THE DIAGNOSTIC USE OF MEASUREMENTS OF MEMBRANE BINDING SITES IN DEATH BY POISONING

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ABBREVIATIONS

ATP	adenosine triphosphate
B-CCB	n-butyl-B-carboline-3-carboxylate
B-CCE	ethyl-B-carboline-3-carboxylate
B_{max}	total number of binding sites
BZ	benzodiazepine
BZ-R	benzodiazepine receptor
CAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CCS	corticosterone
CDP	chlordiazepoxide
CGS 8216	2-phenyl-2,5-dihydro-pyrazolo-{4,3-C)-quinolin-3(3H)one
CI	chemical ionisation
CNS	central nervous system
CRF	corticorophic releasing factor
DBI	diazepam binding inhibitor
DMCM	methyl-6,7-dimethoxy-4-ethyl-b-carboline-3 carboxylate
DZP	diazepam
FG 7142	N-methyl-B-carboline-3-carboxamide
FNL	flumazenil
FNZ	flunitrazepam
GAB_A	α-amino butyric acid
GABA-T	GABA-transaminase
GAD	generalised anxiety disorder
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
h	hour
HPLC	high performance liquid chromatography
i.d	inner diameter
i.p	interperitoneal
I.s	internal standard
i.v	intravenous
IC_{50}	concentration to inhibit 50% of 3H-FN2 binding to BZ-R
KD	equilibrium dissociation constant
l	litre

L/L	liquid/liquid
mg	milligram
min	minute
ml	millilitre
NA	noradrenaline
ng	nanogram
NSB	non-specific binding
o.d	outer diameter
ODN	octadecaneuropeptide
PBS	peripheral binding sites
pg	picogram
PK 8165	phenyl-2[(piperidinyl-4)-2-ethyl]-4-quinoline
PK 9084	1-(-2-chlorphenyl)-N-(1-methylpropyl)-3-isoquinoline carboxamide
RO 5-4864	4,-chlordiazepam
SB	specific binding
SE	solvent extraction
SPE	solid-phase extraction
SR 95531	2'-(3'-carboxy-2',3'propyl)-3-amino-6-P-methoxyphenyl pyrazinium bromide
$t_{1/2}$	half life
TBPS	t-butyl bicyclophosphorothionate
TNM	tetranitromethane
TPZ	triazolopyridine
v/v	volume/volume
vol	volume
w/w	weight/weight

1. AIMS

The aims of this research were:

1. To find the optimal incubation time and to obtain the association curve where benzodiazepine receptor and ^{3}H flunitrazepam reach equilibrium and to determine the stability period of receptor/ligand equilibrium.

2. To determine the location of the highest densities of benzodiazepine receptors in human brain tissue.

3. To study the effects of factors such as age, sex, causes of death and post-mortem conditions (delay-time interval between death and autopsy) which might affect the receptor molecules.

4. To study the effect of benzodiazepine drugs on benzodiazepine receptor densities.

5. To develop a single solid-phase extraction method for the determination of benzodiazepine drugs in human post-mortem whole blood.

6. To develop a single solid-phase extraction method for the determination of benzodiazepine drugs in human post-mortem brain tissue.

7. To compare solid-phase extraction methods with conventional solvent extraction methods.

8. To compare and correlate the concentrations of benzodiazepine drugs in human post-mortem blood samples from different sites (jugular vein, axillary vein, femoral vein and heart).

9. To compare and correlate the concentrations of benzodiazepine drugs in human post-mortem blood samples to their concentrations in brain tissue.

2. SUMMARY

A brief introduction is given to benzodiazepine drugs and benzodiazepine receptors, particularly in human brain tissue.

Human post-mortem brain tissue samples and their authentic blood samples were collected at the City Mortuary, Glasgow.

Benzodiazepine receptor binding sites were studied in human brain tissue samples. The study revealed that the incubation period for maximum benzodiazepine binding was between 30 and 60 minutes. The maximum regional distribution of benzodiazepine receptors was investigated and the highest densities of bound drug receptors were found to be in the frontal cortex area. Intermediate densities were found in most other areas (post-central, occipital and hippocampus) and the lowest densities were found in the cerebellum cortex and temporal cortex. No significant statistical variations in concentrations of receptor binding sites were found with age or sex. A considerable drop in the binding site concentration in subjects who died from a variety of causes of death was found only where they were shown to have been treatd with benzodiazepine drugs prior to death.

Methods for extraction and analysis of benzodiazepine drugs were investigated. The developed methods were quick, easy and reproducible.

A solid-phase extraction procedure was developed to detect, identify and quantify the benzodiazepine drugs (triazolam, temazepam, desmethyl-

diazepam and diazepam) in whole post-mortem blood samples using High Pressure Liquid Chromatography. The extraction was performed by elation of the drugs with chloroform from buffered blood adsorbed on Bond Elut C_8 columns. The separation time for the five chosen drugs was less than 14 minutes on HPLC operated at a flow rate 1 ml/minute. From a total of 1 ml of post-mortem blood, the lower detection limit was 50 ng/ml for all the drugs investigated. The relative recoveries were $101 \pm 3\%$ for triazolam, $88 \pm 3.5\%$ for temazepam, $86 \pm 2\%$ for desmethyldiazepam and $93 \pm 4\%$ for diazepam at that level.

Comparison of the extraction efficiency of the developed method (solid-phase extraction) with liquid-liquid extraction method was carried out for human post-mortem blood samples results. The solid phase method was found to be more efficient than liquid-liquid extraction.

Having established the highest densities of benzodiazepine receptor sites in human brain tissue, the concentration of benzodiazepine drugs in human brain tissue samples was determined by HPLC. The levels found were compared to the levels found in human post-mortem blood samples taken from the same subjects. For this purpose a solid-phase extraction procedure for human brain tissue was developed. It can be used to detect, identify and quantify benzodiazepine drugs in human brain tissue samples using HPLC. The drugs were eluted with chloroform from buffered brain tissue (one gram) adsorbed on Bond Elut C_8 columns. As before, the separation time was less than 14 minutes on the HPLC system operated at flow rate 1 ml/minute. From a total of one gram of human brain (pre-central cortex tissue) the lower detection limit was 10 ng/gram for all the

drugs investigated. The relative recoveries were 101 $\pm$ 2.9% for triazolam, 84 $\pm$ 7% for temazepam, 95 $\pm$ 8% for desmethyldiazepam and 95 $\pm$ 7.5% for diazepam. Higher benzodiazepine concentrations were obtained from blood compared to brain samples.

3. BENZODIAZEPINES - LITERATURE REVIEW

3.1 INTRODUCTION

The first benzodiazepines were synthesized in the 1930s but were not systematically evaluated until 20 years later. The prototype, chlordiazepoxide, tamed animals and had muscle relaxant and sedative properties. Given to chronic schizophrenic patients, it alleviated their anxiety without altering their psychotic features. Chlordiazepoxide was extensively evaluated in anxious patients and marketed in 1960 (Lader Malcolm, 1983). Diazepam was marketed 3 years later. Since that time a large number of benzodiazepines have been synthesized and tested for activity, and more than 20 have been introduced into clinical use.

One of the most fascinating phenomena related to the benzodiazepines has been the rapid acceptance of these drugs by clinicians and by patients. Only 16 years after the introduction of the prototype, chlordiazepoxide or only 13 years after the introduction of diazepam, the latter was itself ranked second amongst the most prescribed drugs in England, only surpassed by paracetamol (Blaha & Bruckmann, 1983). Six benzodiazepines accounted for 7% of all prescriptions in England in 1976 (Blaha & Bruckmann, 1983). Similar data have been reported for many other western countries (Blaha & Bruckmann, 1983; Muller-Oerlinghausen, 1986). Estimates prepared for several European countries suggest that between 10 and 20% of the adult population take a benzodiazepine drug at least once a year, with quite impressive differences between some of the countries (Lader, 1978,

Bellantuono et al., 1980; Blaha & Bruckman, 1983; Muller Oerlinghausen, 1986). It seems highly unlikely that any use of drugs to such an extent would be justified medically. Accordingly, several authors have expressed major concerns about what they call a medical misuse of benzodiazepines (Nolan & O'malley, 1988; Lader, 1978; Tyrer, 1984; Catalan & Gath, 1985). The concern about the obvious misuse of benzodiazepines has been greatly enhanced by the realization during the last few years that problems of drug dependence occur more frequently than had been thought for the many years of clinical use of these drugs (Schoff, 1983; Tyrer et al., 1983; Ashton, 1984; Tyrer, 1984; Catalan & Gath, 1985).

3.2 INDICATIONS

It is obvious that rapid acceptance by the medical profession would not have taken place if the benzodiazepines were not highly active and also relatively safe. They are used today for a broad spectrum of therapeutic indications:(Table 1) (Shader & Greenblatt, 1981; Shader, 1981; Gilman et al., 1985; Gilman et al., 1990; Lader, 1989; Livingston, 1991,; Katzung, 1987; Gillies, 1986).

1. Anxiolytic

Although such hypnosedatives as meprobamate and the barbiturates are powerful agents, they are effective only in doses which produce drowsiness and impairment of mental and physical performance. The benzodiazepines exert a more specific effect on the limbic system, and can therefore reduce anxiety in doses which do not produce somnolence. Nevertheless, large doses are sedative and one of their main uses is as hypnotics.

2. Anticonvulsant

Benzodiazepines raise the threshold for electrically induced fits and those due to Leptazol or local anaesthetic in experimental animals. In clinical practice diazepam, clonazepam, lorazepam and nitrazepam are effective in several forms of epilepsy.

3. Muscle Relaxant

Decreased voluntary muscle tone occurs in normal individuals and in patients with spasticity. In therapeutic doses, neuromuscular transmission is not affected but there is inhibition of polysynaptic reflexes within the spinal cord. This muscle relaxation is due to facilitation of inhibitory GABA receptors in the cord. Although this action may be beneficial in spasticity and muscle spasm due to pain, post-anaesthetic respiratory depression can be prolonged in patients given curare and a benzodiazepines.

Table 1 : The Most Prescribed Benzodiazapines.

Drug	Daily dose mg.	Time half -life Parent compound (h)	Indications
Short acting			
Triazolam	tb. o.125 - 0.25	2 - 4	Hypnotiic
Midazolam	i.v 2.5 - 7.5	2	Psychosedation
Lormetazepam	0.5 - 1	10	Hypnotic
Intermediate acting			
Temazepam	5 - 60	5 - 20	Hypnotic
Oxazepam	tb. 30 - 180	5 - 20	Anxiety
Lorazepam	tb. 3-10,i.v & i.m 1 - 4	10 - 20	Anxiety, premedication anticonvulsant
Long acting			
Diazepam	tb. 6 - 40, i.v 5 - 30	20 - 50	Anxiety, hypnotic, muscle relaxant, anaesthesia.
Clonazepam	tb. 4 - 8, i.v 1	30	Anticonvulsant
Chlordiazepoxide	10 - 25	30	Hypnotic, premedication
Nitrazepam	tb., cap., 2.5 - 10	24	Hypnotic
Prazepam	tb. 10 - 60	40 - 100	Anxiety

3.3 MECHANISM OF ACTION

Since an exact knowledge about the mechanism of action is one of the fundamentals of rational drug therapy, especially if the drugs are as frequently used as the benzodiazepines, to know how benzodiazepines act is important even for their therapeutic use. Moreover, the search for the molecular mechanism of action of the benzodiazepines was always motivated by the hope of finding a key for a better understanding of the biological basis of action.

The mechanism of action of benzodiazepines was not known until 1977, when Mohler and Okado and Squires and Braestrup described the characteristics of a benzodiazepine binding site in the rat brain, known as a benzodiazepine receptor. Shortly after it was recognized that these sites are present in high densities in some regions of the human brain (Braestrup et al, 1977). The benzodiazepine receptor have been intensively studied in laboratory animals and have been characterized in terms of their pharmacology, physiology, anatomy, immunology and biochemistry (Mohler et al., 1986).

Much less is known about this receptor in the human brain (Zezula et al., 1988). Biochemical assays have demonstrated the presence of benzodiazepine receptors in human brain with pharmacological and molecular properties very similar to those described in the rat brain (Braestrup et al., 1977, Mohler & Okado, 1978, Richards et al., 1986, Speth et al., 1978)

3.4 PHARMACOKINETICS

The pharmacokinetics of benzodiazepines (Figures 1, 2, 3) are described in detail in several recent reviews (Klotz, 1983, Greenblatt et al., 1981, Greenblatt et al., 1983, Laurence and Bennett, 1987, Levine and Clark, 1983, Lewis, 1980, Modell, 1984). The various benzodiazepines (Table 1) can be classified according to their plasma half lives as short, intermediate and long-lived drugs.

Four routes of metabolism - dealkylation, oxidation, reduction, and glucuronidation - account for the principal metabolic pathways of various benzodiazepines. Several routes may be employed with some compounds, producing a succession of active metabolites. Chlordiazepoxide, for instance, undergoes first dealkylation, then oxidation and then reduction to form a common metabolite of many of these agents, N-desmethyldiazepam (nordiazepam).

Some drugs on the market are essentially pro-drugs of nordiazepam, very quickly being converted from parent compound to that active metabolite. This may occur either by dealkylation (prazepam) or by hydrolysis (Clorazepate dipotassium). Drugs that have a hydroxy group at the 3-position, such as oxazepam and lorazepam, form no active metabolites but are directly conjugated.

Figure 1 Biotransformation of Triazolam.

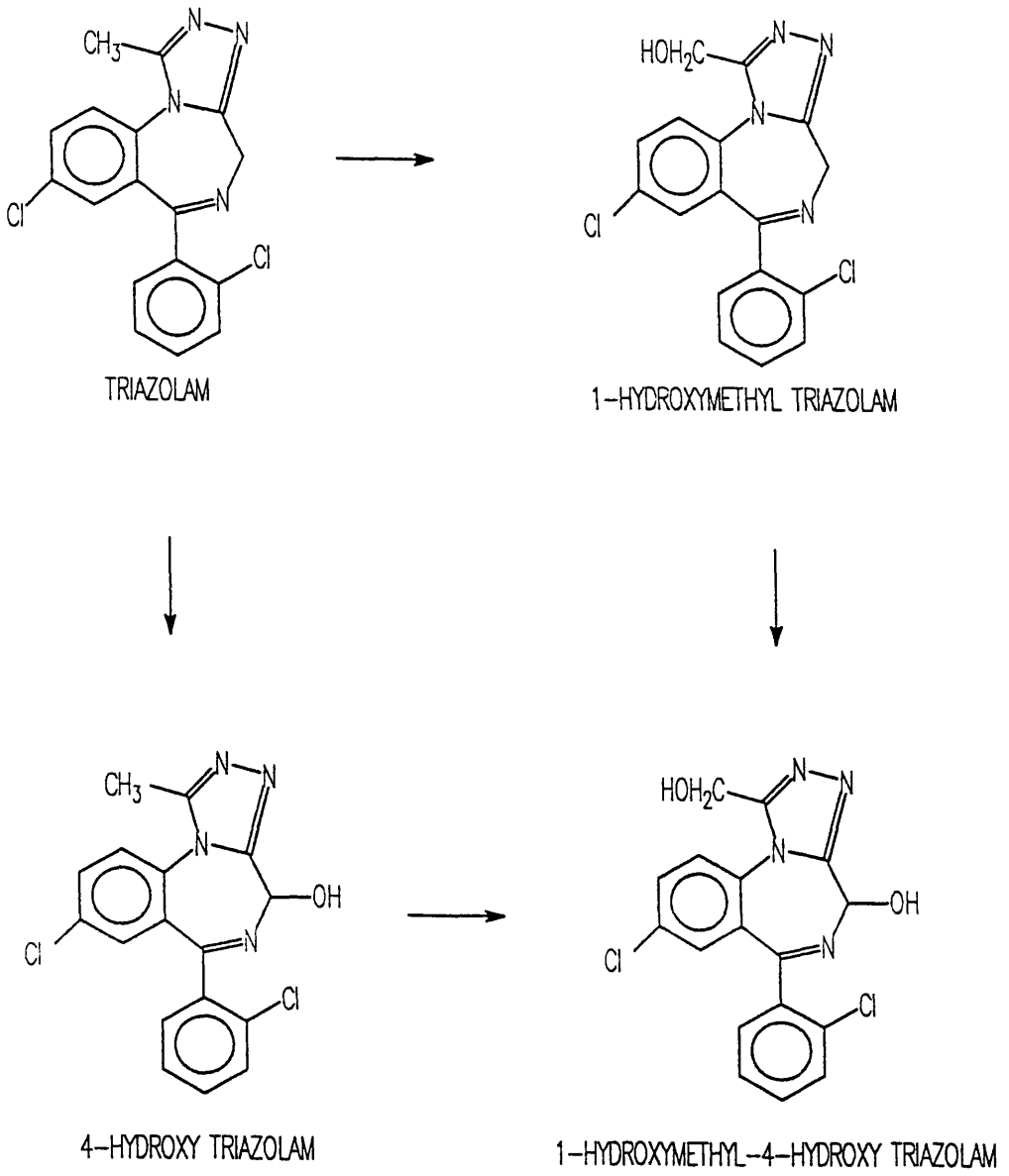


Figure 2 Biotransformation of Diazepam.

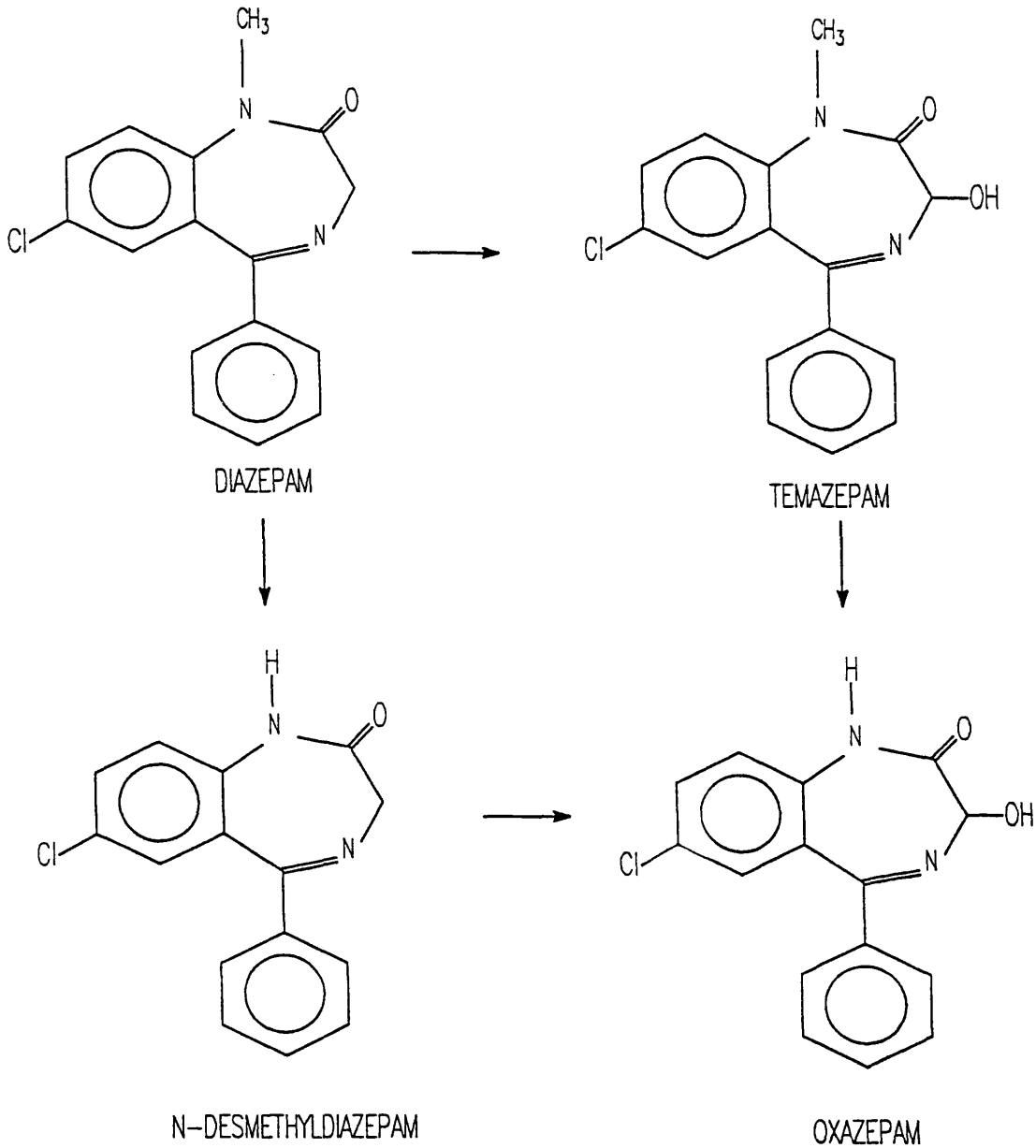
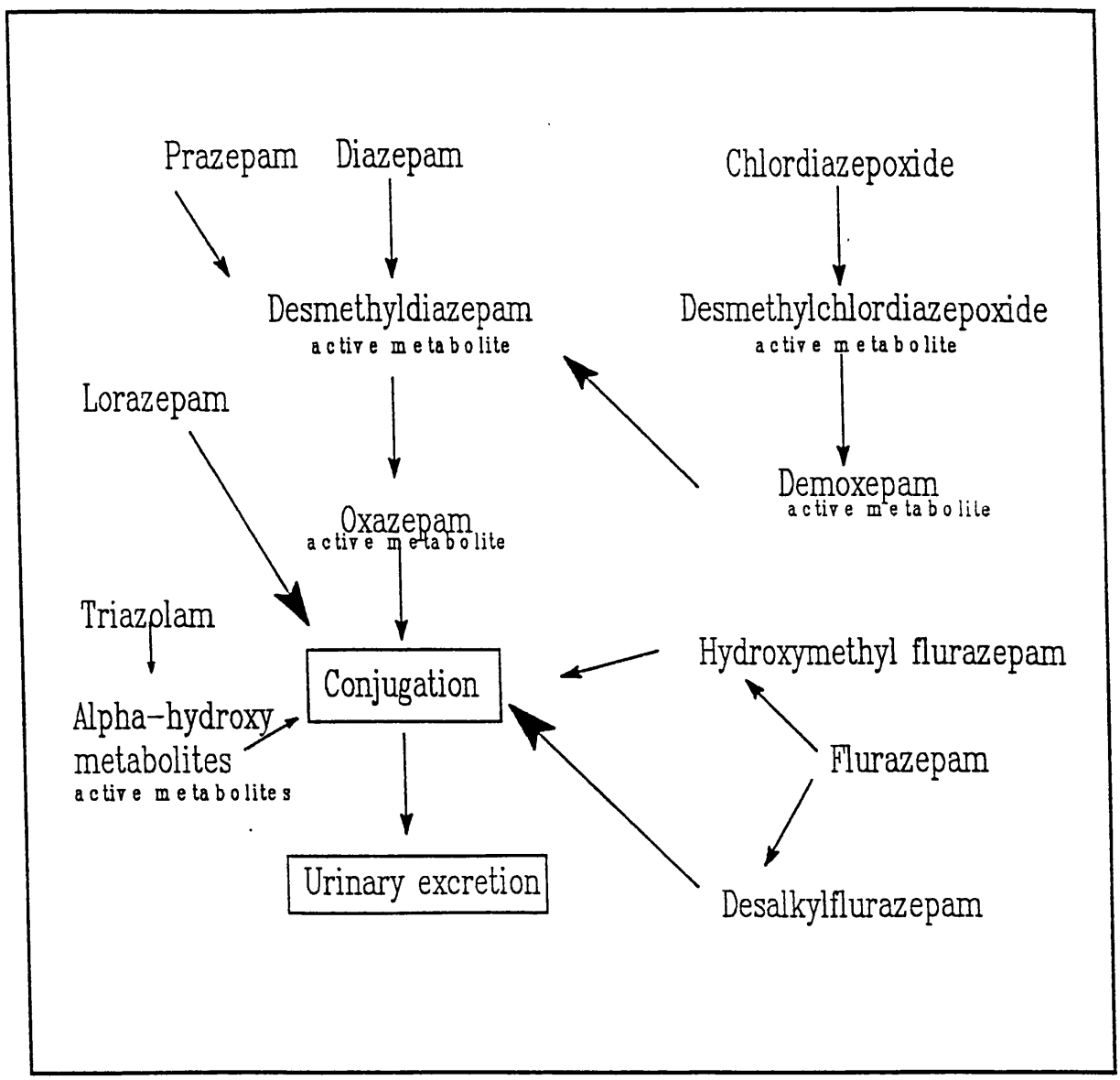


