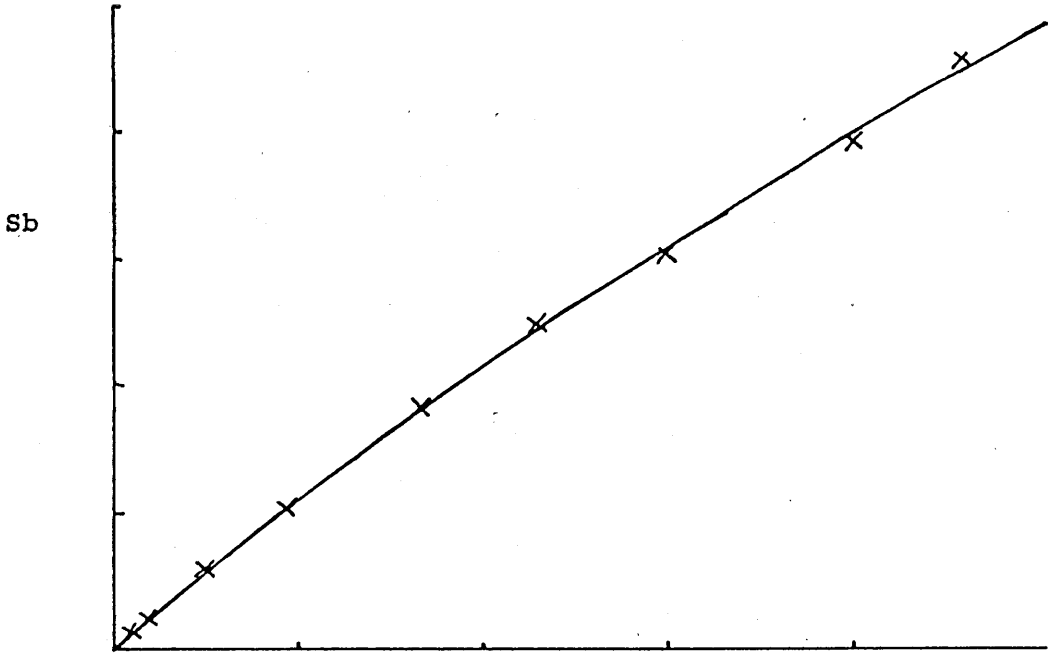
Figures 2:4 and 2:5 are theoretical plots which demonstrate the above points [148].

The Combicept program™ incorporates both an orthogonal weighting scheme and the Rosenthal correction for NSB subtraction [149]. The former statistically eliminates some of the errors encounted during data transformation to the Scatchard form; however the saturation curve is still recommended as the best way of analysing data from a binding study.

Papers are also available which demonstrate varied ways of analysing data from receptor assays, the use of computer modelling to obtain the optimum conditions for RRA and also programs to calculate the errors involved, [150,151,152,153].

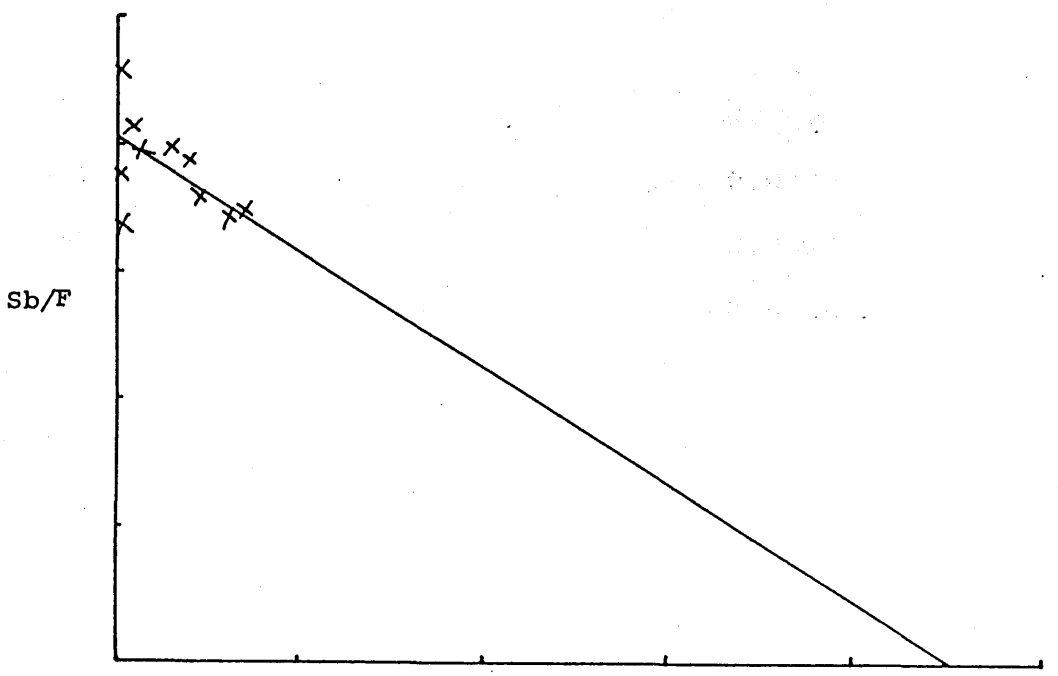
Experimental data obtained from a binding study, protein concentration - 0.12mg/ml, is shown in table 2:7; and plotted both as a Scatchard plot and a saturation curve which are shown by figures 2:6 and 2:7.

Figure 2:4 Theoretical saturation plot to demonstrate the relatively even spacing of the points.



Sb - Specifically bound tritiated flunitrazepam.

Figure 2:5 Theoretical Scatchard plot to demonstrate how the points are clustered near the top part of the graph.



Sb/F - Specifically bound tritiated flunitrazepam over free tritiated flunitrazepam (pM/mg pt/nM).

* - Due to a fault in the commercial program this second point was discounted when the saturation and Scatchard plots were drawn.

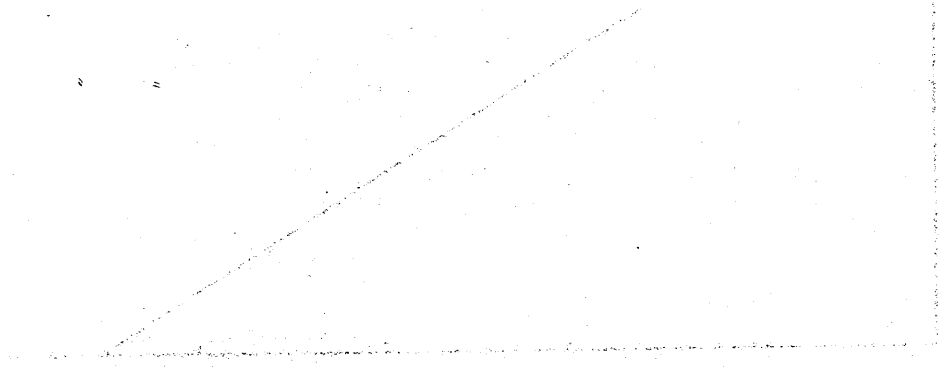


Table 2:7 Experimental Binding Assay Data.

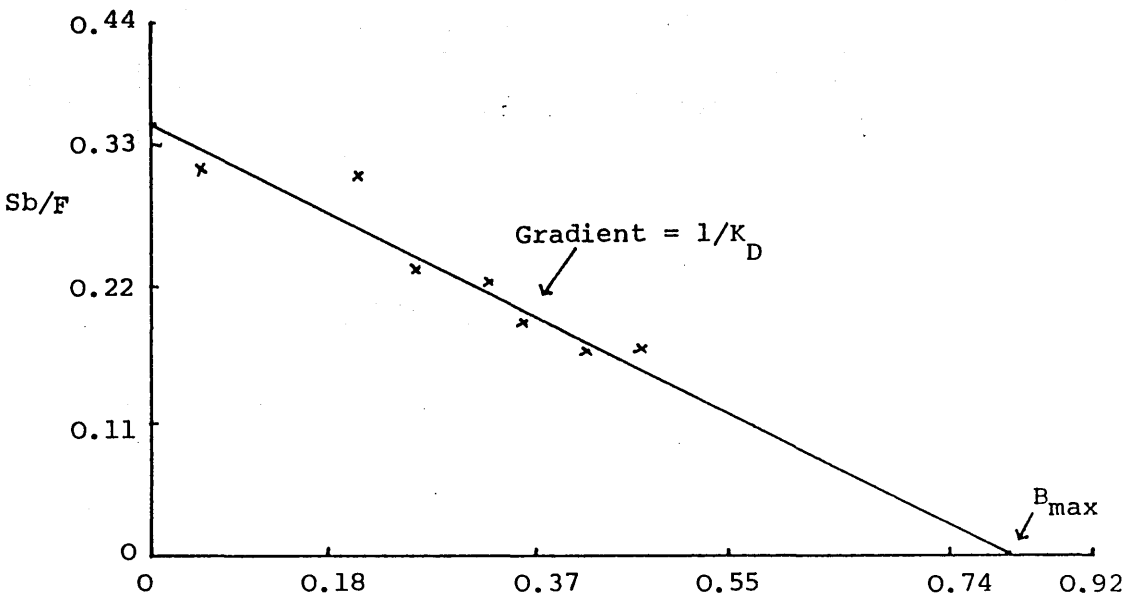
[^{3}H]FNZ (nCi)	Sb [^{3}H]FNZ pM/mg protein	Sb/F pM/mg protein/nM
0.0306	0.0015	0.0520
0.0071	0.0034	0.9347*
0.1401	0.0044	0.0323
0.3360	0.0101	0.0313
0.6669	0.0201	0.0312
1.1514	0.0258	0.0230
1.5888	0.0332	0.0215
2.0651	0.0372	0.0184
2.7668	0.0433	0.0160
3.1141	0.0486	0.0159

The K_D and B_{max} results obtained from this data are as follows:

	Scatchard plot	Saturation Curve
K_D	2.36nM	2.32nM
B_{max}	962fmol/mg pt	938fmol/mg pt

There is significant correlation between the points forming the Scatchard plot, as the regression coefficient = 0.961.

Figure 2:6 Scatchard plot from a set of experimental data, protein concentration 0.12mg/ml.

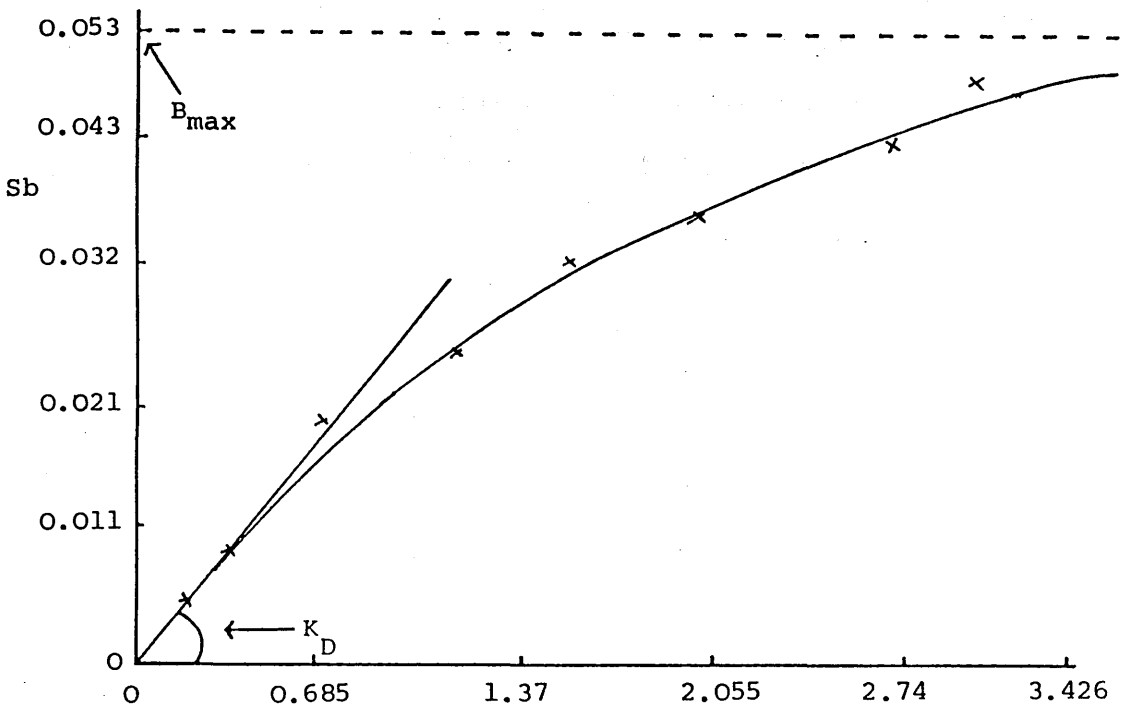


Sb/F - Specifically bound tritiated flunitrazepam over free tritiated flunitrazepam (pM/mg pt/nM).

B_{max} - The intersection with the X axis.

K_D - The reciprocal of the gradient of the line.

Figure 2:7 Saturation plot from a set of experimental data, protein concentration 0.12mg/ml.



Sb - Specifically bound tritiated flunitrazepam.

B_{max} - The horizontal assymptote to the curve.

K_D - This is proportional to the slope of the curve at the origin.

Binding studies were carried out with varying concentrations of protein, the results of which are shown in table 2:8. Protein concentrations below 1mg/ml gave results consistent with literature values, better correlation between data points and greater consistency between binding studies. Results from assays where the protein concentration was above 1mg/ml were more variable. This was thought to be because of the increased number of receptors enabling binding of both labelled and unlabelled ligand in a less competitive manner. Therefore the extrapolation of the results to saturation will be less accurate.

Table 2:8 A Compilation of Several Sets of Results from Scatchard and Saturation Plots with Varying Protein Concentrations.

Protein ug pt/ml	Saturation		Scatchard		Correlation coefficient
	K_D	B_{max}	K_D	B_{max}	
4.5	9.73	713	14.40	1011	0.52
3.5	2.10	17	3.20	23	0.656
2.3	9.73	1003	14.60	2000	0.524
1.7	1.81	59	1.58	55	0.9074
0.12	2.32	938	2.36	962	0.961
0.06	1.96	1710	1.51	1516	0.9079
0.01	1.81	10000	1.58	9400	0.9074

Experiments carried out to determine the length of time the receptors could be stored resulted in a series of graphs, of which figure 2:1 is an example. As the curve is linear over the chosen standard range it is possible to calculate the gradients of each line for comparison. Results for assays carried out over a 6 week period are shown in table 2:9 and are the averages of such data gained from 3 experiments for every 7 day interval, following receptor preparation.

Table 2:9 Data Relating to Radioreceptor Assays using Receptor Preparations up to Six Weeks After Preparation.

Triazolam (ng/ml)	C_o/C_x Week no. 1	2	3	4	5	6
1.0	1.2	1.1	1.3	1.1	1.2	1.0
5.0	1.8	1.4	1.7	1.6	1.6	1.1
10.0	3.4	1.0	2.9	2.5	3.1	1.8
15.0	6.4	4.4	4.7	4.0	4.7	2.8
20.0	9.4	7.4	7.4	6.8	4.8	1.8
25.0	11.0	10.4	12.9	9.5	6.5	2.2
35.0	13.4	21.4	16.5	15.3	12.8	3.0
50.0	21.4	22.7	21.5	26.1	17.0	2.7

Combinations of each weeks results are shown in table 2:10 as average values.

Table 2:10 The Combined Results of Weeks 1 to 6 Listed as Average Values.

Triazolam (ng/ml)	Average C_o/C_x values Weeks combined 1+2	1-3	1-4	1-5	1-6
1.0	1.15	1.2	1.2	1.18	1.0
5.0	1.6	1.63	1.6	1.62	1.53
10.0	2.2	1.63	2.4	2.58	2.45
15.0	5.4	5.16	4.8	4.84	4.5
20.0	8.4	8.1	7.7	7.16	6.26
25.0	10.7	11.4	10.9	10.1	8.75
35.0	17.4	17.1	16.6	15.9	13.7
50.0	22.1	21.8	22.9	21.74	18.6

From these 'average value' results it is difficult to determine any significant changes with time. Therefore the paired t-test [154] was used to statistically compare results from one week with those of previous weeks. This method is preferred to a comparisom of means as the

differences in the results are comparatively large especially when week 6 is considered. By taking the null hypothesis approach, the means of the differences between pairs of results can be tested, with the following formula, to determine whether they significantly differ from zero.

$$t = \frac{(x-u)}{n/s}$$

x = mean of samples

u = mean of population

s = standard deviation of samples, with $s_{(n-1)}$

n = sample size

t = the quantity used in significance testing and has (n-1) degrees of freedom when p = 0.05

Therefore $$t = \frac{xd}{\sqrt{n/s_d}}$$

x = mean of differences

s_d = standard deviation of differences

The various comparisons of the results from weeks 1 to 6 are listed below and the corresponding values of t are shown in table 2:11.

1 Week 1 v. week 2.

2 Weeks 1 & 2 v. week 3.

3 Weeks 1 - 3 v. week 4.

4 Weeks 1 - 4 v. week 5.

5 Weeks 1 - 4 v. week 6.

Table 2:11 A Statistical Comparison of the Results of the Storage Study.

Week number	Values of t
1 v 2	0.35
1+2 v 3	0.00
1-3 v 4	0.84
1-4 v 5	9.16*
1-4 v 6	49 36*

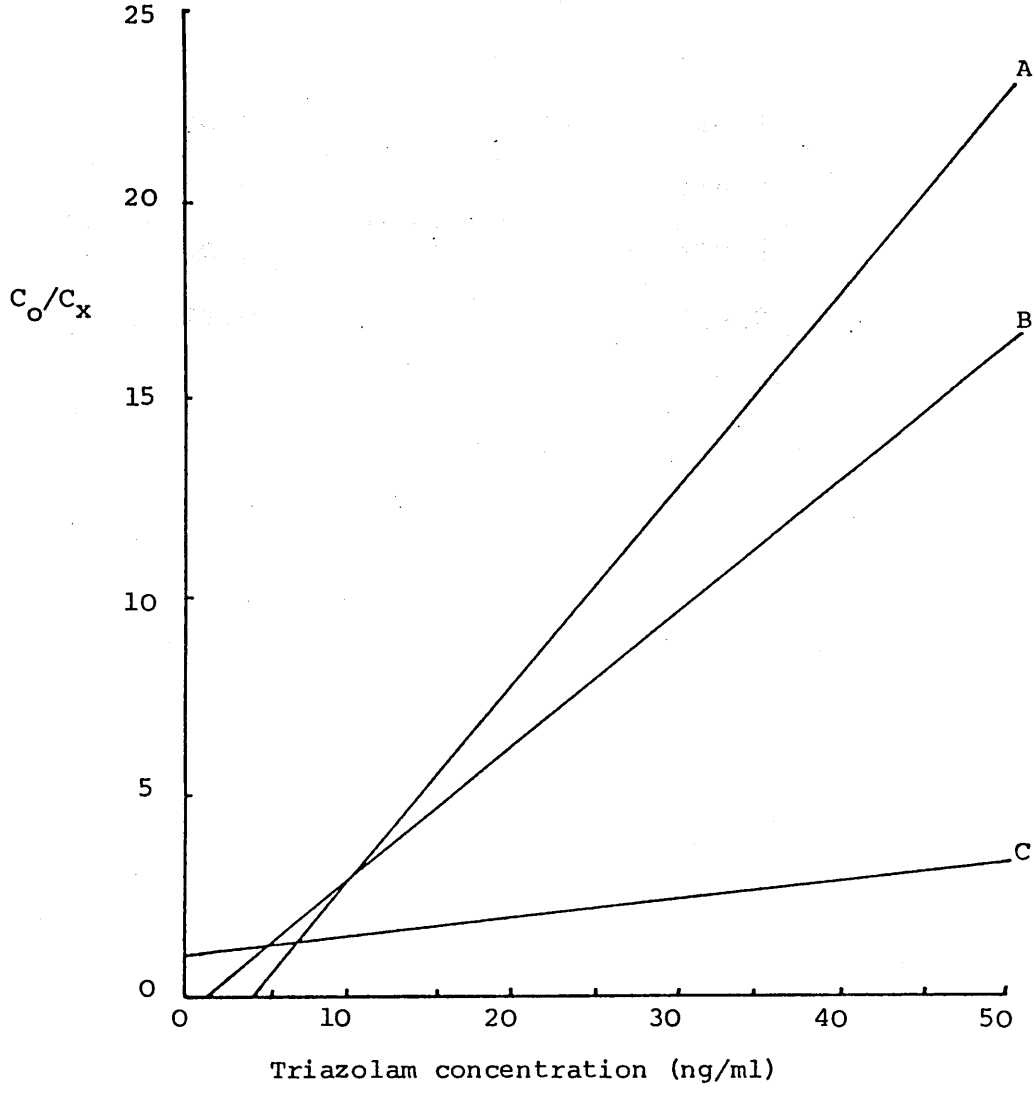
Values of t above 2.31 constitute a statistically significant result, (*).

Figure 2:8 demonstrates the results of table 2:9 graphically where the a decrease in receptor viability with time is seen by a decrease in the gradient of the standard curve. This is presumed to be due to disruption of the proteins which form the receptors which decreases their ability to bind with the ligand/radioligands. Line A represents the average of results from weeks 1 to 4 and indicates no loss of receptor viability up to 28 days after receptor preparation. After this period there is a significant decrease in the C_o/C_x values leading to shallower curves, line B = week 5 and line C = week 6.

Therefore the maximum time period for preparing receptors prior to use was set at 4 weeks. Rat brain tissue stored for 12 months at -20^oC, prior to homogenisation, gave a curve consistent with that for weeks 1 to 4, as shown in figure 2:8, provided the prepared receptors were used within 28 days. Whole rat brains can therefore be stored at -20^oC for at least a year with no loss of binding ability. The 3 dimensional structure of the receptors is retained when the whole brains are frozen down immediately they are excised. Storage time of prepared receptors is reduced to 4 weeks as the action of preparation disrupts the tissue surrounding the receptors thus increasing their fragility.

Results from the specific binding v. time experiment shown in table 2:12 and by figure 2:9, indicate the length of time required by the receptors and radioligand/ligand to reach equilibrium.

Figure 2:8 A series of triazolam standard curves which demonstrate the decrease in gradient with time.

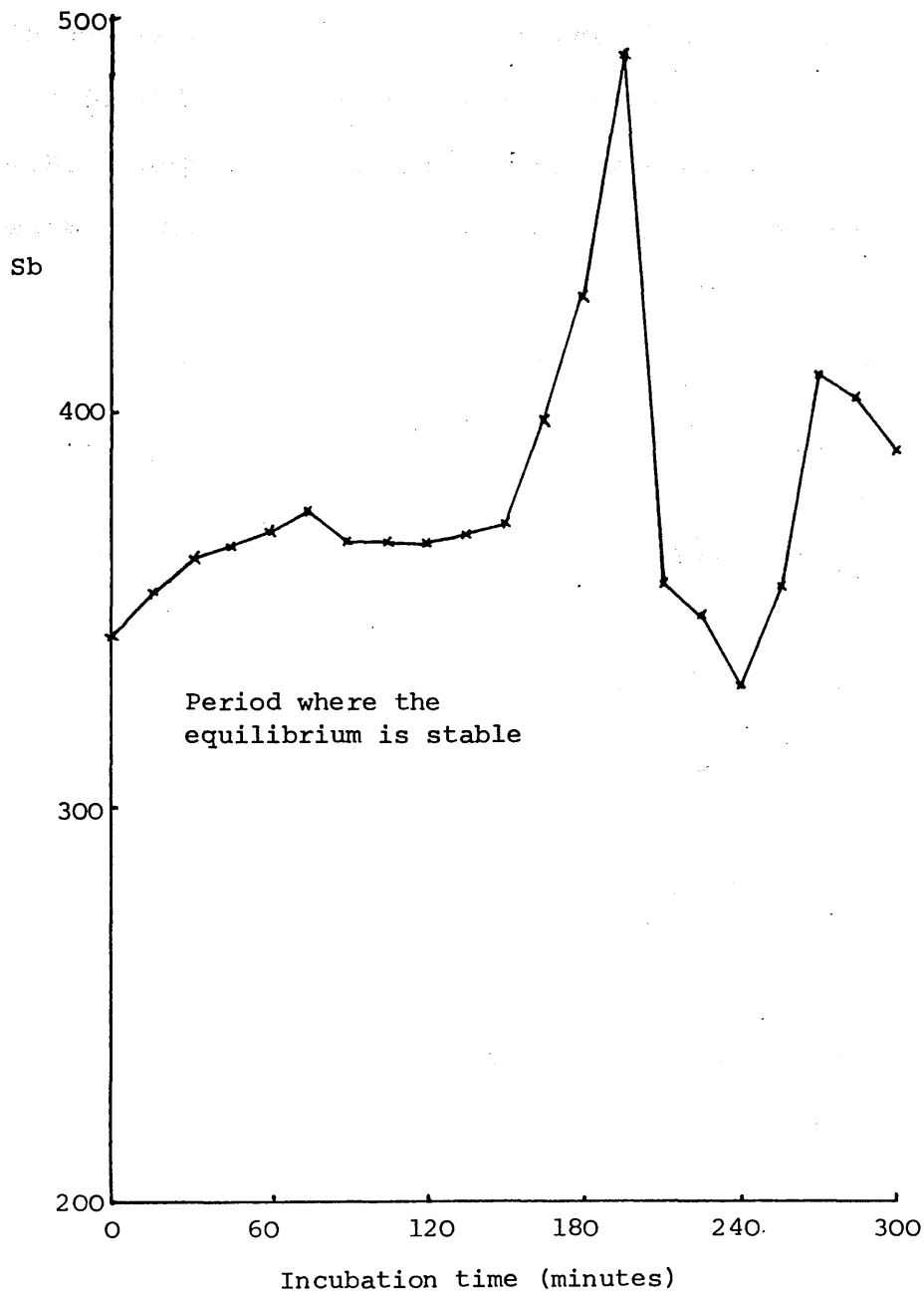


A - Results from weeks 1 to 4.
B - Results from week 5.
C - Results from week 6.

Table 2:12 DPM Results for Each Time Interval Between t_0 and t_{300}.

Time (minutes)	Average DPM
0.0	343.9
15.0	355.3
30.0	364.8
45.0	366.7
60.0	370.5
75.0	375.0
90.0	368.6
105.0	368.4
120.0	368.6
135.0	370.4
150.0	372.4
165.0	399.0
180.0	431.3
195.0	494.0
210.0	357.2
225.0	349.6
240.0	332.5
255.0	357.2
270.0	410.4
285.0	406.6
300.0	391.4

Figure 2:9 Demonstration of the equilibrium disruption with time when incubated at 25°C.



Sb - Specifically bound tritiated flunitrazepam (DPM/assay).

They also reveal how long the incubates could be left before the equilibrium began to disrupt and also how it can be reversed by the addition of excess unlabelled ligand. Once defrosted the BZ-R remain viable up to three hours, at room temperature, after which they begin to degenerate. This time scale allows an assay to be set up, incubation to take place and the bound and free fractions to be separated, providing the assay is begun immediately the BZ-R have completely defrosted.

N.B. Any attempt to speed up the BZ-R defrosting with warm or hot water will disrupt the integrity of the BZ-R.