Figure 3 Biotransformation of Benzodiazpines.



3.5 ABUSE OF BENZODIAZEPINES

The first report about the abusers of benzodiazepines stated that up to 40% of narcotic abusers were 'diazepam users' (Woody et al., 1975). Many studies were carried out later indicated that most, if not all, of the benzodiazepines that are readily available, are abused (Marks, 1978, updated in 1983, Marks, 1983a, b). Benzodiazepines are the UK's most prescribed drugs. They are mainly prescribed as tranquillisers for anxiety problems but also as hypnotics for insomnia. Combined with other medicines, benzodiazepines are also used to treat a host of physical conditions in which anxiety and emotions are thought to play a part (The Institute for the Study of Drug Dependence, 1991).

Most benzodiazepine prescriptions are long term repeat prescriptions (Cooper, 1987). In 1988 the Committee on the Safety of Medicines recommended that benzodiazepines be prescribed only in the lowest possible doses for up to four weeks (I.S.D.D., 1987). It has been estimated that a quarter of UK adults take benzodiazepines at sometime during a year (Ashton et al., 1989) and that 3% - 1.2 million people - take hypnotics or sedatives (around 90% of these are benzodiazepines (Cooper, J., 1987) throughout the year. The proportion of women taking benzodiazepines long term (4%) is twice that for men (Ashton et al., 1989). It is widely held that much benzodiazepine prescribing is unjustified, inappropriate and, if prolonged, ineffective for the treatment of anxiety.

Growing awareness among GPs of the limitations and risks of benzodiazepines has resulted in a fall in the number of prescriptions in Great

Britain every year since 1979, from just over 30 million to just over 18.5 million in 1990 (Personal Communication from Department of Health, 1991).

Navaratnam, 1982, found that 22 out of 57 countries indicated the existence of some benzodiazepine abuse, but of these only 16 suggested that there was a problem causing public health concern. The abuse involved 22 different benzodiazepines (if illicit traffic is taken as an indication of abuse). The data tends to indicate that benzodiazepine abuse is minor compared to the general drug abuse problem or even opiate abuse. Several countries stressed that benzodiazepine abuse was a secondary component to the problems associated with narcotic drugs.

It has been known that alcoholics are users of sedative compounds for some time now (previously barbiturates - Devenyi and Wilson, 1971). However, the extent of benzodiazepine use along with alcohol abuse has only recently been quantified (Busto et al., 1983a). Alcoholics are normally unreliable witnesses (Devenyi and Wilson, 1971, Orrego et al., 1979) as are narcotic abusers (Dally, 1983).

3.6. BENZODIAZEPINES AND THE LAW

The majority of countries, both industrially developed and those of the Third World, now have laws which cover control of benzodiazepine use in clinical medicine. The regulations usually restrict availability to prescription, either not repeatable; limited in the number of repeats; or limited as to the period over which repeats may be dispensed by pharmacists (Marks, 1985). In the United Kingdom benzodiazepines are prescription only medicines and are controlled under Class C of the Misuse of Drugs Act, 1971 (Japp et al., 1988).

3.7 CONCLUSIONS

No data are available concerning benzodiazepine receptor and clinical effects in man and since we know from clinical experience that ataxia and muscle relaxation are usually seen only after high doses of benzodiazepines, it might be speculated that similar, but certainly not identical correlations exist between receptor occupancy and clinical effects or side effects in man.

It has now become possible to study the receptors occupancy in the human brain, which is the critical determinant for biological response. This will help to determine to what extent blood level measurements are a predictor for receptor occupancy in the brain.

Only a few workers have dealt directly with the development of reliable methods for the routine screening and subsequent confirmatory and quantitative analysis for benzodiazepines in whole post-mortem blood and human brain tissue (pre-cortex area, frontal lobe) samples.

The objective of this study was to deal directly with the development of reliable methods for the routine screening and subsequent confirmatory and quantitative analysis for benzodiazepines in whole blood and human brain tissue (pre-cortex area) samples.

3.8 BENZODIAZEPINE RECEPTORS IN HUMAN BRAIN

3.8.1 RECEPTOR COMPONENTS

Receptors may be defined as macromolecules to which a hormone, neurotransmitter, or neuromodulator attaches. Receptors are located either in association with the plasma membrane, such as those for neurotransmitters and some hormones, or with cytoplasmic or nuclear compartments, such as steroid hormone and T3 receptors, respectively. Receptor activation regulates a variety of cellular activities, including the degree of ion permeability, enzyme activity, or the rate or type of transcription and translation. Thus, receptors may be viewed as the primary site for regulating cellular activity in all organs and tissues.

Neurotransmitter receptor proteins are synthesized in the neuron, transported in small vesicles and inserted into the plasma membrane. Eventually the receptors are returned to the cytosol by endocytosis and destroyed by lysosomal enzymes. A common end point for neurotransmitter receptor function is a change in membrane permeability to ions. The basic units of a receptor are the transmitter recognition site and an ion channel. It is unclear whether the recognition site and ion channel are parts of the same molecule, or are separate constituents of a supramolecular complex. Evidence suggests that ion channels may be associated with more than one type of recognition site. There are at least two ways in which recognition site activation leads to channel opening. One is through a direct coupling between

the recognition site and the ion channel, and the other by way of a cyclic nucleotide system.

It also appears that there are membrane constituents that can regulate recognition site number and affinity. These phospholipid or protein modulators are capable of rapidly altering the availability of receptors for neurotransmitters. Because receptors are composed of a number of interacting units, there are a number of ways in which they can be modified (Enna, 1983, Ganong, 1981, Devlin, 1986).

3.8.2 BENZODIAZEPINE RECEPTORS

In 1977 two groups reported specific, high affinity receptors for benzodiazepines (Braestrup and Squires, 1977; Mohler and Okada, 1977). These receptors definitely have been shown to be functionally linked to GABA receptors. GABA and its analogues enhance the binding of benzodiazepines to their receptors (Tallman et al., 1978; Supavilai and Karobath, 1980), and benzodiazepines enhance GABA activation of chloride channels (Gallager, 1978; Study and Barker, 1981). Under certain conditions it also appears that benzodiazepines may be able to enhance GABA receptor binding (Guidotti et al., 1978; Skerritt et al., 1982). Benzodiazepine binding is also enhanced by chloride ion (Costa et al., 1979), and both GABA and benzodiazepine agonists are able to protect the receptors for other classes of compounds against heat inactivation (Gavish and Snyder, 1980).

Several compounds have been found to interact with the benzodiazepine receptors in quite interesting ways. The benzodiazepine Ro 15-1788 appears to antagonize the pharmacological effects of the classical benzodiazepines, such as diazepam, being able to reverse their anticonvulsant, sedative, and ataxic effects (Hunkeler et al., 1981). The compound CGS 8216 appears to behave in a very similar manner (Czrink et al., 1982). Another group of compounds, the beta-carboline-3-carboxylate esters, not only antagonize several effects of benzodiazepines, but can act as convulsants or preconvulsants on their own (Oakley and Jones, 1980; Cowen et al., 1981; Braestrup et al., 1982). Actions of these (inverse agonists) can also be blocked by the (antagonists) such as Ro 15-1788 (Ploc et al., 1982). It has also been found that GABA, which enhances the binding of benzodiazepine agonists, decreases the binding of the inverse agonists to the benzodiazepine receptor (Braestrup and Neilsen, 1981).

The interactions of some of these compounds with the benzodiazepine receptor have revealed that at least two subtypes of this binding site exist in brain. Specifically, the triazolopyridazine anxiolytics, CL218,872, and some beta-carboline-3-carboxylate esters inhibit benzodiazepine binding in a biphasic manner (Klepner et al., 1979; Neilsen and Braestrup, 1980). Both types of ligand bind with greater affinity to the receptor subtype that predominates in the cerebellum, termed (Type 1 or benzodiazepine 1). Certain benzodiazepine agonists also bind preferentially to the type 1 receptor subtype, which appears to correspond to distinct peptide on SDS gels (Sieghart et al., 1983). The two subpopulations differing in affinity for triazolopyridazines also appear to be two different proteins on the basis of ontogeny, regional localization (Young et al., 1981) detergent solubilization

properties, and sensitivity to modulation by ions. When investigated by radioligand, the betacarbolines directly demonstrate biphasic dissociation curves in those brain regions which show both Bz_1, and Bz_2 receptors, such as the hippocampus and the cerebral cortex (Gee et al., 1983). GABA was found to enhance the affinity of benzodiazepine agonist binding to the Bz1 receptor of the cerebellum and cortex as well as the Bz2 receptors of the hippocampus and cortex, while the Bz1 receptors in the hippocampus were apparently less affected (Gee et al., 1983). Several workers (Gee et al., 1983, Braestrup et al., 1982) interpret the apparent heterogeneity of benzodiazepine receptors on the basis of different allosteric states of a single receptor protein, modulated by ligands differing in efficacy.

Insights into the molecular nature of the benzodiazepine receptors have been provided by the ability of certain benzodiazepines to be used as specific, irreversible photoaffinity labels of the receptor. Benzodiazepines with a nitro group in the 7 position of the conjugated ring, such as flunitrazepam and clonazepam (Mohler et al., 1980; Sieghart and Mohler, 1982), are chemically activated by ultraviolet light and bind covalently to the receptor (Sherman-Gold, 1983). When maximal amounts of [^{3}H] flunitrazepam are incorporated into membrane-bound benzodiazepine receptors in this way, about 25% of the total number of receptors present are labelled with tritium (Mohler et al., 1980 Karobath and Supavilai, 1982). The remaining receptors cannot be detected upon assay of the membranes with labelled benzodiazepine agonists (nonradioactive flunitrazepam being used for photoaffinity labelling). If, however, labelled Ro15 1788, CGS 8216, or a beta-carboline is used to assay the membranes, the receptors can still be detected and show unaltered affinity and a decrease of only 25% or less in numbers (Hirsch, 1982; Thomas and

Tallman 1983). Benzodiazepine agonists are able to displace radioactive beta-carboline binding to photoaffinity-labelled membranes, but with greatly reduced affinity compared to their effects in control membranes. Thus it appears that the benzodiazepine agonist interacts with these receptors in a way that involves a functional complex of several binding sites, with the photolabelling of one site leading to a decreased affinity for agonists in three more sites. The antagonists or inverse agonists do not seem to invoke such receptor interactions. Indeed, when an antagonist is used for photolabelling, more than 90% of the sites could be labelled, and sites not labelled had unaltered affinities for both agonists and antagonists (Mohler et al., 1984a). A further interesting interaction is that the 25% covalent incorporation 75% loss of affinity is not seen in detergent - solubilized extracts, where greater than 50% of the available sites are covalently photolabelled with [^{3}H] flunitrazepam (Thomas and Tallman, 1981).

The assignment of different benzodiazepine actions to receptor subtypes is further undetermined by the introduction of imidazopyridines, zolpidem and alpidem. Zolpidem shows a seventy-fold selectivity for type I receptors, but is hypnotic. Alpidem is a non-sedative anxiolytic with high affinity for both type I central receptors and the 'peripheral type' receptors (Langer et al., 1990). It would seem that there is some evidence for bz receptor multiplicity. The selectivities of ligands for the proposed subtypes are, however, not very high. Furthermore, different benzodiazepine ligands seem to have subtly different selectivities for different variants of the subunits of the GABA receptor complex (Luddens and Wisden, 1991). It may be that subunit composition is responsible for the benzodiazepine receptor for the

benzodiazepine receptor subtypes, and that the situation is far more complex than that outlined above.

3.8.3 ALTERATION OF BENZODIAZEPINE RECEPTORS IN MAN IN PATHOLOGICAL CONDITIONS

Because of the clinical efficacy of benzodiazepines and GABA-mimetics, it has been postulated that an alteration of one or more components of the benzodiazepine/GABA receptor complex may occur in some of the disease states where these drugs are of therapeutic value. Consequently, an understanding of these receptor changes in the central nervous system (CNS) might provide mechanistic insight into the disease process and lead to more effective therapeutic interventions. Several approaches have been taken to test whether alteration in benzodiazepine or GABA binding are affected in clinical entities. One approach attempts to measure the binding of different ligands to these receptors in animal models of disease. A second approach is to perform similar binding studies in human tissue obtained either post-mortem or through surgical resection. In both animal and human studies, receptor binding can be assayed either in homogenates of tissue obtained form different brain regions, or by quantitative autoradiographic techniques. A third approach, is the direct and noninvasive measurement of in vivo receptor binding characteristics in the patient using appropriately labelled ligands and currently available imaging techniques, such as a positron emission tomography (Phelps and Mazzoiotta, 1985).

Using one or more of these approaches, altered benzodiazepine and/or GABA receptor properties have been sought in a number of clinical disorders which include Huntington's disease, various types of epilepsy, Alzheimer's disease, alcoholism, Parkinson's disease and others. Table 2 shows the changes in the density of benzodiazepine receptor (B_{max}) in post-mortem brain samples of patients suffering from different mental disorders (Muller, 1987).

Table 2: Changes in the density of the Benzodiazepine receptor (B_{max}) in post-mortem brain samples of patients suffering from different mental disorders (From Walter Muller, 1987).

DISEASE	BRAIN REGION	% CHANGE OF BMAX
Depression	Frontal Cortex	±0
	Hippocampus	±0
	Frontal Cortex	±19
	Temporal Region	±0
Lesch-Nyhan Syndrome	Occipital Cortex	±0
	Temporal Cortex	±0
	Frontal Cortex	±0
	Parietal Cortex	±0
Alcoholism	Frontal Cortex	±0
Schizophrenia	Frontal Cortex	±0
Senile Dementia Alzheimer Type	Temporal Cortex	±13

3.8.4 GAMMA-AMINOBUTYRIC ACID RECEPTORS

GABA represents the most important inhibitory neurotransmitter. By using a simple chromatographic technique in 1949, Gamma-Aminobutyric Acid (GABA) was first isolated from mammalian central nervous systems. For the past twenty years few have doubted its importance as a major inhibitory neurotransmitter in the mammalian central nervous system (Roberts, 1984). All brain regions contain GABA, particularly abundant in the substantia nigra, globus pallidus and hypothalamus (Iversen and Bloom, 1972, Ottersen and Storm-Mathison, 1984). GABA is localized in nerve terminals (Neal and Iverson,, 1969) from which it can be released by depolarizing stimuli in a calcium dependent manner (Bradford, 1970). The majority of these sites involve release from interneurons, though there are some long projection pathways which use this transmitter, such as the cerebellar output neuron, the Purkinje cell (ten Brugencate and Engberg, 1969), the striatonigral pathway (Precht and Yoshida, 1971; Nagai et al 1978) and the nigrotectal pathway (Garcia-Munoz et al 1977) (for reviews see Snodgrass 1983, Roberts, 1984, and Martin, 1990).

Most of the GABA in the central nervous system is derived from glutamic acid through a reaction catalysed by glutamic acid decarboxylase (GAD). Like other neurotransmitters GABA is stored in nerve terminals and is released by a calcium dependent process following depolarization (Ryan and Roskoski, 1975). The liberated GABA is re-accumulated by a sodium-dependent, high affinity uptake system located on nerve terminals and glia (Iversen and Neal, 1968). Some GABA is metabolized to succinic semialdehyde by GABA transaminase (GABA-T). The functional significance

of these biochemical events is indicated by the fact that agents which inhibit GAD significantly reduce brain GABA levels and decrease the seizure threshold (Swaya et al, 1978). Conversely, drugs which inhibit GABA-T increase the GABA content, depress central nervous system activity, and raise the seizure threshold (Meldrum, 1978). Likewise, drugs inhibiting GABA transport are central nervous system depressants and provide protection against chemically induced seizures (Brehm et al., 1979). These data support the contention that GABA is an important inhibitory neurotransmitter in the brain.

Given the distribution of GABAergic neurons in brain, the selectivity of GABAergic drugs must be due to neurological differences rather than anatomical location. It is possible that more active GABAergic pathways would presumably be more sensitive to drugs inhibiting GABA syntheses, metabolism or re-uptake, with the response most likely reflecting a functional modification in these synapses. Selectivity may also occur at the level of the neurotransmitter receptor. Because GABA is a relatively flexible entity, it can assume a variety of conformations, making it conceivable that different GABA receptors recognize different molecular configurations. GABA receptors were initially defined on the basis of their sensitivity to inhibition by bicuculline and picrotoxin (Curtis et al., 1971). Recently, evidence has accumulated that there may be a group of bicuculline insensitive GABA receptors (Bowery et al., 1982). These sites are defined on the basis of their sensitivity to β-P-Chlorophenyl GABA (Baclofen), a GABA receptor agonist. Accordingly, bicuculline-sensitive, baclofen-insensitive GABA receptors are defined as GABA sites, whereas bicuculline-insensitive, baclufen-sensitive response are said to be mediated by GABA-B receptors. The third type of GABA receptors,

autoreceptor is very similar to the A subtype though delta-amino laevulinic acid is a weak agonist at the autoreceptor, but not at the GABA-A receptor (Brennan and Cantrill, 1978, Brennan et al., 1981). This third type of GABA receptor, autoreceptor, appears to function as the one which controls the release of GABA from its presynaptic terminals (Mitchell and Martin, 1978a, Snodgrass, 1978., Arbilla et al 1979, Brennan et al 1981). Other investigators have described autoreceptors of the GABA-A type (Mitchell and Martin 1978; Brennan et al 1981; Ennis and Minchin, 1988) although whether these are coupled to benzodiazepine receptors is unclear; however other results support the existence of autoreceptors of GABA-B type (Pittaluga et al 1987). Both types may occur; Floran et al, 1988 have presented evidence for differential modulation of GABA release by GABA-A and GABA-B autoreceptors in the pars compacta and pars reticulata respectively, of the rat substantia nigra.

3.9 THE BENZODIAZEPINE RECEPTOR AS A MODULATORY UNIT OF GABAergic NEUROTRANSMISSION

GABAergic inhibitory neurotransmission is mediated in the mammalian central nervous system by several kinds of GABAergic neurons and by two different types of neuronal response. If GABAergic neurons synapse pre-synaptically to axons of other neurons (axo-axonic synapses), GABA acts as a presynaptic inhibitory transmitter, probably by depolarizing the receptive axon. This kind of pre-synaptic inhibition has been found on the endings of primary afferents of spinal cord and cranial nerves. All other kinds of GABAergic synapses are axo-somatic or axo-dendritic, where GABAergic neurons might be small interneurons (Collateral inhibition or recurrent inhibition) or projecting principal neurons (Miller 1987). The GABAergic synapses in this second group mediate so-called post-synaptic inhibition and account for the majority of GABAergic synapses in the central nervous system (Simmonds, 1984). Both kinds of GABAergic inhibition (pre- as well as post-synaptic) are mediated by the same GABA receptor subtype, which operates by gating a chloride channel of the neuronal membrane. The electrophysiological response, however, is different in the case of presynaptic inhibition (depolarization) from the case of postsynaptic inhibition (hyperpolarization) due to different intracellular chloride concentration at the receptive neurons (Simmonds, 1984; Snodgrass, 1983). However, in most cases of GABA-mediated responses in the mammalian central nervous system, benzodiazepines have been shown to enhance the effects of GABA in spite of its pre- or post-synaptic locus of action (Simmonds, 1984).

Since GABAergic inhibition is mediated by changes in the chloride conductance of the neuronal membrane, benzodiazepines increase only the

frequency of chloride channel openings (Barker et al., 1984), while the duration of opening events and the channel conductance are not altered. Thus, even at the level of the GABA gated chloride channel, the effect of benzodiazepines is very specific, in as much as only one out of three functional parameters of the chloride channel is altered (Enna and Karbon, 1986).

Pharmacological and biochemical evidence also points to an involvement of GABA in the mechanism of action of the benzodiazepines (Schallek et al; 1970; Haefely et al 1981). Benzodiazepines are usually more potent antagonists against convulsions induced by agents impairing GABAergic neurotransmission than against those induced by convulsants acting via other neuronal systems (e.g. strychnine as a glycine receptor antagonist). Moreover, a large variety of effects of benzodiazepines at the electrophysiological, biochemical and pharmacological levels can be blocked by relatively low doses of GABA antagonists such as bicuculline and pictrotoxin. Finally, in a variety of experimental settings, benzodiazepine activity is profoundly lowered when the brain levels of GABA are reduced by inhibitors of GABA synthesis (Muller, 1987).

Histological data also indicates that the presence of the benzodiazepine receptor in all brain regions correlates strongly with the presence of GABAergic nerve terminals (Placheta and Karobath, 1979; Mohler and Richards 1983). The final evidence for co-localization of the benzodiazepine receptor and the GABA receptor come from the isolation and purification of a synaptic macromolecule containing both the benzodiazepine and GABA receptor (Schoch et al 1984; Haring et al 1985).

3.10 THE DISTRIBUTION OF BENZODIAZEPINE RECEPTORS

3.10.1 INTRODUCTION

The benzodiazepines (BZs) are clinically important anxiolytic, muscle-relaxant and anticonvulsant drugs (Haelfery et al., 1981). Localization of the sites of action of these drugs could provide important clues to defining neural pathways involved in the action of benzodiazepines (Nichoff and Whitehouse, 1983).

3.10.2 REGIONAL DISTRIBUTION OF CNS

Almost contemporaneously, Squires and Braestrup (1977) Mohler and Okada (1977) reported recognition sites on brain neuronal membranes in vitro, to which radio-labelled benzodiazepines (e.g. ^{3}H-diazepam) bind with high affinity and selectivity. These sites did not recognise any known neurotransmitter, including GABA. The presence of such a neuronally-localised entity in the central nervous system, apparently recognising this obscure drug molecule, suggested that benzodiazepines might exert their action (S) via the so-called benzodiazepine receptor (Waddington, 1982). In phylogenetic studies the benzodiazepine receptor appears to have a late evolutionary appearance, being absent in invertebrates and lower vertebrates but present from higher bony fishes to mammals (Neilsen et al., 1978). While originally identified in the rat, it has been characterised in the human brain (using post-mortem human brain tissues) and appear to be the most dense in

the cerebral and cerebellar cortex, of intermediate density in the hippocampus, hypothalamus and caudate/putamen and of low density in the pons and medulla; there are few sites in white matter such as the corpus callosum (Braestrup et al., 1977). However no major differences are present between benzodiazepine receptors in animal and human brains. This is supported by the data of Sieghart et al. (1985), who compared the affinity and density of benzodiazepine receptors in human and rat cortex membranes using two different radioligands and he found no differences in ligand affinity. Only small differences in maximal binding capacity were found between human and rat cortex. Moreover, when the substrate specificities of the benzodiazepine receptors in human and animal brain were compared using the inhibition constants of a variety of benzodiazepines or related compounds for specific radioligand binding, very similar K_1 (inhibition constant): values were usually found. There is a close correlation between the IC_{50} values (inhibitory concentration 50%) against specific [^{3}H] flunitrazepam binding of a large variety of benzodiazepine receptor agonists, antagonists and inverse agonists in three areas of rat and human brain. Similar findings have been reported by Mohler and Okada (1978).

Braestrup and Neilsen (1982) have emphasised however, on classical pharmacological grounds, that before such a binding protein can be validly interpreted as a receptor, some functional consequences of its occupation by a ligand must be demonstrable. The benzodiazepines exert no readily identifiable neurophysiological or neurochemical sequelae at the cellular level of their recognition site analogous to those of the known neurotransmitters. However, in animal studies the rank order of potency of a wide range of benzodiazepines in exerting several pharmacological actions in vivo correlates

well with their affinity for the brain benzodiazepine recognition site in vitro (Braestrup and Squires, 1979). These analyses have been extended to include correlations between (1) Clinical anxiolytic potency in man and affinity for the human brain benzodiazepine recognition site (Braestrup et al., 1977 and (2) Pharmacological potency and the occupancy of binding sites in animals in vivo (Paul et al., 1979; Braestrup and Neilsen, 1982). An important criterion for the functional relevance of a binding site is its stereospecificity; the pharmacologically active isomer of a compound existing as enantiomeric pairs should have the greater binding affinity. This has been confirmed in animal studies (Waddington and Owen, 1978) which have been able to show that such stereospecific benzodiazepine binding affinity extends to pairs whose anxiolytic activity in man resides preferentially in one isomer.

Such evidence does suggest that this recognition/binding site for benzodiazepines can be referred to as a receptor, and that it may be intimately involved in the mediation of many of the pharmacological and clinical actions of benzodiazepines. It is interesting to note that only a small fraction of the total number of rodent brain benzodiazepine receptors (25-50%), (Paul et al., 1979); Braestrup and Neilsen, 1982) need to be occupied for a complete anticonvulsant effect. With regard to the clinical anxiolytic, hypnotic and anticonvulsant actions of benzodiazepines, the extent of receptor occupation required for their manifestation remains unclear (Waddington, 1982)

Figure 4 Chemical Structure of Clonazepam.

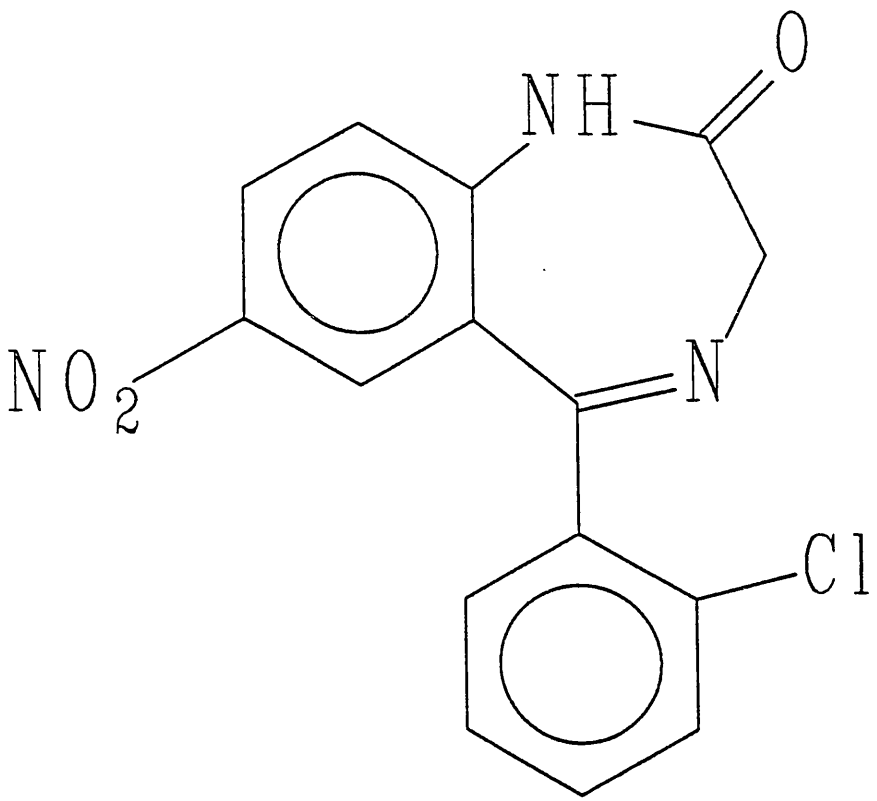
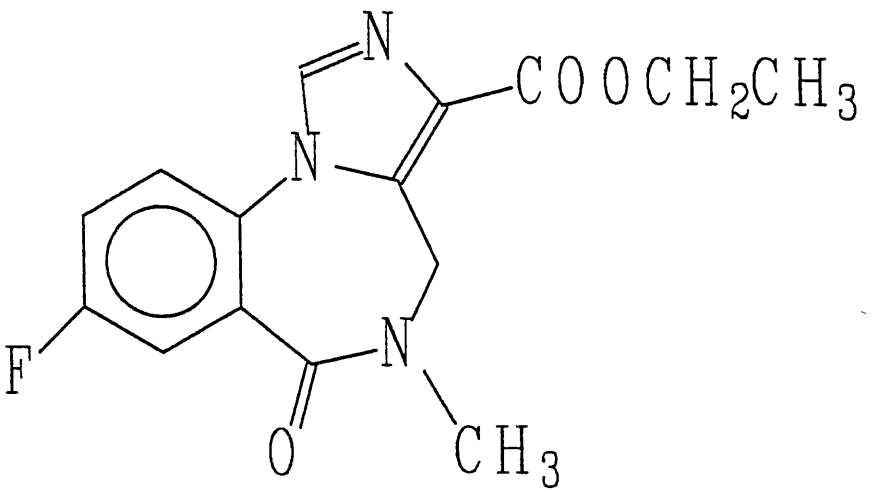


Figure 5 Chemical Structure of Ro 15-1788.



3.10.3 PERIPHERAL BENZODIAZEPINE BINDING SITES

High affinity binding sites for both ^{3}H-diazepam and ^{3}H-flunitrazepam are found in the peripheral spinal cord (Young & Kuhar, 1979), human retina (Borbe et al., 1982), pineal gland (Lowenstein et al., 1984), human pituitary (Gradison et al., 1982) and in liver, kidney, lung, heart and peritoneal mast cell (Braestrup and Squires, 1977, Mohler and Okado, 1977; Taniguchi et al., 1989; Davies and Huston, 1981; Gallager et al., 1981; Regan et al., 1981).

Peripheral binding sites are distinct from the central receptors in their subcellular localization (Anholt et al., 1986) and lack of coupling to GABA and chloride channels (Marangos et al., 1982). Both RO 5-4864 (4-chlordiazepam) and PK 11195 (an isoquinoline carboxamide derivative) bind with high affinity to the peripheral sites while their binding to the central benzodiazepine receptor is negligible (Benavides et al 1983). The binding sites in the peripheral tissues (liver, kidney, lung, heart and peritoneal mast cells), however, exhibit quite different recognition characteristics from those in the central nervous system. Clonazepam (Figure 4) with a very high affinity for the central sites, exhibits a very low affinity for the peripheral type site, while the reverse is the case for RO 5-4864 (Figure 5) a close structural analogue of diazepam that is inactive centrally. It showed a high affinity for the peripheral binding site, whereas clonazepam and the benzodiazepine antagonist RO 15-1788, centrally very potent drugs, showed no binding affinity to the peripheral site (Braestrup and Squire 1977, Mohler et al 1981, Regan et al 1981, Rosenberg et al 1979, Taniguchi et al 1980). No function has yet been proposed for these

peripheral type binding sites though they appear to be associated with the mitochondrial outer membrane (Anholt et al, 1986). The peripheral type binding site is found in the central nervous system though it may easily be resolved in standard binding assay protocols by the use of clonazepam to define non-specific binding. ^{3}H-RO-4864 has been used to study the peripheral type of benzodiazepine binding site (Marangos et al, 1982, Schoemaker et al., 1983, Miongeon et al., 1983, Beaumont et al., 1984). This isoquinoline carboxamide derivative PK11195 has also been used for the same purpose (Benavides et al, 1983).

Peripheral-type benzodiazepine binding sites exist in human brain, with some evidence for a lower density when compared with the density of experimental animals (Schoemaker et al., 1982; Owen et al, 1983). The density of these sites is slightly elevated in the frontal cortex of Alzheimer patients (Owen et al., 1983) and in the putamen of patients who died of Huntington's chorea (Schoemaker et al., 1982), but it is unchanged in the frontal cortex of patients who died of dialysis encephalopathy (Kish et al., 1985b). Since peripheral benzodiazepine binding sites in the central nervous system might occur preferentially on glial cells, their increased levels in the brains of some patients have been interpreted as a sign of gliosis (Owen et al., 1983).

3.10.4 ALBUMIN

Benzodiazepines bind with high degree of specificity, even stereoselectivity, to a single site of the human serum albumin molecule

with particularly high affinities. Since the same single site mediates the stereospecific binding of tryptophan and related compounds, this site has been termed the indole and benzodiazepine binding site (Muller and Wollerty, 1979). This site is of considerable pharmacokinetic relevance since it represents one of the two major drug binding sites of human serum albumin which are responsible for the interaction of nearly all drugs with the albumin molecule. The general properties of this site and their pharmacokinetic relevance for benzodiazepines and many other drugs have been reviewed (Muller and Wollert, 1979, Sellers et al., 1983, Muller et al., 1986a). This site is not involved in any of the pharmacological properties of the benzodiazepines except that the plasma binding of these drugs contributes to their pharmacokinetics in man (Klotzz, 1984). Thus, the indole and benzodiazepine binding site of human serum albumin clearly represents a silent receptor (a binding site without any physiological response, regardless of whether the ligand is endogenous on a drug) and represents an excellent example that specific and even stereospecific binding are not per se indicative of biological function.

3.10.5 CONCLUSIONS

Most of benzodiazepine receptors are co-localized with GABA receptors. On the other hand, since the central nervous system in man contains more GABA receptors than benzodiazepine receptors, it is generally assumed that the majority of GABA receptors are not linked to benzodiazepine receptors (Bowery et al., 1984).

The GABA receptors associated with the benzodiazepine receptors are always GABA-$_A$ subclass, which can be specifically antagonised by bicuculline. No evidence exists that GABA-$_B$ receptors (which can be specifically activated by the drug baclofen) are associated with benzodiazepine receptors (Bowery et al., 1984).

4. MEASUREMENT OF MEMBRANE BINDING SITES

4.1 EQUIPMENT AND MATERIALS

Clonazepam	Roche Products Limited
Sodium dihydrogen Orthophosphate	B.D.H. Chemicals Limited, Poole, England
Human post-mortem brains	City Mortuary, Glasgow
Teflon glass homogeniser	Jacons England
Stirrer	Griffin & George Limited, England
Sodium Carbonate	B.D.H. Laboratory Chemicals Division, England.
Copper Sulphate	B.D.H. Chemicals Limited, Poole, England
Sodium Potassium Tartrate	B.D.H. Chemicals Limited, Poole, England
Bovine Serum Albumin	Sigma Chemical Company
Sucrose	Formachem (Research International) Limited
Folin and Ciocalteu's Phenol Reagent	Sigma Chemical Company
Centrifuge	Beckman JA 2-21 with JA 20 rotor
Scintillation Counter	Packard 2200 CA Tri-Carb^{R}
Data Processing	Binding Assay - Packard Combicept^{tm} program for IBM^{R}-PCAT. RIA - Packard Securia T program for IBM^{R}-PCAT

Radioligand	N-Methyl [^{3}H]Flunitrazepam Amersham International
Filters	Whatman GF/B glass microfibre filters.
Scintillant	Ecoscint^R, National Diagnostics
Pico 'Hang-in' vials	Packard^R.
LP3 tubes	Luckham Limited., Disposable Laboratory Plastics

4.2 BENZODIAZEPINE RECEPTOR BINDING SITES STUDY

Human post-mortem brain tissue samples (43) and their authentic blood samples were collected at the City Mortuary, Glasgow. The collection of the samples was done over the period of this research to obtain both cases where death was due to benzodiazepine drugs overdose and those where no benzodiazepine drugs was involved. The collection of samples entailed visits to the City Mortuary on a daily basis, both in the morning and in the afternoon. Cases were rejected for a number of reasons including, hepatitis, AIDS, lack of blood or where only blood or brain tissue was available.

Blood samples were collected from each case under sterile conditions at the time of the autopsy in order to preclude or exclude the presence of benzodiazepine drugs.

The distribution of benzodiazepine receptors in human brain tissue (frontal cortex, precentral cortex, temporal cortex, occipital cortex, cerebellum cortex and hippocampus) was investigated to find the area with the highest benzodiazepine brain receptor concentration. Factors which might affect the binding site concentration of benzodiazepine receptors in the post-mortem human brain tissue such as incubation period, age, sex, cause of death, time of death, time interval between death and autopsy and subjects who were on benzodiazepine drug therapy prior to death were investigated.

After the protein concentration in each gram of brain tissue had been determined, estimation of the receptor densities (B_{max}) could be studied. This

was carried out by binding assay using the Packard Combicept program to translate the data for each binding.

The binding technique of high affinity binding is quite simple. The labelled ligand was incubated with suitable tissue homogenate. It uses the so-called P_2-fraction from the brain which is a relatively crude membrane preparation containing synaptic plasma membranes. After incubation, the free and bound ligand were separated by filtration through glass fibre filters. Biological membranes together with bound ligand were retained on the glass fibre filters by adsorption. The amount of bound ligand was measured by scintillation counting.

Specific binding is the amount of binding expected to occur at the receptor. Non-specific binding is the remaining binding, i.e. adsorption of the ligand to glass filters, the amount of ligand dissolved in the biological membranes, and is usually defined as the amount of bound labelled ligand which cannot be displaced by excess of unlabelled ligand. Specific binding thus, is defined as total binding minus non-specific binding.

4.2.1 PROPERTIES OF AUTHENTIC RECEPTORS

1. Saturability

The great majority of receptors are on the surface of a cell. Since there are a finite number of receptors per cell, it follows that a dose-response curve for the binding of a ligand should reveal saturability. In

general, receptor binding is characterized by a high affinity and low capacity whereas non-specific binding usually exhibits high capacity and low affinity binding which is virtually nonsaturable.

2. Specificity

This is one of the most difficult and important criteria to fulfil, not only because of the enormous mass of non-specific binding sites compared to receptor sites in tissue but also because of the avidity with which inert surfaces bind ligands. For example, substance A binds tenaciously to glass and insulin can bind to calcium powder in the nanomolar range. With agents that exist as optical isomers, it is of obvious importance to show that the binding of the ligand is stereospecific.

Specificity obviously means that one should find receptors only in cells known to respond to the particular transmitter or hormone under examination. Further, it is a truism that a correlation should be evident between the binding affinity of a series of ligands and the biological response produced by this series. This correlation is the sine qua non for receptor identification.

3. Reversibility

Since transmitters, hormones and most drugs act in a reversible manner, it follows that the binding of these agents to receptors should be reversible. It is also to be expected that the ligand of a reversible receptor should be not only dissociable but recoverable in its natural (i.e. non-

metabolised) form. This last dictum distinguishes receptor ligand interactions from enzyme substrate reactions (Cooper et al 1978).

4.3 PREPARATION OF SYNAPTOSOMAL MEMBRANES

4.3.1 INTRODUCTION

Neural tissue is composed of neurons and their support cells, the glia. Neurones are notable for their highly elaborate shapes and the possession of long and often branching processes which intermingle extensively with each other and with glia. Such cells do not survive homogenization intact; the cell bodies are sheared from their processes which break up into discrete fragments. The plasma membranes of cell fragments may reseal to form osmotically active particles and when such particles contain the organelles of the synapse they are known as synaptosomes (Gordon, 1987).