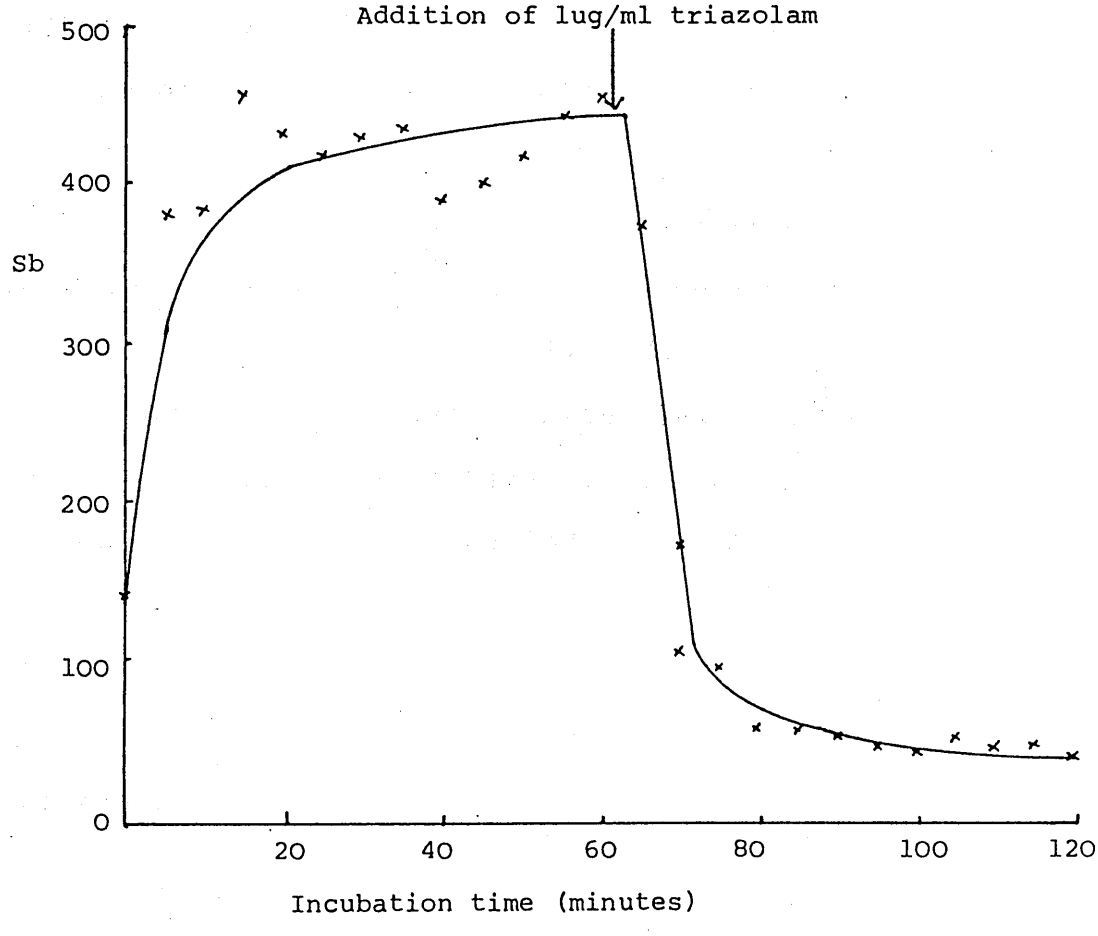
The results in table 2:13, also shown in figure 2:10, demonstrate the time period to reach equilibrium between the receptors and the radioligand and how it can be reversed by excess unlabelled ligand.

Table 2:13 DPM Results for Time Intervals Between t_0 and t_{120}.

Association Curve		Dissociation Curve	
Time (minutes)	average DPM	Time (minutes)	average DPM
0.0	138.7	65.0	375.2
5.0	381.9	70.0	108.3
10.0	383.8	75.0	100.7
15.0	454.1	80.0	60.8
20.0	431.3	85.0	57.0
25.0	418.0	90.0	55.1
30.0	431.3	95.0	49.4
35.0	437.0	100.0	47.5
40.0	389.0	105.0	70.3
45.0	402.8	110.0	51.3
50.0	418.0	115.0	53.2
55.0	446.5	120.0	45.6
60.0	456.0		

The association curve shows that over 90% of the radioligand is bound after 20 minutes and that the equilibrium between the receptors and the ligand remains constant up to 60 minutes. The addition of excess triazolam displaces 95% of the radioligand within 5 minutes reaching NSB levels after 10 minutes. Excess

Figure 2:10 Association and dissociation curves for tritiated flunitrazepam and benzodiazepine receptors incubated at 25°C.



Sb - Specifically bound tritiated flunitrazepam (DPM/assay).

triazolam is added at t_{60} as this falls within the stable period of the equilibrium. If it had been added towards the end of this period, reversal might have been attributed to the start of the equilibrium disrupting as well as by the additional triazolam. From these two experiments the incubation period for a BZ-RRA can be set at 30 minutes minimum and 150 minutes maximum. This two hour period were the equilibrium is stable is sufficient to filter large numbers of samples before the receptor/ligand binding is disrupted.

Separation of the bound and free radioligand by filtration was used throughout, and the NSB reduced by washing the filters. As increased volumes of assay buffer were used to wash the filters, there was a corresponding decrease in the ± values. When the filters were washed with 5 and 10ml, these limits were approximately the same. Accordingly a volume of 5 x 1ml was used for susequent RRA as this enabled the NSB to be minimised whilst keeping the separation time as short as possible. Table 2:14 lists the compiled results for each of the filter/washing experiments, each result is the average of three experiments carried out in duplicate.

Table 2:14 Results from Washing the Filters with Varying Volumes of Assay Buffer.

Wash (ml) Triazolam (ng/ml)	1.0 DPM	3.0 DPM	5.0 DPM	10.0 DPM
0.0	962.3	823.6	953.2	967.2
	±55.1	±52.4	±11.6	±13.7
1.0	789.4	680.2	866.8	842.0
	±63.1	±94.0	±12.0	±15.6
5.0	780.9	566.2	826.6	871.0
	±24.2	±40.3	±17.8	±15.4
10.0	626.0	561.4	616.7	572.9
	±73.8	±14.7	±17.3	±12.7
15.0	531.1	430.3	442.4	467.6
	±65.8	±17.4	±19.6	±17.8
25.0	453.1	348.6	332.6	293.5
	±41.6	±4.03	±7.4	±16.2
50.0	128.2	212.8	120.1	117.3
	±32.2	±20.1	±10.4	±3.3

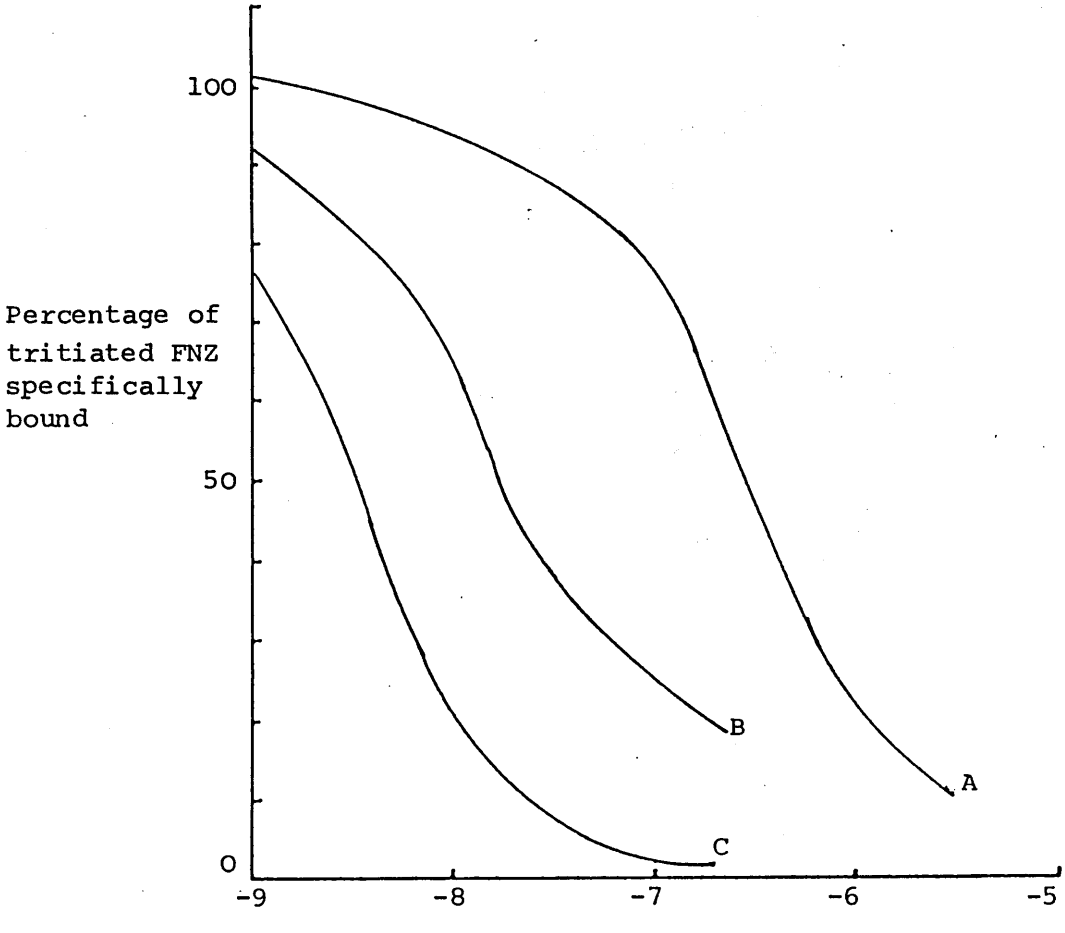
RRA results from the assay of various benzodiazepines were graphed in order to obtain the K_i values. The results from three examples - bromazepam, flunitrazepam and triazolam, are shown in table 2:15 and plotted out in figure 2:11.

Table 2:15 Three Examples of Benzodiazepines used to Determine K_i Values.

Concentration $\log_{10}M$	Percentage of [^{3}]FNZ specifically bound.		
	Bromazepam	FNZ	Triazolam
-9	101	91	75
-8.3	94	76	40
-8.0	96	64	18
-7.7	90	42	8
-7.3	87	31	5
-7.1	74	26	3
-7.0	78	23	2
-6.7	51	18	1
-6.0	20	-	-

The standards are calculated as a percentage of the zero standard to give the % of [^{3}H]FNZ specifically bound to the receptors. From IC_{50} values, K_i values are calculated which indicate the potency of a particular drug to displace [^{3}H]FNZ from the receptors. The IC_{50} and K_i values for the benzodiazepines assayed are given in Table 2:16 and follow published trends.

Figure 2:11 Curves demonstrating the concentration of benzodiazepine to cause 50% inhibition of tritiated flunitrazepam binding to the receptors.



A - Bromazepam.
B - Flunitrazepam.
C - Triazolam.

Table 2:16 IC_{50} and K_i Values for a Selection of Benzodiazepines.

Benzodiazepine	IC_{50} M	K_i nM
Alprazolam	$1.8x10^{-8}$	12.2
Bromazepam	$2.2x10^{-7}$	149.6
Clonazepam	$1.3x10^{-8}$	8.8
Diazepam	$7.6x10^{-8}$	51.7
Desmethyl Diazepam	$2.3x10^{-7}$	156.4
3-OH-Diazepam	$1.0x10^{-7}$	68.0
Flunitrazepam (FNZ)	$1.4x10^{-8}$	9.5
Desmethyl FNZ	$2.5x10^{-8}$	17.0
7-amino FNZ	$3.9x10^{-6}$	2700.0
Lorazepam	$1.0x10^{-8}$	6.8
Nitrazepam	$8.7x10^{-8}$	59.1
Oxazepam	$3.4x10^{-7}$	231.0
Triazolam	$2.7x10^{-9}$	1.8
1-OH Triazolam	$3.2x10^{-8}$	21.7
4-OH Triazolam	$6.9x10^{-8}$	46.9

$$K_i = IC_{50}/(1 + C/K_D)$$

C = Concentration of $[^3H]$FNZ - 1.11nM.

K_D = 2.34nM.

Similar assays were carried out with a cross section of non-BZ, representing most pharmaceutical classes, to investigate their lack of cross reactivity. Of all the drugs assayed only caffeine and amphetamine showed any cross reactivity, at levels of 25ug/ml and over as seen in table 2:17 and 2:18 and by figures 2:12 and 2:13.

Table 2:17 Diazepam Control Curve.

Drug	Average DPM	C_x	C_o/C_x
Diazepam (ng/ml)			
0.0	635.4	565.2	
10.0	473.4	403.2	1.4
25.0	457.2	387.0	1.46
50.0	297.0	226.8	2.49
100.0	199.8	129.6	4.36
200.0	141.7	71.5	7.9
NSB	70.2		

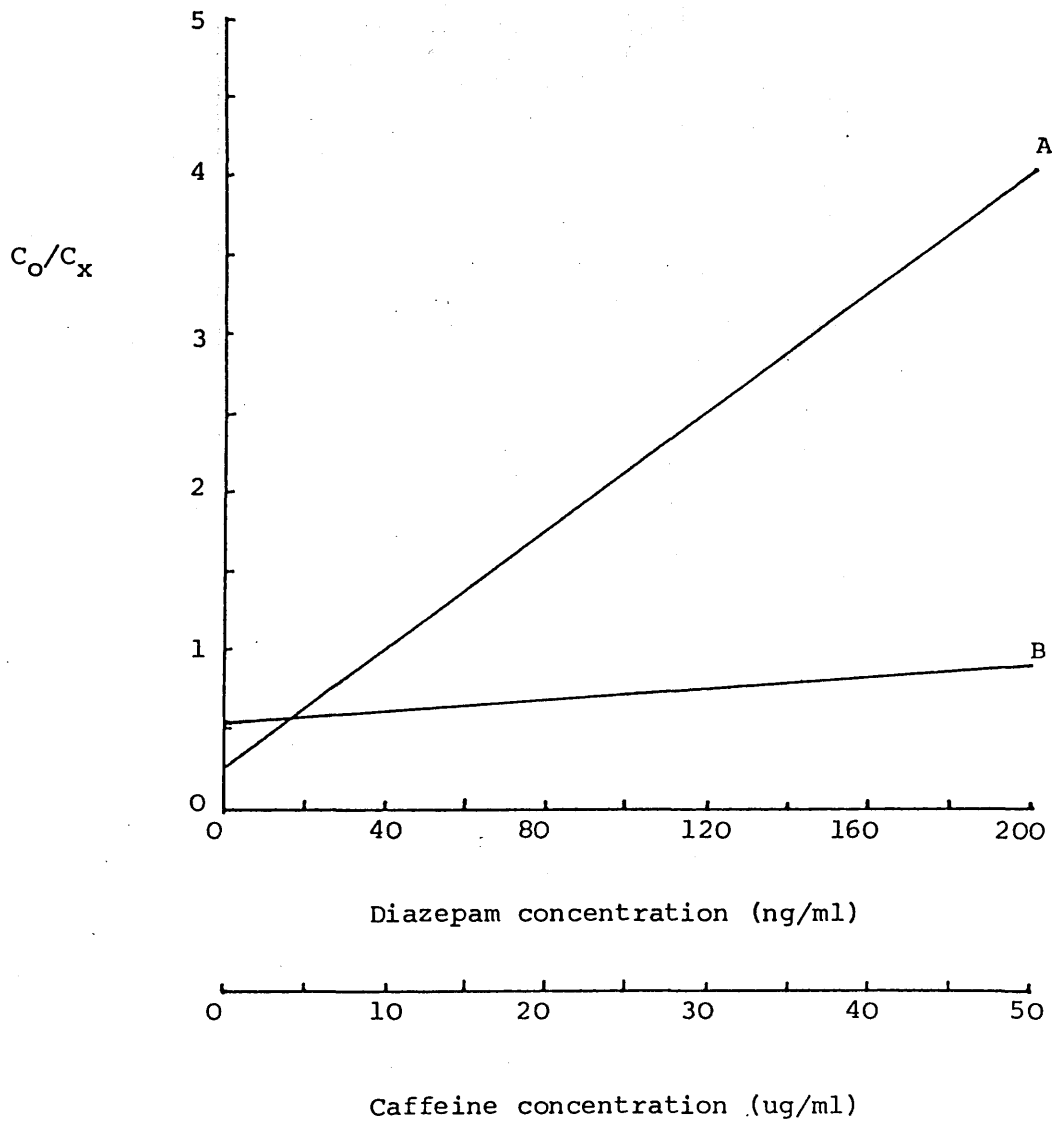
N.B. Diazepam NSB result used to calculate C_x values for caffeine and amphetamine.

Table 2:18 Non-benzodiazepine Results.

Drug	Average DPM	C_x	C_o/C_x
Caffeine			
(ug/ml)			
1.0	730.8	660.6	0.85
5.0	669.6	599.4	0.94
10.0	585.0	514.8	1.09
25.0	549.0	478.8	1.18
30.0	516.6	446.4	1.26
45.0	484.2	414.5	1.36
50.0	477.0	406.8	1.39
Amphetamine			
(ug/ml)			
1.0	572.4	502.2	1.12
5.0	558.0	487.8	1.16
10.0	491.4	421.2	1.34
25.0	442.8	372.6	1.51
30.0	415.8	345.6	1.63
45.0	383.4	313.2	1.80
50.0	406.8	336.6	1.68

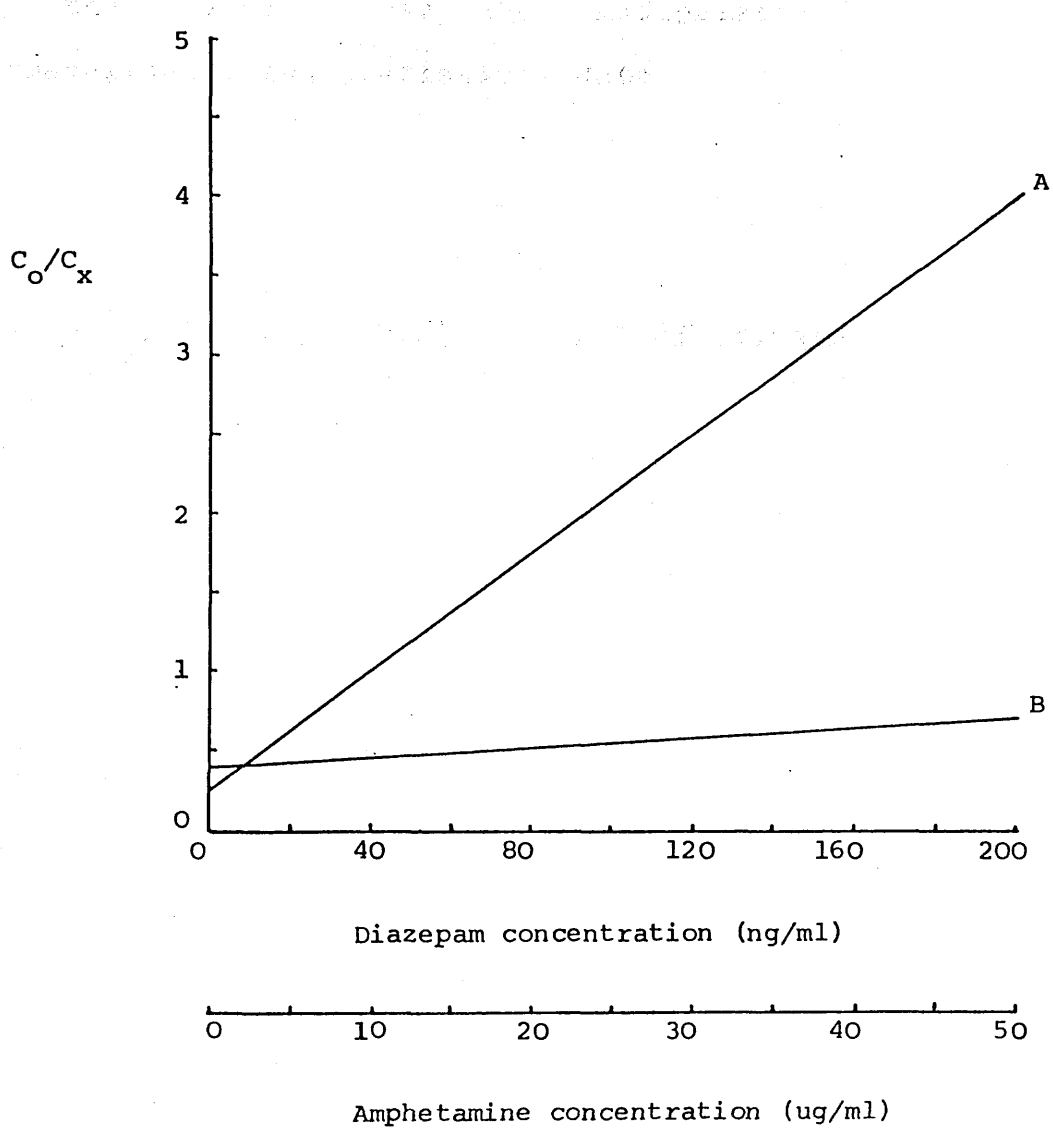
As the BZ-RRA is used to detect benzodiazepines in the ng range, any other drugs present would need to have a very high concentration in order to interfere with the assay.

Figure 2:12 RRA results indicating slight cross reaction between caffeine and BZ-receptors above 25ug/ml.



A - Diazepam.
B - Caffeine.

Figure 2:13 RRA results indicating slight cross reaction between BZ-receptors and amphetamine, 25ug/ml and above.



A - Diazepam.
B - Amphetamine.

2:5 Conclusions

This chapter has covered the various parts of the RRA, setting out the areas which have to be investigated in order to establish a RRA. A working protocol can now be drawn up for the BZ-RRA, which is the protocol followed when the greyhound urine samples were assayed for either triazolam or flunitrazepam. The method is easy to set up and the results can be manipulated to provide pharmacokinetic and statistical data.

Protocol

400ul Receptor preparation - 1mg/ml of protein.
100ul [^{3}H]FNZ - 50nCi.
50ul Standard or sample.

Vortex
Incubate for 30 minutes, 25^{o}C.
Separate bound and free fractions by filtration.
Wash filters with 5 x 1ml assay buffer.
Place in Pico vials and add 4ml scintillant/vial.
Shake vigorously to disperse filter in scintillant.
Count DPM of bound [^{3}H]FNZ for 1 minute.

Standards: Triazolam - 0, 1, 5, 10, 25, 35, 50ng/ml.
in drug Flunitrazepam - 0, 10, 25, 50, 100, 200ng/ml,
free urine.

For each assay a standard curve was established with the appropriate standards, plus zero standards - 50 ul assay buffer, and NSB standards. All the standards and samples are assayed in duplo. The results are expressed as C_o/C_x. C_o/C_x for the standards graphed against concentration (ng/ml) gives a straight line with a reproducible gradient, as shown by graph 2:1. From this the concentrations of triazolam or flunitrazepam in the urine samples can be estimated.

As increasing numbers of standard curves were prepared the gradients were collated and statistically analysed to provide the limits within which the line must lie. Any line whose gradient is outside of these limits cannot be used to determine benzodiazepine concentrations in greyhound urine samples. The usual cause of this is the degradation of the standards with time. To prevent this, the standards were made up at the start of each week and stored at 4 to 6^{o}C. Below is the gradient data collated from RRA of the greyhound urine samples:

Triazolam standard curves

n = 62 x = 0.48, limits = 0.1.

Flunitrazepam standard curves

n = 9 x = 0.06, limits = 0.008.

CHAPTER THREE

Analysis of Greyhound Urine Samples for Selected Benzodiazepines by Radioreceptor Assay and Radioimmunoassay.

3:1 Introduction

The benzodiazepine-greyhound studies involved the collection of urine samples following single oral dosing of each greyhound with a benzodiazepine. Prior to dosing a blank urine sample was collected and during the study any behavioural changes by the greyhounds, such as drowsines, were noted. A pilot study was carried out with diazepam to establish the dosing, sample collection and analysis procedures. Once the experimental procedures had been established, the main trials were carried out with triazolam and flunitrazepam.

Triazolam and flunitrazepam were studied as they are relatively new to the pharmaceutical market and consequently there is little information regarding their effect on dogs, especially greyhounds. As they are more potent than the more established benzodiazepines, lower dosage forms can be used to obtain the required response [155]. Examples of the varying doses prescribed are shown in table 3:1:

Table 3:1 A Variety of Benzodiazepines and their Active Metabolites Illustrating Differences in Dosage.

Benzodiazepine	To treat	Dose (mg)	$t_{1/2}$ (hours) (man)
Triazolam	Insomnia	0.25	1.5 - 3
FNZ	Insomnia	0.5 to 1.0	10 - 70
Nitrazepam	Insomnia*	5.0 to 10.0	18 - 38
Diazepam	Insomnia*	6.0 to 30.0	20 - 100
Desmethyl diazepam			40 - 100
CPX[a]	Insomnia*	30.0	5 - 30
Demoxepam[b]			14 - 95
Desmethyl diazepam[c]			40 - 100

[a] - Chlordiazepoxide.

[b] & [c] - Chlordiazepoxide metabolites.

* - Causes daytime sedation due to a long half-life.

The smaller effective doses of triazolam and flunitrazepam result in lower amounts available in urine samples for detection by laboratories. Also the time scale during which urine samples, containing evidence of the drug, can be collected is less due to the much shorter half-lives of triazolam and flunitrazepam. Therefore sensitive analytical methods are required to detect these benzodiazepines in greyhound urine samples.

Before the urine samples could be analysed, three additional experimental methods had to be established and evaluated:

a) Hydrolysis of the urine samples.

b) Extraction of the benzodiazepines and their metabolites from the unhydrolysed and hydrolysed urine samples.

c) Radioimmunoassay (RIA), of both the unhydrolysed and hydrolysed urine samples, to confirm the presence of benzodiazepines.

Radioreceptor assay of samples detects only the benzodiazepine and its phase I metabolites, which are pharmacologically active. The assay will not detect any phase II metabolites conjugated as the glucuronide or sulphate, or any phase I metabolites which are not pharmacologically active. With the purpose of phase II metabolism being to increase the water solubility of the drug and its phase I metabolites, a high percentage of the dose excreted in the urine will be in the conjugated form. Diazepam, triazolam and flunitrazepam conjugates are excreted in the urine as the following percentages of the total dose given:

Diazepam - approximately 70%.

Triazolam - approximately 80%.

Flunitrazepam - approximately 80%.

Thus, conjugated as the glucuronide or the sulphate, rapid elimination of the drug from the body is ensured. The urine samples may contain some free parent drug and phase I metabolites at very low levels [87,156]:

Diazepam - trace amounts.

Triazolam - less than 1%.

Flunitrazepam - less than 1%.

As diazepam, flunitrazepam and triazolam metabolites are excreted mainly as the glucuronide, mild hydrolysis of the urine samples with ß-glucuronidase, from Helix pomatia was used to release the benzodiazepine metabolites from their conjugated form (157).

Extraction of benzodiazepines from biological samples is a logical step to obtain a more accurate assesment of the amount of drug present. In the case of RRA it generally involves the removal of protein constituents which would interfere with ligand/BZ-R binding. Three organic solvents were used to extract a series of benzodiazepine standards and the results assessed to determine the viability of including this extra step in the RRA. The three solvents were; methanol, chosen as it is the standard laboratory solvent to extract samples prior to radioimmunoassy, ethyl acetate (33,35,158,159,160,161,162,163,164) and dichloromethane (165,166).

Radioimmunoassay (RIA) was used as the second method to confirm the presence of diazepam, triazolam and flunitrazepam in the urine samples. It was chosen as it is both a well established and reliable analytical method, as well as being sufficiently similar to radioreceptor assay to allow confident comparison of the results (157).

The data generated by RRA and RIA of all unhydrolysed and hydrolysed urine samples from each triazolam and flunitrazepam greyhound study was collated. This enabled the benzodiazepine urine excretion profiles for triazolam and flunitrazepam plotted, which demonstrate the increase in metabolites detected by RRA and RIA after hydrolysis.

The data generated during the triazolam/flunitrazepam greyhound trials also enabled limmited pharmacokinetic studies to be carried out. Pharmacokinetics enables the breakdown of the physical processes of drug absorbtion, distribution and elimination to be expressed as mathmatical functions involving time and concentration. Such data is used by doctors and pharmacists to predict the effect of a particular dose of a drug and to obtain correct dosing regimes. Should the intervals between doses become too long, the drug concentrations at the site of action will repeatedly fall below effective levels. If the dose is repeated too frequently though and the concentration of the drug will increase to toxic levels and have a detrimental effect.