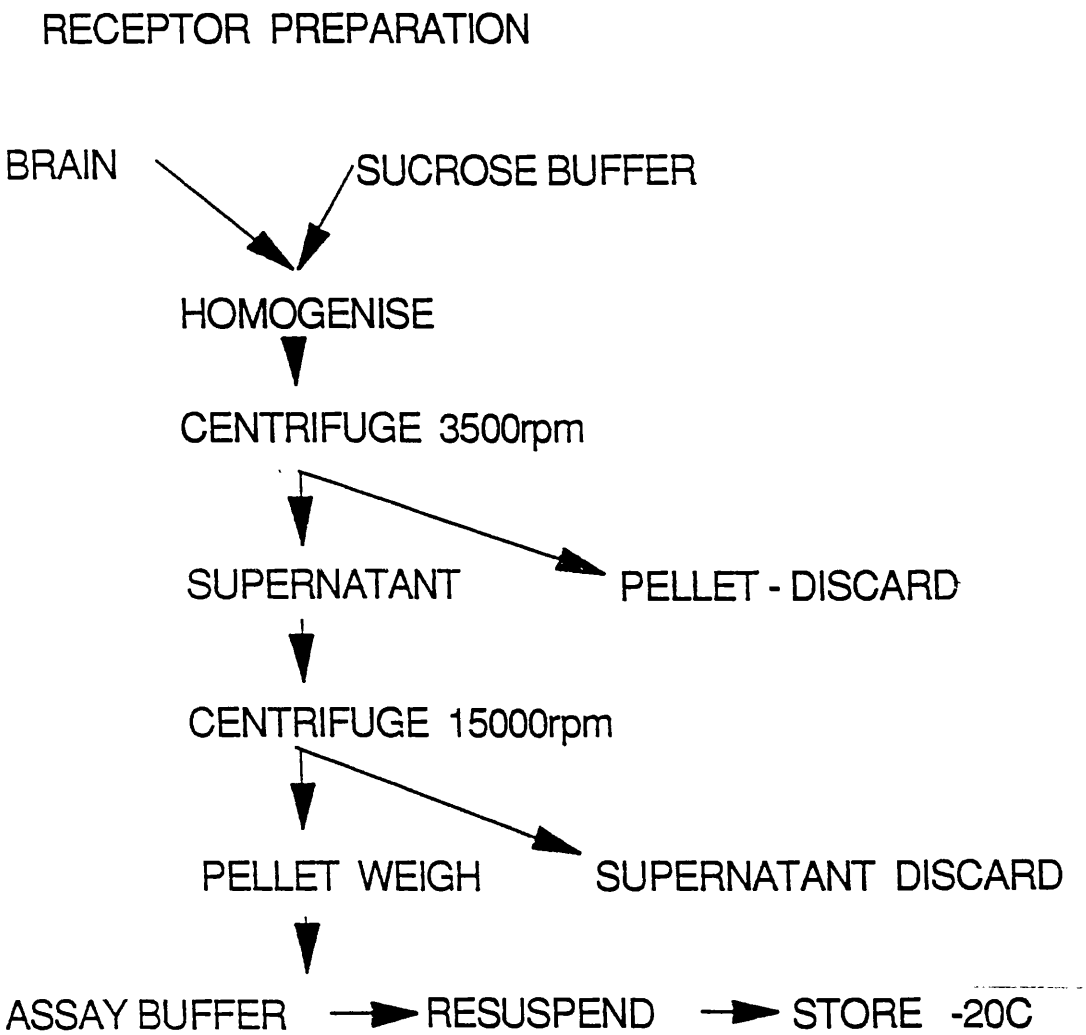
Subcellular fractions enriched in synaptosomes are sufficiently pure to permit the study of certain physiological and pharmacological aspects of synaptic function. These fractions are not, however, completely free of contaminating particles particularly axonal, myelinated and unmyelinated, and mitochondria, nor are they homogeneous in one type of synapse (Cotman, 1974). This has important consequences for the interpretation of experiments using synaptosomes for it is extremely difficult to assign a particular property of the fraction to a constituent, except under certain conditions such as when investigating the release of radiolabelled neurotransmitter that is known to accumulate only in that constituent.

Unlike synaptosomal fractions, subcellular components derived from synaptosomes such as post-synaptic densities and synaptic vesicles are sufficiently pure for biochemical analysis.

4.3.2 PREPARATION

1. One gram of post-mortem human brain tissue was homogenised in 10 *ml* of ice-cold 0.32M sucrose solution using 20 strokes of a teflon-glass homogeniser. (Sucrose is ideal in many respects as an isolation medium, the concentration used to isolate synaptosomes is hypertonic and consequently synaptosomes lose water and are very shrunken when recovered from the gradient).
2. Centrifuge at 3500 rpm for 10 minutes at 4°C.
3. Discard the pellet, the remaining supernatant is centrifuged for 20 minutes, spin speed 15,000 rpm, at 4°C.
4. Discard the supernatant and measure the weight of the pellet.
5. Homogenise the pellet in 10 *ml* of ice-cold assay buffer (25 mm sodium phosphate assay buffer, pH 7.4)
6. Transfer to a suitable vial and store at -20°C until required (see Figure 6).

Figure 6 Preparation Of Synaptosomal Membranes.



4.4 PROTEIN DETERMINATION

4.4.1 INTRODUCTION

Proteins are readily determined by the colorimetric method of Lowery et al (1951). It is carried out using bovine serum albumin to prepare the standard curve. The coloured complex which is measured in a spectrophotometer directly relates to the protein concentration. It is due to tyrosine and tryptophan residues in the protein complexing with the alkaline copper-phenol reagent. For each tissue sample, determine the protein present (in duplicate) and a standard curve (in duplicate).

4.4.2 REAGENTS REQUIRED

- Sodium carbonate, 2% w/V, in 0.1M NaOH.
- Copper sulphate (hydrate), 1% w/v, in water.
- Sodium potassium tartrate, 2% w/v, in water.

4.4.3 METHOD

(a) Tissue Sample

1. Dilute the tissue sample 1:100 with 0.5M NaOH.

2. Take 0.2, 0.4, 0.6, 0.8 and 1.0 *ml* of the diluted tissue sample and make up to a final volume of 1ml with 0.5M NaOH. Prepare a blank containing 1.0 *ml* of 0.5M NaOH instead of the tissue sample.

3. Add 5 ml of copper reagent to all samples (including the blank), mix thoroughly by vortexing, and allow to stand for 10 minutes.

4. Add 0.5 ml of 1N Folin reagent, mix immediately and completely and stand for 30 minutes.

5. Read absorbance at 540 nm on a spectrophotometer, after zeroing the instrument on the blank.

6. The tissue protein content can then be directly interpolated from the standard curve below.

(b) **Bovine Serum Albumin Standard Curve**

1. Make a stock solution of bovine serum albumin (100 $\mu g/ml^{-1}$) in 0.5M NaOH.

2. Take 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the above solution (equivalent to 0,20, 40, 60, 80, 100 ug protein ml^{-1} respectively) and make up to a final volume of 1 ml with 0.5 NaOH.

3. Process the standard curve as described on (a) above starting from (3.) and continuing to (5.).

4. Construct the standard curve by plotting absorbance against bovine serum albumin (ug) per assay (Figure 7 and Table 3).

Figure 7 Protein Concentration Curve.

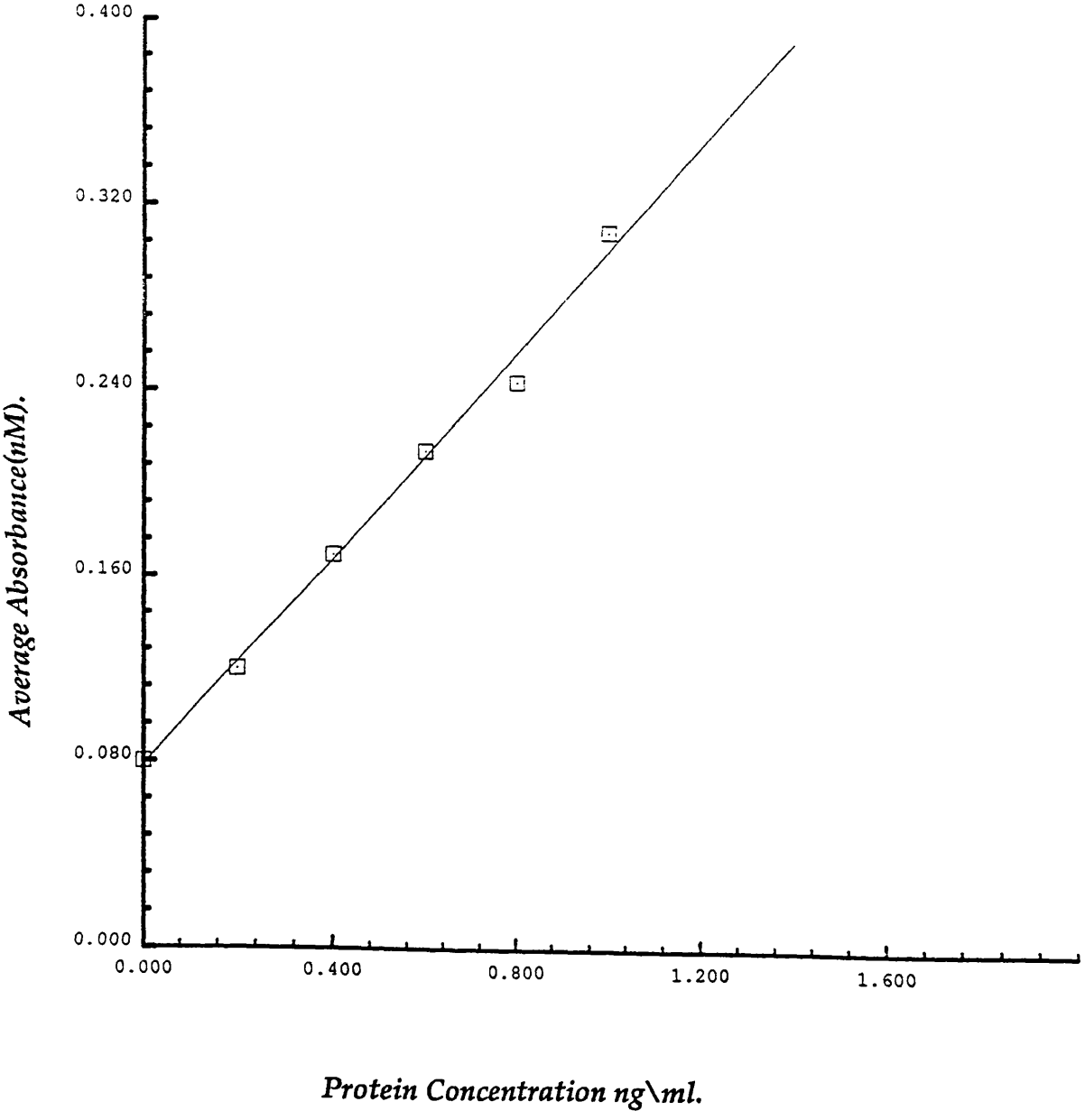


Table 3: Data For Standard Curve For Protein Assay

µg/protein/*ml*	Absorbance	Average Absorbance
0 - 0 0 - 0	0.08 0.08	0.08
0.2 0.2	0.12 0.12	0.12
0.4 0.4	0.17 0.17	0.17
0.6 0.6	0.20 0.20	0.20
0.8 0.8	0.24 0.25	0.255
1.0 1.0	0.32 0.30	0.31

NOTES

1. Prepare the stock bovine serum albumin and copper reagent fresh on day of use.
2. If the tissue protein content absorbance values read higher than the standard curve range, further dilute the tissue sample to 1:50 and repeat the determination.
3. If a spectrophotometer is not available to read 450 nm, absorbances can also be read at 750 nm.
4. Copper reagent is prepared fresh by mixing the above sodium carbonate, copper sulphate and sodium potassium tartrate solutions in the ratios of 200:2:2 by volume respectively).

Bovine serum albumin, 100 $\mu g/ml^{-1}$ in 0.5M NaOH.

OTHER MATERIALS

- vortex mixer.
- spectrophotometer capable of reading absorbance at either 750 nm or 540 nm.

After the human brain tissue was homogenised and centrifuged twice as described earlier, the weight of each pellet was determined and 5 ml assay buffer (25 mM sodium dihydrogen orthophosphate, pH 7.4) was added to each pellet and vortexed well in order to display protein concentration in each pellet (P_2 = which contain synaptic membrane) using the Lowery method.

Estimation of protein concentration of each pellet (from human brain tissue) was determined at two dilution levels (using 0.5M NaOH), $^{1}/_{10}$ and $^{1}/_{50}$, in order to obtain a more accurate determination of its concentration. Tables 4 and 5 indicate the figures from the two dilutions $^{1}/_{10}$ and $^{1}/_{50}$ of the human brain receptor preparation used, from which human brain protein concentration of the pellet can be calculated.

Table 4: U.V. Absorbance of Known Dilutions of the Receptor Preparation Pellet (protein content of 1/10 dilution of receptor preparation pellet)

Samples 1/10				
Volume of Pellet Solution *	Absorbance	Average Absorbance	mg pt/*ml*	Protein content of original sample dilution mg/ml
0.2	0.15	0.145	31	155
0.2	0.14			
0.4	0.19	0.195	54	135
0.4	0.20			
0.6	0.26	0.26	83	138
0.6	0.26			
0.8	0.32	0.315	107	133.7
0.8	0.31			
1.0	0.38	0.375	134	134
1.0	0.37			

Average 139.2

*** this volume was diluted to 1 ml with 0.5M NaOH**

Table 5: U.V. Absorbance of Known Dilutions of the Receptor Preparation Pellet (protein content of 1/50 dilution of receptor preparation pellet)

Samples 1/50				
Volume of Pellet Solution *	**Absorbance**	**Average Absorbance**	**mg pt/*ml***	**Protein content of original sample dilution mg/ml**
0.2 0.2	0.09 0.09	0.09	7	35
0.4 0.4	0.10 0.09	0.095	9	22.5
0.6 0.6	0.11 0.10	0.105	14	23.3
0.8 0.8	0.11 0.12	0.115	18	22.5
1.0 1.0	0.13 0.13	0.13	25	25

Average 25.67.

*** this volume was diluted to 1 ml with 0.5M NaOH**

4.5 EQUILIBRIUM STUDY (INCUBATION PERIOD)

4.5.1 INTRODUCTION

The object of the experiment was to find the optimal incubation time and to obtain the association curve for where the benzodiazepine receptors and ^{3}H flunitrazepam reach equilibrium. It was also designed to determine the stable period for the receptor/ligand equilibrium.

Human post-mortem brain tissue sample was obtained at autopsy from a female aged 28 years, who died from chest and abdominal injuries (RTA). A blood sample was taken from the deceased and analysed for the presence of benzodiazepine drugs using both radio-immunoassay and HPLC with negative results. The grey matter from the frontal cortex was freshly dissected taking care to avoid contamination by white matter.

4.5.2 BUFFER AND STANDARD PREPARATION

Sodium dihydrogen orthophosphate anhydrous (NaH_2P0_4 - m.wt 119.98) buffer was made up by dissolving 39.1 grams of NaH_2P0_4 in deionised water and made up to one litre producing a buffer concentration of 0.5M. This was adjusted with sodium hydroxide (Na0H) to pH 7.4. Further dilutions with deionised water were made to produce a working solution of 25 mM.

All [^{3}H] flunitrazepam (0.63-20nM) dilutions were made up with 25 mM sodium dihydrogen orthophosphate buffer, pH 7.4.

A stock solution containing clonazepam was prepared by dissolving 10 mg of clonazepam drug in a few drops of methanol and then diluting to 10 ml with deionised water to produce a stock standard clonazepam solution of 1 mg/ml. Further dilution with deionised water was made to produce a working solution of 20 uM.

4.5.3 REMOVAL OF BRAIN

The final part of the autopsy is the removal of the brain. The incision on either side of neck is continued upwards and slightly posteriorly over the top of the head. It is important to part the hair anteriorly and posteriorly on either side of the incision to avoid cutting off large pieces of hair. The front and rear flaps of the skin of the scalp are then reflected.

An incision is then made around the insertion of the temporalis muscle into the side of the skull.

The vault of the skull is removed by a saw cut through the calvaria which should be made immediately above the supra-orbital ridge and continued horizontally round the skull to the occiput until the underlying dura is reached. An electric or handsaw can be used for this purpose.

A T-piece key will usually prise the vault off the base of the skull, although a mallet and chisel might be needed to loosen imperfectly sawn places. If the dura mater adheres to the inner aspect of the vault, it can sometimes be separated with a flat dissector or the dura may have to be incised and removed with the vault. A midline sagittal incision into the dura should then be made, to open the superior longitudinal sinus. The dura is then removed by cutting into its lateral parts. The cuts are made laterally where the brain is least in contact with the dura, and is consequently less likely to be damaged as the dura is removed.

Tilting the head away from the lateral incision further prevents injury to the brain which falls away from the cut. The dura is then reflected over the top of the brain and its attachment to the skull base anteriorly in the middle line is divided. It can then be pulled backwards over the back of the brain. The brain is then removed.

This is achieved by gently raising the frontal poles and with even greater care, the delicate olfactory nerves are lifted off the base of the skull. This is the first cranial nerve and the remaining eleven pairs are divided successively.

After this has been done, the vertebral arteries on either side are cut and the lower medulla oblongata (the upper end of the spinal cord) is transected.

The brain can then be lifted gently out of the skull. Before further dissection of the brain at the time of autopsy it is first inspected carefully, then the upper pons is divided freeing the hind brain. The cerebral hemispheres

are examined by transverse cuts across them. The slices of brain can be laid out sequentially on a large tray for demonstration purposes (Gresham and Turner, 1979).

4.5.4 PROTEIN DETERMINATION

Protein concentration in each gram of human frontal cortex tissue (grey matter) was determined by the colorimetric method of Lowery as described in 4.4

4.5.5 SATURATION CURVE ANALYSIS OF [^{3}H] FLUNITRAZEPAM BINDING

The receptor binding assay used in this study for investigating the binding site concentration has been modified from Horton et al., 1982, 1988. The protein concentration of the homogenate was adjusted by dilution with 25 mM sodium dihydrogen orthophosphate buffer to produce 0.15 mg/ml.

Total Binding Assay

12 pairs of LP3 tubes were set up to enable each [^{3}H] flunitrazepam concentration to be assayed in duplicate. 100 ul ^{3}H-flunitrazepam (concentration ranging from 0.6-20 nM) was incubated with 800 ul (approximately 0.15 mg protein) of membrane preparations (see page ??) and 100 ul of 25 mM sodium phosphate buffer (pH 7.4) after each tub was

vortexed, to ensure thorough mixing. They were incubated at room temperature (20°C) for different times 0, 30, 60, 90, 120 and 150 minutes. The incubation mixture was rapidly filtered through a Whatman GF/C glass fibre filter with suction. The filter and tube were washed with an additional 5 ml of 25 mM sodium phosphate buffer (pH 7.4). The radioactivity retained on the filters were placed into pico vials, 4 ml of scintillant (Ecoscint) was added and vials mixed well. Each tube which contained the radioactivity retained on the filter was estimated by counting for one minute and gave the DPM of the bound radioligand (see Figure 8).

Non-Specific Binding Assay

Twelve pairs of LP3 tubes were set up to enable each [^{3}H] flunitrazepam concentration to be assay in duplicate. 100 ul ^{3}H-flunitrazepam (concentration ranging from 0.6-20 nM) was incubated with 800 ul (approximately 0.15 mg protein) of membrane preparations and 100 ml of clonazepam (2 uM) after each tub was vortexed, to ensure through mixing. They were incubated at room temperature (20°C) for different times 0, 30, 60, 90, 120 and 150 minutes. The incubation mixture was rapidly filtered through a Whatman GF/C glass fibre filter with suction. The filter and tube were washed with an additional 5 ml of 25 mM sodium phosphate buffer assay (pH 7.4). The radioactivity retained on the filters were placed into pico vials, 4 ml of scintillant (Ecoscint) was added and vials mixed well. Each tube which contained the radioactivity retained on the filter was estimated by counting for one minute and gave the DPM of the bound radioligand (Figure 8).

Figure 8.

BINDING ASSAY

MEMBRANE HOMOGENATE

PROTEIN CONCENTRATION

(1) HOMOGENATE+FNZ+ASSAY BUFFER

(2) HOMOGENATE+FNZ+CLONAZEPAM

MIX &INCUBATE

FILTER

WASH FILTER

COUNT TRAPPED RADIO-ACTIVITY

DATA PROCESS-COMBICEPT

4.5.6 RESULTS AND DISCUSSION

0.15 mg/ml protein concentration was used as a working concentration for the benzodiazepine binding assay study to assess the suitable incubation time of prepared receptors.

The receptor binding assay consisted of an osmotically shocked crude synaptosomal preparation which was incubated for different times 0, 30, 60, 90, 120 and 150 minutes at room temperature. After incubation the receptor - ligand complex was separated by filtration through glass fibre filters and measured to determine the maximal binding concentration (Bmax) by the combicept program which incorporates both an orthogonal weighting scheme and the Rosenthal correction for NSB (non-specific binding) subtraction (Combicept, Packard).

Subtracting the non-specific DPM results from the total bound DPM results gives the DPM for the specifically bound [^{3}H]flunitrazepam as revealed in Table 6.

These results together with the DPM of the total radioligand added are transformed by the Combicept Program, to determine maximal binding concentration as shown in Figure 9 (from data in Table 7). Using the orthogonal weighting scheme it statistically eliminates some of the errors encountered during data transformation to the saturation curve which is still recommended as the best way of analysing data for a binding study.

Figure 10 (from data of Table 8) demonstrates how long the incubates take to reach equilibrium (which is 30 minutes) and how long the incubates could be left before the equilibrium began to disrupt (which is after 60 minutes).

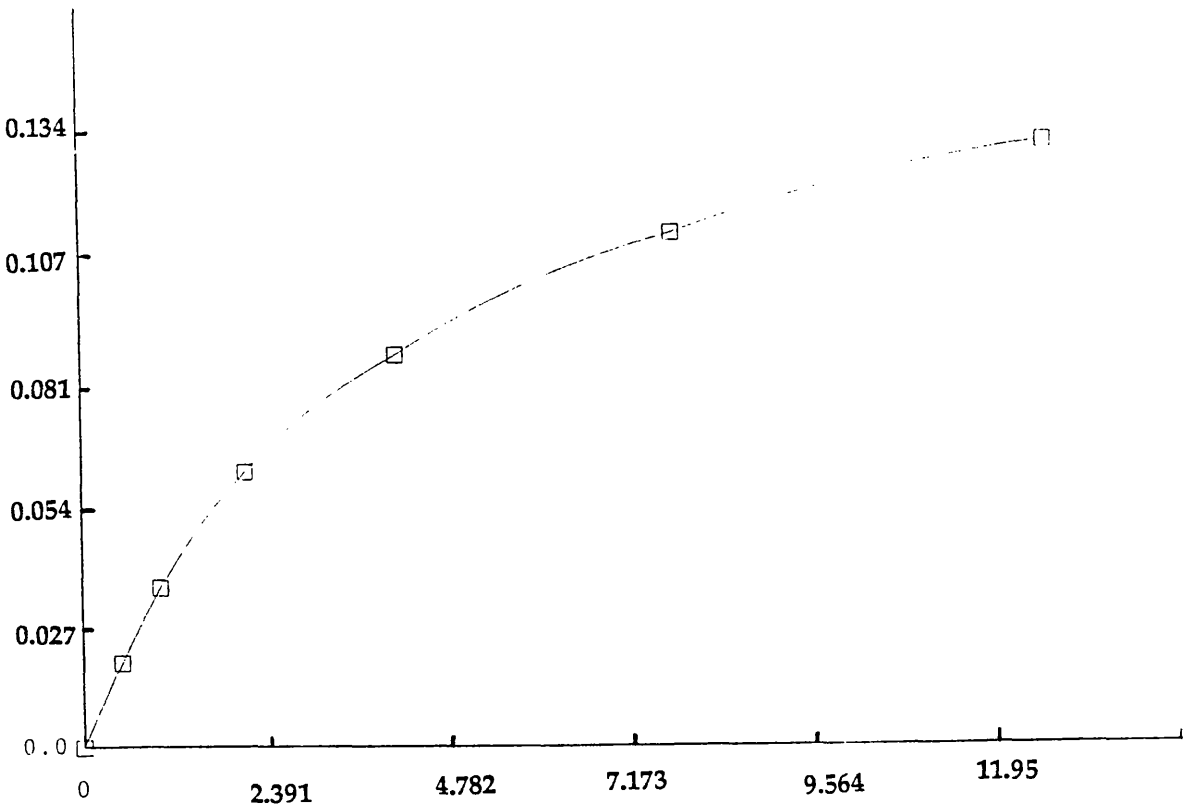
It can be seen from this experiment that the incubation period of benzodiazepine binding can be started investigated 30 minutes minimum and at 60 minutes maximum (receptor viability). This 30 minute period where the equilibrium is stable is sufficient to filter a large number of samples before the receptor/ligand binding is disrupted (radiolysis).

Table 6: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue. (Frontal cortex region).Incubation 30 minutes.

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	2512.7	χ=2644.8	81.6	χ=98.2	χ=2546.6
1	2776.8		114.7		
2	4919.4	χ=5009.9	126.6	χ=102.3	χ=4907.6
2	5100.3		77.9		
3	9215.6	χ=9147.6	125.3	χ=144.6	χ=9003
3	9079.6		163.8		
4	13631.0	χ=13496.8	324.7	χ=452.6	χ=13044.2
4	13362.6		580.6		
5	17129.4	χ=16551.5	768.1	χ=818.8	χ=15732.7
5	15973.6		869.5		
6	26734.6	χ=24117.0	1424.1	χ=1287.3	χ=22829.7
6	21499.4		1150.5		

χ= Average.
DPM= Disintegration/minute.

Figure 9 Saturation Curve
(Clonazepam).



sb-Specifically bound tritiated flunitazepam.

st-total tritiated flunitazepam.

Table 7 Experimental Binding Assay.Incubation 30 Min.
(Rosenthal NSB Correction)

Point	Sb	St
1	0.140	0.334
2	0.264	0.681
3	0.0482	1.345
4	0.071	2.581
5	0.085	5.295
6	0.122	10.868

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 10 Binding Site Concentration Against Time.

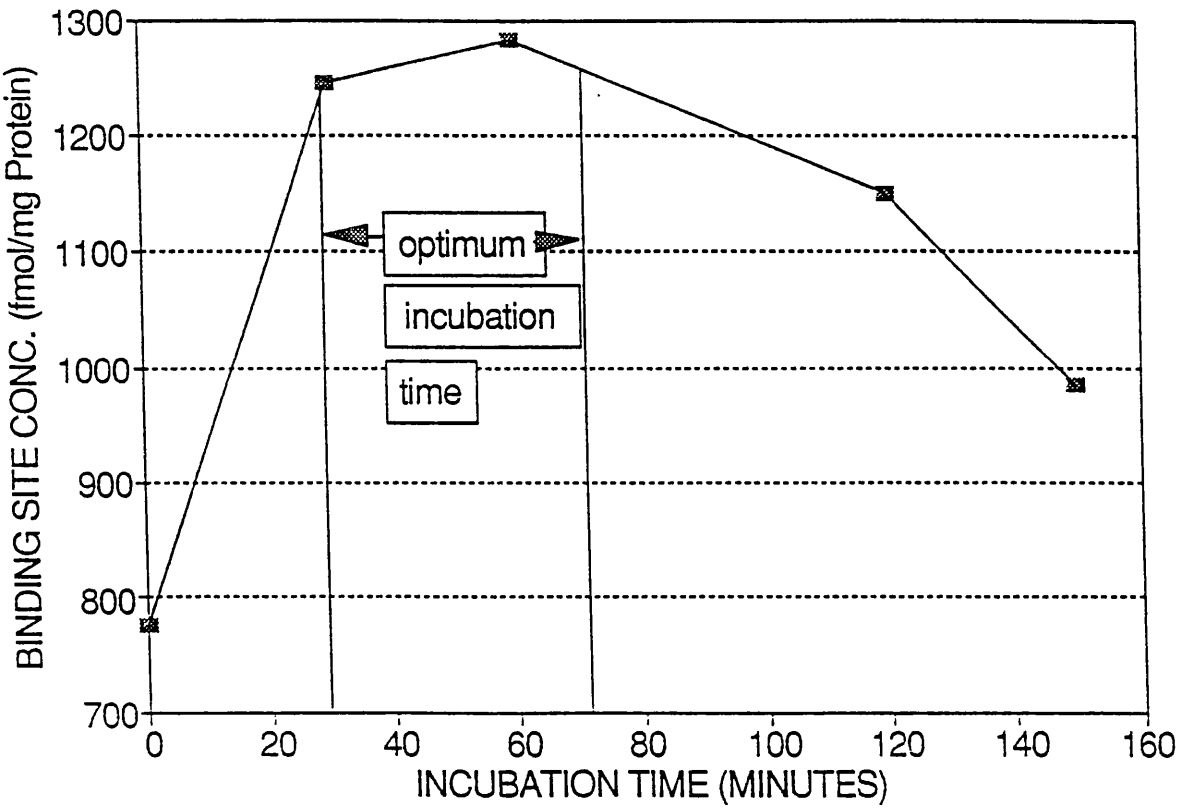


Table 8 Effects of incubation time against binding site concentration.

Time (min)	Binding site concentration (fmol/mg protein)	Average
0	800 750	775
30	1253 1238	1245.5
60	1286 1276	1283
120	1138 1162	1150
150	1067 900	983.5

4.6 REGIONAL DISTRIBUTION OF BENZODIAZEPINE RECEPTORS IN HUMAN BRAIN TISSUE

4.6.1 INTRODUCTION

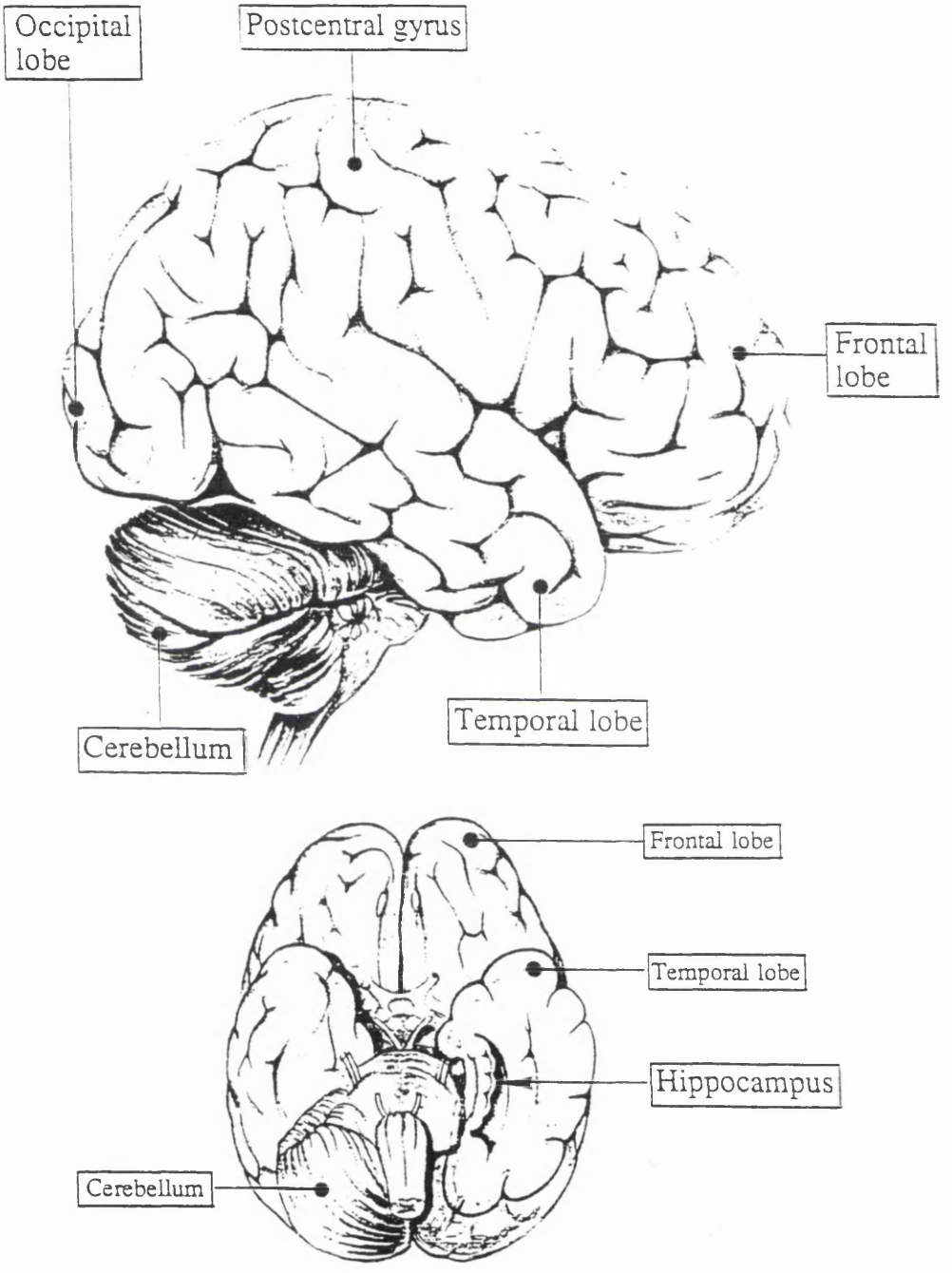
Human brain tissue was obtained at autopsy from four females and two males (all died for a variety of reasons including multiple injuries due to road traffic accident, hypertension, coronary artery atheroma, acute alcohol intoxication, hanging and acute myocardial infarction. Their ages ranged from 15 to 68 years). All were shown to be free of benzodiazepine drugs by case history and analysis using HPLC and RIA.

In order to find the site of highest densities of benzodiazepine receptors in human brain tissue, six sites were studied - frontal lobe cortex, postcentral cortex, temporal cortex, occipital cortex, cerebellum and hippocampus (Figure 11).

4.6.2 REMOVAL OF BRAIN

Six human brain tissue samples were removed (as described in 4.5.3) and dissected rapidly. The cortices were collected under sterile condition (white matter was avoided).

Figure 11.


Occipital lobe
Postcentral gyrus
Frontal lobe
Cerebellum
Temporal lobe
Frontal lobe
Temporal lobe
Hippocampus
Cerebellum

4.6.3 PREPARATION OF SYNAPTOSOMAL MEMBRANES

Each gram of brain tissue was homogenized in 10 ml of ice cold 0.32M sucrose solution using 20 strokes of a Teflon-glass homogeniser. The homogenate was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was centrifuged again at 15000 rpm at 4°C. The resultant pellet (crude p_2 fraction) was suspended in 10 ml assay buffer (25 mM dihydrogen-orthophosphate buffer pH 7.4). The membranes were frozen until they were used for the binding studies.

4.6.4 PROTEIN DETERMINATION

Protein concentration in each gram of human brain area (frontal cortex, post central cortex, occipital cortex, temporal cortex, cerebellum cortex and hippocampus) was determined by the colorimetric method of Lowery as described in 4.4.

4.6.5 BUFFER AND STANDARD PREPARATION

Buffer and standard preparation was carried out as described in 4.5.2.

4.6.6 BINDING STUDY

The receptor binding assay study used in this research, has been modified from the technique of Horton et al. (1982, 1988). It was used to determine the number of receptors available for binding in a preparation of brain tissues (frontal cortex, post central cortex, occipital cortex, temporal cortex, cerebellum and hippocampus).

Non-Specific Binding

Aliquots of homogenate were incubated in duplicate for 30 minutes at room temperature 20 ^{o}C in a total volume of 1 ml, as follows:

	Final Concentration
800 ul homogenate	0.15-0.20 mg/ml
100 ul clonazepam	2 uM
100 ul ^{3}[H] FNZ	0.6-20 nM

^{3}[H] FNZ (85 Ci/mmol) was obtained from New England nuclear. Clonazepam (Roche) was initially dissolved in ethanol (1 mg/ml) and made up to the required concentration (2 uM) with buffer (25 mM sodium dihydrogenorthophosphate buffer pH 7.4).

Bound radioactivity was separated on Whatman GF/B glass fibre filters under vacuum and the filters were washed with 5 x 1 ml of assay buffer (25 mM sodium dihydrogenorthophosphate buffer pH 7.4). The filters were

placed into Pico vials, 4 ml of scintillant (Ecoscint) was added and the trapped radioactivity determined by liquid scintillation counting (Packard) to find the DPM of the bound radioligand.

Total Binding

Aliquots of homogenate were incubated in duplicate for 30 minutes at room temperature (20°C) in a total volume of 1 ml as follows:

	Final Concentration
800 ul homogenate	0.15-0.2 mg/ml
100 ul assay buffer	
100 ul [^{3}H] FNZ	0.6-.20 nM

Non-specific binding was treated as total binding (as mentioned above).

12 pairs of LP3 tubes were set up to enable each [^{3}H] flunitrazepam concentration to be assayed in duplicate. They were incubated at room temperature (20 °C) for 30 minutes. The incubation mixture was filtered through a Whatman GF/C glass filter with suction. The filter and tube were washed with an additional 5 ml of 25 mM sodium phosphate buffer assay (pH 7.4). The filters were placed into pico vials, 4 ml of scintillant (ecoscint) was added and vials mixed well. The trapped radioactivity was estimated by counting for one minute as before to find the DPM of the bound radioligand.

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

4.6.7 RESULTS AND DISCUSSION

Using the general binding method described above, the distribution of benzodiazepine receptors has been investigated in human brain tissue. The receptor binding assay consisted of an osmotically shocked crude synaptosomal preparation which was incubated for 30 minutes at room temperature. After incubation the receptor-ligand complex was separated by filtration through glass fibre filters and measured to determine the maximal binding concentration in frontal cortex, post-central cortex, occipital cortex, hippocampus, cerebellum cortex and temporal cortex by the Combicept^{R} programme as before.

Subtracting the non-specific DPM results from the total bound DPM results gives the DPM for the specifically bound [^{3}H]flunitrazepam as highlighted in Tables 10-15.

The results together with the DPM of the total radioligand added are transformed by the Combicept program, to determine maximal binding concentration (Bmax) as illustrated in Figures 13-18 from data in Tables 16-21.

This experiment showed that highest densities of binding sites were to be found in the frontal lobe cortex (grey matter) area. Intermediate densities were found in most other areas (post-central cortex, occipital cortex, hippocampus) and low densities were found in cerebellum cortex and temporal cortex (Table 9 and Figure 12). This study agrees with other investigators and supports the concept that benzodiazepines are bound to specific receptor sites in areas of the central nervous system, which are most likely their sites of action.

Table 9 Regional distribution of benzodiazepine receptors in human brain tissue.

No	Brain region	Maximum specific binding site (fmol/mg protein)						statistics
1	Frontal cortex	1341	1216	1325	1362	1126	1300	χ=1278.4 S.D=90.2 c.v=7.05%
2	Postcentral cortex	1050	981	1062	1170	1098	998	χ=1059.8 S.D=68.89 c.v=6.50%
3	Occipital cortex	1016	1092	927	1060	1090	960	χ=1024.2 S.D=69.05 c.v=6.74%
4	Hippocampus	841	910	807	980	896	900	χ=889 S.D=6 c.v=0.7%
5	Cerebellum	662	542	613	590	642	660	χ=618.2 S.D=46.6 c.v=7.5%
6	Temporal cortex	638	700	590	610	561	531	χ=605 S.D=59.6 c.v=9.8%

Figure 12 Regional Distribution of Benzodiazepine Receptors in Human Brain Tissue

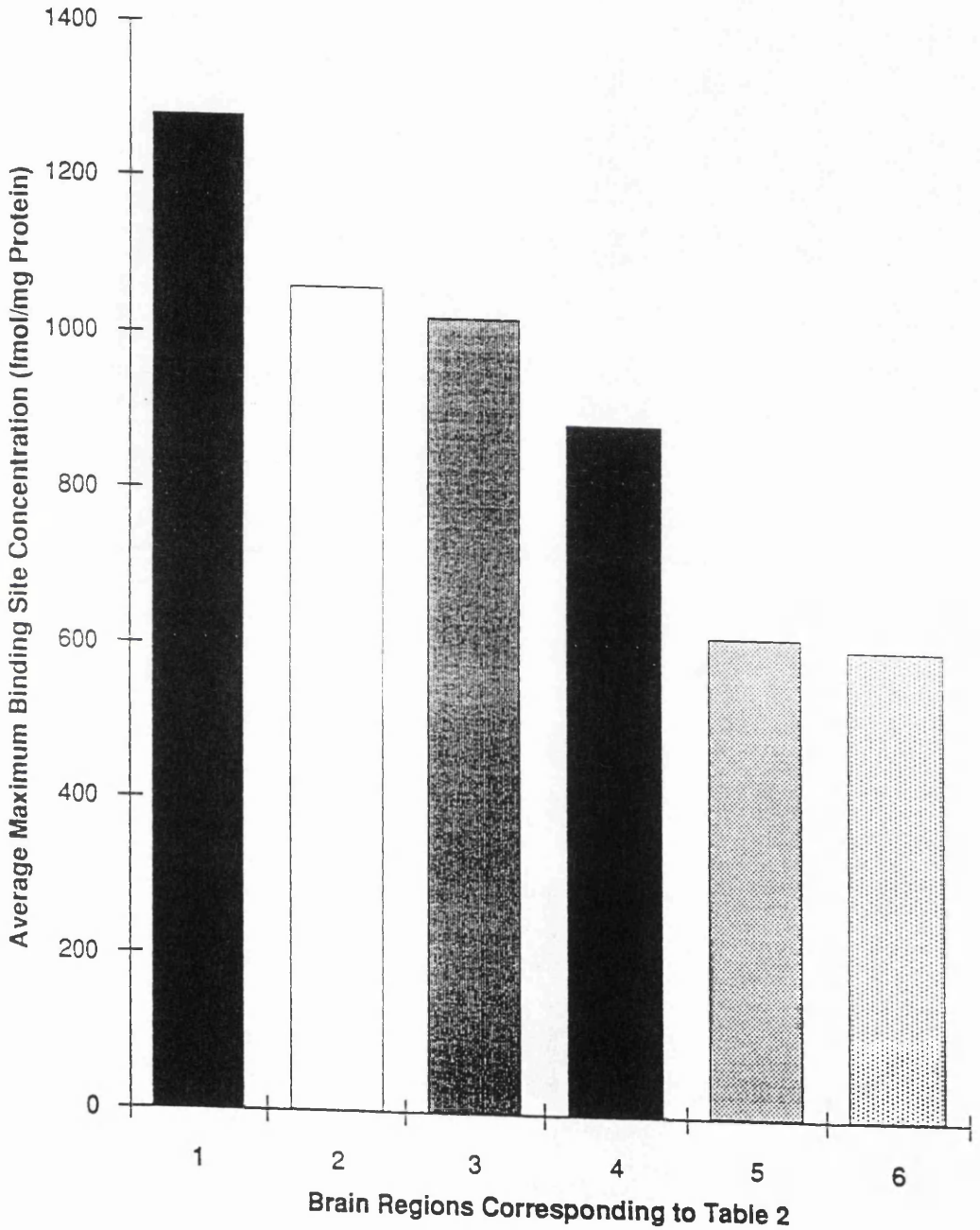


Table 10: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue.(Frontal cortex region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	2512.7	χ=2644.8	81.6	χ=98.2	χ=2546.6
1	2776.8		114.7		
2	4919.4	χ=5009.9	126.6	χ=102.3	χ=4907.6
2	5100.3		77.9		
3	9215.6	χ=9147.6	125.3	χ=144.6	χ=9003
3	9079.6		163.8		
4	13631.0	χ=13496.8	324.7	χ=452.6	χ=13044.2
4	13362.6		580.6		
5	17129.4	χ=16551.5	768.1	χ=818.8	χ=15732.7
5	15973.6		869.5		
6	26734.6	χ=24117.0	1424.1	χ=1287.3	χ=22829.7
6	21499.4		1150.5		

χ=Average
DPM= Disintegration/minute.