The benzodiazepine results for each set of hourly samples obtained by both assay methods were compared statistically by the paired t-test [154]. This particular test was used as the samples contained substantially different amounts of the benzodiazepine given depending on the time of collection and the volume collected. Usual statistical methods whereby the means of two samples are compared cannot be used due to the large differences in the results which make significant variations between the means difficult to detect. By looking at the difference between pairs of results at each time interval and by adopting a null hypothesis approach, it is possible to test whether the means of the differences significantly differ from zero.

3:2 Materials

Triazolam Tablets 0.25mg	Donated by Messrs. Upjohn Ltd.
Flunitrazepam Tablets, 1mg	Donated by Messrs. Hoffmann-La Roche.
ß-Glucuronidase	Obtained from Sigma Chemical Company® U.S.A. 90,000 ß-glucuronidase units/ml, 400 sulphatase units/ml.
Emit® d.a.u.™ BZ Assay kit	Obtained from Syva Company.
Sheep serum	Scottish Antibody Production Unit, Law Hospital, Lanarkshire.
Donkey-anti-Sheep antibody	Scottish Antibody Production Unit, Law Hospital, Lanarkshire.
Assay Buffer throughout	25mM sodium phosphate buffer, pH 7.4.

All other chemicals and solvents were of analytical grade.

3:3 Methods

3:3:1 Hydrolysis

Aliquots of each urine sample were hydrolysed as follows:- 2ml aliquots of each urine sample were placed in glass screw top vials with 0.5ml, 0.1M, sodium acetate buffer, pH 5 and 20ul ß-glucuronidase. After thorough mixing the vials were incubated for 24 hours at 37^{o}C. The enzymic activity was stopped after this period by freezing the contents of each vial. These hydrolysed urine samples were stored at -20^{o}C together with the unhydrolysed samples.

3:3:2 Sample Extraction

Diazepam and triazolam standards were prepared with drug free greyhound urine and extracted with each of the three solvents: methanol, ethyl acetate and dichloromethane. The range of standards were:

Diazepam - 0, 10, 25, 50, 100, 200ng/ml.

Triazolam - 0, 1, 5, 10, 15, 20, 25, 35, 50ng/ml.

Method

The standard was extracted in duplicate by adding 0.5ml of each to 2ml of solvent. After thorough mixing and centrifugation, the organic layer was removed and evaporated to dryness. When required for assay the standards were reconstituted with 0.5ml of assay buffer.

The reconstituted standards were assayed together with unextracted diazepam or triazolam urine standards, which acted as controls. The results were plotted as C_o/C_x against benzodiazepine concentration. This enabled plots of the extracted standards to be visually compared with those of the controls.

3:3:3 Radioimmunoassay

Both the hydrolysed and unhydrolysed urine samples were analysed by this method. A reagent solution was prepared by mixing equal volumes of the following:

1 0.05M sodium bicarbonate buffer, pH 9.0.

2 [^{3}H]FNZ 50nCi.

3 Benzodiazepine specific antibody (4% solution), from the EMIT & BZ assay kit.

4 A 0.125% solution of sheep serum.

Standards - all made up in drug free greyhound urine.

Diazepam - 0, 10, 25, 50, 100, 200ng/ml.

Triazolam - 0, 1, 5, 10, 15, 20, 25, 35, 50ng/ml.

Flunitrazepam - 0, 10, 25, 50, 100, 200ng/ml.

Method

LP3 tubes were set up in duplicate, one pair for each standard or sample and the following constituents of the assay were added to each: 500ul reagent solution, 100ul standard or sample and 200ul of donkey anti-sheep antibody (12.5%) solution. The tubes were vortexed and incubated overnight at $4^{o}C$. To separate the bound and free fractions the tubes were centrifuged at 3000rpm for 15 minutes.

400ul of the supernatant was removed and thoroughly mixed with 4ml of scintillant in a Pico vial and counted for 1 minute to determine the DPM for each standard or sample. A plot of standard DPM against benzodiazepine concentration gave a calibration curve could be used to determine the benzodiazepine concentrations in the samples. The resulting curve is linear over the standard range used and enables the sample concentrations, in ng/ml, to be read directly from the curve.

The total amount of radiolabel available, the total counts, was estimated by the addition of 125ul of label to 675ul buffer, with incubation and separation as for the standards. The percentage binding was estimated by the following equation:

$$\% \text{ binding} = \frac{1 - 0\text{ng/ml CPM}}{\text{Total CPM}} \times 100$$

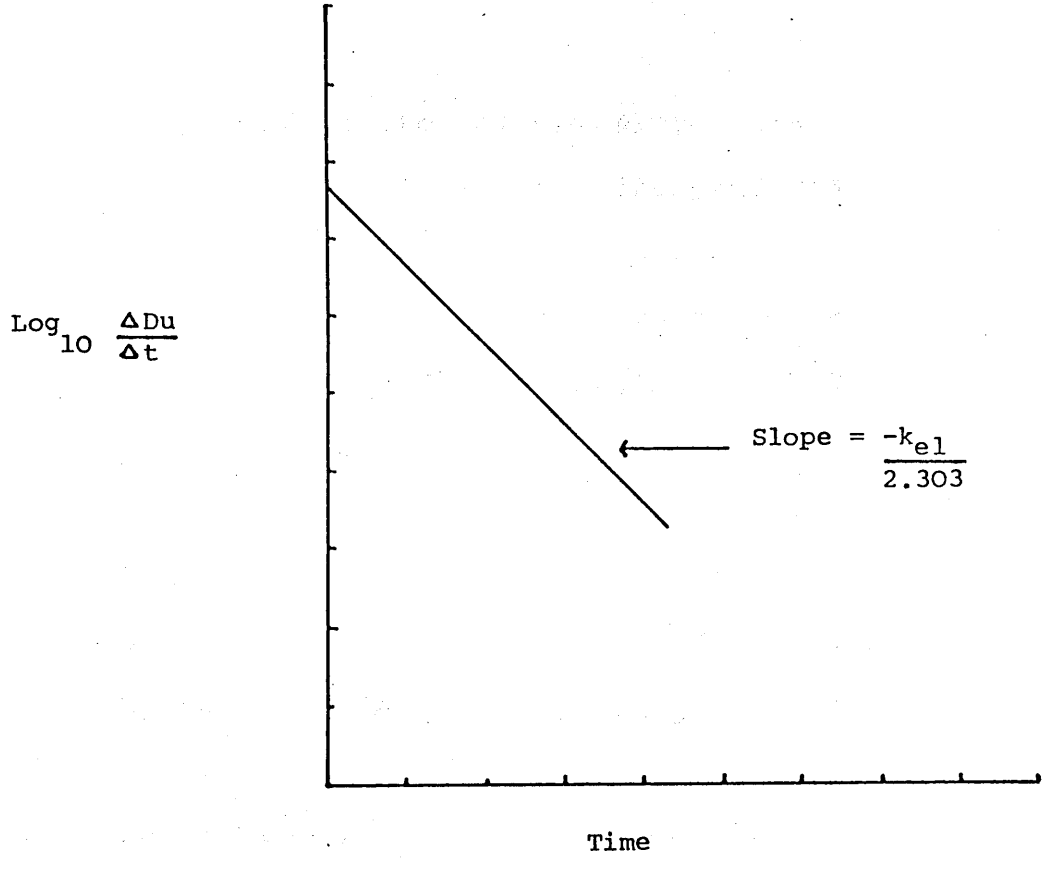
3:3:4 Pharmacokinetics of Urinary Excretion - Calculation of the Elimination Rate Constant and Half-Life

Renal clearance is the rate of filtration plus the rate of secretion of urine, minus the rate of aqueous reabsorbtion by the kidney. Glomerular filtration and diffusion is a first order process so the rate of transfer of drug is related to its concentration in the plasma which in turn is related to the concentration of the drug in the body. The appearance of the drug in the urine reflects its disapperance from the plasma/body. It was not possible to obtain complete urinary excretion data for some of the greyhound trials. Due to either the absence of, or incomplete collection of samples. A semi-log plot of $\Delta Du/\Delta t$ against time was used to estimate the elimination rate constant (k_{el}) and therefore calculate the half-life ($t_{½}$).

It gives a straight line, the gradient of which = $-k_{el}/2.303$ and from this it is possible to calculate the half-life as $t_{½} = 0.693/k_{el}$, as illustrated by figure 3:1.

Du is the cummulative amount of drug excreted. This usually refers to the excretion of the parent drug, but in the case of triazolam and flunitrazepam it refers to the metabolites excreted as only a very small percentage of the parent drug is excreted.

Figure 3:1 Illustration of a semi-log plot from which the elimination rate constant and the half life can be determined.



3:3:5 Statistical Comparisons

A paired t-test was used to test whether the mean of the difference between each set of results compared differs significantly from zero [154]. The following sets of results were statistically compared:

1 Triazolam

a Unhydrolysed RIA and hydrolysed RIA.

b Unhydrolysed RRA and unhydrolysed RIA.

c Hydrolysed RRA and hydrolysed RIA.

d Unhydrolysed RRA 1 and Unhydrolysed RRA 2.

e Hydrolysed RRA 1 and hydrolysed RRA 2.

2 Flunitrazepam

a Unhydrolysed RIA and hydrolysed RIA.

b Unhydrolysed RRA and unhydrolysed RIA.

c Hydrolysed RRA and hydrolysed RIA.

N.B. RRA 1 refers to urine samples assayed within 2 days of collection.

RRa 2 refers to samples assayed after being stored at $-20^{o}C$ for 12 months.

3:4 Protocol for Greyhound Trials

The following benzodiazepines were used to dose the greyhounds:

Diazepam	1 x 5mg tablet.
Triazolam	1 x 250ug tablet or 2 x 250ug tablets.
Flunitrazepam	1 x 1mg tablet or 2 x 1mg tablets.

A blank urine sample was collected from each greyhound prior to oral dosing with one of the three BZ selected. Subsequent urine samples were collected at the following hourly intervals:

1, 2, 3, 4, 5, 6, 7, 24, 25, 26, 27, 28, 29, 30, & 31.

The samples from each BZ-greyhound trial were treated and assayed as follows:

1 After collection, the total volume of each sample was measured and recorded.

2 2ml of each sample was removed and subjected to enzymic hydrolysis.

3 The remainder of each sample was aliquoted into 5ml volumes for analysis by RRA and RIA.

4 The unhydrolysed and hydrolysed samples were stored at $-20^{o}C$ until required for analysis.

3:5 Results and Discussion

3:5:1 Hydrolysis of the Urine Samples

Mild hydrolysis with ß-glucuronidase increases the concentration of pharmacologically active components available to interact with the BZ-R which effectively makes the assay more sensitive. It also enables benzodiazepine metabolites to be detected in samples collected up to 24 hours after dosing. This is an important factor in the analysis of samples from race tracks as the greyhound may have been dosed prior to arrival at the track.

Diazepam is excreted in the urine as the parent compound only in trace amounts. The majority, 70%, of the dose is excreted as the metabolites: N-desmethyl diazepam, 3-hydroxy diazepam and 3-hydroxy-desmethyl diazepam. The latter two are prescribed in their own right as the tranquillizers temazepam and oxazepam. Figure 3:2 illustrates the metabolic pathway of diazepam. The majority of the excreted metabolites are in the urine conjugated with glucuronic acid. Also the urine samples contain a small percentage of non-pharmacologically active metabolites which are not detected by the RRA. The increase in compounds detectable by BZ-RRA is demonstrated by figure 3:3 which plots an example of an excretion profiles of diazepam, both before and after hydrolysis. The corresponding RRA results are given in table 3:2 below.

Figure 3:2 The metabolic pathway of diazepam.

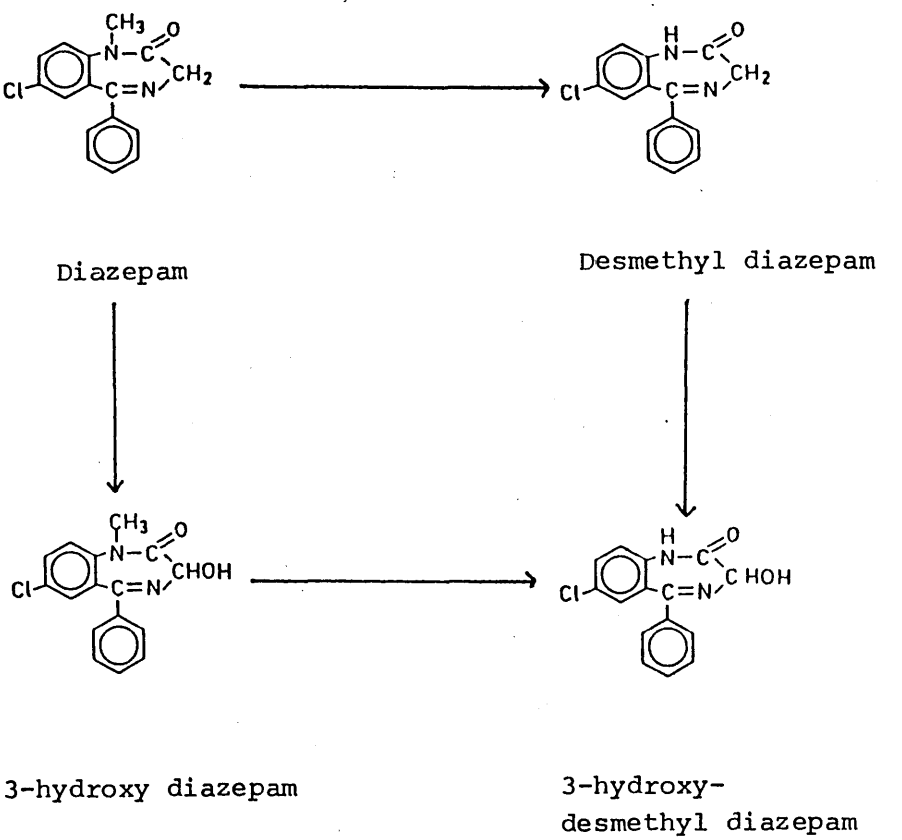
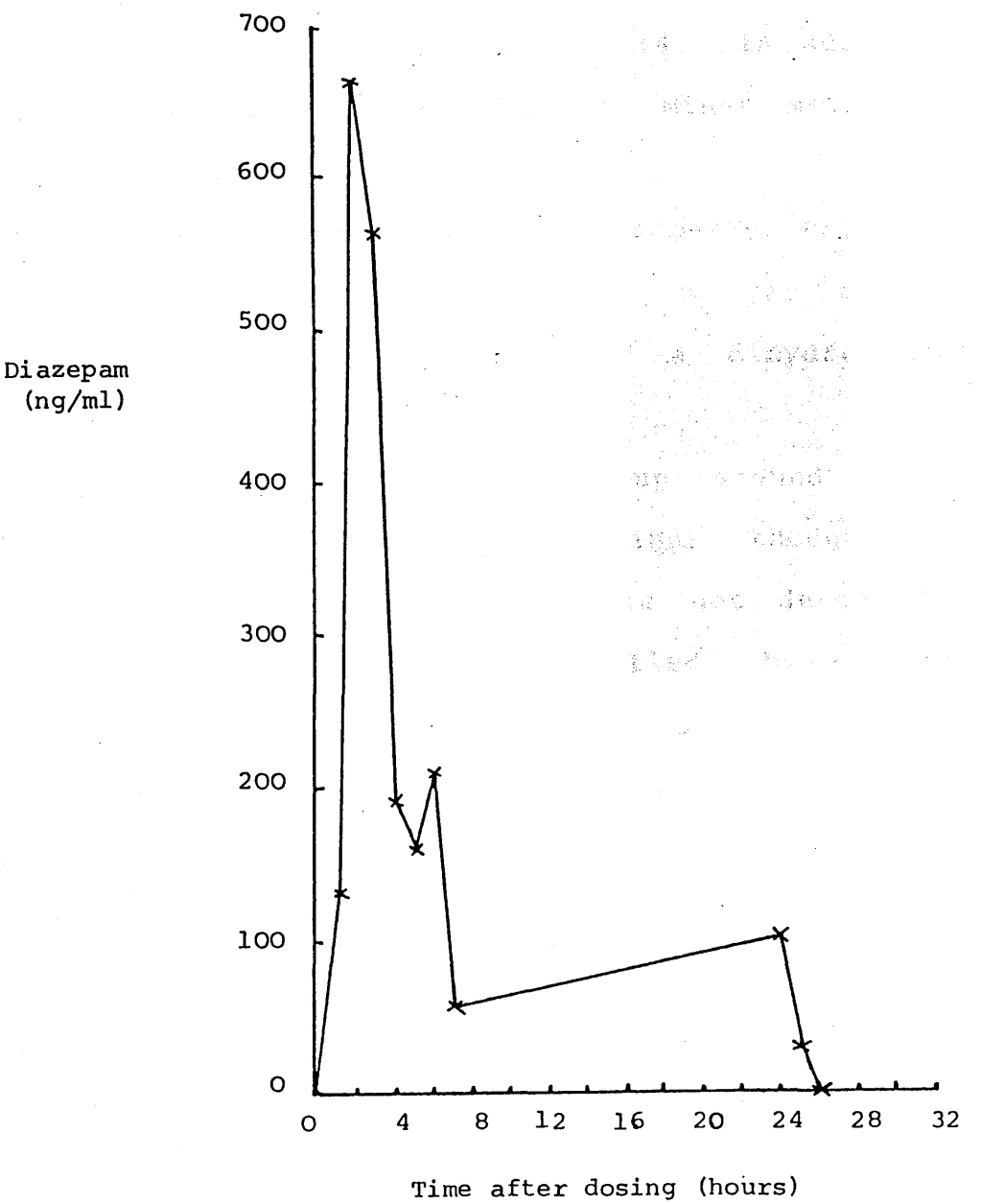


Table 3:2 RRA Results for Urine Samples Collected after Dosing the Greyhound with 5mg of Diazepam.

Time after dosing (hrs)	Diazepam ng/ml	
	Unhydrolysed	Hydrolysed
1	0.0	132.0
2	0.0	668.0
3	0.0	564.0
4	0.0	192.0
5	0.0	164.0
6	0.0	208.0
7	0.0	60.0
24	0.0	100.0
25	0.0	28.0
26	0.0	0.0
27	0.0	0.0
28	0.0	0.0
29	0.0	0.0
30	0.0	0.0
31	0.0	0.0

Figure 3:3 An example of an excretion profile for diazepam, both before and after hydrolysis.



The main metabolic pathway to remove triazolam from the body is by hydroxylation and its major metabolites are 1'-hydroxy triazolam and 4-hydroxy triazolam. 80% of a dose of triazolam is excreted in the urine with the remainder being lost via the faeces. To date nine triazolam metabolites have been found in dog urine [85,87]. The metabolic pathway of triazolam and its major metabolites is shown in figure 3:4. In addition to the major metabolites, the following minor metabolites have also been identified:

1',4-hydroxy triazolam, 1-desmethyl triazolam,

2 monohydroxy analogues + a dihydroxy, a monohydroxymonomethoxy and a dihydroxymonomethoxy triazolam analogue.

These minor metabolites make up around 10% of the triazolam excreted in the urine, though not being pharmacologically active they are not detected by RRA. They were originally identified by administering [^{14}C]triazolam followed by TLC and autoradiography of the urine samples. Further identification was made by GC-MS of the various fractions. Tables 3:3 and 3:4 contain examples of radioreceptor assay results following oral dosing with either 0.25 or 0.5mg of triazolam. Figures 3:5, 3:6, 3:7, 3:8, 3:9 and 3:10 show the excretion profiles of triazolam and its metabolites, both before and after hydrolysis plotted from the data given in tables 3:3 and 3:4.

Figure 3:4 The metabolic pathway and major metabolites of triazolam.

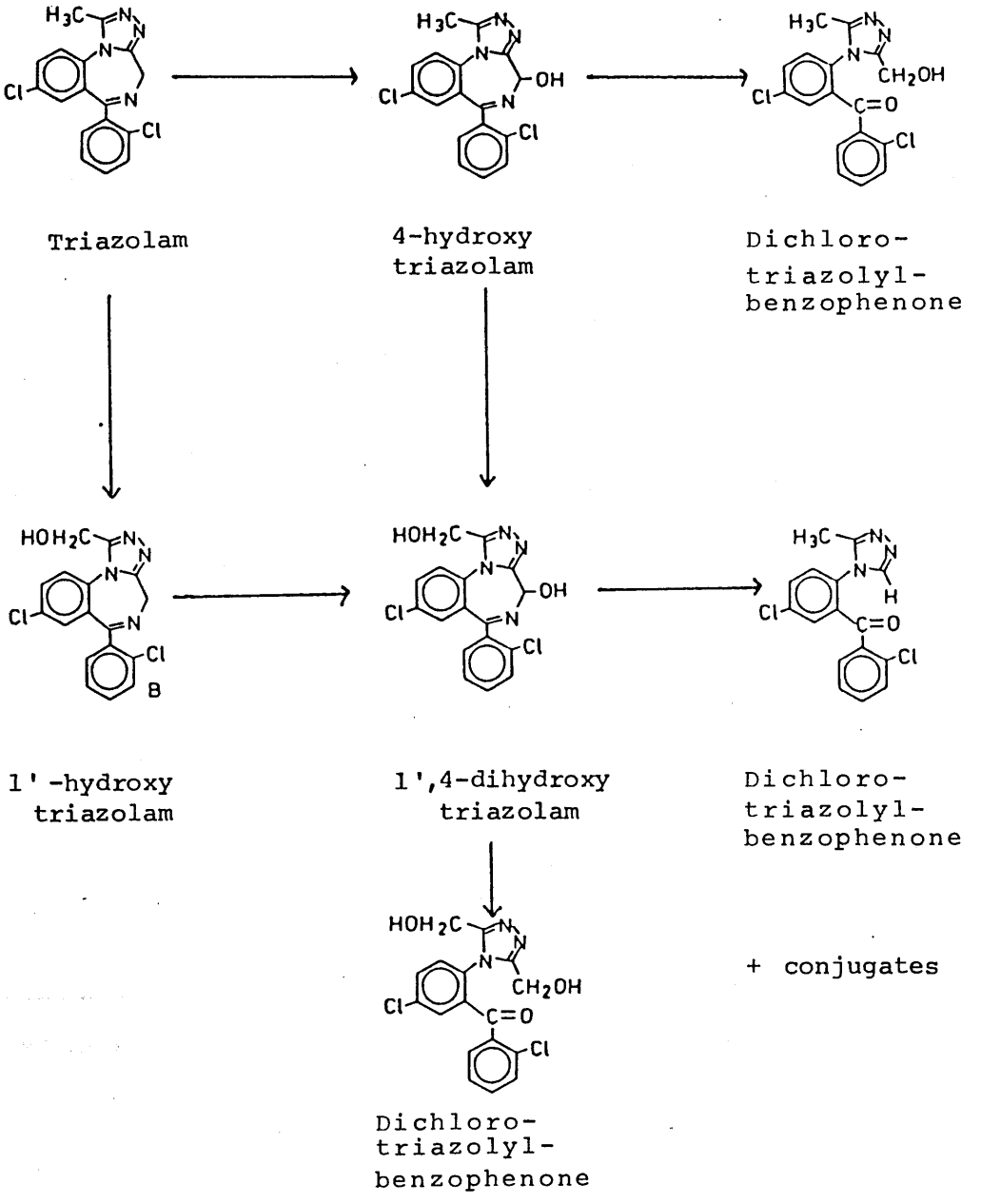


Table 3:3 Three Sets of Radioreceptor Assay Results for Urine Samples Collected after Dosing the Greyhound with 0.25mg of Triazolam.

Time after dosing (hrs)	1 (ng/ml)		2 (ng/ml)		3 (ng/ml)	
	U/H	H	U/H	H	U/H	H
1	7.0	7.0	2.0	10.0	5.0	0.0
2	12.5	212.5	2.5	23.0	6.0	65.0
3	9.5	72.5	2.0	21.0	5.5	14.5
4	8.0	50.0	2.0	13.5	5.0	6.5
5	8.0	40.0	2.0	8.0	5.0	6.0
6	8.5	70.0	2.0	14.5	5.0	6.0
7	-	-	-	-	0.0	6.0
24	7.5	28.5	2.0	6.5	5.0	5.7
25	7.5	27.5	0.0	0.0	5.5	5.5
26	7.5	10.5	0.0	0.0	5.5	0.0
27	-	-	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	5.5
29	1.5	0.0	0.0	0.0	-	-
30	-	-	0.0	0.0	0.0	0.0
31	0.0	0.0	-	-	0.0	0.0

- no sample collected.

U/H - Unhydrolysed urine sample.

H - Hydrolysed urine sample.

Figure 3:5 Excretion profile 1 following a single oral dose of 0.25mg of triazolam.

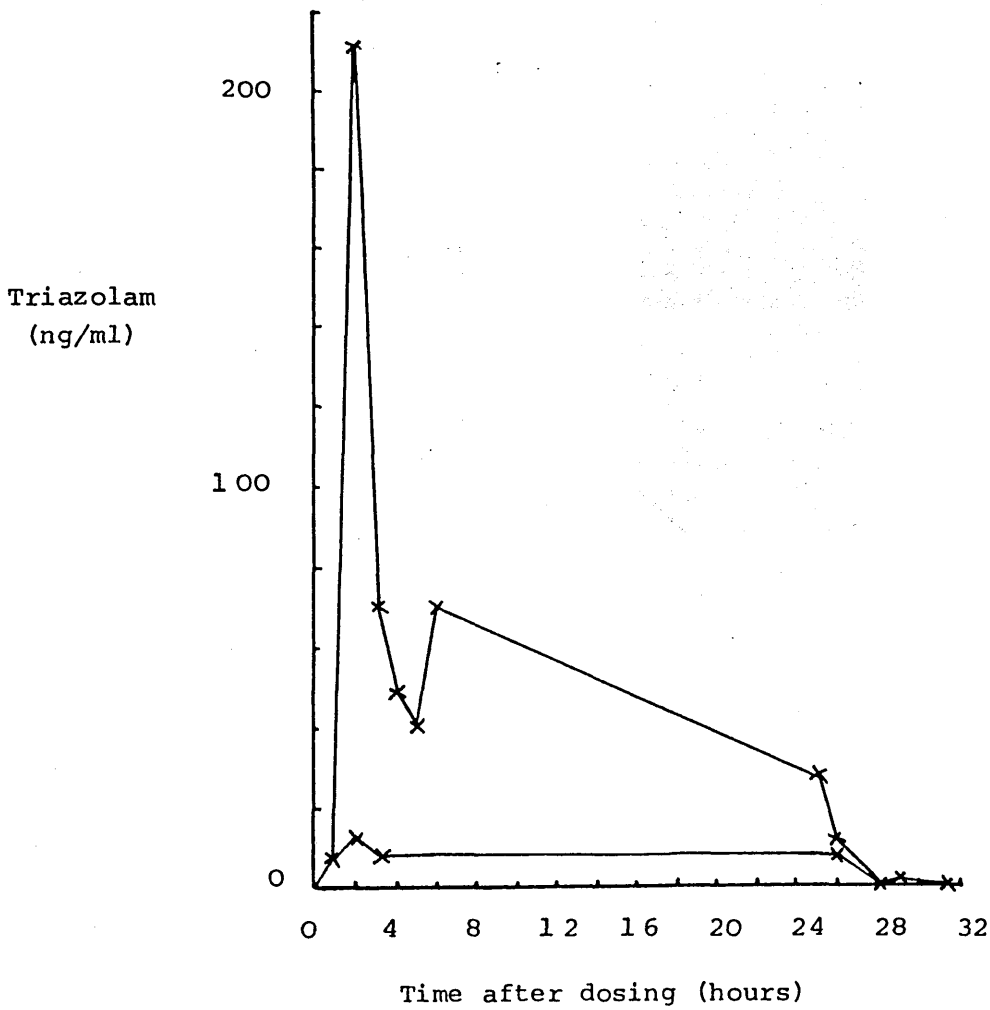


Figure 3:6 Excretion profile 2 following a single oral dose of 0.25mg of triazolam.