Table 11: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue (Post-central cortex region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	1539.8	χ=1633.8	59.8	χ=80.4	χ=155304
1	1727.8		101.1		
2	3236.6	χ=3180.5	181.5	χ=142.1	χ=3038.4
2	3124.4		102.7		
3	4830.7	χ=5308.9	141.2	χ=176.8	χ=5132.1
3	5787.0		212.4		
4	8506.5	χ=8537.0	458.9	χ=472.2	χ=8064.8
4	8567.6		485.5		
5	12719.4	χ=12078.9	842.6	χ=1043.6	χ=11035.3
5	11438.3		1244.5		
6	14211.9	χ=14459.1	1836.8	χ=1639.5	χ=12819.6
6	14706.4		1442.2		

χ=Average.
DPM= Disintegration/minute.

Table 12:Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue (Occipital cortex region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	1673.6	χ=1818.9	223.9	χ=223.2	χ=1595.7
1	1964.2		222.5		
2	2762.4	χ=2796.2	501.2	χ=371.5	χ=2424.7
2	2830.1		241.9		
3	4092.6	χ=4534.9	759.9	χ=768.5	χ=3766.4
3	4977.3		777.1		
4	7297.0	χ=6449.5	1624.5	χ=1550.8	χ=4898.7
4	5601.9		1477.2		
5	9607.6	χ=9493.7	4137.2	χ=2698.9	χ=6794.8
5	9379.9		1260.5		
6	11788.4	χ=12564.6	3593.8	χ=3558.0	χ=90116
6	13340.8		3522.2		

χ=Average.
DPM= Disintegration/minute.

Table 13: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue (Hippocampus region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	1351.7	χ=1242.4	156.0	χ=151.7	χ=1090.7
1	1133.1		147.4		
2	2143.9	χ=2069.4	297.8	χ=275.7	χ=1793.7
2	1994.8		253.6		
3	3025.5	χ=3106.4	1190.2	χ=865.5	χ=2240.9
3	3187.2		540.7		
4	4413.8	χ=4715.5	957.0	χ=996.5	χ=3719.0
4	5017.3		1036.0		
5	7015.2	χ=7083.4	1539.0	χ=1560.9	χ=5522.5
5	7151.5		1582.9		
6	9659.3	χ=10616.7	3261.9	χ=3693.4	χ=6923.3
6	11574.0		4124.9		

χ=Average.
DPM= Disintegration/minute.

Table 14: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue (Cerebellum cortex region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	5208.2	χ=5208.2	127.0	χ=127.2	χ=5081
1	5208.2		127.3		
2	7979.2	χ=7988.6	591.9	χ=634.6	χ=7354
2	7998.1		677.4		
3	12178.6	χ=11878.0	270.6	χ=294.4	χ=11583.6
3	11577.4		318.3		
4	17539.3	χ=18241.0	2515.9	χ=1766.7	χ=16474.3
4	18942.8		1017.5		
5	23028.6	χ=23687.8	1285.0	χ=1285.2	χ=22402.6
5	24347.1		1285.3		
6	28488.0	χ=28488.0	1174.6	χ=1651.4	χ=26836.6
6	28488.0		2128.2		

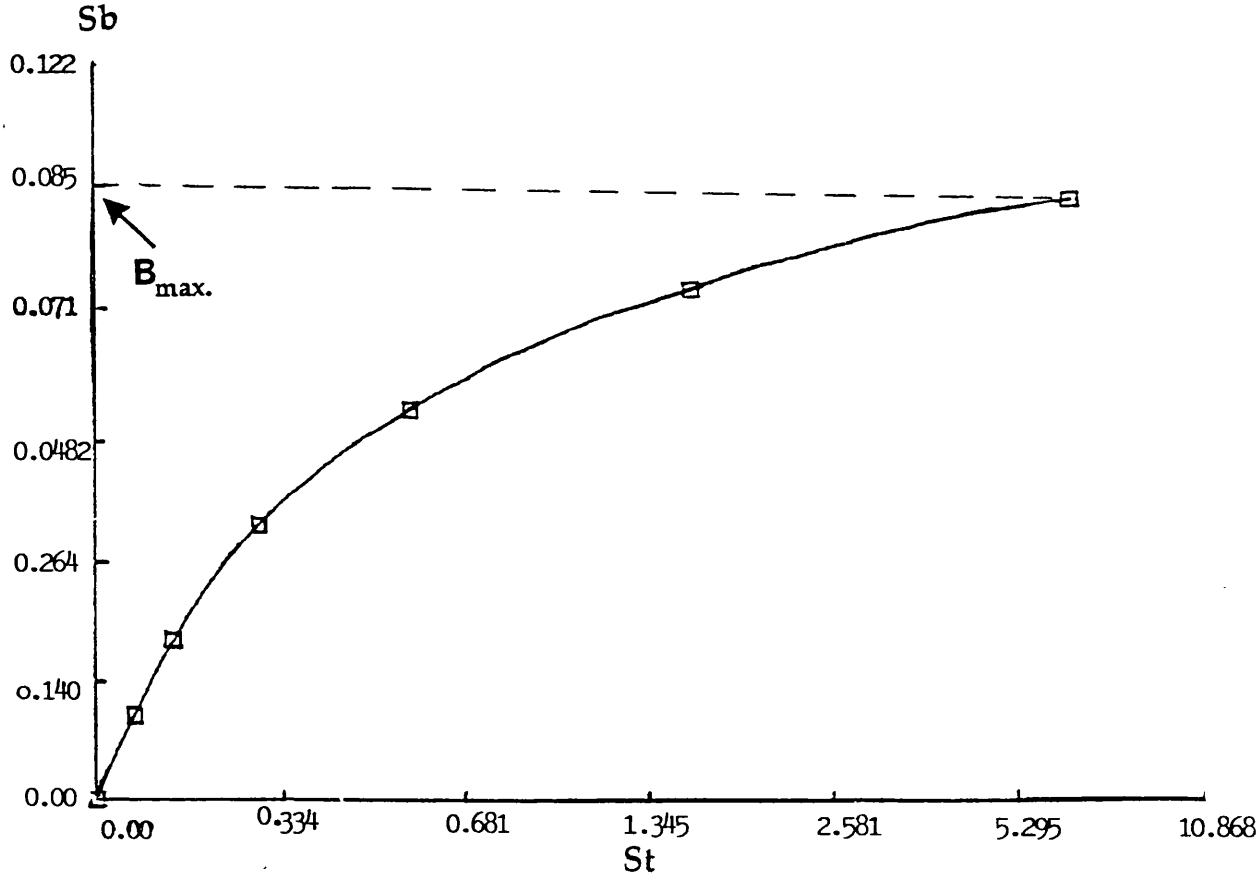
χ=Average.
DPM= Disintegration/minute.

Table 15: Binding assay for total, non-specific and specific binding of [3H] Flunitrazepam to benzodiazepine receptors in human brain tissue (Temporal cortex region).

No. Std.	Total bound DPM		Non-specifically bound DPM		Specifically bound DPM
1	1051.5	χ=967.2	70.0	χ=60.3	χ=906.9
1	885.0		50.5		
2	1660.0	χ=1707.2	51.9	χ=56.2	χ=1651
2	1754.4		60.4		
3	2605.3	χ=2677.4	144.9	χ=134.1	χ=2543.3
3	2749.6		123.2		
4	4531.7	χ=4190.4	244.5	χ=354.9	χ=3835.5
4	3849.1		465.3		
5	5935.0	χ=6149.0	444.1	χ=483.8	χ=5665.2
5	6363.0		523.5		
6	8457.1	χ=7950.4	11089.0	χ=1230.6	χ=67198
6	7443.7		1372.3		

χ=Average.
DPM= Disintegration/minute.

Figure 13 Saturation Curve.Frontal Cortex.



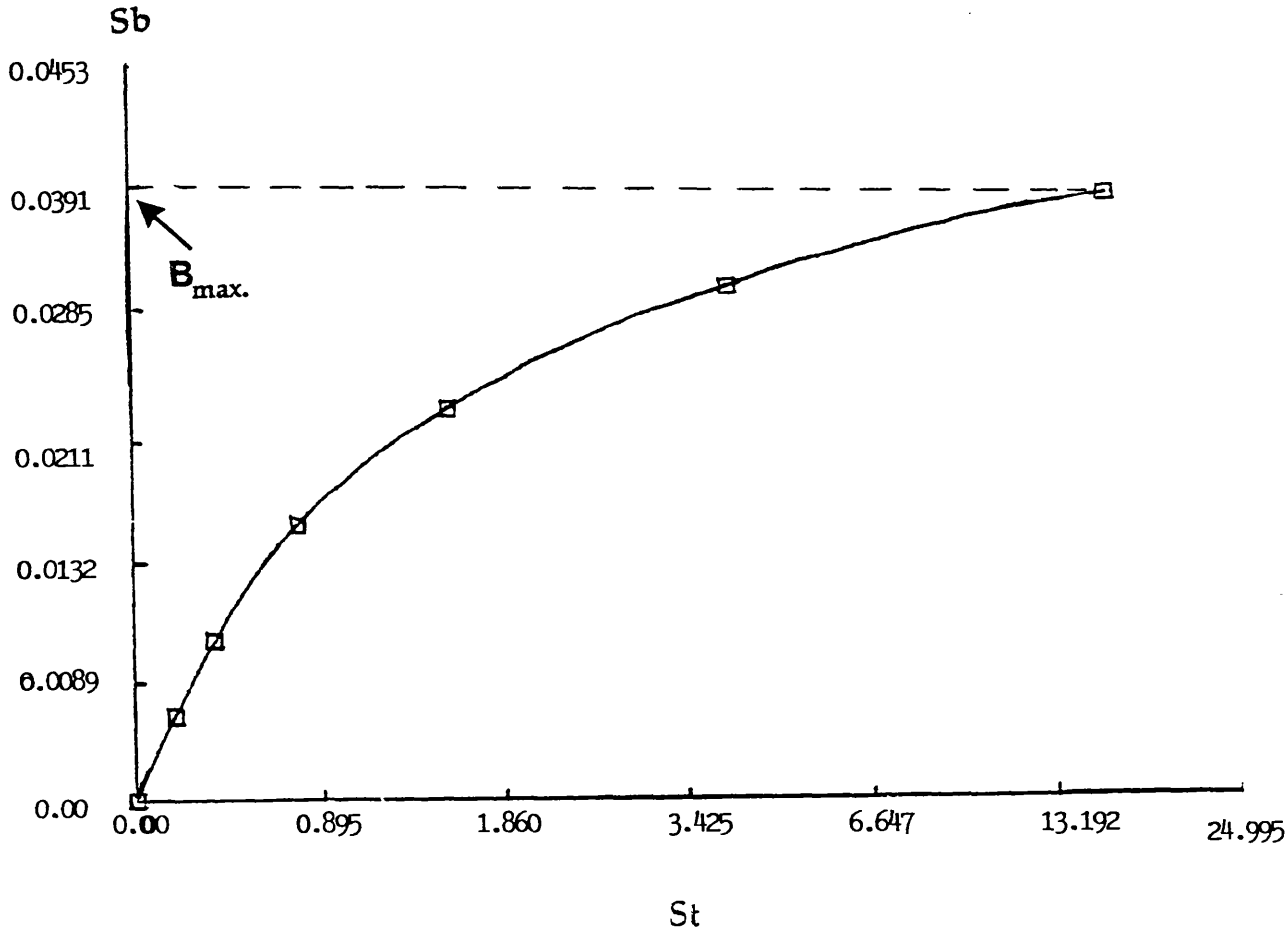
st-Total tritiated flunitrazepam Added (nM).
sb-Specifically bound tritiated flunitrazepam.

Table 16 Experimental Binding Assay.Frontal Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.140	0.334
2	0.264	0.681
3	0.0482	1.345
4	0.071	2.581
5	0.085	5.295
6	0.122	10.868

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 14 Saturation Curve.Post-central Cortex.



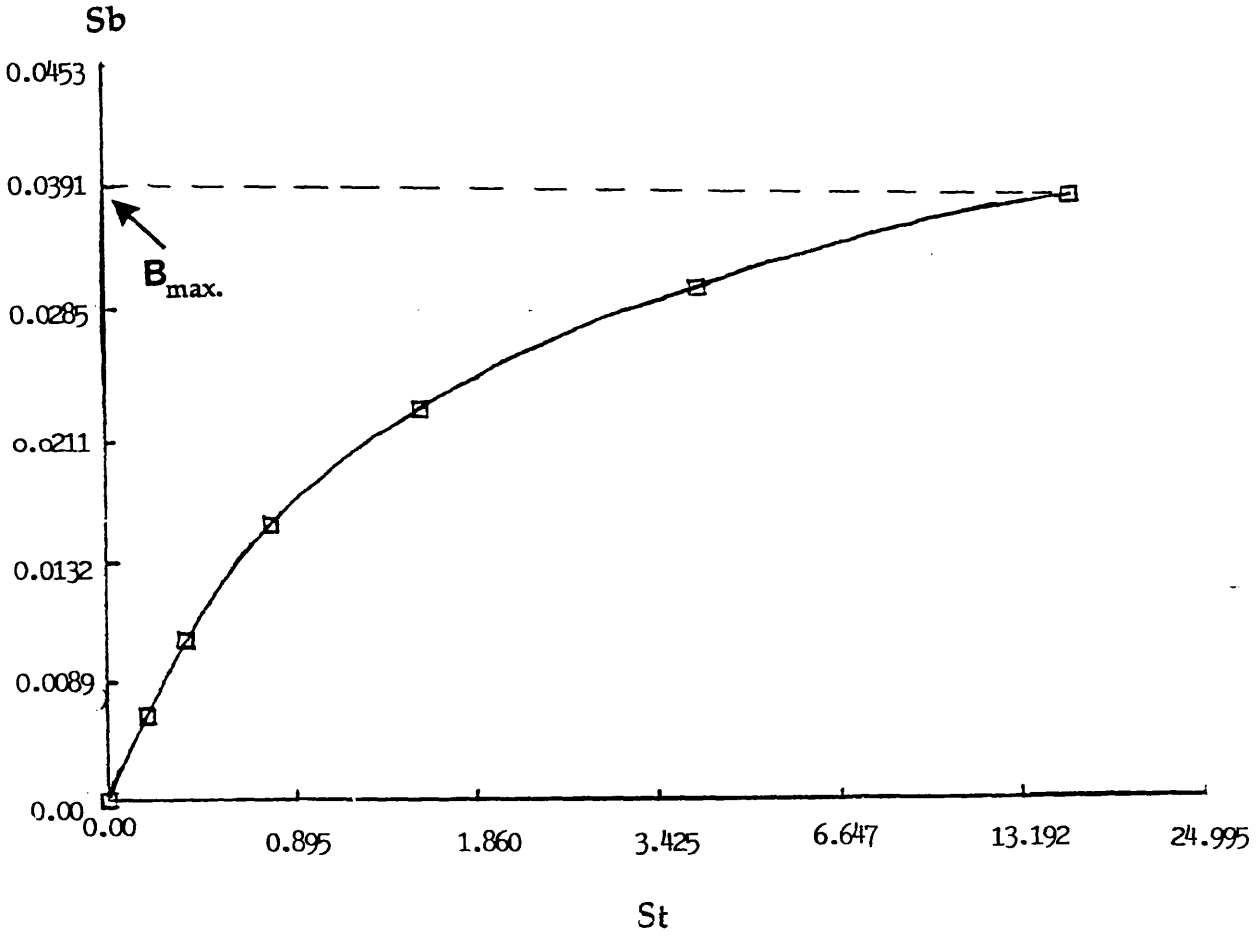
st-Total tritiated flunitrazepam Added (nM).
sb-Specifically bound tritiated flunitrazepam.

Table 17 Experimental Binding Assay.Post-central Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.0086	0.3364
2	0.0168	0.7381
3	0.0278	1.528
4	0.0443	3.144
5	0.0612	6.538
6	0.0694	13.616

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 15 Saturation Curve.Occipital Cortex.



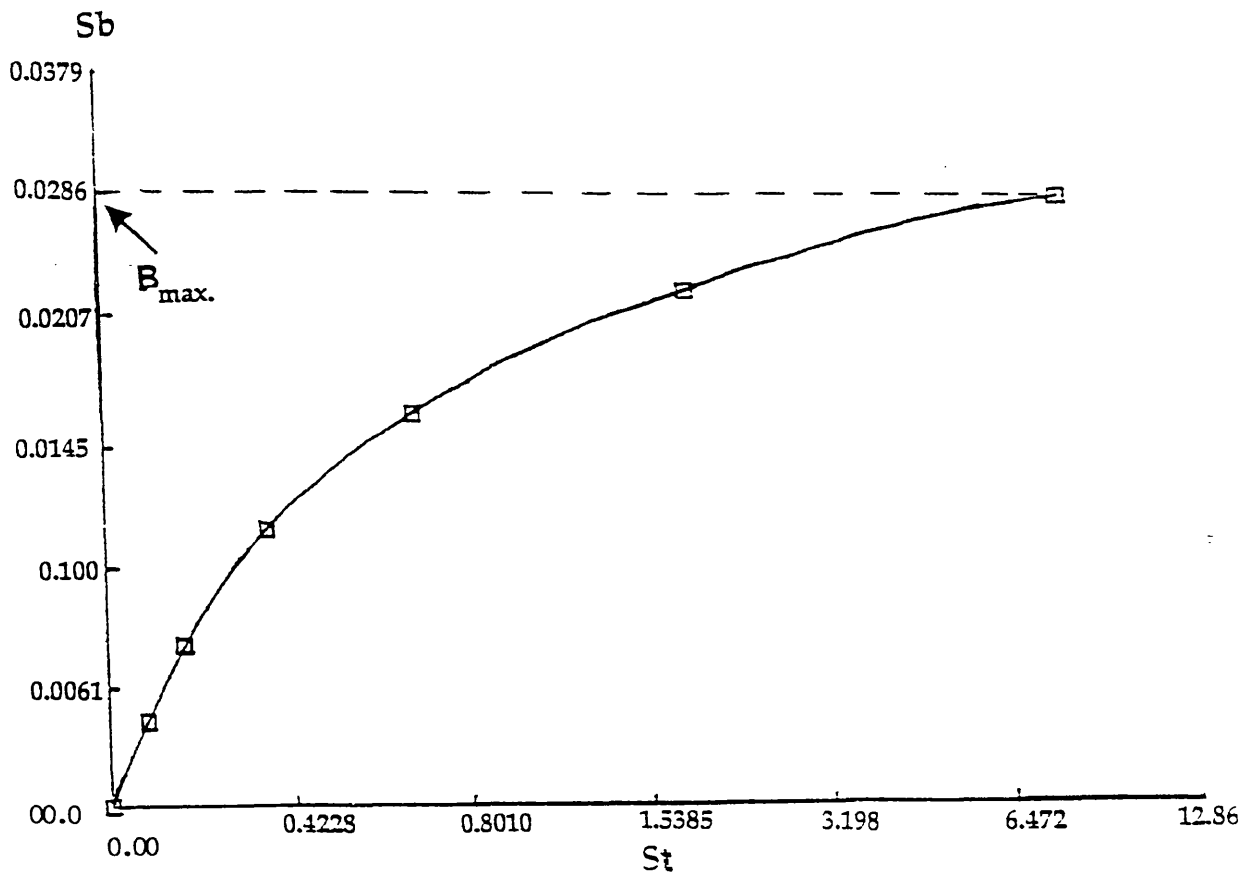
st-Total tritiated flunitrazepam Added (nM).
sb-Specifically bound tritiated flunitrazepam.

Table 18 Experimental Binding Assay.Occipital Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.0089	0.895
2	0.0132	1.860
3	0.0211	3.425
4	0.0285	6.647
5	0.0391	13.192
6	0.0453	24.995

St-Total tritiated flunitrazepam Added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 16 Saturation Curve.Hippocampus.



sb-Specifically bound tritiated flunitazepam.

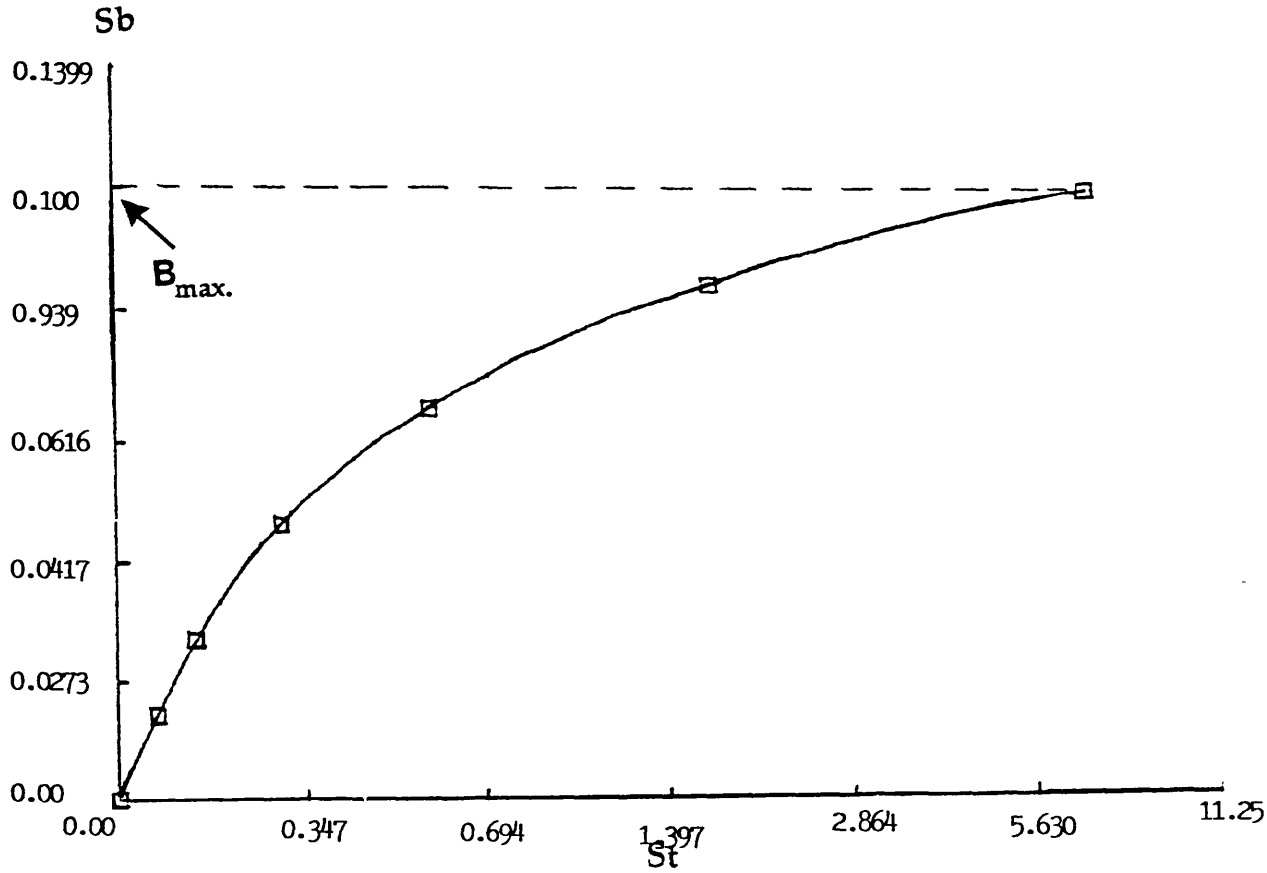
st-total tritiated flunitazepam.

Table 19 Experimental Binding Assay.HIPPOCAMPUS. (Rosenthal NSB Correction)

Point	Sb	St
1	0.0061	0.4228
2	0.100	0.8010
3	0.0145	1.5385
4	0.0207	3.198
5	0.0286	6.472
6	0.0379	12.867

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 17 Saturation Curve.Cerebellum Cortex.



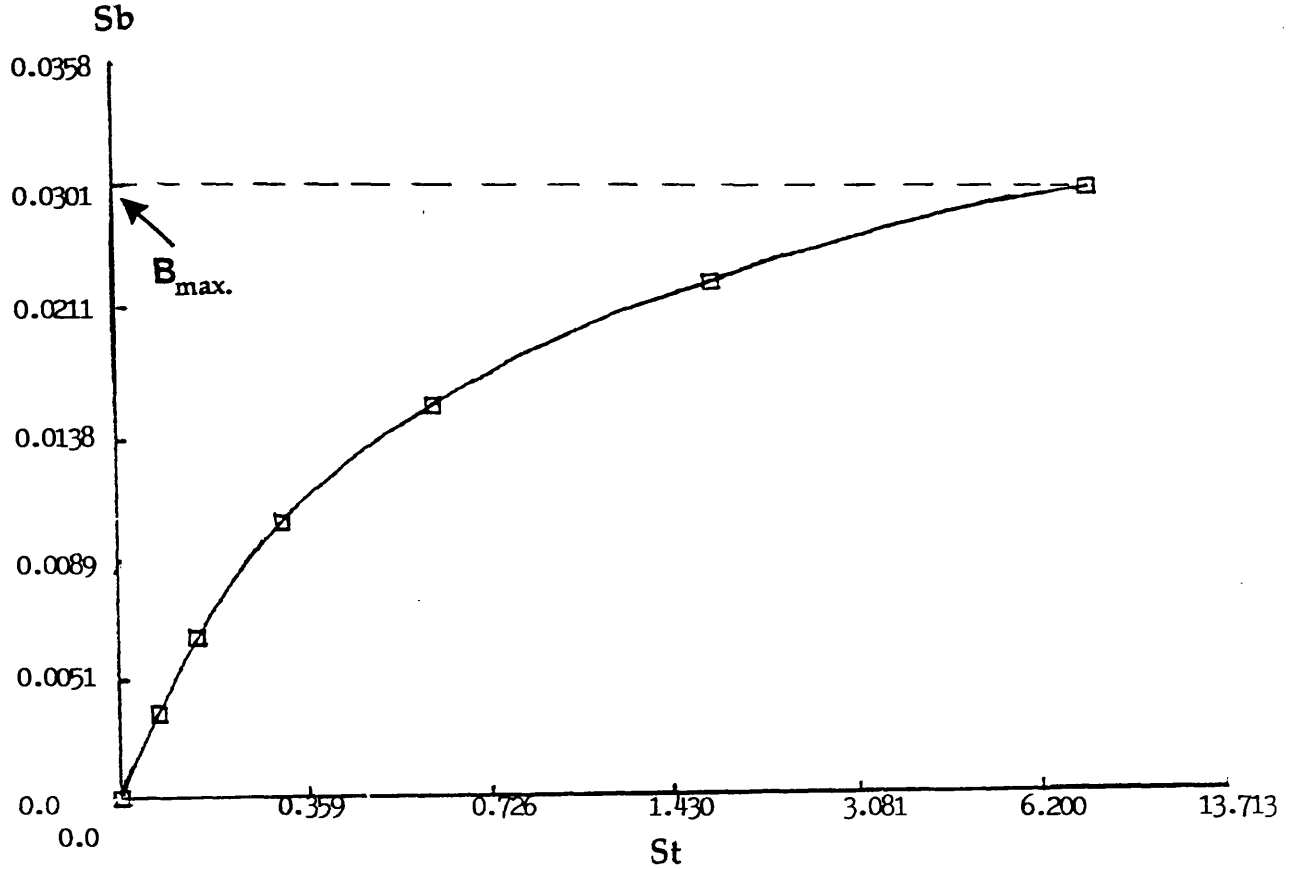
st-Total tritiated flunitrazepam Added (nM).
sb-Specifically bound tritiated flunitrazepam.

Table 20 Experimental Binding Assay.Cerebellum Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.0273	0.347
2	0.0417	0.694
3	0.0616	1.397
4	0.939	2.864
5	0.100	5.630
6	0.1399	11.246

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Figure 18 Saturation Curve.Temporal Cortex.



st-Total tritiated flunitrazepam Added (nM).
sb-Specifically bound tritiated flunitrazepam.

Table 21 Experimental Binding Assay.Temporal Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.0051	0.359
2	0.0089	0.726
3	0.0138	1.430
4	0.0211	3.081
5	0.0301	6.200
6	0.0358	13.713

St-Total tritiated flunitrazepam Added (nM).
Sb-Specifically bound tritiated flunitrazepam.

4.7 INFLUENCE OF AGE, SEX, CAUSES OF DEATH AND TIME INTERVAL BETWEEN DEATH AND AUTOPSY

4.7.1 INTRODUCTION

Estimation of the characteristics of membrane binding sites in the human brain post-mortem contributes to the understanding of the pathophysiology of diseases of the central nervous system and may suggest new therapeutic strategies. Alterations in neurotransmitter receptors have been described in post-mortem human brain in a wide variety of neurodegenerative disorders including Parkinson's disease (Olsen et al, 1980), Huntington's disease (Whitehouse et al, 1985), Alzheimer's disease (Hardy et al, 1985) and in psychiatric disorders such as schizophrenia (Owen et al, 1978, Seeman, 1984) and depression (Meyerson et al, 1982, Stanley et al, 1982 and Perry et al, 1983). Interpretation of binding data is problematic, however, due to the rather small variations observed in pathological brains compared to controls, and factors which may affect the characteristics of binding sites must be excluded before the changes in receptor density and/or affinity observed in human brain post-mortem can be attributed to the pathological process.

The present study is concerned with the effects of factors such as age, sex, causes of death and post-mortem conditions (delay time interval between death and autopsy) that might affect the receptor molecules (Perry and Perry, 1983).

4.7.2 REMOVAL OF BRAIN

Thirty-six human post-mortem brain tissue samples were removed as described in (4.5.3) and dissected rapidly. The frontal cortex tissue (grey matter) were collected from these samples under sterile conditions (white matter was avoided). All were shown to be free of benzodiazepine drugs by analysis of their blood samples using HPLC and radio-immunoassay.

4.7.3 PREPARATION OF SYNAPTOSOMAL MEMBRANES

One gram of human brain tissue (frontal cortex) was homogenized in 10 ml of ice cold 0.32M sucrose solution using 20 strokes of Teflon-glass-homogeniser. The homogenate was centrifuged at 3500 rpm for 10 minutes at 4 ^{o}C. The supernatant was centrifuged again at 15000 at 4 ^{o}C. The resultant pellet (crude P2 fractions) was suspended in 10 ml assay buffer (25 mM dihydrogenorthophosphate buffer pH 7.4). The membranes were frozen until used.

4.7.4 PROTEIN DETERMINATION

Protein concentration in each gram of human frontal cortex was determined by the method of Lowery et al as described in 4.4 using bovine serum albumin as standard.

4.7.5 BUFFER AND STANDARD PREPARATION

Buffer and standard preparation was carried out as described in 4.5.2.

4.7.6 BINDING STUDY

The maximum specific binding site concentration (using saturation curve analysis) was determined in all 36 of the human brain tissue samples (frontal cortex). The method used in this study has been modified from Horton et al (1982, 1988).

Total Binding Assay

Total Binding Assay was determined as described in 4.5.5.

Non-Specific Binding

Non-specific binding was determined as described in 4.5.5.

4.7.7 RESULTS AND DISCUSSION

The benzodiazepine receptor concentration in the frontal cortices was determined for 36 post-mortem cases all of whose blood samples had been tested for and found to be negative. Table 22 summarizes the characteristics of these cases with respect to age group (<40 years, 40-60 years and >60 years). The number of benzodiazepine receptors in the frontal cortex was measured (using Saturation Curve) as 1305 $\pm$ 92 (male), 1268 $\pm$ 30.7 (female) for age group one. For group 2 (40-60) this was 1267 $\pm$ 90 (male), 1250 $\pm$ 6.1 (female) and for group 3 (>60) the number of benzodiazepine receptors in frontal cortex was 1166 $\pm$ 90 (male), 1268 $\pm$ 47 (female).

This study reveals no significant statistical variations in cortical synaptosomal binding in all three groups of age and these results are in agreement with the reports of Pedigo et al, 1981 and Tsang et al., 1982, on experimental animals. However, conflicting data have been claimed age-related changes: an increase (hippocampus only) (Memo et al., 1981) and a decrease (hippocampus and cortex) (DeBlasi et al, 1982) in cortical synaptosomal binding have been found for rats of different strains (Fischer 344 or Sprague-Dawery). Although a final interpretation of this data is difficult due to many factors which might be responsible for alterations such as cell membranes, nuclei, chromatin, cyclic nucleotide metabolism and various cellular enzyme systems.

Several studies have reported age-related changes in receptor binding within the central nervous system. There are for example, papers

describing decreased (Thal et al., 1980, Severson and Finch, 1980, Misra et al., 1980 and Memo et al., 1980) or increased (Marquis and Pelham, 1981) dopamine (DA) antagonist receptors, decreased β noradrenergic receptors (Misra et al, 1980, Greenberg and Weiss, 1978, Maggi et al 1979) decreased alpha receptors (Misra, 1980), unchanged (Memo et al, 1980, Pediog et al, 1981, Heusner and Bosmann 1981) or increased (Memo et al., 1981, Reeves and Schweizer 1983, Dr Blasi et al 1982) benzodiazepine receptors, unchanged (Maggi et al., 1979) or decreased (Calderini et al., 1981) gamma-aminobutyric acid (GABA) receptors with aging. Unfortunately each receptor type was investigated separately and the differences in species, strains and age of the animals used by these authors make it difficult to assess whether age-related changes in receptor binding are a general phenomenon or whether some classes (or subclasses) of brain receptors are more affected than others by aging.

Most of the studies reviewed have focussed on changes in the density of the benzodiazepine receptor in animals. In humans more studies are urgently needed to ascertain parameters which effect the changes in the density of benzodiazepine receptors and to divide the data into genetically determined and presumably pathologically determined alteration, as was done for animal data.

Tables 23-26 list the binding site concentrations measured for 36 brains from patients who had not received benzodiazepine drugs before death in males and females with respect to causes of death and the time interval between death and autopsy.

With respect to the delay between time of death and post-mortem, the results show no significant effect. This is possibly due to storage of bodies in a refrigerator prior to autopsy. Also, the cause of death shows no significant change in the receptor density.

Table 22 : Binding site concentration of benzodiazepine receptors in the human frontal cortex area against sex and age group.

Binding site concentration (fmol/mg protein)					
Age group	Case No.	Male		Female	
< 40 years	n1	1328	χ 1305 SD 92 cv7%	1281	χ 1268 SD 30.7 cv 2.4%
	n2	1375		1250	
	n3	1270		1305	
	n4	1210		1220	
	n5	1230		1290	
	n6	1217		1260	
40 - 60 years	n1	1272	χ 1267 SD 90 cv7%	1215	χ 1250 SD 6.1 cv 4.9%
	n2	1157		1210	
	n3	1212		1320	
	n4	1193		1185	
	n5	1230		1236	
	n6	1290		1333	
> 60 years	n1	1275	χ 1166 SD 90 cv 4.5%	1212	χ 1209 SD 47 cv 3.9%
	n2	1113		1216	
	n3	1168		1300	
	n4	1172		1180	
	n5	1232		1190	
	n6	1208		1157	

Table 23 Effects of age cause of death on binding site concentration in females.

Case No.	Age	Cause of death	Binding site concentration
1	18	Chest&Abdominal injuries(RTA)	1220
2	31	Myocarditis.	1305
3	33	Phenobarbitone Intoxication	1260
4	36	Acute Alcohol Intoxication(RTA).	1250
5	37	Spontaneous subarachnoid haemorrhage due to rupture of cerebral artery aneurysm	1290
6	38	Acute peritonitis due to chronic pancreatitis.	1281
7	46	Myocardial infarction.	1333
8	47	Scalding due to hot water.	1215
9	47	Coronary thrombosis	1185
10	48	Acute myocardial infarction.	1236
11	53	Ischaemic heart disease.	1320
12	59	Hypertension & Ischaemic heart disease.	1216
13	62	Ischaemic heart disease	1210
14	68	Lobar pneumonia	1190
15	68	Acute myocardial infarction	1330
16	68	Haemopericardium due to myocardial infarction	1157
17	78	Ischaemic heart disease coronary artery atheroma.	1212
18	85	Metastatic large bowel carcinoma&pulmonary thromboembolism.	1180

Table 24 Effects of time interval (death&autopsy) on binding site concentration in females.

Case No.	Age	Time interval (death&autopsy)	Binding site concentration
1	46	12 hours	1333
2	62	12 hours	1210
3	53	14 hours	1320
4	68	17 hours	1330
5	68	19 hours	1190
6	85	24 hours	1181
7	31	24 hours	1305
8	18	34 hours	1220
9	33	34 hours	1260
10	38	34 hours	1281
11	47	52 hours	1185
12	37	54 hours	1290
13	78	73 hours	1212
14	47	74 hours	1215
15	48	76 hours	1236
16	59	80 hours	1216
17	36	86 hours	1250
18	68	88 hours	1157

Table 25 Effects of time interval (death&autopsy) on binding site concentration in males.

Case No.	Age	Time interval (death&autopsy)	Binding site concentration
1	49	18 hours	1272
2	68	23 hours	1168
3	19	30 hours	1341
4	64	36 hours	1263
5	58	38 hours	1157
6	48	49 hours	1272
7	19	72 hours	1362
8	55	72 hours	1325
9	76	72 hours	1136
10	38	82 hours	1126
11	37	84 hours	1300
12	74	84 hours	1113
13	75	84 hours	1194
14	37	86 hours	1375
15	73	95 hours	1119
16	37	97 hours	1328
17	46	102 hours	1223
18	48	141 hours	1121

Table 26 Effects of age and cause of death on binding site concentration in males.

Case No.	Age	Cause 0f death.	Binding site concentration
1	19	Multiple injuries,Road traffic accident.	1341
2	19	Acute alcohol intoxication.	1362
3	37	Acute pancreatitis.	1300
4	37	Coronary artery atheroma.	1328
5	37	Chest&abdominal injuries,Road traffic accident.	1375
6	38	Hanging.	1126
7	46	Cerebral abscess.	1223
8	48	Hanging.	1272
9	48	Acute artery atheroma.	1121
10	49	Acute myocardial infarction.	1272
11	55	Coronary artery atheroma,Bronchitis&emphysema .	1325
12	58	Choking.	1157
13	64	Acute myocardial infarction.	1263
14	68	Ischaemic heart disease.	1168
15	73	Ruptured aortic aneurysm.	1119
16	74	Pulmonary thrombeoembolism.	1113
17	75	Acute bronchopneumonia.	1194
18	76	Haemopericardium due to myocardial infarction.	1136

4.8 THE EFFECT OF BENZODIAZEPINE DRUGS

4.8.1 INTRODUCTION

The toxicological investigation of death by poisoning relies primarily on the analysis of a biological sample (blood, urine, liver, etc.) for the presence of a suspected drug. As a result, a level can be measured which can be compared with findings from previous cases. The finding will lie within the fatal, toxic, therapeutic, or sub-therapeutic range. Increased sophistication in this interpretation has been reported at international conferences where reported use has been made of the comparison of drug levels measured in samples taken from different sites in the body. This research presents the results of a preliminary investigation of the potential use of drug receptors as a means of diagnosing death by poisoning.

4.8.2 BRAIN SAMPLES

Six human post-mortem brain tissue samples (frontal cortex area, white matter was avoided) were gathered at autopsy as described in (4.5.3), from deaths due to various causes (males and females). Post-mortem blood samples were obtained under sterile conditions and examined for the presence of benzodiazepine drugs using HPLC and radio-immunoassay as detailed in Table 28.

4.8.3 PREPARATION OF SYNAPTOSOMAL MEMBRANES

One gram of human brain tissue sample (frontal cortex) was homogenized in 10 ml of ice cold 0.32 M sucrose solution using 20 strokes of a Teflon homogeniser. The homogenate was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was centrifuged again at 15000 rpm at 4°C. The resultant pellet (crude P2 fraction) was suspended in 10 ml assay buffer (25 mm sodium dihydrogen orthophosphate buffer pH 7.4). The membranes were frozen until use.

4.8.4 BUFFER, LIGAND AND COLD LIGAND PREPARATION

Buffer, ligand and cold ligand preparation were carried out as described in 4.5.2.

4.8.5 PROTEIN DETERMINATION

Protein concentration in each gram of human brain tissues (frontal cortex) was determined by the colorimetric method of Lowery as described in 4.4.

4.8.6 BINDING STUDY

The receptor binding assay used, has been modified from Horton et al. (1982, 1988) in order to determine the number of receptors available for binding in a preparation of brain tissue (frontal cortex).

Total Binding

Total binding was determined as described in 4.5.5.

Non-Specific Binding

Non-specific binding was determined as described in 4.5.5.