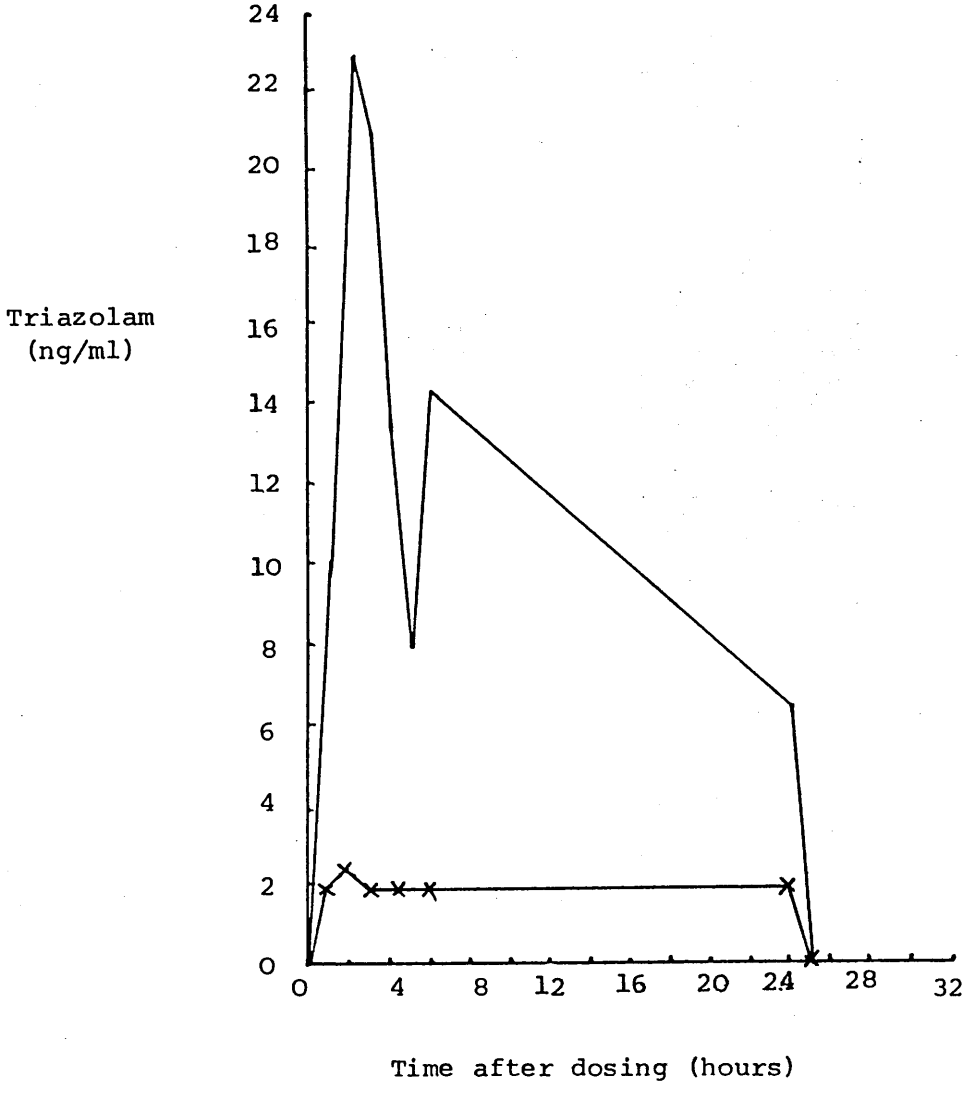


Figure 3:7 Excretion profile 3 following a single oral dose of 0.25mg of triazolam.

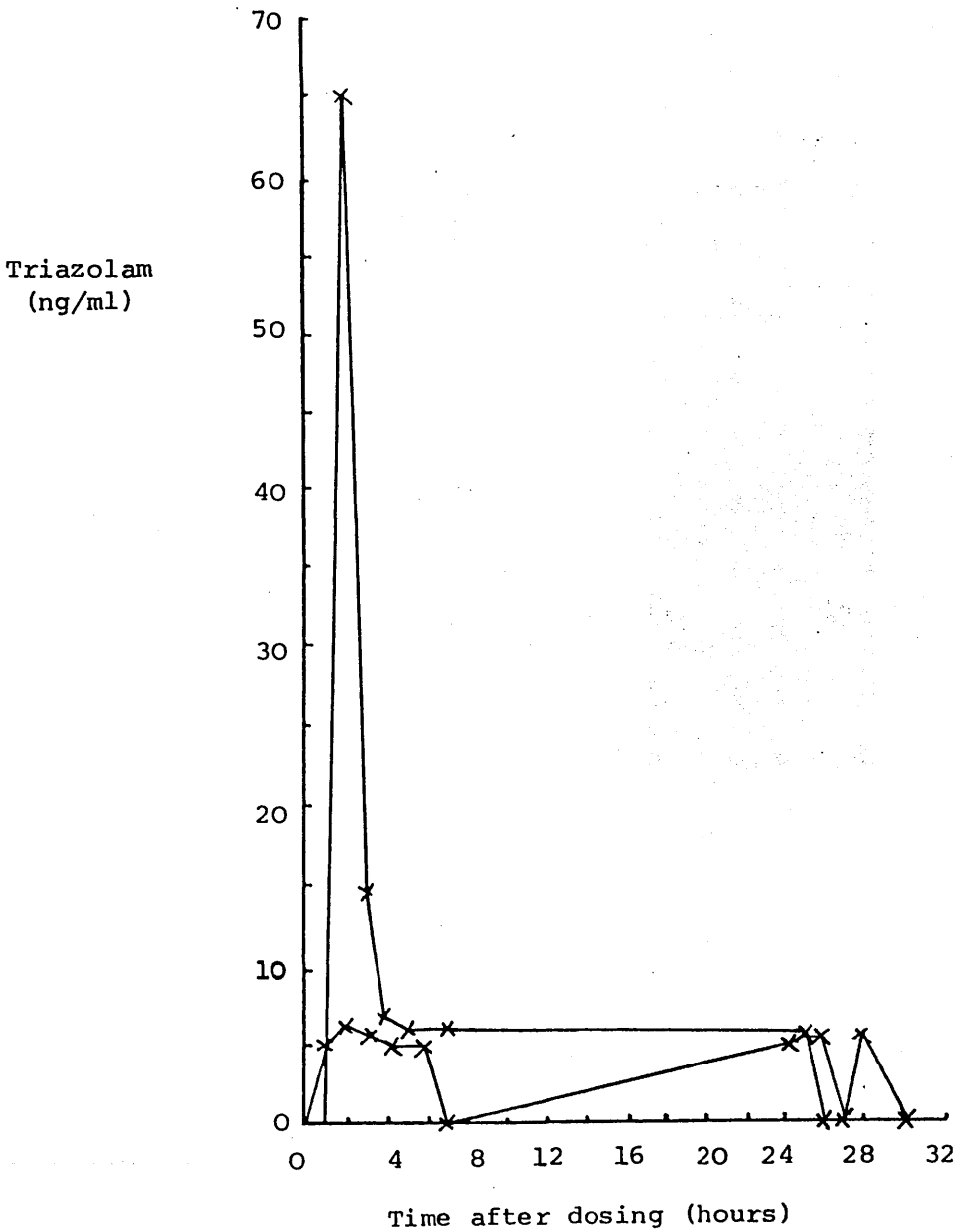


Table 3:4 Three Sets of Radioreceptor Assay Results for Urine Samples Collected after Dosing the Greyhound with 0.5mg of Triazolam.

Time after dosing (hrs)	1 (ng/ml)		2 (ng/ml)		3 (ng/ml)	
	U/H	H	U/H	H	U/H	H
1	10.5	210.0	3.2	110.0	13.0	112.5
2	4.5	62.0	2.2	42.5	6.0	53.7
3	0.0	27.5	1.5	12.5	0.0	37.5
4	0.0	7.5	0.0	6.2	0.0	5.5
5	0.0	7.0	0.0	9.7	0.0	2.0
6	0.0	21.5	0.0	18.5	0.0	2.5
7	0.0	16.5	0.0	6.5	0.0	2.0
24	1.5	5.5	1.5	5.5	0.0	2.0
25	0.0	5.0	0.0	0.0	0.0	0.0
26	0.0	0.0	0.0	2.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0

- no sample collected.

U/H - Unhydrolysed urine sample.

H - Hydrolysed urine sample.

Figure 3:8 Excretion profile 1 following a single oral dose of 0.5mg of triazolam.

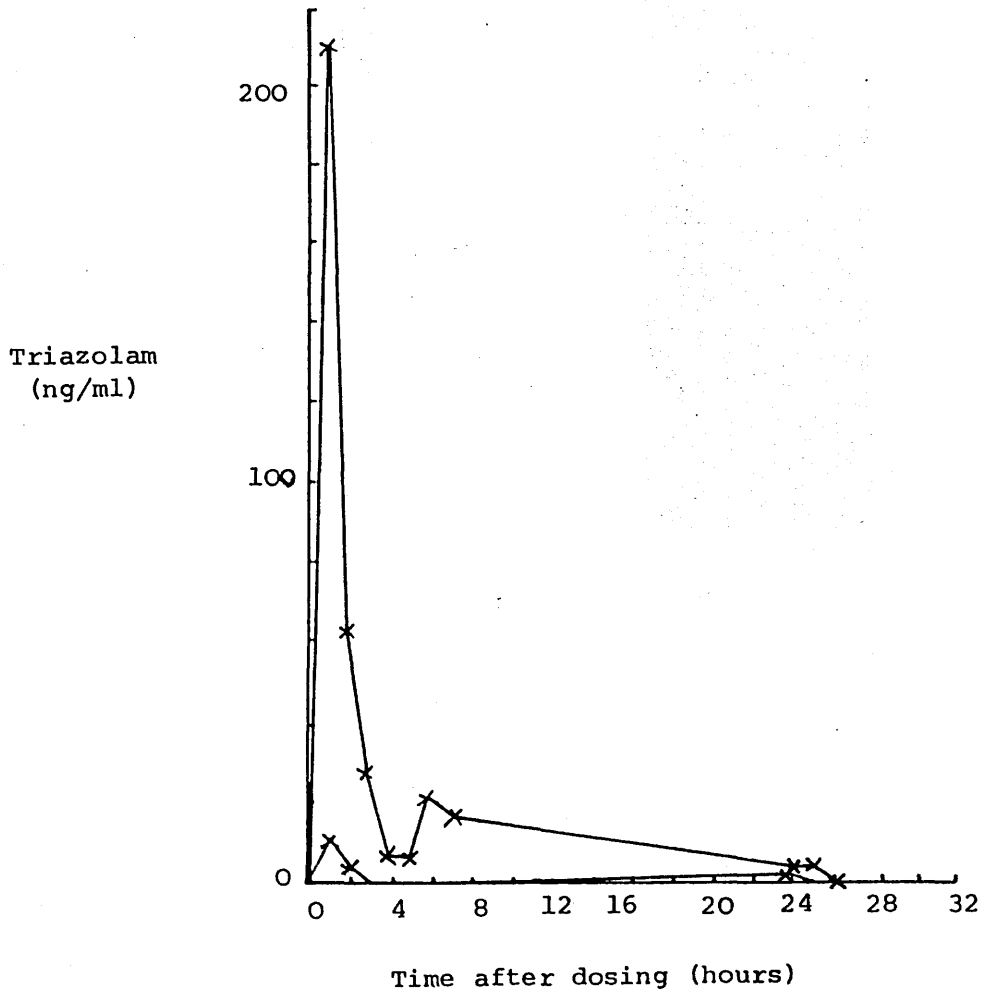


Figure 3:9 Excretion profile 2 following a single oral dose of 0.5mg of triazolam.

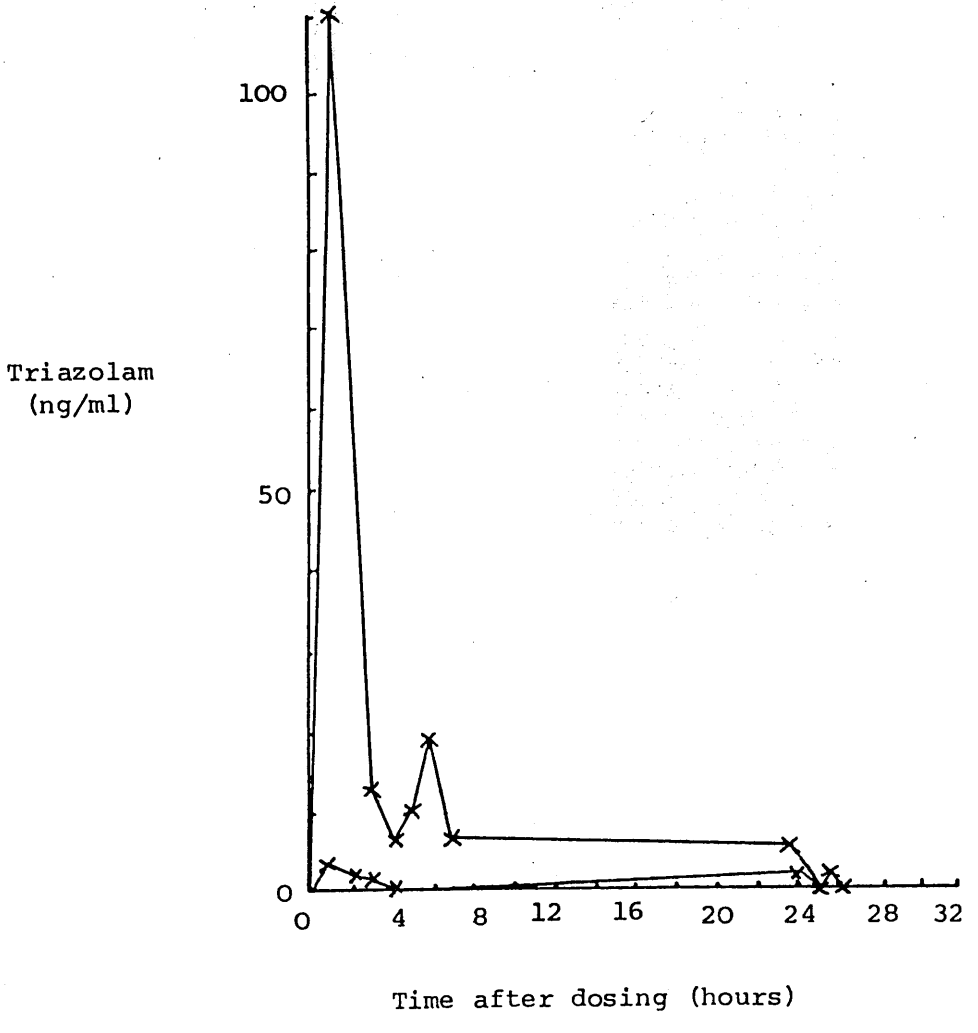
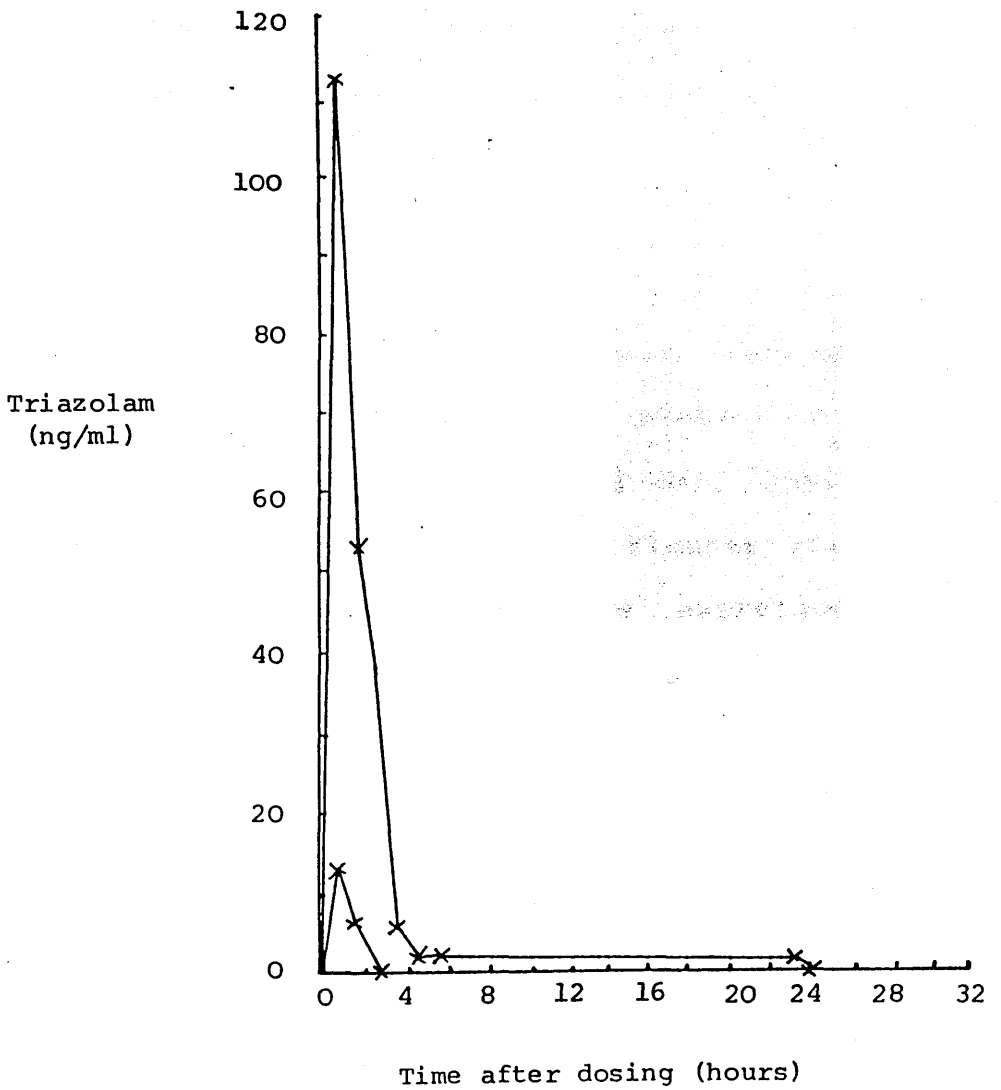


Figure 3:10 Excretion profile 3 following a single oral dose of 0.5mg of triazolam.



Flunitrazepam is excreted in the urine as desmethyl flunitrazepam and 7-amino flunitrazepam, its major metabolites which are pharmacologically active. Less than 1% of that excreted in the urine is as unchanged parent drug. The metabolic pathway of flunitrazepam is shown in figure 3:11. Other minor metabolites excreted in this way include:

3-hydroxy flunitrazepam.

3-hydroxy-7amino flunitrazepam.

7-amino-1-desmethyl flunitrazepam.

7-acetamido flunitrazepam.

The metabolites excreted in the urine constitute 90% of the dose with the remaining 10% excreted in the faeces. Tables 3:5 and 3:6 contain a selection of receptor assay results following oral dosing with either 1.0 or 2.0mg of flunitrazepam. Figures 3:12, 3:13, 3:14, 3:15, 3:16 and 3:17 show the excretion profile for flunitrazepam, both before and after hydrolysis, for both dose levels.

Figure 3:11 The metabolic pathway of flunitrazepam.

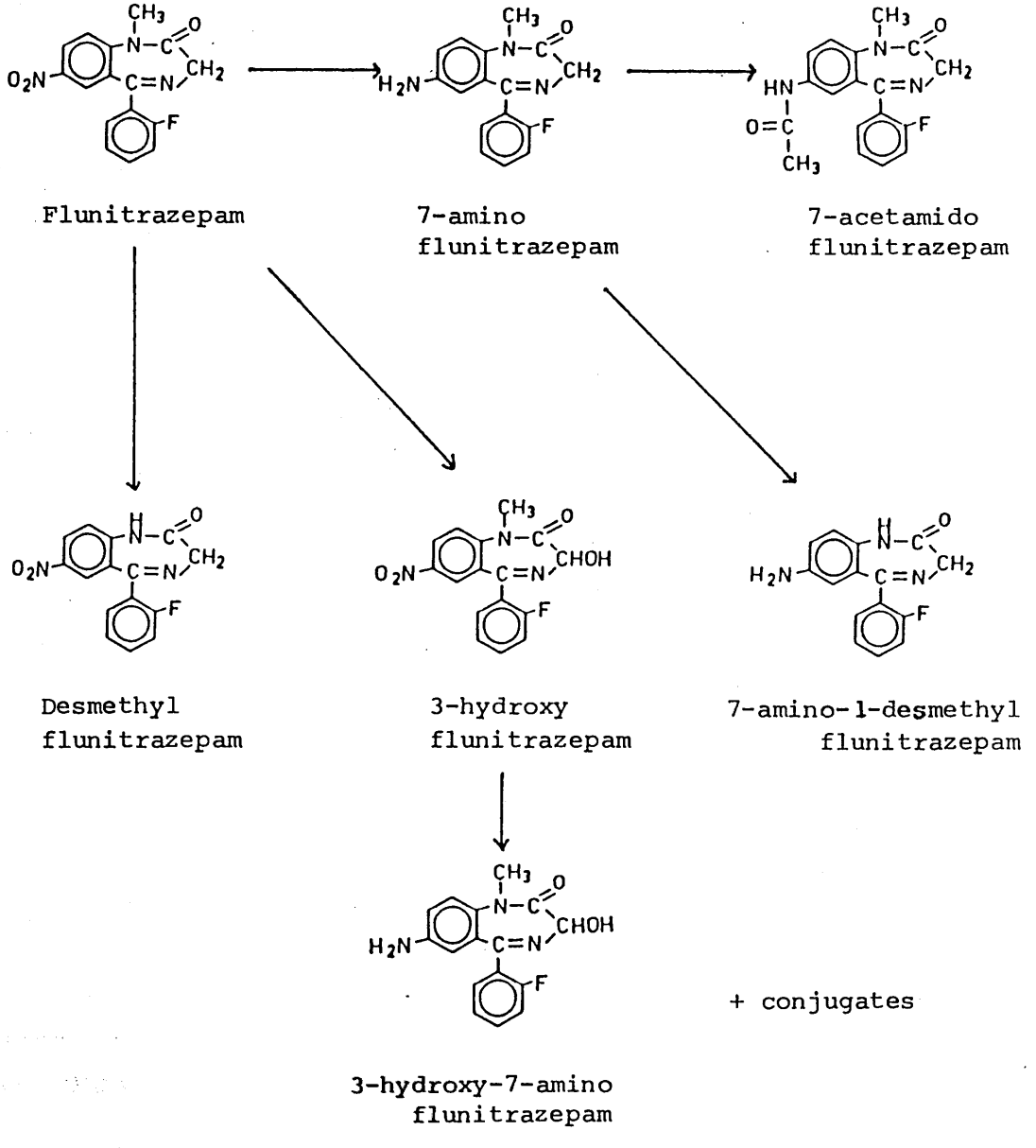


Table 3:5 Three Sets of Radioreceptor Assay Results for Urine Samples Collected after Dosing the Greyhound with 1.0mg of Flunitrazepam.

Time after dosing (hrs)	1 (ng/ml)		2 (ng/ml)		3 (ng/ml)	
	U/H	H	U/H	H	U/H	H
1	0.0	33.0	4.0	2.0	0.0	26.5
2	9.0	60.0	6.0	22.0	0.0	296.0
3	0.0	0.0	4.0	14.0	0.0	366.0
4	0.0	11.0	4.0	11.0	15.5	1276.0
5	8.0	0.0	2.0	5.0	0.0	141.0
6	0.0	0.0	4.0	15.0	0.0	61.0
7	0.0	0.0	4.0	17.0	0.0	61.0
24	9.0	9.0	7.0	8.0	0.0	2.8
25	0.0	0.0	4.0	8.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0

U/H - Unhydrolysed urine sample.

H - Hydrolysed urine sample.

Figure 3:12 Excretion profile 1 following a single oral dose of 1.0mg of flunitrazepam.

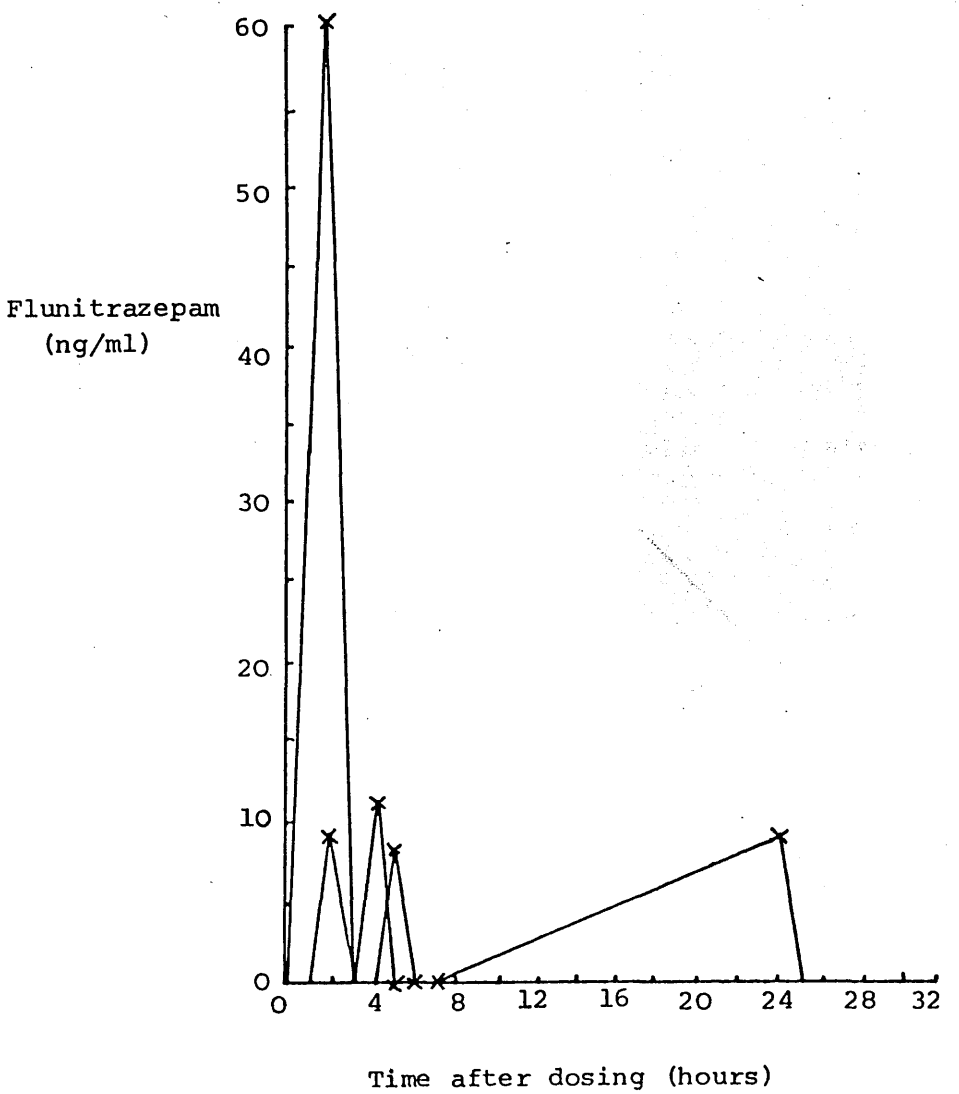


Figure 3:13 Excretion profile 2 following a single oral dose of 1.0mg of flunitrazepam.

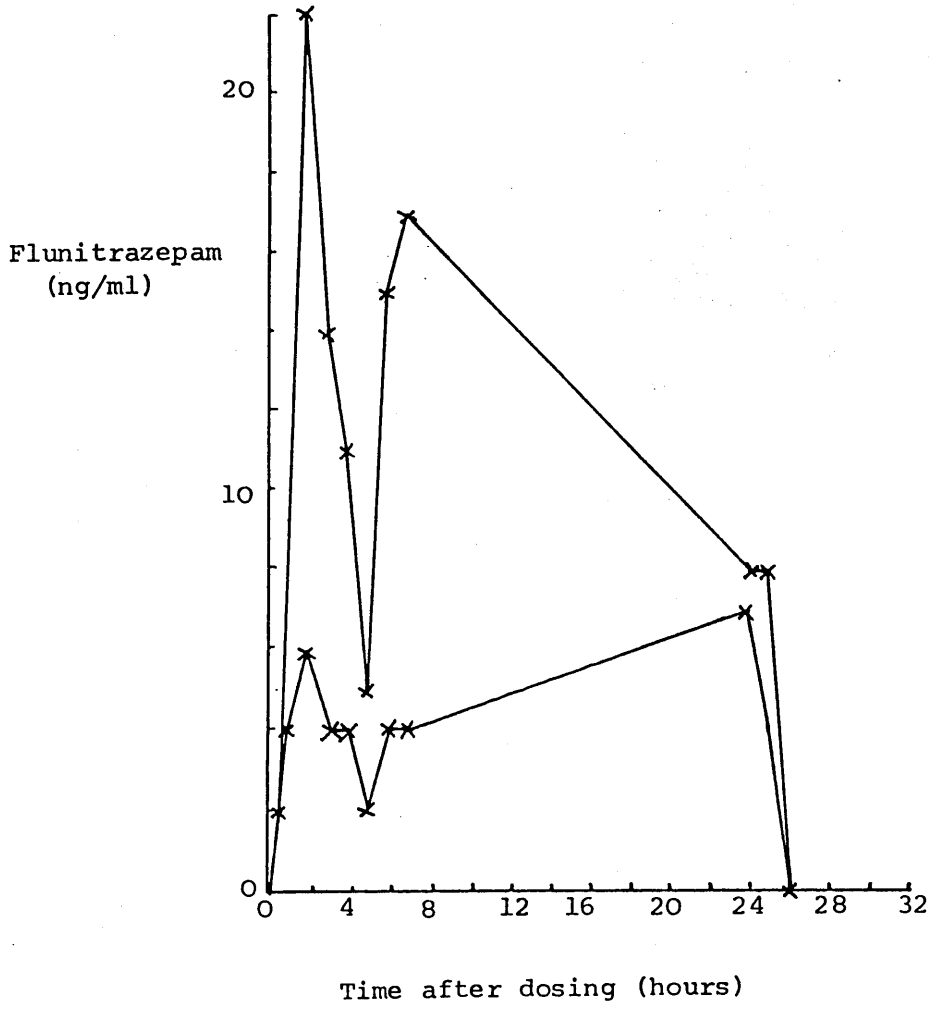


Figure 3:14 Excretion profile 3 following a single oral dose of 1.0mg of flunitrazepam.

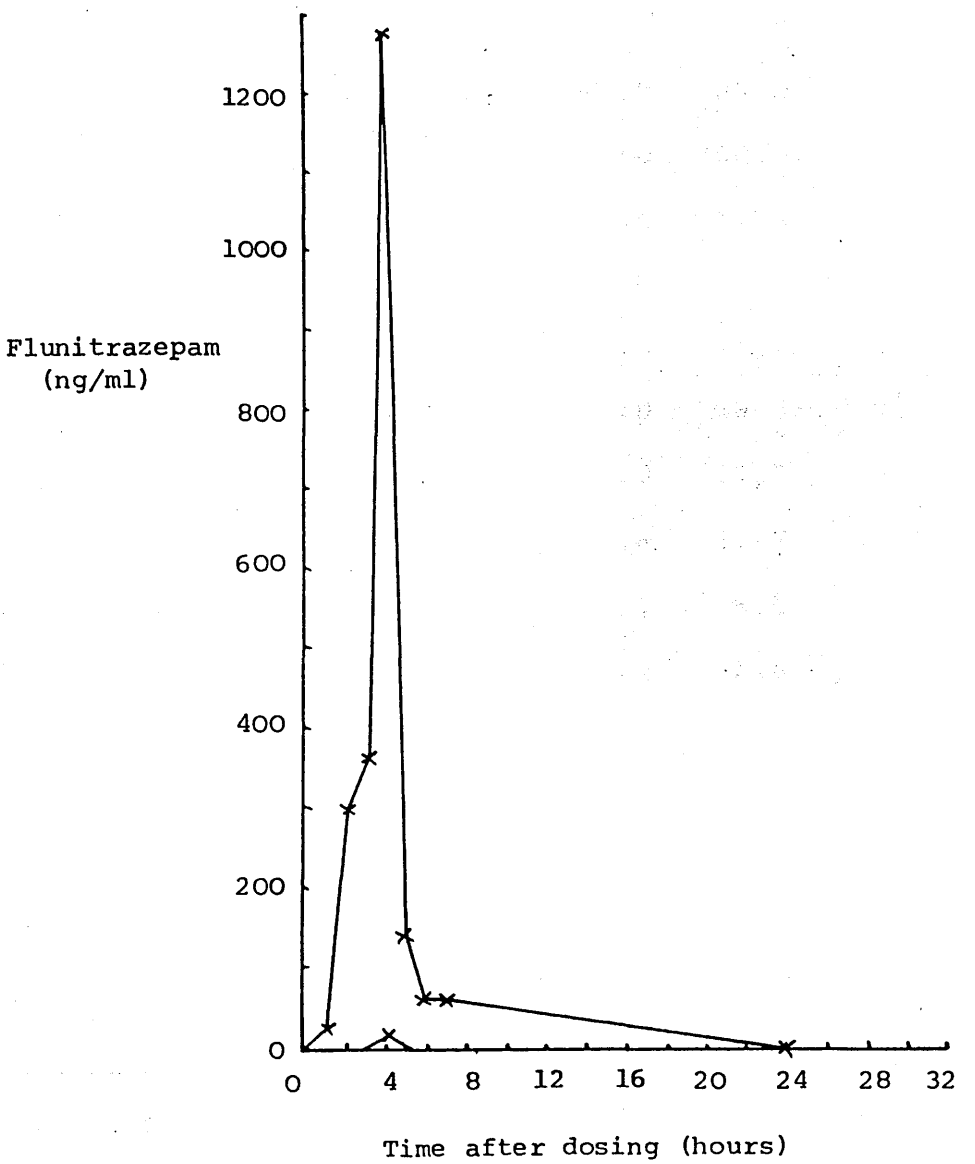


Table 3:6 Three Sets of Radioreceptor Assay Results for Urine Samples Collected after Dosing the Greyhound with 2.0mg of Flunitrazepam.

Time after dosing (hrs)	1 (ng/ml)		2 (ng/ml)		3 (ng/ml)	
	U/H	H	U/H	H	U/H	H
1	0.0	4.2	67.0	103.0	0.0	1.5
2	26.7	1.5	10.0	280.0	-	-
3	53.3	570.0	10.0	66.0	0.0	143.0
4	0.0	100.0	20.0	26.6	0.0	43.0
5	0.0	60.0	0.0	95.6	0.0	103.0
6	0.0	0.0	0.0	53.3	0.0	76.6
7	0.0	56.7	0.0	26.7	0.0	43.3
24	0.0	0.0	2.8	26.7	0.0	26.6
25	0.0	0.0	4.0	8.0	0.0	0.0
26	0.0	0.0	0.0	6.6	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0

- no sample collected.

U/H - Unhydrolysed urine sample.

H - Hydrolysed urine sample.

Figure 3:15 Excretion profile 1 following a single oral dose of 2.0mg of flunitrazepam.

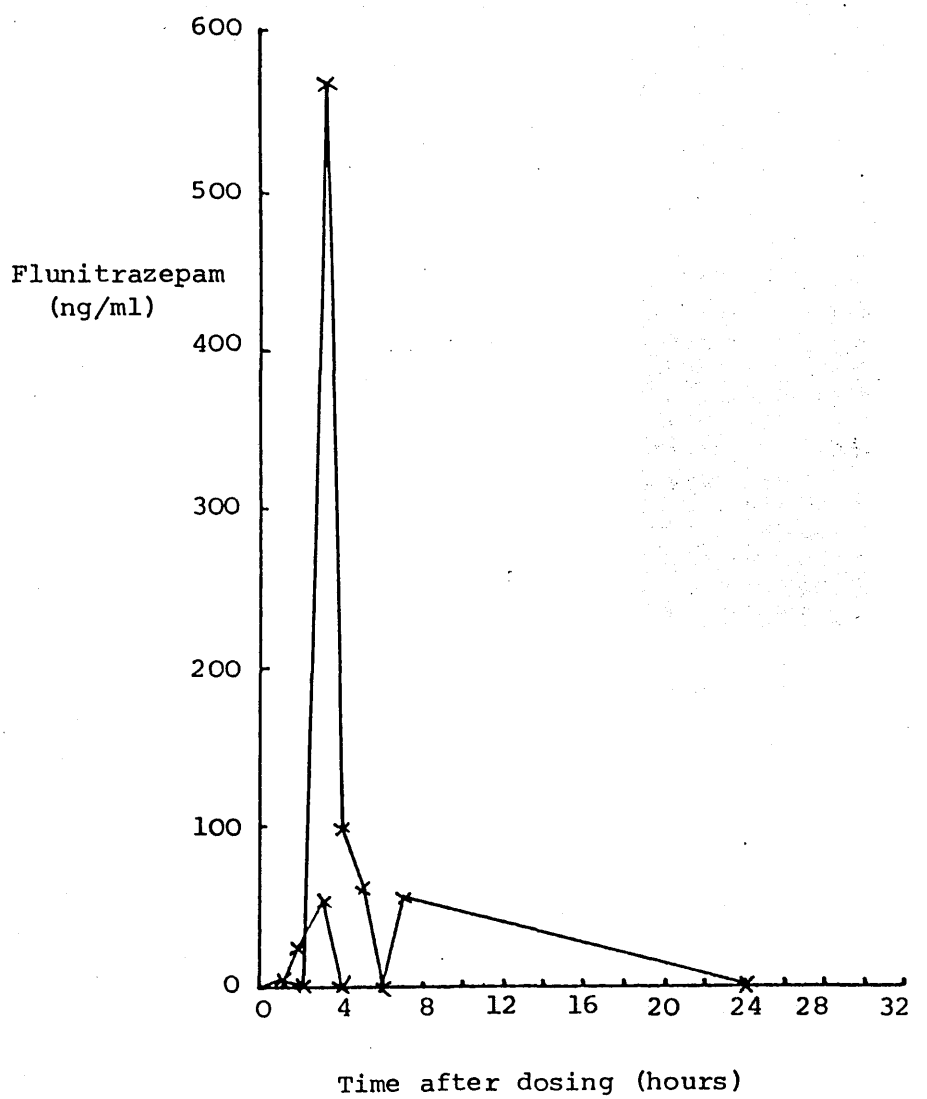


Figure 3:16 Excretion profile 2 following a single oral dose of 2.0mg of flunitrazepam.

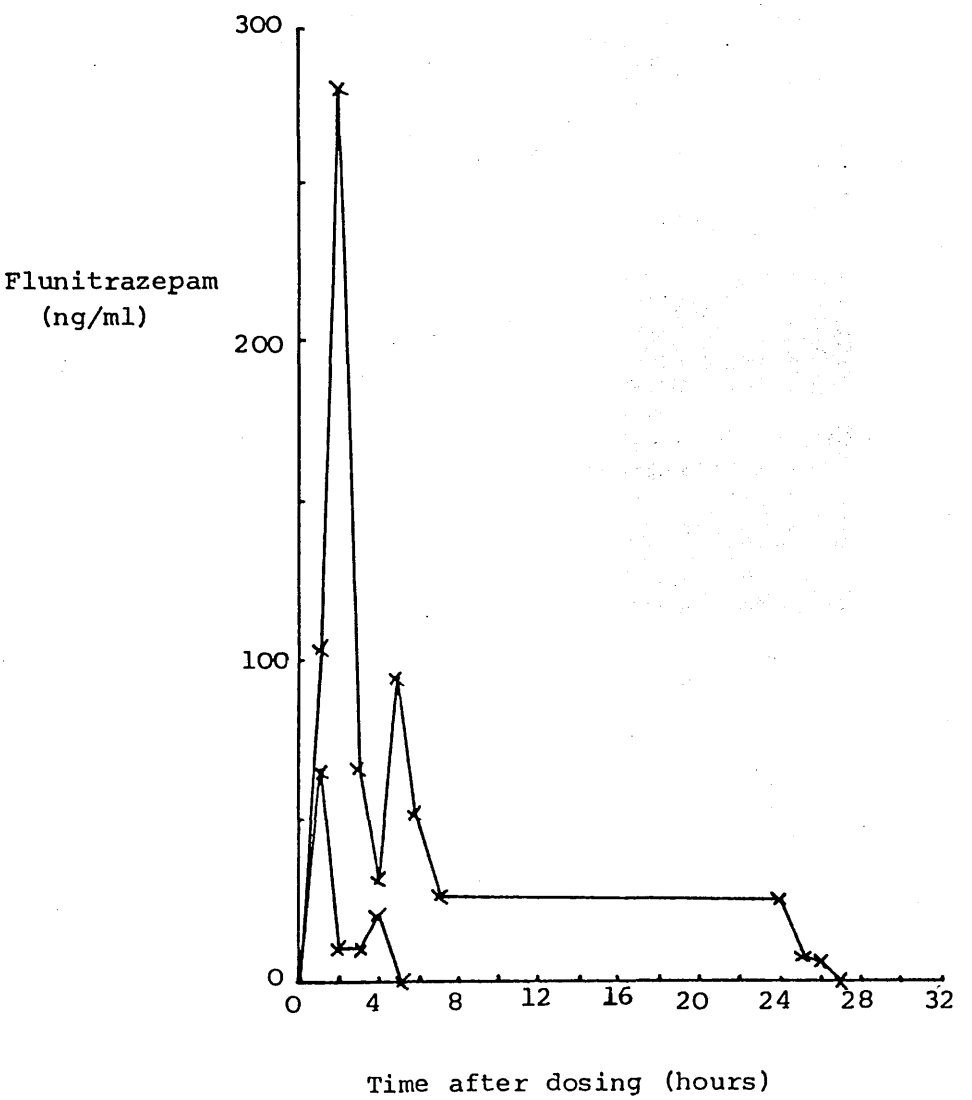
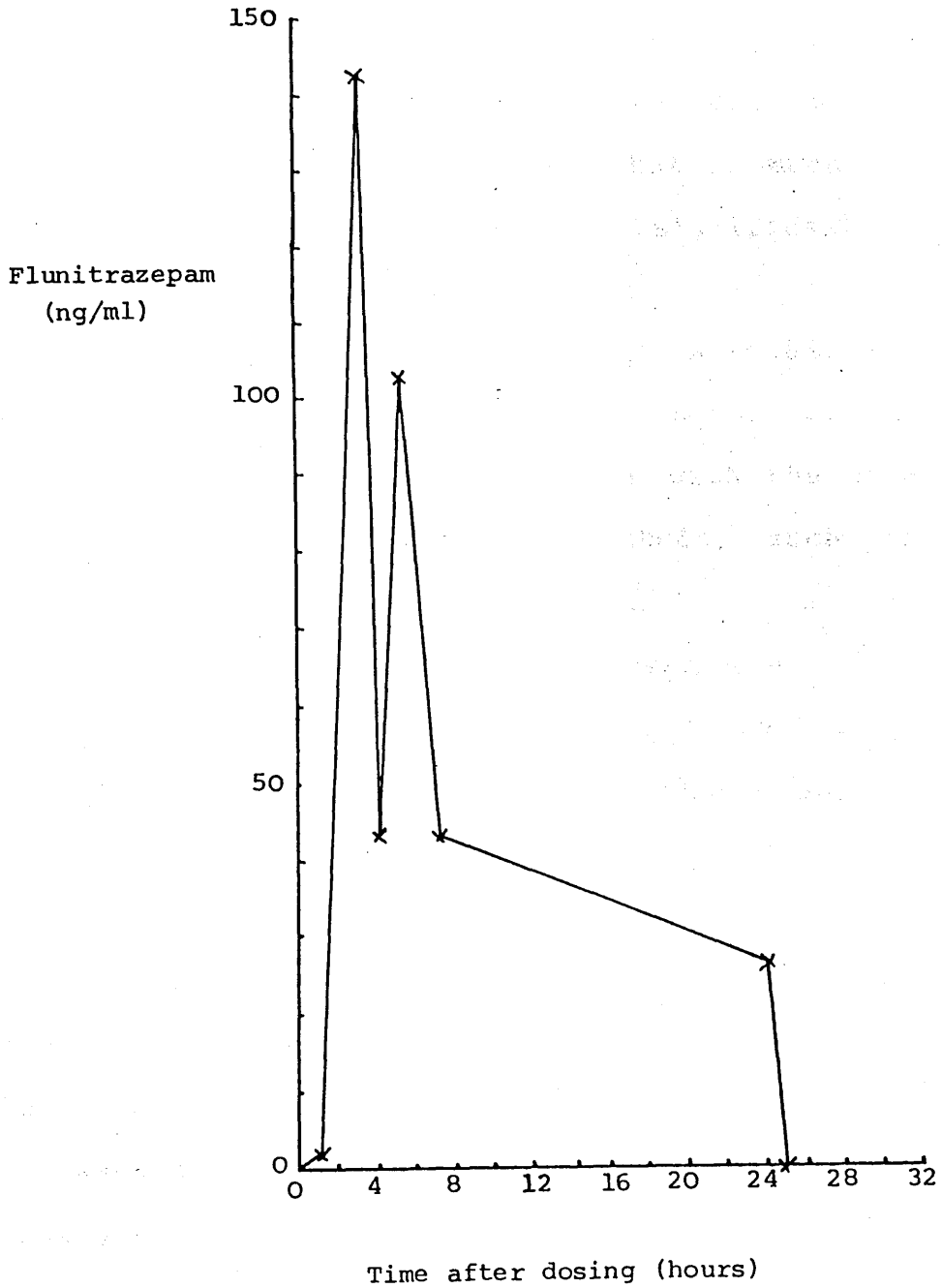


Figure 3:17 Excretion profile 3 following a single oral dose of 2.0mg of flunitrazepam.



Though two different dose levels of triazolam and flunitrazepam were given to the greyhounds there was little difference in the RRA results shown in tables 3:3, 3:4, 3:5 and 3:6; and in figures 3:5, 3:6, 3:7, 3:8, 3:9, 3:10, 3:12, 3:13, 3:14, 3:15, 3:16, and 3:17. Some increase in the amounts of benzodiazepines detected had been expected, though as the greyhounds were not drowsy after either dose it follows that a much higher level would have to be given to see a significant increases in the RRA results.

As can be seen from the preceeding tables and figures hydrolysis increases the amount of benzodiazepam metabolites available to interact with the BZ-R. Whereas conventional chromatographic methods, such as HPLC and GLC, seperate the parent drug and its metabolites into individual peaks; RRA only measures the total amount of pharmacologically active components in each sample.

As it is known which benzodiazepine was given to each greyhound, it follows that the metabolites subsequently excreted in the urine can be predicted [5,12,155,167,168]. As the increase in the amount of benzodiazepine detected after hydrolysis is due only to an increase in pharmacologically active primary metabolites, released from the conjugates, it is possible to estimate what percentage of the increase is due to each metabolite.

The K_i values for triazolam, flunitrazepam and their respctive metabolites are given below:

Triazolam	1.8nM.
1'-hydroxy triazolam	21.7nM.
4-hydroxy triazolam	46.9nM.
Flunitrazepam	9.5nM.
Desmethyl flunitrazepam	17.0nM.
7-amino flunitrazepam	2700.0nM.

They show that higher concentrations of the metabolites are required to displace [^{3}H]FNZ than their parent benzodiazepine. This was confirmed by a series of RRA carried out with either flunitrazepam or triazolam as the standards and their appropriate metabolites as the samples. Thus the metabolites can be equated with their parent benzodiazepine in their ability to displace [^{3}H]FNZ. The results are given in tables 3:7, 3:8 and 3:9.

Table 3:7 Triazolam Metabolite RRA Results Equated with Triazolam.

Metabolite (ng/ml)	Triazolam (ng/ml)		Triazolam Equivalent
1'-hydroxy triazolam			
75	5.2		69.3
100	10.3		103.0
200	30.1		150.5
1000	101.0		101.0
		average	105.9
4-hydroxy triazolam			
100	1.8		18.0
200	5.9		29.5
1000	72.9		72.9
		average	40.0

106ng triazolam is equivalent to 1000ng of 1'-hydroxy triazolam.
Or 1'-hydroxy triazolam is approximately 10x less potent than triazolam.

40ng triazolam is equivalent to 1000ng 4-hydroxy triazolam.
Or 4-hydroxy triazolam is approximately 25x less potent than triazolam.

Table 3:8 Desmethyl Flunitrazepam RRA Results Equated with Flunitrazepam.

Desmethyl FNZ ng/ml	FNZ ng/ml		FNZ Equivalent
1	1.0		1000.0
5	2.2		440.0
10	9.6		960.0
25	18.6		744.0
50	32.2		644.0
75	47.0		627.0
100	118.4		1884.0
200	1742.0		8710.0
		average	1876.0

lug flunitrazepam = 1.8ug desmethyl flunitrazepam.

Or desmethyl flunitrazepam is approximately 1.8x less potent than flunitrazepam.

Table 3:9 7-amino Flunitrazepam RRA Results Equated with Flunitrazepam.

7-amino FNZ ng/ml	FNZ ng/ml		FNZ Equivalent
100	2.6		260.0
500	5.6		112.0
1000	4.2		42.0
2000	13.0		65.0
4000	25.8		64.5
5000	46.4		92.8
6000	31.6		52.6
8000	37.4		46.7
10000	46.5		46.5
		average	86.9

10000ng 7-amino flunitrazepam = 87ng flunitrazepam.
Or 7-amino flunitrazepam is approximately 115x less potent than flunitrazepam.

From the K_i values and the metabolite/benzodiazepine standard RRA graphs it is possible to determine the contribution made by each of the major metabolites to the amount of benzodiazepine detected after hydrolysis.

To calculate the affinity of triazolam and FNZ metabolites in relation to their parent compound.

	K_i (nM)
Triazolam	1.8
1'-hydroxy triazolam	21.6
4-hydroxy triazolam	46.6

Ratio: triazolam:1'-hydroxy triazolam:4-hydroxy triazolam

1 12 26

These results compliment those from the RRA results. Therefore presume that for every 1g of triazolam detected, 10g and 25g of 1'-hydroxy and 4-hydroxy triazolam are detected.

	K_i (nM)
Flunitrazepam	9.5
Desmethyl flunitrazepam	17.0
7-amino flunitrazepam	2700.0

Ratio: flunitrazepam:desmethyl flunitrazepam:7-amino flunitrazepam

1 1.8 284

These result compliment the RRA results in demonstrating that this metabolite has ~ 100x less affinity for the benzodiazepine receptors than flunitrazepam or desmethyl flunitrazepam. So for every 1g of flunitrazepam detected, 1.8g of desmethyl flunitrazepam are detected.

To determine what percentage of the original dose is excreted in each sample, estimates of the contribution to the RRA result made by each benzodiazepine metabolite have to be made. The two major metabolites of triazolam have different affinities for the BZ-R so before recalculating the hydrolysis results, the proportion they are excreted in has to be known. From previous benzodiazepine trials with dogs, 1'-hydroxy and 4-hydroxy triazolam are excreted in the proportions of 10:7.5. So for each hydrolysed triazolam urine sample, of the increase after hydrolysis, 57% is assumed to be due to 1'-hydroxy triazolam with the remaining 43% assumed to be due to 4-hydroxy triazolam.

As the 7-amino flunitrazepam metabolite has 100x less affinity for the receptors than flunitrazepam and desmethyl flunitrazepam, it was assumed that the higher concentrations detected were due only to an increase in the latter metabolite. Though both would become unconjugated during hydrolysis, desmethyl flunitrazepam would bind preferentially due to its higher affinity for the receptors. By using the assumption made above it is possible to recalculate the results after sample

hydrolysis. This enables a more accurate picture of the greyhound excretion profiles of flunitrazepam and triazolam to be drawn. This was done as follows:

For a particular time interval the unhydrolysed RRA result was subtracted from the hydrolysed RRA result, thus giving the increase in metabolites (ng/ml). For triazolam, 57% of this increase was due to 1'-hydroxy triazolam which has 10x less affinity for the BZ-R than triazolam; so 57% of the increase was multiplied by 10. The remaining 43% was due to 4-hydroxy triazolam which has 25x less affinity for the BZ-R; So this percentage was multiplied by 25.

In the case of flunitrazepam only desmethyl flunitrazepam is considered and it has 1.8x less affinity for the BZ-R. Therefore the increase after hydrolysis was multiplied by 1.8. Examples for each benzodiazepine are given in tables 3:10 and 3:11 to illustrate the calculations made.

3:5:2 Extraction

Extraction of diazepam and triaolam by the three solvents involved a simple, though time consuming method. Examples of RRA results of extracted diazepam and triazolam standards are shown in tables 3:12 and 3:13.

TABLE 3:10

Examples of Experimental Data to Illustrate the Recalculation of Triazolam RRA Results following Hydrolysis of the Greyhound Urine Samples.

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
Triazolam															
U/H[a] (ng/ml)	2.0	2.5	2.0	2.0	2.0	2.0	-	2.0	0.0	0.0	0.0	0.0	0.0	0.0	-
H[b] (ng/ml)	10.0	23.0	21.0	13.5	8.0	14.5	-	6.5	0.0	0.0	0.0	0.0	0.0	0.0	-
Increase (ng/ml)	8.0	20.5	19.0	11.5	6.0	12.5	-	4.5	0.0	0.0	0.0	0.0	0.0	0.0	-
% as 1'-OH T[c]	4.7	12.0	11.1	6.7	3.5	7.3	-	2.6	0.0	0.0	0.0	0.0	0.0	0.0	-
% as 4-OH T[d]	5.3	8.4	7.8	4.7	2.4	5.1	-	1.8	0.0	0.0	0.0	0.0	0.0	0.0	-
Recalculated x 10	47.0	120.0	111.0	67.0	35.0	73.0	-	26.0	0.0	0.0	0.0	0.0	0.0	0.0	-
Recalculated x 25	132.0	211.0	195.5	118.0	61.7	128.0	-	46.0	0.0	0.0	0.0	0.0	0.0	0.0	-

- no sample collected.

U/H[a] Unhydrolysed urine sample. H[b] Hydrolysed urine sample.
1-OH T[c] 1'-hydroxy triazolam. 4-OH T[d] 4-hydroxy triazolam.

TABLE 3:11

Examples of Experimental Data to Illustrate the Recalculation of Flunitrazepam RRA Results following Hydrolysis of the Greyhound Urine Samples.

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
Flunitrazepam															
U/H[a] (ng/ml)	6.7	10.0	10.0	20.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
H[b] (ng/ml)	103.0	280.0	6.6	26.6	995.6	53.3	26.7	26.7	0.0	6.6	0.0	0.0	0.0	0.0	0.0
Increase (ng/ml)	96.3	270.0	0.0	6.6	95.6	53.3	26.7	16.7	0.0	6.6	0.0	0.0	0.0	0.0	0.0
Recalculated x 1.8	173.3	271.0	0.0	11.8	172.1	95.9	48.0	30.0	0.0	11.8	0.0	0.0	0.0	0.0	0.0

U/H[a] Unhydrolysed urine sample.
H[b] Hydrolysed urine sample.

Table 3:12 Examples of Experimental Data from the RRA of Control and Extracted Diazepam Standards.

Diazepam (ng/ml)	C_o/C_x Control	Methanol	Ethyl Acetate	Dichloro-methane
0	1.13	0.93	1.19	1.22
10	1.34	1.35	1.18	1.78
25	1.94	1.79	1.41	1.66
50	2.07	2.96	1.74	2.29
100	5.24	3.21	3.93	3.40
200	9.53	5.64	4.64	7.32

Table 3:13 Examples of Experimental Data from the RRA of Control and Extracted Triazolam Standards.

Triazolam (ng/ml)	C_o/C_x Control	Methanol	Ethyl Acetate	Dichloro-methane
0	0.98	1.70	1.01	1.19
1	1.61	1.67	1.45	1.51
5	2.69	2.74	2.47	2.14
10	6.29	4.95	5.59	5.29
25	10.78	8.36	4.37	20.13
50	30.20	10.40	18.87	17.76

The three extracted sets of standards gave results which fell below those of the unextracted standards, as can be seen in figures 3:18 and 3:19. This is due to the proportional extraction of diazepam and triazolam by each organic solvent. The amount extracted is dependant on the solubility of the benzodiazepines in each solvent. Diazepam and triazolam are equally soluble in the three solvents used in this experiment, though an unknown benzodiazepine in a sample could have a different solubility in the solvents and may not be extracted.

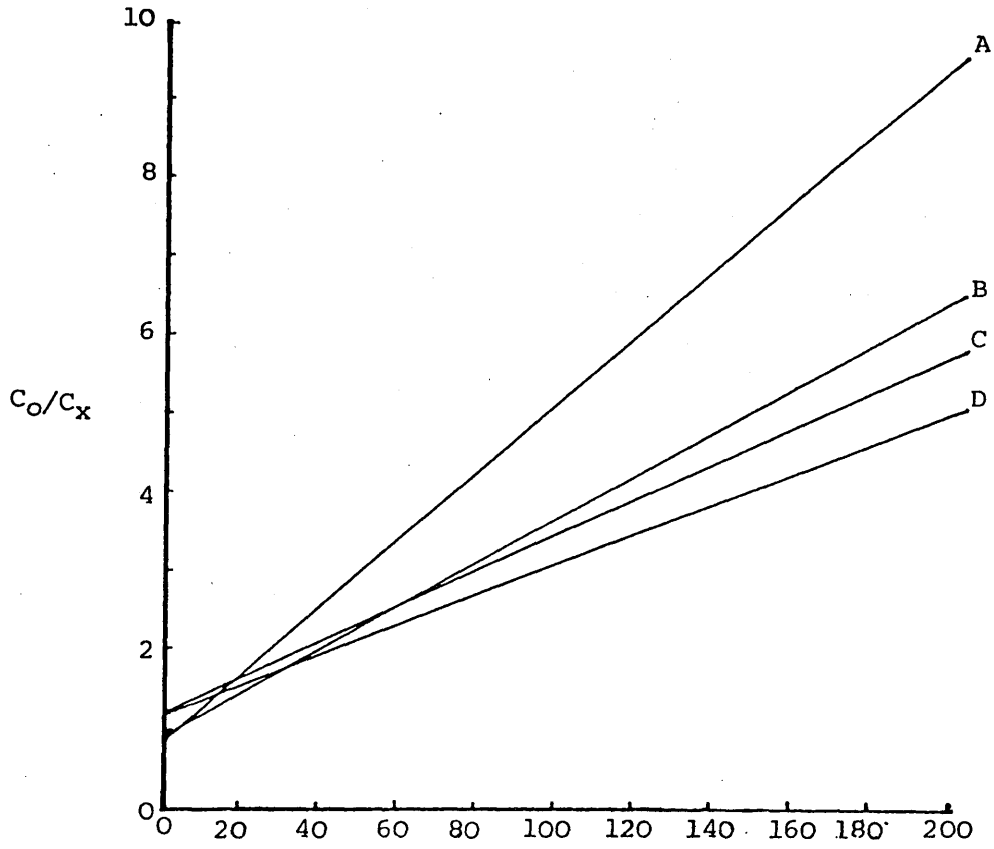
Repeated re-extraction of the a sample followed by pooling of the extracts increases the amount extracted, however 100% extraction can not be acheived. It is also time consuming, and detracts from the quick analytical method RRA was developed as. As extraction consistantly gave lower results, it was not included in the RRA of urine samples. Very turgid samples were centrifuged for 5 minutes prior to assay to remove any proteins wwhich could cross react with the receptors (34, 38, 308).

Published papers refer only to extraction when plasma or whole blood is being analysed, usually only as a way of precipitating the protein fraction. Examples of such methods are as follows:

Addition of 2M perchloric acid followed by centrifugation, (29,30)

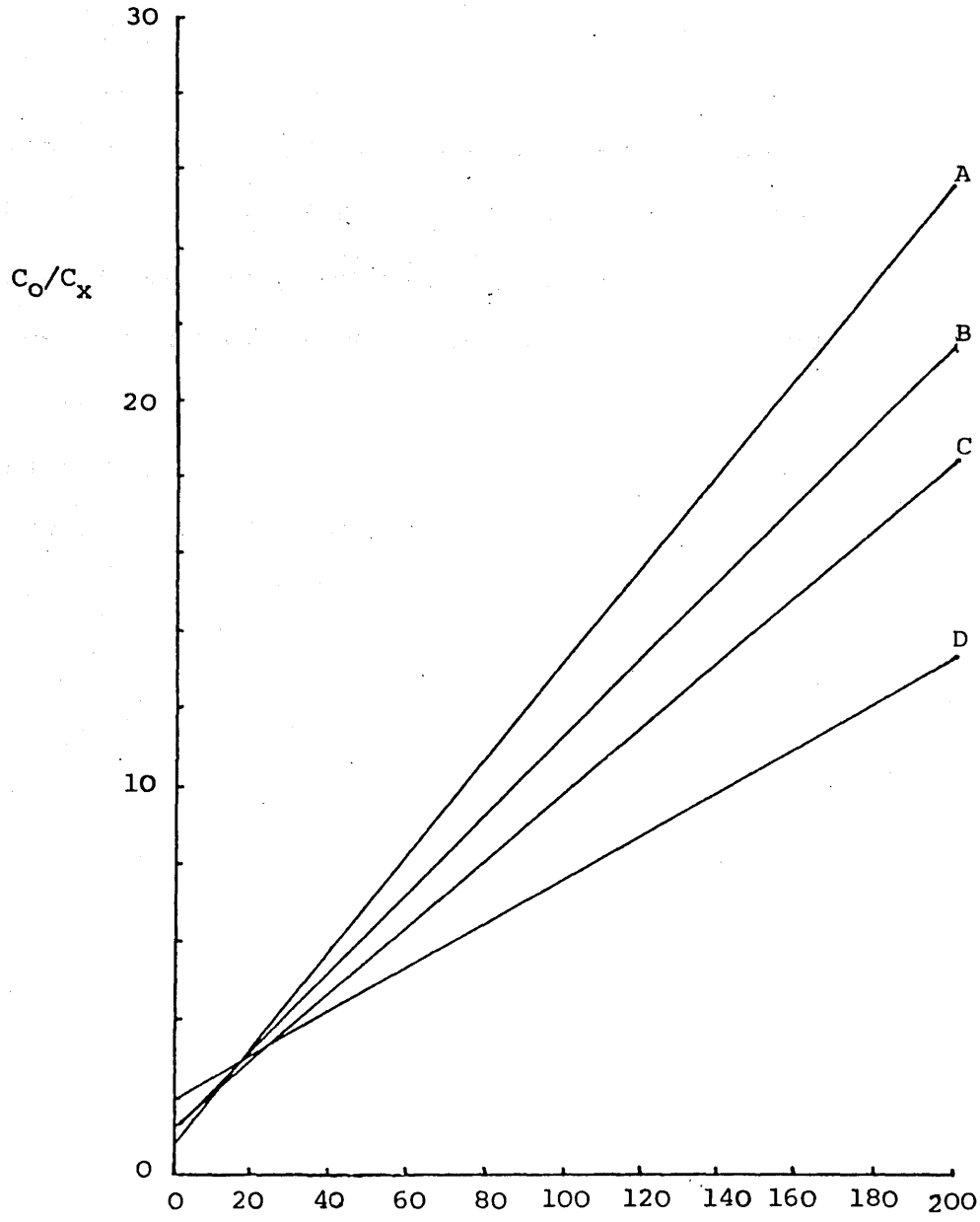
Addition of 1N NaOH followed by extraction with ether, (31, 32 & 303).

Figure 3:18 An example of experimental data from RRA of control and extracted diazepam standards.



A - Unextracted standards (controls).
B - Dichloromethane extracted standards.
C - Methanol extracted standards.
D - Ethyl acetate extracted standards.

Figure 3:19 An example of experimental data from RRA of control and extracted triazolam standards.



A - Unextracted standards (controls).
B - Dichloromethane extracted standards.
C - Ethyl acetate extracted standards.
D - Methanol extracted standards.

3:5:3 Radioimmunoassay

Radioimmunoassay (RIA) is a well established routine method for the analysis of drugs in biological fluids. It is a very similar technique to RRA in that it depends on the competition between the drug and the radiolabelled drug for a binding site. They differ as with RRA the binding site is part of the receptor complex, where as with RIA it forms part of an antibody. The outcome of each method is the same as the bound and free labelled drug is separated, either by centrifugation or filtration, followed by either fraction being counted in order to construct a standard curve. It is the antibody component of RIA which can present problems and which sets the two apart from each other. In order to establish the RRA method all that is required is an appropriatly labelled drug and a receptor preparation. The latter is usually made from brain tissue as it contains all the receptors for the major drug groups.

To set up a RIA, the appropriate antibody has first to be generated. This is done by injecting a low concentration of the drug into the animal, a sheep or rabbit, where it acts as an antigen and antibodies are raised against it. Often the molecular weight of the drug is too low to cause an immune response so it has to be coupled to a larger protein, a hapten, usually albumin. Once established, blood is withdrawn and the antibodies

can be separated and stored until required. It is this time period which can hold up experiments, as it can take several weeks to raise the antibodies and to purify the serum enough for use. Purification is necessary as the antiserum requires a certain degree of specificity in order that it only recognises the group of drugs to which it was raised. The antibody titre is generally high enough that it can be serially diluted to a point where 50% of labelled drug will bind to the antibodies.

The source of antisera can also affect the results as antibodies from different manufacturers will vary in the degree of specificity for the drug group required. By using commercial preparations of antisera from the same source for each assay it is possible to obtain reproducible results. As RRA and RIA are similar analytical methods it was therefore possible to confirm the RRA results, that benzodiazepines are present in the urine samples, and to compare RRA and RIA results to demonstrate the specificity of each method for benzodiazepines only.

3:5:4 Diazepam Pilot Study

Diazepam was used to establish the greyhound trial procedures as it is one of the most widely studied benzodiazepines. There are many papers available relating to clinical studies and pharmacokinetics of diazepam [65,66,67,68,3169,170,171,172,173,174,175]; also the analysis of diazepam and its metabolites in plasma and urine: by TLC [176,177,178,179], GLC [180,181,182,183,184, 185,186,187,189], HPLC [190,191,192,193,194,195,196], RIA [172,197,198,199], and RRA [4,5,6,7,10,11].

As diazepam has a long half-life of 2 to 4 days it can be detected in urine samples, by RRA, up to 28 hours after dosing. The relatively high recommended dose of 1 to 5 mg for children, [155], which was used to dose the greyhounds enables relatively easy detection of metabolites. As the majority of diazepam metabolites are pharmacologically active, this also increases the liklihood of the RRA detecting benzodiazepines in urine samples.

The greyhounds were not affected by the single oral 5mg dose of diazepam in that they were not noticeably drowsy and there was no difficulty in collecting the samples. The dose remained at 5mg for the pilot study as at this level the metabolites could be easily detected by RRA and RIA.

By carrying out four trials with diazepam, the whole experimental procedure from dosing the greyhounds to assaying the samples was tested out. It enabled any problems associated with the procedures to be ironed out before the main triazolam and flunitrazepam trials were begun. The dosing and sample procedure was kept simple and presented few problems, the main one being to persuade the dog to swallow the tablets. This was overcome by placing the tablets directly to the back of the greyhounds throat and encouraging it to swallow, rather than by mixing them in the food where they could be easily nosed out.

The daytime hourly sample collections were made easier by the use of a metabolic cage with the dogs being returned to their kennels overnight. Little sample was lost as the dogs did not foul their sleeping area and it was possible to collect a total overnight sample when the dogs were exercised first thing every morning.

As the greyhounds were accustomed to the sample collection procedure carried out at the race tracks by 'dexterous use of a bowl', this method was employed during the trials and caused little distress or discomfort. It also meant there was no need for cathaterisation. Though the dogs were kept in the metabolic cage during the day, they were exercised at hourly intervals which generally resulted in the sample being being collected. Each greyhound trial was carried out separatly to enable the animal nurses to focus their full attention on the

particular dog being dosed, and also avoided any mix up with samples. It also enabled each greyhound to have a 3 to 4 week recovery period between dosing which ensured there was no residual triazolam or flunitrazepam in the dog when the next dose was given. With diazepam this period between trials was extended to 6 weeks due to its longer half-life.

Once a full set of samples for a particular trial had been collected they were aliquoted in to 2 and 5 ml portions. The 2 ml aliquot was hydrolysed according to the methods given in section 3:3:1 and the samples were stored at -20^{o}C until they were required for assay.

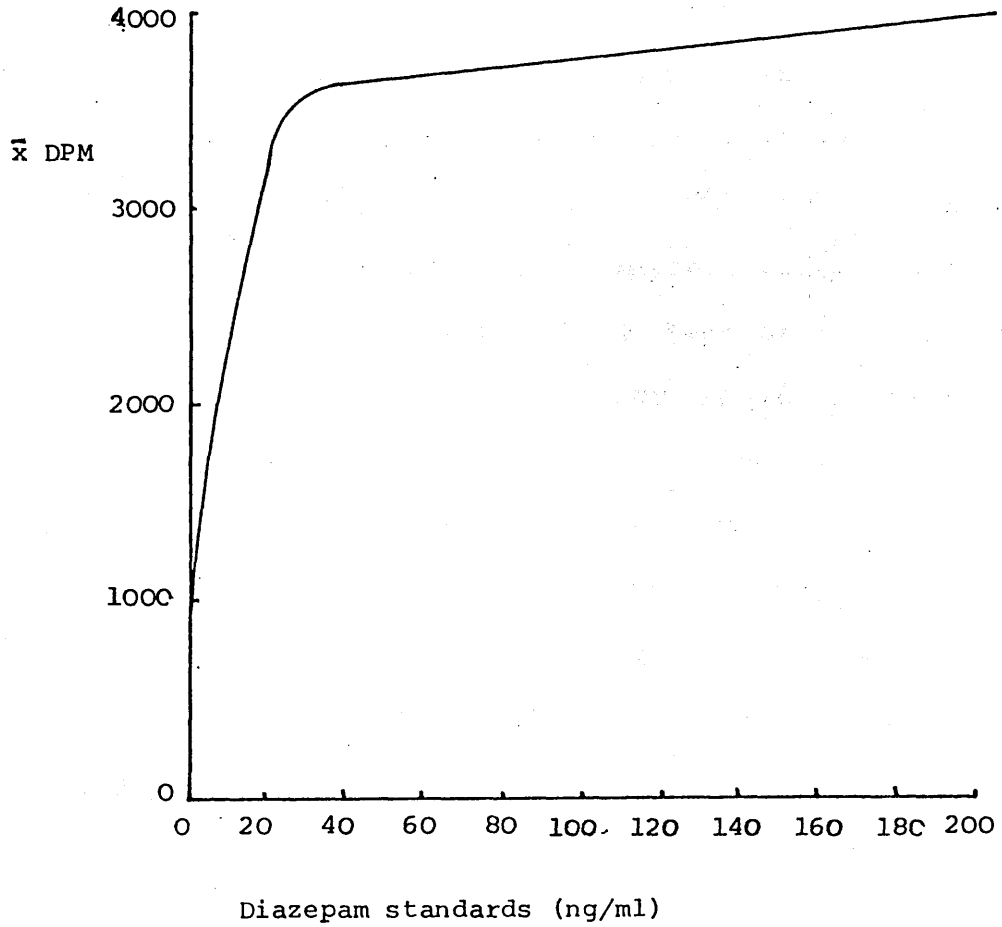
As the diazepam dose was relatively large, this made it easy to set up a RRA for the samples. The experimental procedures had already been established for the standards and spiked urine samples, so this pilot study presented a way of discovering any problems with the RRA of actual samples. In practice this proved to be no different to the RRA of standards/spiked samples. Any samples which had excessive amounts of proteinaceous material and other particulate matter were centrifuged prior to assay. A blank urine sample was always taken prior to dosing so any variation between greyhounds as to possible cross reactivity with the RRA could be taken into account. The RRA result of the blank urine sample also served as a cut off point whereby any results below this result were taken as not containing any benzodiazepine.

The samples from the diazepam trials were assayed both before and after hydrolysis and the results are shown in table 3:2 and figure 3:2. Figures 3:2 demonstrates how there is an increase in metabolites detected after hydrolysis. This is due to the release of the phase I metabolites from their glucuronide conjugates. The samples were also analysed by RIA which confirmed the presence of benzodiazepines in the urine. Table 3:14 lists a set of RIA results which constitute a standard curve, as an example, and figure 3:20 demonstrates them graphically.

Table 3:14 Example of Radioimmunoassay Results to Form a Diazepam Standard Curve.

Diazepam (ng/ml)	Average DPM
0	999
10	2545
25	3421
50	3544
100	3537
200	3861

Figure 3:20 An example of a standard curve following radioimmunoassay of diazepam standards.



Once this pilot study had established a workable dosing regime and sample collection, followed by successful RRA of the samples; further studies were set up to dose the greyhounds with triazolam and flunitrazepam. The results of these studies were used to obtain pharmacokinetic data relating to the interaction of benzodiazepines and greyhounds, and to show excretion profiles. The latter also demonstrate the increase in benzodiazepine metabolites available to the receptors after hydrolysis, thus effectively increasing the sensitivity of the assay. The results were also used to statistically compare RIA and RRA of benzodiazepines enabling similarities and differences in the two assays to be described. The triazolam urine samples were reassayed after they had been stored at $-20^{o}C$ for 12 months. The results were statistically compared with those obtained from RRA of the samples within 2 days of collection. This indicated whether such conditions would cause any change in the benzodiazepine content of the samples. Such a comparison was carried out to determine that the analysis of stored samples reflects the true benzodiazepine concentration from when the sample was taken. This is important in cases where samples cannot be analysed within a few days of collection.

3:5:5 Pharmacokinetics

During the greyhound trials only a single dose was given, to mimick attempted doping, rather than to carry out clinical trials. Therefore the pharmacokinetic data generated enables only two parameters to be calculated. These are the elimination rate constant and the half-life. They can be used to determine the extent to which it is possible to detect the presence of a benzodiazepine in a urine sample from a dog suspected of being drugged.

The results from the unhydrolysed samples were not used to determine pharmacokinetic data as RRA did not detect benzodiazepines at enough time intervals to plot satisfactory graphs. Before the results from the hydrolysed samples were graphed as a semi-log plot, they were recalculated so the metabolites were equated to their parent benzodiazepine in their affinity for the BZ-R. They were then used to calculate the elimination rate constants and half-lives for triazolam and flunitrazepam in greyhounds. Below, in tables 3:15 and 3:17, are examples of experimental data from the RRA of hydrolysed samples, one set for each benzodiazepine together with tables 3:16 and 3:18 which contain the same sets of data calculated in order to draw semi-log plots.

Table 3:15 RRA Results from a Triazolam Trial with the Metabolites Recalculated to Equal the Affinity of Triazolam for the BZ-R.

Time after dose (hrs)	Increase (ng/ml)	Proportion as 1-OH T	4-OH T	Recalculated (ng/ml) x 10	x 25	Urine volume (ml)	Final concentration (ug) x 10 (A)	x 25 (B)
1	106.7	62.7	47.2	627	1181.0	15	9.4	17.71
2	40.25	23.7	16.6	237	414.0	35	8.3	14.5
3	11.0	6.4	4.5	64	113.2	56	3.6	6.34
4	6.25	3.6	2.6	36	66.2	180	6.5	11.92
5	9.75	5.7	4.0	57	100.3	115	6.55	11.54
6	18.5	10.9	7.6	109	190.0	25	2.72	4.76
7	6.5	3.8	2.7	38	67.0	23	0.8	1.5
24	4.0	2.4	1.6	24	41.1	60	1.4	2.5
25	0.0	0.0	0.0	0	0.0	22	0.0	0.0
26	2.0	1.1	0.8	11	20.6	31	0.34	0.64

The addition of A and B gives the ΔDu values for Table 3:16.

Table 3:16 Results from Table 3:15 Calculated to Draw a Semi-log Plot.

Time after dosing (hrs)	Δt	ΔDu	$\log_{10}\frac{\Delta Du}{\Delta t}$
1	1	27.16	1.43
2	1	22.88	1.36
3	1	10.02	1.00
4	1	18.42	1.26
5	1	18.09	1.26
6	1	7.48	0.87
7	1	2.3	0.36
24	17	3.99	-0.6
26	2	0.98	-0.31

Table 3:17 RRA Results from a Flunitrazepam Trial with the Metabolites Recalculated to Equal the Affinity of Flunitrazepam for the BZ-R.

Time after dose (hrs)	Increase (ng/ml)	Recalculated (ng/ml) x 1.8	Urine vol. (ml)	Final conc. (ug)
1	1.5	2.7	10	0.027
2	-	-	-	-
3	143.0	257.0	365	93.9
4	43.0	77.4	327	25.3
5	103.0	185.0	123	22.8
6	76.6	137.0	30	4.1
7	43.3	77.9	47	3.6
24	26.6	47.8	774	37.0
25	0.7	1.26	20	25.2

- no sample collected.

Table 3:18 Results from Table 3:17 Calculated to Draw a Semi-log Plot.

Time after dosing (hrs)	Δt	ΔDu	$\log_{10}\frac{\Delta Du}{\Delta t}$
1	1	27.0	1.43
3	2	93.9	1.67
4	1	25.3	1.40
5	1	22.8	1.35
6	1	4.1	0.61
7	1	3.6	0.55
24	17	37.0	0.33
25	1	25.9	1.41

Once the above data is plotted it is possible to calculate k_{el} and $t_{½}$. Tables 3:19 and 3:20 summerise this data obtained from all the triazolam and flunitrazepam greyhound trials. These results are within the literature values available for triazolam and flunitrazepam in dogs [85,86,90].

Table 3:19 Summary of Kinetic Data following Single Oral Dosing with Triazolam.

Trial	k_{el} (hr^{-1})	$t_{½}$ (hrs)
01	0.53	1.29
02	0.50	1.36
03	0.49	1.39
04	0.66	1.03
05	0.64	1.09
06	0.48	1.43
07	0.67	1.02
08	0.82	0.85
09	0.74	0.93
10	0.39	1.77
11	0.39	1.77
12	0.47	1.44
13	0.67	1.03
14	0.66	1.04
15	0.53	1.31
average	0.57 ± 0.12	1.25 ±0.28

Table 3:20 Summary of Kinetic Data following Single Oral Dosing with Flunitrazepam.

Trial	k_{el} (hr^{-1})	$t_{½}$ (hrs)
16	0.91	0.76
17	0.43	1.58
18	1.18	0.58
19	0.97	0.70
20	0.56	1.23
21	0.50	1.36
22	0.41	1.67
23	0.94	0.70
24	0.84	0.82
25	0.76	0.91
26	0.64	1.07
average	0.74 ± 0.25	1.03 ±0.4

3:5:6 Statistics

The radioreceptor assay and radioimmunoassy results from the analysis of unhydrolysed and hydrolysed triazolam urine samples are shown in tables 3:21, 3:22, 3:23 and 3:24. A comparison of the RIA and RRA results for the unhydrolysed urine samples is shown in table 3:25 and demonstrates the lack of correlation between the two sets of results. The RRA results demonstrate that low concentrations of triazolam are excreted in the urine as free drug. The maximum levels seen were 30 to 35ng/ml, but more commonly the levels were below 10ng/ml. As the RIA antibody will bind to any benzodiazepine-type compound, concentrations recorded varied from 20 to 100ng/ml for the early samples and 5 to 30ng/ml for the samples up to 30 hours after oral dosing.

Following comparison of the RIA and RRA results for the hydrolysed urine samples, shown in table 3:26, there is still a significant difference between the results up to 31 hours. This was unexpected as hydrolysis releases the conjugated triazolam metabolites thus increasing the amounts available to the receptor preparation. Therefore levels detected by RRA should approach those detected by RIA. However only some of the metabolites released are pharmacologically active and can be detected by RRA. The RIA antibody will bind to all the metabolites thus indicating higher levels of triazolam metabolites are present in urine samples.

TABLE 3:21

RADIORECEPTOR ASSAY RESULTS OF THE UNHYDROLYSED URINE SAMPLES

Triazolam concentrations in urine (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	2	7	–	0	2	2.5	–	10.5	10	21.5	3.25	2	13	2	0	6.3	5
2	3	12.5	0	0	2.5	3	12.5	4.5	38.5	23.5	2.25	1.75	6	2	0	5	6
3	2	9.5	–	4.5	2	6.5	0	0	2.5	21.5	1.5	2	0	1.5	0	0	5.5
4	0	8	1.5	4	2	3	0	0	1	21.5	0	0	0	0	0	0	5
5	0	8	1.5	0	2	–	0	0	7	2.5	0	0	0	0	0	5	5
6	0	8.5	0	0	2	3	0	0	14.5	4	0	0	0	0	0	0	5
7	–	–	–	–	–	–	0	0	2.5	2	0	0	0	0	0	0	0
24	2.5	7.5	0	4.5	2	0	2.5	0	2	0	1.5	1.5	0	2	0	5.5	5
25	0	7.5	0	4.5	0	2.5	2	0	0	0	0	3.5	0	0	0	5	5.5
26	2.5	7.5	0	0	0	0	0	0	1.5	0	0	0	0	0	3.5	0	5.5
27	–	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
29	0	0	1.5	–	0	–	0	0	0	0	0	0	0	0	0	0	–
30	2.5	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	–	–	0	–	–	0	0	0	0	0	0	0	0	0	0	0	0

– no sample collected.

TABLE 3:22

RADIORECEPTOR ASSAY RESULTS OF THE HYDROLYSED URINE SAMPLES

Triazolam concentrations (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	35	7	–	3	10	–	–	210	5	19	110	6.3	112	285	15	5.5	0
2	41	212	15	5	23	10	38.5	62	40	11.5	42.5	11.5	53	90	35	7.5	65
3	20	72	–	22.5	21	37.5	12	27.5	16.5	16.5	12.5	38	37.5	55	24	7.5	14.5
4	4.5	50	7.5	20	13.5	47.5	6.5	7.5	0	12.5	6	20	5.5	16	7.5	12.5	6.5
5	7	40	11	12.5	8	–	32.5	7	5	11	10	10	2	5.5	3.5	9	6
6	12	70	6	6	14.5	48	6.5	21.5	7.5	12	18.5	15	2.5	9.5	0	5.5	6
7	–	–	–	–	–	–	7.5	16.5	4	7.5	6.5	11	2	7.5	0	5	6
24	4	28.5	0	0	6.5	17	10	14.5	2.5	4	5.5	10	2	5.5	0	10	5
25	0	27.5	0	0	0	7	3	5	1	0	0	6.5	0	3.5	0	6	5.5
26	4	10	0	0	0	0	0	0	0.5	0	2	2.5	0	2	0	5	0
27	–	–	0	0	0	0	0	0	0	0	0	2.5	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	02.5	0	1.5	0	0	5.5	0
29	0	0	0	0	0	–	0	0	0	0	0	2	0	0	0	5	–
30	0	0	–	0	0	0	0	0	0	0	0	0	0	1.5	0	0	0
31	–	–	0	–	–	0	0	0	0	0	0	0	0	0	0	0	0

– no sample collected.

TABLE 3:23

RADIOIMMUNOASSAY RESULTS OF THE UNHYDROLYSED URINE SAMPLES

Triazolam concentrations (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	23	2.5	–	1	21.5	10	–	100	26	2.5	97.5	2.5	120	120	0	3	0
2	180	97.5	102	14.5	13.5	30	272	162	82.5	80	127	20	100	90	9.5	23.5	127
3	25	152	–	27	15	125	18	45	25	26.5	32.5	27.5	20	60	9	5.5	102
4	6.5	125	52	16	10.5	120	11.5	22.5	18	15	37.5	12.5	0.5	11	0	23	26
5	8	102	8.5	4.5	1	–	25.5	19.5	27	26	29	21.5	0	2.5	0	31	12
6	10	110	17.5	4	10	36	6.5	30	30.5	22.5	25.5	11.5	0	4.5	0	14	12
7	–	–	–	–	–	–	10.5	32.5	50.5	32.5	18.5	25.5	1.5	5.5	0	2	7.5
24	0.5	14.5	5.5	12.5	7	36	5.5	19.5	9.5	16	12	7.5	0	5.5	0	11	3.5
25	0	13	5.0	9	4	13.5	0	–	9.5	36	7	5	1.5	0.5	0	0	3.5
26	0	12	2.5	1.5	4	14.5	2.5	21.5	10.5	7	5	1.5	0	1.5	0	3	2
27	–	–	2.5	3.5	4	17.5	5	11.5	14	8.5	0	1	0	6	0	0	0
28	0	2.5	0.5	3	4	5	2.5	5.5	8.5	0	–	0	0	0.5	0	2.5	0.5
29	0	5.5	0	4	2.5	–	7	9	0	8	6.5	0	0	0	0	0	–
30	0	0	–	1.5	2	5.5	8.5	6	4	8	0	0	0	0.5	0	0	0
31	–	–	0	–	–	5	4	7	7.5	0	0	0	0	0	0	0	1

– no sample collected.

TABLE 3:24

RADIOIMMUNOASSAY RESULTS OF THE HYDROLYSED URINE SAMPLES

Triazolam concentrations (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	26	7.5	-	7.5	28	-	-	975	26	20	92.5	1.5	210	245	0	3.5	0
2	165	172	255	29.5	34	26.5	207	130	202	72.5	55	49	210	200	25	14	142
3	41	105	-	34	32.5	92.5	25	50	27.5	35	22.5	57.5	97.5	30	22	16	120
4	6	112	50	35	26.5	27.5	12	24	21.5	49	39.5	80	3	7.5	0	72.5	19
5	12.5	29	13.5	5	26.5	-	30	26.5	19	26	27	40	0	3	0	37.5	11
6	14	50	5	4.5	31.5	120	8.5	30.5	33	17	23	36	4	4.5	0	50	5.5
7	-	-	-	-	-	-	13.5	43	55	24	12	49	12.5	4	0	17.5	5.5
24	0	25	2.5	17.5	12	16.5	14.5	30.5	17	7	13.5	26	0	3	0	12.5	2.4
25	0	41	0	11.5	8.5	10.5	3.5	21.5	16.5	8	0	16.5	4	4.5	0	0	1
26	0	17.5	1	3	7.5	10	5	14	7	0	0	8	3	0.55	0	0	3.5
27	-	-	0	0	1.5	10	2	7.5	12.5	0	5	7	0	0	0	0	0
28	0	0	0	0	7.5	6.5	0	5	18.5	0	-	0	0	0	0	0	3
29	0	4	0	0	5.5	-	5	7	0	9.5	0	0	0	5.5	0	0	-
30	0	0	-	1	2.5	6.5	4.5	7	0	0	6.5	6.5	0	2	0	0	0.5
31	-	-	0	-	-	7.5	4	7	0	0	0	0	0	0	0	0	0

- no sample collected.

TABLE 3:25

STATISTICAL COMPARISON: Radioimmunoassay Results for Unhydrolysed Triazolam Urine Samples versus Radioreceptor Assay Results for Unhydrolysed Triazolam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA U/H[b] vs RRA U/H	64.6*	40.8*	25.3*	18.4*	8.2*	15.4*	13.8*	10.3*	5.1*	6.5*	8*	5.3*	5.5*	4.6*	3.8*

t [a] has (n-1) degrees of freedom; P = 0.05.

U/H[b] Unhydrolysed urine samples.

* = a statistically significant result.

TABLE 3:26

STATISTICAL COMPARISON: Radioimmunoassay Results for Hydrolysed Triazolam Urine Samples versus Radioreceptor Assay Results for Hydrolysed Triazolam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA H^c vs RRA H^c	15*	39*	17*	16*	8.9*	9.2*	11*	6.6*	7.6*	6.2*	5.5*	3.3*	3.8*	4.5*	3.0*

t [a] has (n-1) degrees of freedom; P = 0.05.

H^c Hydrolysed urine samples.

* A statistically significant result.

The comparison of results from RIA of the hydrolysed and the unhydrolysed samples, are shown in table 3:27 and show the difference between RIA of the two sets of samples. In theory the two sets of results should be similar as the benzodiazepine antibody is reasonably non-specific and will bind to any benzodiazepine, its metabolite(s) and glucuronide conjugates. Experimentally however a significant difference was noted up to and including the 24 hour sample. The samples obtained after 25 hours had no significant differences in their results, as they only contained negligible amounts of triazolam. The significant difference in the early sets of results was unexpected as the Emit® antibody is specific only for benzodiazepines, but appears to be non-specific for the form of the benzodiazepine. The difference in the results may be due to the increased concentration of triazolam and metabolites present in the hydrolysed samples. Therefore the antibody must have some specificity, and not bind to conjugated glucuronide metabolites, though it appears not to differentiate between the unconjugated active or inactive metabolites of triazolam.

The flunitrazepam RRA and RIA results for both unhydrolysed and hydrolysed samples are shown in tables 3:28, 3:29, 3:30 and 3:31. Comparison of the unhydrolysed RRA and RIA results, shown in table 3:32, reveals the expected lack of correlation. The highest levels recorded

TABLE 3:27

STATISTICAL COMPARISON: Radioimmunoassay results for Hydrolysed versus Unhydrolysed Triazolam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA H[c] vs U/H[b]	19.6*	13.7*	5.3*	3.3*	3.6*	4.1*	4.8*	3.6*	1.3	1.1	1.1	0.8	1.4	0.1	1.1

t [a] has (n-1) degrees of freedom; P = 0.05.

U/H[b] Unhydrolysed urine samples.
H[c] Hydrolysed urine samples.

* = a statisticallly significant result.

TABLE 3:28

RADIORECEPTOR ASSAY RESULTS OF THE UNHYDROLYSED URINE SAMPLES

Flunitrazepam concentrations in urine (ng/ml)

Hours after dose	Number of trial											
	1	2	3	4	5	6	7	8	9	10	11	12
1	0	0	14	10	4	0	0	0	29.6	6.7	0	23.3
2	9	11	0	17	6	791	0	26.7	27.6	10	-	30
3	0	0	0	11	4	308	0	53.3	36.7	10	0	0
4	0	0	0	-	4	0	15.5	0	-	20	0	0
5	8	0	0	0	2	32	0	0	0	0	0	0
6	0	0	0	0	4	65	0	0	0	0	0	0
7	0	0	0	-	4	19.5	0	0	0	0	0	0
24	9	0	11	11	7	0	0	0	0	10	0	0
25	0	7	10	15	4	11.5	0	0	25	0	19.3	0
26	0	0	9	0	0	0	0	0	-	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0

- no sample collected.

TABLE 3:29

RADIORECEPTOR ASSAY RESULTS OF THE HYDROLYSED URINE SAMPLES

Flunitrazepam concentrations in urine (ng/ml)

Hours after dose	Number of trial											
	1	2	3	4	5	6	7	8	9	10	11	12
1	33	9	14	88	2	182	26.5	130	810	103	15	533
2	60	0	21	11	22	462	296	30	39	280	–	0
3	0	0	13	0	14	84	366	570	61.3	6.6	143	0
4	11	0	13	–	11	64.5	1276	100	–	26.6	43	110
5	0	0	0	0	5	22	141	60	0	95.6	103	86.7
6	0	0	0	0	15	15.5	61	0	0	53.3	76.6	83.3
7	0	0	0	–	17	22	61	56.7	73.6	26.7	43.3	150
24	9	10	16	0	8	34	2.8	0	46.6	26.7	26.6	50
25	0	0	11	14	8	4	0	0	25	0	20	0
26	0	0	9	0	0	0	0	0	–	6.6	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	25.6	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0

– no sample collected.

TABLE 3:30

RADIOIMMUNOASSAY RESULTS OF THE UNHYDROLYSED URINE SAMPLES

Flunitrazepam concentrations in urine (ng/ml)

Hours after dose	Number of trial											
	1	2	3	4	5	6	7	8	9	10	11	12
1	4	0	4	0	0	9	0	12	36	16	0	39
2	6	18	4	22	5	109	6	0	32	22	–	64
3	6	6	0	9	6	79	14	31	29	24	40	28
4	4	7	0	–	5	6	14	0	–	32	32	20
5	0	5	4	11	7	24	9	0	16	22	28	8
6	2	3	3	4	1	10	0	0	16	9	30	14
7	4	0	0	–	0	3	4	0	20	16	40	20
24	0	0	0	0	0	0	0	0	10	4	0	4
25	0	0	0	0	0	0	0	0	6	22	27	6
26	0	0	3	0	0	0	0	0	–	16	30	6
27	0	0	3	0	0	0	0	0	8	19	24	0
28	0	0	0	0	0	0	32	0	20	14	24	4
29	0	0	0	0	0	25	0	0	8	20	28	2
30	0	0	0	0	0	0	0	0	15	13	26	2
31	0	0	0	0	0	4	7	0	11	12	24	4

– no sample collected.

TABLE 3:31

RADIOIMMUNOASSAY RESULTS OF THE HYDROLYSED URINE SAMPLES

Flunitrazepam concentrations in urine (ng/ml)

Hours after dose	Number of trial											
	1	2	3	4	5	6	7	8	9	10	11	12
1	8	0	2	11	0	20	0	27	72	41	18	62
2	8	6	10	8	8	131	54	0	20	44	–	24
3	5	4	3	8	9	94	45	56	29	33	41	18
4	3	4	2	–	8	27	60	10	–	41	25	20
5	2	0	3	5	4	36	18	13	18	28	29	12
6	3	0	4	4	5	31	18	11	19	23	33	20
7	4	0	0	–	8	19	9	0	38	28	31	19
24	0	0	7	0	0	3	13	0	0	28	20	4
25	0	0	0	0	0	0	0	0	0	24	20	4
26	0	0	0	0	0	0	0	0	–	28	14	8
27	3	0	0	0	0	0	0	0	16	25	16	2
28	0	0	0	0	0	0	15	0	17	27	0	9
29	0	0	0	0	0	0	0	8	0	22	0	6
30	0	0	0	0	0	0	0	0	0	35	0	3
31	0	0	0	0	0	0	0	00		28	16	26

– no sample collected.

TABLE 3:32

STATISTICAL COMPARISON: Radioimmunoassay Results for Unhydrolysed Flunitrazepam Urine Samples versus Radioreceptor Assay Results for Unhydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA U/H[b] vs RRA U/H[b]	3.26*	8.6*	5.2*	7.8*	8.0*	1.1*	5.4*	3.8*	2.6*	5.6*	5.4*	6.8*	7.3*	5.5*	6.6*

t [a] has (n-1) degrees of freedom; P = 0.05

U/H[b] Unhydrolysed results.

* = a statistically significant result.

after RRA were seen 2 and 3 hours after dosing though no flunitrazepam was detected in samples collected from 26 to 31 hours after dosing. However RIA detected the benzodiazepine in samples at later time intervals, up to 31 hours after dosing for some trials.

Comparison of the RRA and RIA results for the hydrolysed urine samples, shown in table 3:33, revealed a sgnificant difference between the results up to 31 hours. As RIA detects all the flunitrazepam metabolites, whether pharmacologically active or otherwise, it was expected that the increase in compounds detected by RRA following hydrolysis would reduce the differences in the two sets to an insignificant level. However this did not happen and demonstrates further how the RIA benzodiazepine antibody is relatively non-specific.

A comparison of the unhydrolysed and hydrolysed RIA results is shown in table 3:34. As before, no significant difference was expected between the two sets of results. However, for the majority of the time intervals there was a significant difference between the results. The two sets of results from samples collected 2 hours after dosing, where the highest levels of flunitrazepam and metabolites were found, were found not to be significantly different. This is likely due to the high concentration of pharmacologically active metabolites present in the unhydrolysed samples which balance any metabolites release by hydrolysis. For the other time intervals where there was no significant differences between the results, 25,

TABLE 3:33

STATISTICAL COMPARISON: Radioimmunoassay Results for Hydrolysed Flunitrazepam Urine Samples versus Radioreceptor Assay Results for Hydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA H^Cvs RRA H^C	32.1*	24.5*	20.3*	23.6*	19.9*	7.4*	13.9*	10.4*	2.8*	4.8*	6.1*	4.0*	2.9*	3.4*	6.1*

t [a] has (n-1) degrees of freedom; P = 0.05.

H^C Hydrolysed results.

* = a statistically significant result.

TABLE 3:34

STATISTICAL COMPARSON: Radioimmunoassay Results for Unhydrolysed versus Hydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA U/H[b] vs RIA H[c]	12*	1.56	6.09*	6.4*	3.9*	8.2*	5.1*	5.3*	0.16	0.59	1.6	2.4*	4.87*	1.6	0.9

t [a] has (n-1) degrees of freedom; P = 0.05

U/H[b] unhydrolysed result.
H[c] Hydrolysed result.

* = a statistically significant result

26, 27, 30 and 31 hours after dosing, it was due to negligble amounts of flunitrazepam detected.

The RRA results of the unhydrolysed and hydrolysed triazolam urine samples reassayed after twelve months at $-20^{o}C$ are shown in tables 3:35 and 3:36. Statistical comparison of these with the corresponding RRA results from analysis of the samples within two days of collection, shown in tables 3:21 and 3:22, are listed in tables 3:37 and 3:38. The comparison of the unhydrolysed sets of samples revealed significantly different results 2 and 25 hours after dosing, whereas comparison of the hydrolysed results showed significant differences 4, 5 and 6 hours after dosing. At all the time points where such a difference was seen, it was due to an overall increase in the amount of triazolam and metabolites detected by the second assay. This increase is most likely due to chemical breakdown of the glucuronide metabolite, which releases the triazolam primary metabolites. Therefore RRA of the samples stored for any length at time may not give the actual levels at the time of collection.

TABLE 3:35

RADIORECEPTOR ASSAY RESULTS OF THE UNHYDROLYSED URINE SAMPLES REASSAYED AFTER 12 MONTHS AT -20°C

Triazolam concentrations in urine (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	2.5	1.5	–	0	3	0	–	16.5	8	15	7.5	0	11	4	0	2.8	0
2	0	7	2.5	0	3.2	3	14	10	43	72.5	5.5	6.5	58	3.5	0	3.5	4
3	0	3.5	–	0	2.6	4.5	0	0	7	5.5	4	6.5	10	3	0	0	0
4	0	2.5	1.8	0	2.6	3	0	0	6.5	25	0	0	0	0	0	3	0
5	0	2	0	0	2.8	–	0	0	7.5	4	0	0	0	0	0	3.2	0
6	0	3	0	0	0	3	0	0	10	4.5	0	0	0	0	0	0	0
7	–	–	–	–	–		0	0	7.5	5	0	0	0	0	0	0	0
24	0	2	1.5	0	0	3.5	0	0	6.5	3.5	4.5	6.5	0	3	0	3.4	0
25	0	2.1	0	0	0	0	0	0	0	0	0	5.5	0	3	0	0	0
26	0	1.5	0	0	0	0	0	0	0	4.5	0	0	0	3	0	0	0
27	–	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	00	0	0	0	0	–	0	0	0	0	0	0
29	0	0	0	0	0–	0	0	0	0	0	0	0	0	0	0	0	–
30	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	–	–	0	–	–	0	0	0	0	0	0	0	0	0	0	0	0

– no sample collected.

TABLE 3:36

RADIORECEPTOR ASSAY RESULTS OF THE HYDROLYSED URINE SAMPLES REASSAYED AFTER 12 MONTHS AT -20°C

Triazolam concentrations in urine (ng/ml)

Hours after dose	Number of trial																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	14.5	8	-	2.5	6.8	-	-	215	9	19	82.5	7.5	31.5	370	6.5	4.5	0
2	1	100	45.2	3.5	22	4.5	96	107.5	57	17.5	27.5	37.5	36	115	66.5	17.5	76
3	3.8	52.5	-	43	12.2	35.2	7.5	25	8.5	23	32.5	80	5	35	11.5	7	34
4	0	45	17	11.6	8.8	70	7	6.5	0	25	21	42.5	0	3	3	26.2	3.5
5	1	62.5	5	2.5	5.2	-	8.5	6.5	6.5	7	12.5	32.5	0	4.5	2.5	39.5	3
6	0	62.5	2.5	3	7.8	37.2	6	14	9.5	9	8.5	32.5	0	5.5	0	4.5	3
7	-	-	-	-	-	-	7	24	8	15	5.5	37.5	0	3	0	0	3
24	0	21	0	3.2	3	12	8.5	11	6.5	6.5	5.5	37.5	0	7.5	2	3	3.5
25	0	27.5	0	0	2.5	3.5	6	7.5	0	0	0	7.5	0	5	0	0	3.5
26	0	7.5	0	0	2.2	2.8	0	5.5	0	0	0	6.5	0	0	0	0	0
27	-	-	0	0	0	2.8	0	0	0	0	0	5	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0
29	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	-
30	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	-	-		0	-	-	2.8	0	0	0	0	0	0	0	0	0	0

- no sample collected.

TABLE 3:37

STATISTICAL COMPARISON: Radioreceptor Assay Results for Unhydrolysed Urine Samples, Assayed Within 2 Days of Receipt versus Radioreceptor Assay Results for Unhydrolysed Urine Samples Reassayed after 12 months at -20°C.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RRA U/H[b] vs RRA U/H[b]	2.01	6.7*	1.42	0.23	2.02	2.8	1.86	0.58	3.06*	1.73	-	0.69	0.61	0.79	-

t [a] has (n-1) degrees of freedom; P = 0.05

U/H [b] Unhydrolysed results.

* = a statistically significant result

TABLE 3:38

STATISTICAL COMPARISON: Radioreceptor Assay Results for Hydrolysed Urine Samples, Assayed Within 2 Days of Receipt versus Radioreceptor Assay Results for Hydrolysed Urine Samples Reassayed after 12 months at -20°C.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RRA H^c vs RRA H^c	2.1	0.25	1.13	3.4*	1.46*	5.04*	2.9	0.41	0.3	0.33	1.41	1.87	1.52	-	-

t [a] has (n-1) degrees of freedom; P = 0.05

H^c Hydrolysed results.

* = a statistically significant result

3:6 Conclusions

Hydrolysis of the urine samples increased the amount of benzodiazepines detected by RRA for the three drugs studied. In some cases benzodiazepines were only detected after hydrolysis indicating how extensive metabolism of a drug could mask its presence in a urine sample. This was independant of the dose. For the triazolam and flunitrazepam trials two dose levels were used and when the higher dose was given, there was little difference in the amounts detected both before and after hydrolysis compared to the lower dose. This was expected as the greyhounds showed no physical signs of having ingested a significantly higher concentration of triazolam or flunitrazepam. Any differences in the overall metabolic profiles was attributed to variation in the rate that each greyhound's metabolic system broke down the benzodiazepines.

Radioreceptor assay of the triazolam and flunitrazepam metabolites, together with the appropriate standards, gave results which complimented those obtained from the IC_{50} experiments. Together with the figures for the percentage of the increase attributed to each

metabolite, it was possible to recalculate the RRA results for the hydrolysed samples. Thus more accurate semi-log plots could be drawn in order to calculate the elimination rate constants and half-lives for triazolam and flunitrazepam in greyhounds. The kinetic results obtained were similar to those quoted in the literature, demonstrating that RRA can be used as an analytical method during pharmacokinetic studies. Triazolam was found to have a half-life of 1.25 hours ± 15 minutes which corresponds to the major peak in each of the plots of RRA urine excretion data shown in figures 3:5, 3:6, 3:7, 3:8, 3:9 and 3:10. The half-life the flunitrazepam metabolites was calculated as being 1.03 hours ± 20 minutes which equates with the major peaks shown in figures 3:12, 3:13, 3:14, 3:15, 3:16 and 3:17.

Conclusions drawn from the extraction experiments showed that the increase in analysis time was not in proportion to any increase in sample detection. Providing a blank greyhound urine sample is assayed alongside the actual samples, it is possible to obtain accurate results for the concentration of benzodiazepine in the sample. Any samples which contain particulate matter were centrifuged to ensure there is no interference with the BZ-receptors.

Radioimmunoassay proved to be a suitable second method to confirm the presence of triazolam and flunitrazepam in the urine samples. Though from the statistical comparisons it was not possible to establish total correlation between RIA and RRA. Results from samples collected when urine concentrations of both benzodiazepines and their metabolites were highest had significant statistical differences when analysed by the two methods. These differences were attributed to the high specificity of the receptor assay for pharmacologically active benzodiazepine metabolites and the relatively non-specific BZ-antibody used in RIA. Therefore RIA could be used to corroborate the presence of benzodiazepines in urine samples, though not to confirm actual levels.

CHAPTER FOUR

CONCLUSIONS

A suitable radioreceptor assay has been established to analyse urine samples following single oral dosing of greyhounds. Isolation of the synaptosomal fraction was achieved by homogenisation and centrifugation of rat brain tissue. These pellets containing the benzodiazepine receptors were found to remain viable for up to 4 weeks when stored at $-20^{o}C$, though the intact brain tissue could be stored for several months. This is probably due to the disruption of connective and supportive tissue surrounding the receptors during preparation. Thus allowing the 3D protein structure of the receptor to unfold until it no longer recognises benzodiazepines. The protein concentration of the pellets containing the receptors consistantly fell between 1 and 2mg/ml, so a final protein concentration of 1mg/ml was used throughout. When the actual number of receptors (B_{max}) and their affinity for benzodiazepines (K_D) was determined, the results were similar to literature values under the same conditions. Again protein concentrations of 1mg/ml, or less, were used during these binding assays in order to obtain more accurate graphs and a better correlation between the points on the Scatchard plot.

It was found that the equilibrium between benzodiazepines and the BZ-R was established within 20 minutes and remained stable for a further 2 hours. This was sufficient to assay many samples and to successfully separate the bound and free radioligand before disruption of the equilibrium begins. When these two fractions were separated it was by filteration and 5 x 1ml volumes of assay buffer were found to be sufficient to reduce the NSB to a minimum. Greater volumes of assay buffer did not significantly alter the levels of NSB, they only served to increase the separation time.

Calculation of the affinity of various benzodiazepines and their metabolites, the K_i value, demonstrated the high affinity of the parent drug for BZ-R. This was especially true for the short acting benzodiazepines, such as clonazepam, flunitrazepam, lorazepam and triazolam, which have the lowest values and therefore the highest potency. Longer acting benzodiazepines, such as diazepam and nitrazepam, have higher values and lower potencies so higher doses are required to gain an effect. The metabolites assayed had higher K_i values than their parent drug, as is expected, as metabolism is the way in which the body alters drug in order that it can be readily excreted.

Having set up the experimental conditions, with standards and spiked "test" urine samples, a pilot greyhound study was carried out with diazepam. This was a siccess and showed there were no problems specific to the RRA of actual samples. Providing a blank urine sample is assayed along with possible BZ-containing samples, there is no requirement to carry out an extraction process. Hydrolysis extended to 24 hours the length of time after dosing during which triazolam and flunitrazepam could be detected in greyhound urine samples. It also increases the sensitivity of the assay by releasing pharmacologically active metabolites from their glucuronide conjugate thus enabling higher concentrations of benzodiazepines to be detected. This increase after hydrolysis was used in order to obtain more accurate pharmacokinetic data. The half-lives for triazolam and flunitrazepam in greyhounds obtained experimentally correlated with literature values for dogs.

When statistical comparisons of RRA and RIA of unhydrolysed and hydrolysed samples for both benzodiazepines were made, there were significant differences at all time intervals after dosing. This demonstrated the difference in specificity between the BZ-R and the BZ-antibodies, with the former having a higher degree of specificity than the latter. The samples were found to have a statistically significant increase in their benzodiazepine concentration when reassayed after 12 months at $-20^{o}C$. This was only seen for samples which

had originally contained high concentrations of triazolam and therefore would also contain relatively high concentrations of triazolam glucuronide conjugates. It is probable that over the 12 month period these conjugates began to breakdown, thus increasing the concentration of triazolam metabolites in the samples. Such a factor would have to be taken in to account when urine samples are assayed after being stored for several months.

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RADIORECEPTOR ASSAY AND RADIOIMMUNOASSAY OF TRIAZOLAM IN URINE SAMPLES FROM RACING GREYHOUNDS

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Summary

Two complimentary assay techniques were used to determine triazolam levels in greyhound urine samples following a single oral dose. The results from the trials were statistically compared. The relative non-specificity of the benzodiazepine antibody used in radioimmunoassay caused a significant difference in the two sets of results. This was independent of hydrolysis.

Key words: Radioreceptor assay; Radioimmunoassay; Triazolam; Greyhound urine

Introduction

Triazolam, a 1,4-benzodiazepine derivative is a hypnotic agent with a half-life of 2.5-3 hours. Urine samples collected from greyhounds following a single dose of this drug were assayed by radioreceptor assay (RRA) and radioimmunoassay (RIA) and the results compared.

Radioreceptor assay uses receptors prepared from rat brain tissue. These are highly specific for benzodiazepines and their pharmacologically-active metabolites. [1,2]. RIA involves antibodies raised against benzodiazepines and consequently is less specific. The samples were assayed by both methods before and after hydrolysis using the enzymatic method by Bloch [3], which involves ß-glucuronidase .

Materials and Methods

The triazolam tablets (0.25 mg) were donated by Messrs. Upjohn Ltd. The tritiated flunitrazepam ([^{3}H]FNZ) specific activity 81.2 Ci/mmol, was obtained from Amersham International plc. The ß-glucuronidase, 90,000 ß-glucuronidase units/ml and 400 sulfatase units/ml was obtained from Sigma Chemical Company® U.S.A. All other chemicals were of analytical quality.

Radioreceptor assay (RRA)

Rat brain preparations were made by the Hunt [1] procedure but with minor adaptions due to equipment and scale. Approximately 1g of brain tissue was homogenised in 5ml of 0.32M ice cold sucrose solution and centrifuged for 10 min at 3500g. The supernatant was removed and further centrifuged for 20 min at 15000g. The pellet formed was resuspended in 5ml of 0.025 M sodium phosphate

buffer, pH 7.4 (assay buffer) and frozen at -20^{o}C until required. The receptor preparation remains viable for up to 6 weeks under these conditions.

The protein concentration of the preparation was estimated by the colourimetric method of Lowrey [4], whereby the protein-copper-Folin reagent complex can be measured in a spectrophotometer at a wavelength of 750nm.

RRA was carried out using 400ul of receptor preparation (aproximately 1mg/ml of protein), plus 50ul of standard or sample and 100ul of [^{3}H]FNZ,(50nCi). The mixtures were incubated for 45 min. at room temperature, and rapidly filtered through Whatman GF/B filters, under vacuum. The filters were washed with 5ml of assay buffer. The bound radiolabel-receptor complex retained by the filters was measured by scintillation counting for one minute. Incubation of the receptors with excess triazolam (1ug/ml) enabled non-specific binding to be determined. Standards were prepared to cover a range from 0 to 50ng/ml of triazolam.

The results from the RRA were calculated by the method of Lund [5], where the specific binding is the total binding minus the binding in the assay blank. The standard triazolam concentrations were plotted against the specific binding in the zero standard divided by the specific binding in the standard or sample. The curve is linear over the range used and this enables the triazolam concentration in each sample to be calculated.

Radioimmunoassay (RIA)

The samples were assayed essentially by the method of Robinson [6] using the antibody from the EMIT® benzodiazepine kit as follows. A reagent solution was prepared by mixing the following in equal volumes.

a) 0.05M sodium bicarbonate buffer, pH 9.0.

b) [^{3}H]FNZ 50nCi.

c) Benzodiazepine specific antibody (4% solution).

d) A 0.125% solution of sheep serum.

500ul of this reagent was added to 100ul of sample or standard together with 200ul of donkey anti-sheep antibody (12.5% solution) and the tubes left to incubate overnight at 4^{0}C. Bound and free fractions were separated by centrifugation. 400ul of the supernatant was added to 4ml of Ecoscint scintillant before counting.

The total amount of radiolabel available was estimated by the addition of 125ul of label to 675ul buffer, followed by incubation overnight at 4^{0}C, centrifugation and removal of 400ul for scintillation counting. The percentage binding was estimated by the following equation:

$$\% \text{ binding} = \frac{1 - \text{0ng/ml CPM}}{\text{Total CPM}} \times 100$$

The RIA results from the standards (0 to 50ng/ml of triazolam) were plotted as average counts per minute against triazolam concentration, from the curve the sample triazolam concentrations were determined.

Urine Samples

Greyhounds were given a single oral dose of triazolam (either one or two 250ug tablets) and urine samples were collected at hourly intervals for 48 hours. These samples were assayed before and after hydrolysis by RRA and RIA. Hydrolysis was carried out on 2ml of sample with the addition of 0.5 ml of acetate buffer (0.1M, pH 5) and 20ul of ß-glucuronidase from Helix pomatia. The mixture was incubated for 24 hours at 37^{o}C.

Results - Data Analysis

The triazolam concentrations for each set of hourly samples obtained by both assay methods were compared statistically by the paired t-test, [7]. This method is preferred to a means comparison as both analytical methods assay samples from different sources (greyhounds) which vary in their triazolam concentration. The paired t-test can deal with results from samples presented over an extended period as occured in this study as each greyhound dosing trial was carried out individually.

Radioimmunoassay (RIA) Results for Hydrolysed versus Unhydrolysed Urine Samples

The comparison of results from radioimmunoassay of the hydrolysed and the unhydrolysed samples, are shown in Table 1 and determines the effect of hydrolysis on the RIA. Hydrolysis is known to increase the amount of unconjugated triazolam and its metabolites in the samples. In theory the two sets of results should be similar as the benzodiazepine antibody is reasonably non-specific and will bind to any benzodiazepine, its metabolite(s) and glucuronide conjugates. Experimentally a significant difference between the two sets of results was noted up to and including the 24 hour samples.

Radioimmunoassay (RIA) Results for Unhydrolysed Urine Samples versus Radioreceptor Assay (RRA) Results for Unhydrolysed Urine Samples

Comparison of the RIA results, shown in Table 2, of the unhydrolysed urine samples and the RRA results for the unhydrolysed urine samples demonstrates the lack of correlation between these two sets of results. This was expected as the RRA only gives the amounts of unconjugated triazolam and metabolites present in the urine samples.

TABLE 1
STATISTICAL COMPARISON:- Radioimmunoassay results for Hydrolysed versus Unhydrolysed Urine Samples.

Values of t [a]

	Hours after dose															
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31	48
RIA H [b] vs U/H [c]	19.6*	13.7*	5.3*	3.3*	3.6*	4.1*	4.8*	3.6*	1.3	1.1	1.1	0.8	1.4	0.1	1.1	0.2

[a] t has (n-1) degrees of freedom ; P = 0.05
[b] H- Hydrolysed urine samples
[c] U/H - Unhydrolysed urine samples

* = a statistically significant result

TABLE 2
STATISTICAL COMPARISON:- Radioimmunoassay Results for Unhydrolysed Urine Samples versus Radioreceptor Assay Results for Unhydrolysed Urine Samples.

Values of t [a]

	Hours after dose															
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31	48
RIA U/H vs RRA U/H	16.9*	36.7*	25.1*	18*	14.9*	13.8*	8.6*	6*	6.4*	8*	5.5*	10*	4.6*	3.9*	1.9	2.13

[a] t has (n-1) degrees of freedom ; P = 0.05

* = a statistically significant result

Radioimmunoassay (RIA) Results for Hydrolysed Urine Samples versus Radioreceptor Assay (RRA) Results for Hydrolysed Urine Samples

Comparison of the the RIA results for the hydrolysed urine samples and the RRA results also for the hydrolysed urine samples is shown in Table 3. Greater correlation was expected between these sets of results as hydrolysis releases the conjugated triazolam metabolites thus increasing the amount available to the receptor preparation. Levels obtained for RRA theoretically would approach those obtained after RIA.

Discussion and Conclusions

By statistical analysis it was possible to identify the samples where there was a significant difference in the results.

There was a significant difference between RIA results from the hydrolysed and the unhydrolysed samples, (Table 1) for each of the hourly urine collections up to and including the seven hour sample,

and for the 24 hour sample. The samples obtained after 25 hours had no significant differences in their results, as they only contained negligible amounts of triazolam. The significant difference in the early sets of results was unexpected as the Emit® antibody is specific only for benzodiazepines, but appears to be non-specific for the form of the benzodiazepine.The difference in the

TABLE 3
STATISTICAL COMPARISON:- Radioimmunoassay Results for Hydrolysed Urine Samples versus Radioreceptor Assay Results for Hydrolysed Urine Samples.

Values of t [a]

	Hours after dose															
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31	48
RIA H vs RRA H	15.5*	29*	16.9*	16.4*	9.8*	9.5*	10.3*	3.6*	6.9*	5.7*	4.3*	3.8*	4.2*	5.4*	3.1*	1.7

[a] t has (n-1) degrees of freedom ; P = 0.05

* = a statistically significant result

results may be due to more triazolam and metabolites being detected in the hydrolysed samples, therefore the antibody must have some specificity for the unconjugated drug only, though it does not differentiate between the unconjugated active or inactive metabolites of triazolam.

The expected significant difference in the RIA and RRA results of the unhydrolysed samples (Table 2) was observed up to and including the 30 hour sample results. The results from the RRA showed a low concentration of triazolam was excreted in the urine as the free drug. The maximum levels seen were 30 to 35 ng/ml, but more commonly the levels were below 10 ng/ml. As the RIA antibody will bind to any benzodiazepine-type compound, concentrations recorded varied from 20 to 100 ng/ml for the early samples and 5 to 30 ng/ml for the samples up to 30 hours after oral dosing.

A greater correlation of the RIA and RRA results was expected following hydrolysis of the samples. However a significant difference was still found between the results up to 31 hours, (Table 3). A possible explanation for this involves the unconjugation of the glucuronide metabolites, even though not all the metabolites released are pharmacologically active. RRA only detects those which are active where as the RIA antibody will bind to both active and inactive metabolites thus indicating higher concentrations of triazolam metabolites in urine samples.

Figure 1 shows how hydrolysis increases the amount of pharmacologically active triazolam and metabolites in the samples, thus increasing the sensitivity of the radioreceptor assay. They also illustrate that the majority of the triazolam metabolites are to be found in the 2 and 3 hour samples. This ties in with the half-life of the drug which is between 2.5 and 3 hours in dogs, as previously established by Kitagawa [8], who studied the absorption, distribution and excretion of triazolam in dogs, rats and monkeys. He also identified the metabolites of triazolam found in dog and rat samples,[9]. The graph of the unhydrolysed samples show the amount of free triazolam excreted in the urine. As expected these levels are not very high as triazolam is extensively metabolised by the liver to form 4-hydroxy triazolam and 1'-hydroxy triazolam, both of which possess pharmacological activity. This metabolic pathway is described by Eberts, [10] in his paper relating to the disposition of triazolam in the dog. However both metabolites are extensively conjugated with glucuronide derivatives thus losing their pharmacological activity. As hydrolysis unconjugates the derivatives found in urine, the amount of pharmacologically-active triazolam metabolites increases, as shown on the graph.

The graph also show how at 24 and 25 hours after oral dosing, triazolam and its metabolites are still detectable by RRA in urine samples. This can be attributed to a combination of two bio-processes.

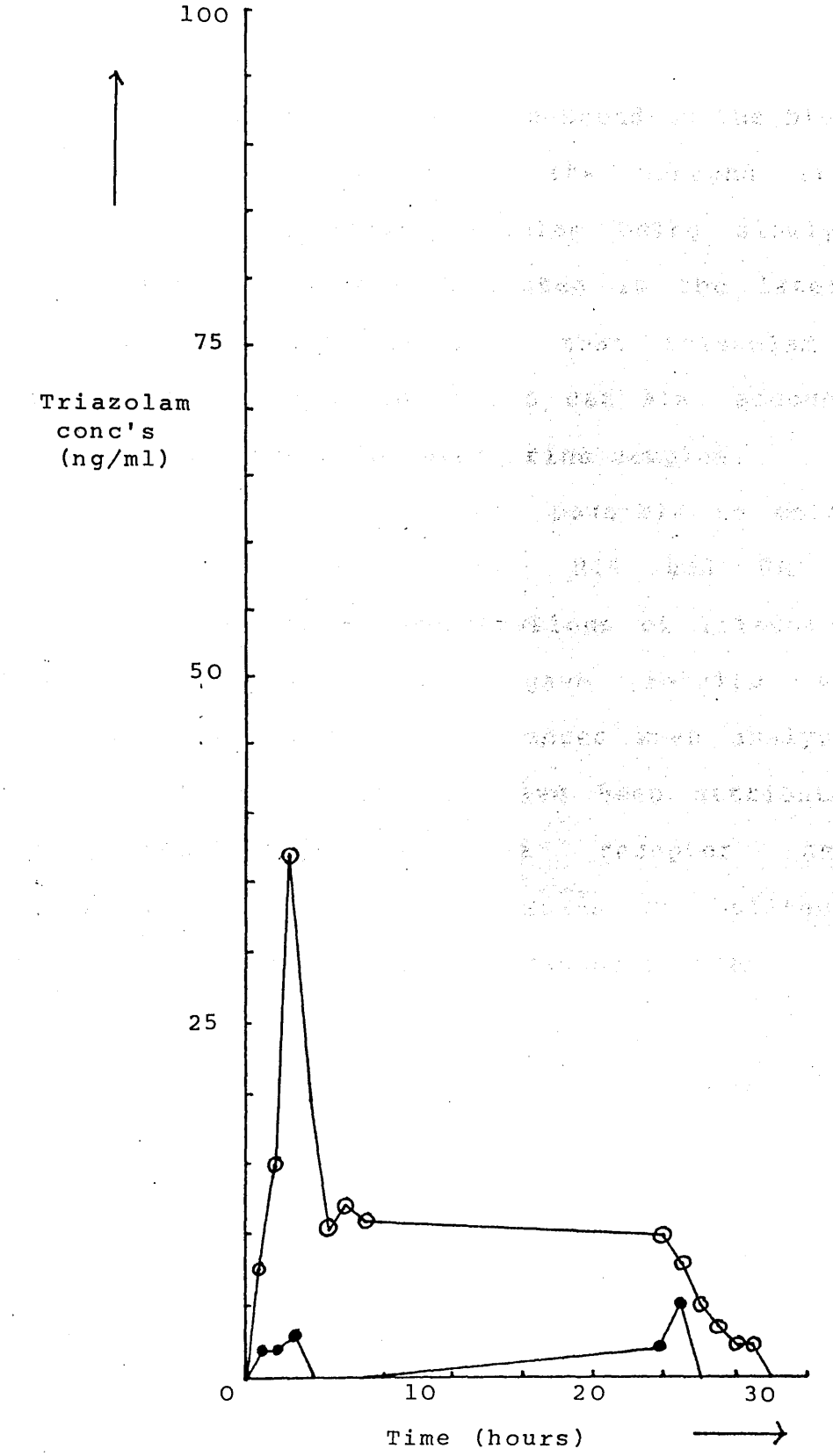

Figure 1. RRA of urine samples immediately after collection.
Unhydrolysed samples - ●
Hydrolysed samples - O

Triazolam is extensively protein-bound in the blood and is released slowly as the unbound fraction is excreted. It is this triazolam being slowly released which accounts for that detected in the later samples. There is also some evidence that triazolam undergoes enterohepatic circulation which can also account for its continued appearance in later urine samples.

To conclude it was not possible to establish any form of correlation between RIA and RRA. Samples collected when urine concentrations of triazolam and its metabolites were highest gave results which had significant statistical differences when analysed by RIA or RRA. These differences have been attributed to the high specificity of the receptor assay for pharmacologically active triazolam metabolites and the relatively non-specific antibody used in RIA.

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RADIORECEPTOR ASSAY AND RADIOIMMUNOASSAY OF FLUNITRAZEPAM IN URINE SAMPLES FROM RACING GREYHOUNDS

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Summary

Two complimentary assay techniques were used to determine flunitrazepam levels in greyhound urine samples following a single oral dose. The results from the trials were statistically compared. The relative non-specificity of the benzodiazepine antibody used in radioimmunoassay caused a significant difference in the two sets of results. This was independent of hydrolysis.

Key words: Radioreceptor assay; Radioimmunoassay; Flunitrazepam; Greyhound urine

Introduction

Flunitrazepam, a 1,4-benzodiazepine derivative is a hypnotic agent with a half-life of 2.5-3 hours, in dog [1]. Urine samples collected from greyhounds following a single dose of this drug were assayed by radioreceptor assay (RRA) and radioimmunoassay (RIA) and the results compared.

Radioreceptor assay uses receptors prepared from rat brain tissue. These are highly specific for benzodiazepines and their pharmacologically-active metabolites. [2,3]. RIA involves antibodies raised against benzodiazepines and consequently is less specific. The samples were assayed by both methods before and after hydrolysis using the enzymatic method by Bloch [4], which involves ß-glucuronidase .

Materials and Methods

The flunitrazepam tablets (1.0 mg) were donated by Messrs. Hoffmann-La Roche. The tritiated flunitrazepam ([^{3}H]FNZ) specific activity 81.2 Ci/mmol, was obtained from Amersham International plc. The ß-glucuronidase, 90,000 ß-glucuronidase units/ml and 400 sulfatase units/ml was obtained from Sigma Chemical Company® U.S.A. All other chemicals were of analytical quality.

Radioreceptor assay (RRA)

Rat brain preparations were made by the Hunt [2] procedure but with minor adaptions due to equipment and scale. Approximately 1g of brain tissue was homogenised in 5ml of 0.32M ice cold sucrose solution and centrifuged for 10 min at 3500g. The supernatant was removed and further centrifuged for 20 min at 15000g. The pellet formed was resuspended in 5ml of 0.025 M sodium phosphate

buffer, pH 7.4 (assay buffer) and frozen at -20^{o}C until required. The receptor preparation remains viable for up to 6 weeks under these conditions.

The protein concentration of the preparation was estimated by the colourimetric method of Lowrey [5], whereby the protein-copper-Folin reagent complex can be measured in a spectrophotometer at a wavelength of 750nm.

RRA was carried out using 400ul of receptor preparation (aproximately 1mg/ml of protein), plus 50ul of standard or sample and 100ul of [^{3}H]FNZ,(50nCi). The mixtures were incubated for 45 min. at room temperature, and rapidly filtered through Whatman GF/B filters, under vacuum. The filters were washed with 5ml of assay buffer. The bound radiolabel-receptor complex retained by the filters was measured by scintillation counting for one minute. Incubation of the receptors with excess flunitrazepam (1ug/ml) enabled non-specific binding to be determined. Standards were prepared to cover a range from 0 to 200ng/ml of flunitrazepam.

The results from the RRA were calculated by the method of Lund [6], where the specific binding is the total binding minus the binding in the assay blank. The standard flunitrazepam concentrations were plotted against the specific binding in the zero standard divided by the specific binding in the standard or sample. The curve is linear over the range used and this enables the flunitrazepam concentration in each sample to be calculated.

Radioimmunoassay (RIA)

The samples were assayed essentially by the method of Robinson [7] using the antibody from the EMIT® benzodiazepine kit as follows. A reagent solution was prepared by mixing the following in equal volumes.

a) 0.05M sodium bicarbonate buffer, pH 9.0.

b) [^{3}H]FNZ 50nCi.

c) Benzodiazepine specific antibody (4% solution).

d) A 0.125% solution of sheep serum.

500ul of this reagent was added to 100ul of sample or standard together with 200ul of donkey anti-sheep antibody (12.5% solution) and the tubes left to incubate overnight at 4^{o}C. Bound and free fractions were separated by centrifugation. 400ul of the supernatant was added to 4ml of Ecoscint scintillant before counting.

The total amount of radiolabel available was estimated by the addition of 125ul of label to 675ul buffer, followed by incubation overnight at 4^{o}C, centrifugation and removal of 400ul for scintillation counting. The percentage binding was estimated by the following equation:

$$\% \text{ binding} = \frac{1 - \text{0ng/ml CPM}}{\text{Total CPM}} \times 100$$

The RIA results from the standards (0 to 200ng/ml of flunitrazepam) were plotted as average counts per minute against flunitrazepam concentration, from the curve the sample flunitrazepam concentrations were determined.

Urine Samples

Greyhounds were given a single oral dose of flunitrazepam (either one or two 1mg tablets) and urine samples were collected at hourly intervals for 31 hours. These samples were assayed before and after hydrolysis by RRA and RIA. Hydrolysis was carried out on 2ml of sample with the addition of 0.5 ml of acetate buffer (0.1M, pH 5) and 20ul of ß-glucuronidase from Helix pomatia. The mixture was incubated for 24 hours at 37^{o}C.

Results - Data Analysis

The flunitrazepam concentrations for each set of hourly samples obtained by both assay methods were compared statistically by the paired t-test, [8]. This method is preferred to a means comparison as both analytical methods assay samples from different sources (greyhounds) which vary in their flunitrazepam concentration. The paired t-test can deal with results from samples presented over an extended period as occured in this study as each greyhound dosing trial was carried out individually.

Radioimmunoassay (RIA) Results for Hydrolysed versus Unhydrolysed Urine Samples

The comparison of results from radioimmunoassay of the hydrolysed and the unhydrolysed samples, are shown in Table 1 and determines the effect of hydrolysis on the RIA. Hydrolysis is known to increase the amount of unconjugated flunitrazepam and its metabolites in the samples. In theory the two sets of results should be similar as the benzodiazepine antibody is reasonably non-specific and will bind to any benzodiazepine, its metabolite(s) and glucuronide conjugates. Experimentally a significant difference between the two sets of results was noted up to and including the 24 hour samples.

Radioimmunoassay (RIA) Results for Unhydrolysed Urine Samples versus Radioreceptor Assay (RRA) Results for Unhydrolysed Urine Samples

Comparison of the RIA results, shown in Table 2, of the unhydrolysed urine samples and the RRA results for the unhydrolysed urine samples demonstrates the lack of correlation between these two sets of results. This was expected as the RRA only gives the amounts of unconjugated flunitrazepam and metabolites present in the urine samples.

TABLE 1

STATISTICAL COMPARSON: Radioimmunoassay Results for Unhydrolysed versus Hydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA U/H[b] vs RIA H[c]	12*	1.56	6.09*	6.4*	3.9*	8.2*	5.1*	5.3*	0.16	0.59	1.6	2.4*	4.87*	1.6	0.9

t [a] has (n-1) degrees of freedom; P = 0.05

U/H[b] unhydrolysed result.
H[c] Hydrolysed result.

* = a statistically significant result

TABLE 2

STATISTICAL COMPARISON: Radioimmunoassay Results for Unhydrolysed Flunitrazepam Urine Samples versus Radioreceptor Assay Results for Unhydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA U/H[b] vs RRA U/H[b]	3.26*	8.6*	5.2*	7.8*	8.0*	1.1*	5.4*	3.8*	2.6*	5.6*	5.4*	6.8*	7.3*	5.5*	6.6*

t [a] has (n-1) degrees of freedom; P = 0.05

U/H[b] Unhydrolysed results.

* = a statistically significant result.

Radioimmunoassay (RIA) Results for Hydrolysed Urine Samples versus Radioreceptor Assay (RRA) Results for Hydrolysed Urine Samples

Comparison of the RIA results for the hydrolysed urine samples and the RRA results also for the hydrolysed urine samples is shown in Table 3. Greater correlation was expected between these sets of results as hydrolysis releases the conjugated flunitrazepam metabolites thus increasing the amount available to the receptor preparation. Levels obtained for RRA theoretically would approach those obtained after RIA.

Discussion and Conclusions

By statistical analysis it was possible to identify the samples where there was a significant difference in the results.

There was a significant difference between RIA results from the hydrolysed and the unhydrolysed samples, (Table 1) for each of the hourly urine collections up to and including the seven hour sample, and for the 24 hour sample. The samples obtained after 25 hours had no significant differences in their results, as they only contained negligible amounts of flunitrazepam. Though a

TABLE 3

STATISTICAL COMPARISON: Radioimmunoassay Results for Hydrolysed Flunitrazepam Urine Samples versus Radioreceptor Assay Results for Hydrolysed Flunitrazepam Urine Samples.

Values of t [a]

	Hours after dose														
	1	2	3	4	5	6	7	24	25	26	27	28	29	30	31
RIA H^c vs RRA H^c	32.1*	24.5*	20.3*	23.6*	19.9*	7.4*	13.9*	10.4*	2.8*	4.8*	6.1*	4.0*	2.9*	3.4*	6.1*

t [a] has (n-1) degrees of freedom; P = 0.05.

H^c Hydrolysed results.

* = a statistically significant result.

significant difference can be seen between samples taken at 28 and 29 hours. This is possibly due to slow release of the protein bound flunitrazepam. The significant difference in the early sets of results was unexpected as the Emit® antibody is specific only for benzodiazepines, but appears to be non-specific for the form of the benzodiazepine.The difference in the results may be due to more flunitrazepam and metabolites being detected in the hydrolysed samples, therefore the antibody must have some specificity for the unconjugated drug only, though it does not differentiate between the unconjugated active or inactive metabolites of flunitrazepam.

The expected significant difference in the RIA and RRA results of the unhydrolysed samples (Table 2) was observed up to and including the 31 hour sample results. The results from the RRA showed a low concentration of flunitrazepam was excreted in the urine as the free drug. The maximum levels seen were up to 790ng/ml, but more commonly the levels were between 10 - 50 ng/ml. As the RIA antibody will bind to any benzodiazepine-type compound, concentrations recorded varied from 20 to 100 ng/ml for the early samples and 0 to 30 ng/ml for the samples up to 31 hours after oral dosing.

A greater correlation of the RIA and RRA results was expected following hydrolysis of the samples. However a significant difference was still found between the results up to 31 hours, (Table 3). A possible

explanation for this involves the unconjugation of the glucuronide metabolites, even though not all the metabolites released are pharmacologically active. RRA only detects those which are active where as the RIA antibody will bind to both active and inactive metabolites thus indicating higher concentrations of flunitrazepam metabolites in urine samples.

Figure 1 shows how hydrolysis increases the amount of pharmacologically active flunitrazepam and metabolites in the samples, thus increasing the sensitivity of the radioreceptor assay. They also illustrate that the majority of the flunitrazepam metabolites are to be found in the first four hours after dosing, which ties in with the half-life of the drug. The graph of the unhydrolysed samples show the amount of free flunitrazepam excreted in the urine. As expected these levels are not very high as flunitrazepam is extensively metabolised by the liver to form N-desmethyl flunitrazepam and 7-amino flunitrazepam, both of which possess pharmacological activity [9]. However both metabolites are extensively conjugated with glucuronide derivatives thus losing their pharmacological activity. As hydrolysis unconjugates the derivatives found in urine, the amount of pharmacologically-active flunitrazepam metabolites increases, as shown on the graph.

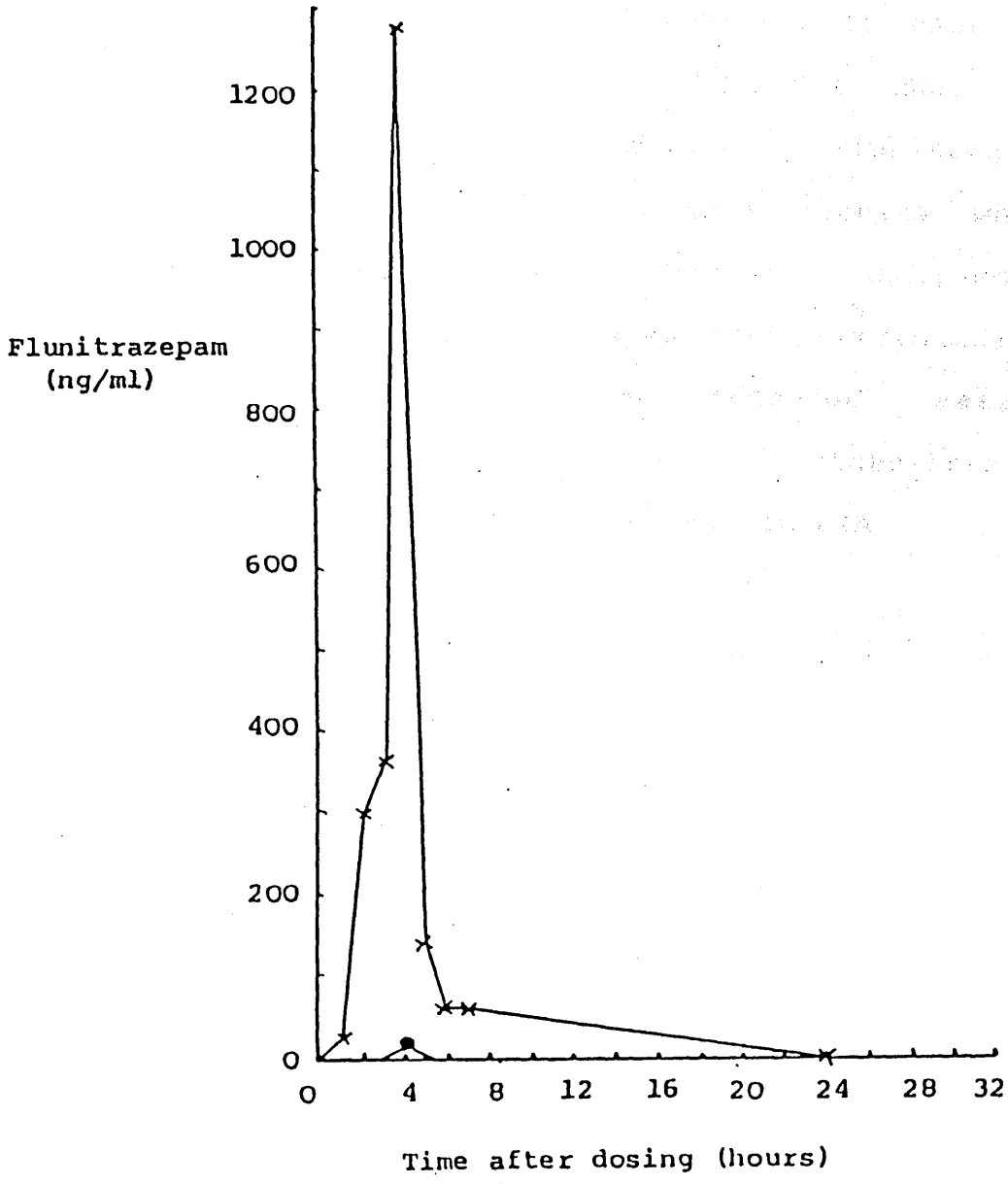

Figure 1 Radioreceptor Assay of Urine Samples for Flunitrazepam.

Unhydrolysed Samples - ●
Hydrolysed Samples - x

To conclude it was not possible to establish any form of correlation between RIA and RRA. Samples collected when urine concentrations of flunitrazepam and its metabolites were highest gave results which had significant statistical differences when analysed by RIA or RRA. These differences have been attributed to the high specificity of the receptor assay for pharmacologically active flunitrazepam metabolites and the relatively non-specific antibody used in RIA.

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