4.8.7 RESULTS AND DISCUSSION

Table 28 and the saturation curve (Figure 19) from the data in Table 27, demonstrates a significant drop in frontal cortex benzodiazepine site concentrations in subjects who have been shown to have taken benzodiazepine drugs before death compared to the control group (Table 22).

Several studies have addressed the effects of chronic treatment with benzodiazepine drugs on animal receptor binding sites (rats, mice, etc.), with conflicting results. Roseborg and Chiu revealed a reduced number of benzodiazepine receptors in rat brain tissue (Chiu and Rosenberg 1978: Rosenberg and Chiu 1981) while employing receptor autoradiography for identifying rats' brain receptors Tietz et al. (1986) and by various investigators using low doses of have shown some reduction in receptor numbers in the brain tissues of mice and rats' (Crawley et al 1982; Abbracchiu et al., 1983; Szcazwinska et al 1988, 1988). In addition, Miller and Co-workers, using a low dose of lorazepam and using in vivo binding to investigate receptor characteristics, have shown down-regulation of the benzodiazepine receptor in the brain tissue of mice (Miller et al., 1988a), and up-regulation on withdrawal (Miller et al., 1988 b). Grimm and Hershkowitz (1981) disclosed reduced benzodiazepine receptor numbers in the cortex and hippocampus of rats treated with low doses of diazepam for two weeks after they had been sacrificed two days after dosing. Some investigators have claimed no change in the benzodiazepine receptor densities in the brain tissues of rats and mice (Mohler et al., 1978; Rauch

and Gallager, 1983; Stephens and Schneider, 1985; Heninger and Gallager, 1988, Brett, 1992).

Other researchers have indicated receptor upregulation after chronic treatment. In a study on rat brain tissue Scharf and Feil (1983) revealed an increase in Bmax 18-25 hours after the last dose of a one week's treatment regime with clonazepam and lorazepam. A similar study on rat brain tissues by DiStefano et al. (1979) found a decrease in benzodiazepine binding 24 hours after cessation of 35 days of exposure to high-doses of diazepam, but an increase in binding 72 hours after the last dose. A similar result was outlined by Rogo et al. (1984), after 10 days of diazepam treatment.

The above conflicting data from animal research may be due to non-comparable studies. The benzodiazepine dose, length of treatment, length of withdrawal period before sacrifice of the animal and method of tissue preparation vary. It has been shown that the duration of action, dose and treatment length are important to correlate any biochemical changes with observed behavioural changes (Brett, 1992). Finally, variations may be due to differences in species, in the absence of systemic or brain concentrations of drug, assay conditions and removal of endogenous GABA.

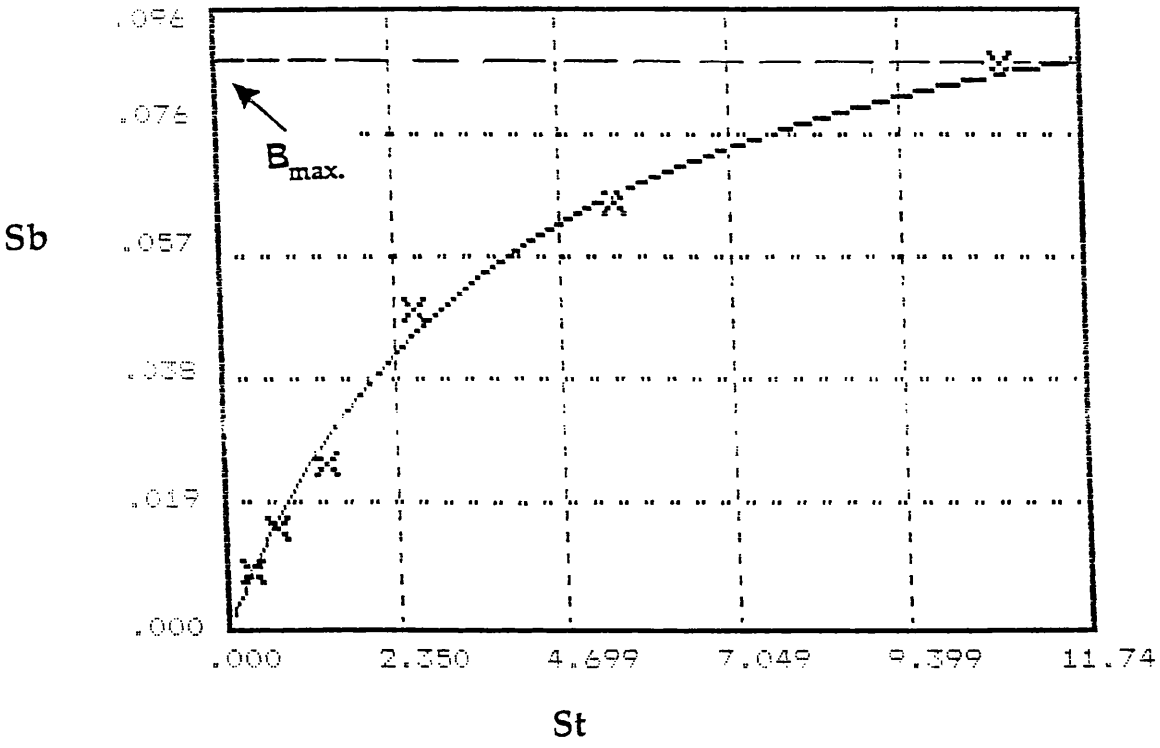
This study has dealt with human samples (brain and blood). The binding studies were performed in the presence of benzodiazepine drugs and were related and compared to a control group (Table 22). Blood and brain tissue benzodiazepine drug concentration (5.2.2.3.A and 5.2.2.3.B.) were measured. These measurements show that in the presence of

benzodiazepine drugs at death there is an apparent decrease in the number of binding sites. The decrease in maximum binding was a specific response to prolonged occupation of binding sites. This specific adaptation to prolonged drug action favours the idea that the high-affinity binding sites represent a site of action of benzodiazepines.

4.8.8 CONCLUSION

In the presence of benzodiazepine drugs there is a decrease in number of benzodiazepine binding receptors in frontal cortex.

Figure 19 Saturation Curve.Pre-central Cortex.



St-Total tritiated flunitrazepam.
Sb-Specifically bound tritiated flunitrazepam.

Table 27 Experimental Binding Assay.Frontal Cortex. (Rosenthal NSB Correction)

Point	Sb	St
1	0.00800	0.3458
2	0.0159	0.6820
3	0.0228	1.3575
4	0.379	2.6052
5	0.0558	5.3227
6	0.0655	10.517

St-Total tritiated flunitrazepam added (nM).
Sb-Specifically bound tritiated flunitrazepam.

Table 28 Changes in the density of the benzodiazepine receptors in the post-mortem brain samples of patients on benzodiazepine treatment.

Case No	Age (years)	Sex	Binding site concentration (fmol/mg protein)	Blood sample analysis (HPLC & RIA)
1	26	male	833	Temazepam, D.M.D. & Diazepam.
2	33	male	772	Temazepam & Diazepam
3	58	female	610	Temazepam.
4	24	male	844	Temazepam, D.M.D. & Diazepam.
5	24	male	976	Triazolam, D.M.D. & Diazepam.
6	20	female	658	Temazepam, D.M.D. & Diazepam

D.M.D: Desmethyldiazpam.

5. BENZODIAZEPINES - EXTRACTION AND ANALYSIS

5.1 CHROMATOGRAPHIC ANALYSIS

Chromatography has been classically defined as a separation technique which enables samples or chemical mixtures to be separated by differential migration of their constituents during passage through a chromatographic column. If the stationary phase is a solid support or a liquid-coated solid support and the mobile phase is a liquid, the technique is known as liquid chromatography. Depending on how the stationary phase is physically applied, there are various forms of liquid chromatography. If the stationary phase is applied as a layer, the technique is referred to as thin-layer chromatography. When the stationary phase is placed in a column, the chromatographic system is liquid column chromatography. In gas chromatography the stationary is a solid support or a liquid-coated solid support and the mobile phase is a gas. The separation process is governed by the distribution of substances between two phases: the mobile phase (moving phase, carrier or eluent) and stationary phase (packing material). The longer this process is allowed to continue, the greater separation is achieved. After the sample has been carried by the moving phase through the chromatographic bed, the liquid that emerges from the column, the column effluent, is a composite of the sample (the eluate) and the mobile phase (the eluent) (Krestulovic and Brown, 1982, Lindsay, 1987). Ideally, the mixture (sample) is completely separated by the time the components reach the end of the column.

The elution of the components from the column is detected electronically by a suitable detector, and signals are sent to a strip chart recorder which displays the chromatogram. This chromatogram consists of a series of peaks, each of which indicates the elution of a component and the amount of component present. The time of elution is used to identify the components of the mixture and is defined as the elution time or retention time for a particular component. The peaks of the chromatograms should be completely separated. The peak width at the base should be narrow, even at the end of the chromatogram.

5.1.1 HPLC

High performance liquid chromatography (HPLC) has developed dramatically over the last fifteen years and is now a highly acclaimed technique in almost all areas of analytical chemistry. Major advantages of HPLC are (Brown, 1973, Hadden et al, 1971, Kirkland, 1971):-

- operation at low temperatures minimizes chances for thermo-decomposition
- applicable to non-volatile and/or polar compounds.
- capable of separating components of widely differing polarities.

When the retention behaviour of components in HPLC is compared with that in other chromatographic techniques, such as GLC and TLC, the

following observations can be made:

In GLC retention behaviour is mainly determined by the stationary phase, as the mobile phase serves merely as a transport medium.

In TLC, the stationary phase, the mobile and the vapour phase all affect the retention behaviour, which is usually a mixture of adsorption and partition processes.

Therefore, changing one or more of the phases may result in large differences in retention behaviour.

In HPLC there are two active phases, the stationary phase and the mobile phase and here too, changing one or both of the phases may have a large impact on the retention behaviour. In addition, in normal phase HPLC, adsorption processes may be assumed to dominate the separation, whereas in reversed-phase HPLC, partition processes are more important.

These advantages seem to justify an important role for HPLC in systematic toxicological analysis, in which it is common practice to use several techniques and systems side-by-side to detect and identify potentially harmful substances.

In high performance liquid chromatography the separation process is governed by the distribution of substances between two phases:

the mobile phase (moving phase, eluent, carrier) and the stationary phase (packing material).

The extent of the interaction that results between the solute molecules and the molecules of each phase is determined by properties of the solute molecules in a given environment.

The basic operating forces exerted on solutes can be due to polarity arising from permanent or induced electric fields (Van der Waals forces) which depend on the relative masses of the solute and solvent molecules. In all forms of chromatography, any variable that can influence the balance of intermolecular forces which are responsible for selective retardation of solute molecules will affect the separation.

Expressions for the resolution, column efficiency and capacity factors are calculated from the widths and retention characteristics of the eluting peaks, i.e. all derived from easily measured parameters taken from the chromatographic trace as capacity factor, selectivity, column efficiency.

a) Capacity factor or capacity ratio (retention factor) ($\bar{K}_1$)

$$\bar{K}_1 = \frac{t_1 - t_0}{t_0} \qquad \textbf{Equation 1.}$$

$$\bar{K}_2 = \frac{t_2 - t_0}{t_0}$$

t_0 = void volume or dead volume = solvent front = is the retention time of an unretained component t_0 (e.g the distance measured from the injection point to the apex of peak t_0),

t_1 = the retention time of compound 1 (e.g. the distance measured from the injection point to the apex of the peak t_1,

t_2 = the retention time of compound 2 (e.g. the distance measured from the injection point to the apex of peak t_2,

optimum $\bar{K}$ values should lie in range $1 < \bar{K} < 10$.

b) The separation or selectivity factor A

A measures the ability of a column to separate components 1 and 2 due to different affinity and therefore retention.

$$A = \frac{K_2}{K_1}$$ **Equation 2.**

K_1: column capacity factor of component 1

K_2: column capacity factor of component 2.

For separation to occur A must be greater than 1.

c) The column efficiency is measured by (H), the height equivalent to theoretical plate (HEPT).

$$H = \frac{L}{N}$$ **Equation 3.**

L: column length.

N: theoretical plate and number.

Efficiency is a function of a column length among other parameters, and, for purpose of comparison, efficiency is commonly expressed in terms of height equivalent to a theoretical plate (HEPT).

(d) Resolution (R) is the quantitative description of the separation obtained between two peaks. This term which is defined by the following relationship:

$$R = \frac{t_2 - t_1}{1/2(W1 + W2)} = \frac{2Dt}{W2-W1} \qquad \textbf{Equation 4}$$

In the **Equation 4,** t_2 and t_1 represent retention times of peaks 1 and 2, while W_1 and W_2 represent peak widths of peaks 1 and 2 respectively.

There are four principal mechanisms in liquid chromatography by which components of samples are selectively retained. These are the exploitation of differences in partition coefficients (liquid-liquid chromatography), adsorption effects on surfaces such as silica gel (liquid-solid chromatography), dissociation of weak or strong electrolytes (ion-exchange chromatography, or in molecular size or shape (steric exclusion chromatography).

5.1.2 H P L C SYSTEM FOR BENZODIAZEPINE ANALYSIS

The method used for benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) analysis was high performance liquid chromatography based on a Gilson 305 Pump with single piston rinsing head. The detector used was a variable wavelength ultraviolet detector (Gilson 115) with an 8 ul flowcell with a path length 10 mm. The column was a 25 cm x 4.6 mm I.D (Internal diameter) cartridge pre-packed with Hypersil 5 um ODS C_{18} (Capital HPLC specialists) fitted with 5 cm 4.6 mm I.D ODS C_{18} guard column. The injector valve was a Rheodyne 7125 with 20 ul loop.

Benzodiazepine drugs were monitored at 230 nm. The chromatograms were recorded on a BBC Goerz Metrawatt SE120 recorder, operated at 1 cm/min and at 10 mV full-scale deflection.

5.1.3 MOBILE PHASE

The mobile phase of 0.01M disodium hydrogen orthophosphate dihydrate ($Na_2HP0_4.2H_20$ M.wt 177.99) (pH8.8) and methanol in the proportions (30:70 v/v) was used. The resulting mobile phase was degassed with helium 10 minutes before use. Disodium hydrogen orthophosphate dihydrate buffer of 0.01 M was prepared by dissolving 1.78 gram of $Na_2HP0_4.2H_20$ in deionised water and making up to one litre (Eppel, 1980).

5.2 METHODS OF EXTRACTION

5.2.1 INTRODUCTION AND LITERATURE REVIEW

The principle behind the different extraction techniques available is basically the same - to retain the analyte(s) of interest and allow interfering compounds to be removed with an appropriate solvent, before quantitatively euluting the drug which can then be analysed by such methods as high-performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (MS).

The phase which retains the analyte can be a water-immiscible solvent , e.g. ethyl acetate as in the popular liquid-liquid extraction (LLE), a solid phase such as XAD-2 a polystyrene medium capable of removing proteins, fats and lipids, on porous polymer beads or inorganic porous materials for the extraction of urine. The last group includes diatomaceous earths, magnesium silicate, alumina and charcoal but the most widely used sorbent is silica.

Liquid-liquid extraction remains the most commonly used sample preparation technique in analytical toxicology. Drugs are extracted from biological samples by partitioning between two immiscible solvents, often adjusting pH to control ionization. Back-extraction at a different pH often improves selectivity and recovery.

The majority of methods used for extracting benzodiazepines from biological fluids have been based on partitioning of the drugs in favour of an organic solvent from a (usually alkaline) buffered aqueous solution.

Buffers have ranged in pH from that of physiological fluids (7.4) to about 10, while the choice of extracting solvent has involved n-hexane (light petroleum) (Verebey and Mule, 1982), toluene, (Lindley, 1979), (Wong, 1983), octanol, (Pegaon et al., 1982), chloroform (Curry, 1976, Slightom, 1982) diethyl ether (McCurdy et al, 1979; Martinez and Grimenez, 1981; Poklis, 1981), n-butyl chloride (Dickson et al. 1980; Peel and Perrigo, 1980), n-butylacetate (Osselton et al, 1980; Rutherford, 1977). Mixed solvents like chloroform-ether (Parry and Ferry 1976) or toluene-n-heptane-isoamyl alcohol (Peat and Kopjak, 1979) have also been used. In an article by Clifford and Smyth over half of the extractions reviewed had been made using diethyl ether (Clifford and Smyth, 1974).

The disadvantages of liquid-liquid extraction are the time required for multi-step extractions, the expense and disposal considerations for large volumes of organic solvents, and the limited precision resulting from non-selective extraction of other compounds that may interfere with analysis and/or cause variable recovery of the analyte (s). In addition, emulsions are often formed that may require additional steps to produce an adequate separation.

More recently, there have been various attempts to replace the time-consuming traditional solvent extraction procedures by liquid-solid solvent extraction. The principle of liquid-solid absorption extraction involves the

absorption of the aqueous phase onto diatomaceous earths, a porous material which acts as a support for the aqueous phase. This provides a large surface area for partition into an eluting solvent, which flows though the immobilized specimen under gravity, eluting the analytes of interest. These materials are capable of being loaded to the limit of their water absorption capacity without releasing any of the water upon elution with solvents. The diatomaceous earth is inactive and chemically inert; the pH value of absorbed aqueous solutions can range between pH1 and 13. It is possible therefore, to elute substances at an optimum pH to separate acidic, neutral and alkaline compounds. Biological samples such as urine, blood, serum, plasma, gastric juice, liquor, and tissue can be extracted by this type of material. Samples extracted through diatomaceous earth do not need any kind of special treatment, such as filtration or centrifugation, before applying the sample.

A method for benzodiazepine drugs extraction using diatomaceous earth has recently been reported (Logan and Stafford, 1989) with a recoveries of 70 $\pm$ 7.6% and 74 $\pm$ 9.5% for diazepam and alprazolam respectively. Lillsunde and Korte (1991) successfully extracted 12 benzodiazepine drugs from urine using diatomaceous earth (Chem-Elut) with recoveries in the range of 60-95% for oxazepam and chlordiazepoxide respectively. While Cordonnier et al. (1987) extracted some benzodiazepines from spiked human liver tissue with recoveries ranging from 69-83% with day to day variation ranging from 3.7-8.7%. The disadvantages of using diatomaceous earth in extracting drugs from biological fluids is the large volume of solvent (10-20 ml) that must be evaporated before the next chromatographic step and identification can take place.

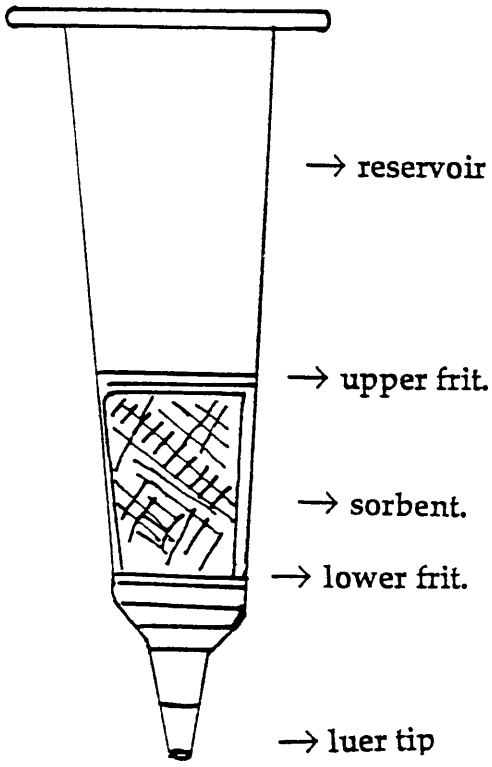
Another type of adsorbent is the cross-liked divinylbenzene-polystyrene resin XAD-2 (Bastos et al, 1972). Urine containing diazepam was extracted by Amberlite XAD-2 and diazepam eluted from the column with 4 x 20 ml portions of methanol. The first 20 ml of the elution solvent (methanol) gave the highest recovery of 61.3 $\pm$ 1% and the lowest was in the fourth 20 ml of the elution solvent with recoveries of 5.7 $\pm$ 1.2% (Hyde, 1985). The total recovery was 96.2 $\pm$ 0.4%. The limitations of using XAD-2 for extracting compounds from biological material are the need for large volumes of elution solvents, usually organic solvents and (Hyde, 1985) the lengthy conditioning prior to use. Also they swell unpredictably in response to organic solvent (Harkey and Stolowitz, 1984).

5.2.2 SOLID PHASE SORBENT

The use of chemically bonded silicas for sample preparation followed their use in high performance liquid chromatography (HPLC) to provide a selective separation of compounds. These materials usually provide a better recovery of drugs from biological matrices and may be more convenient than other procedures used in analytical toxicology (Stewart et al 1984, Hyde 1985).

Bonded silicas are packed either in individual cartridges that can be attached to a syringe or in syringe barrels, as showed in Figure 20. The barrel is made from medical grade polypropylene with the bonded material held in place between two polyethylene fritted disks.

Figure 20 Sorbent Extraction Cartridge.



Solid Phase Sorbent Properties

Bonded silicas are formed by reaction of organosilanes with activated silica. The product is a sorbent with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage. The product is stable within a pH range of approximately 2-7.5. Above pH 7.5 the silica substrate is susceptible to dissolution in aqueous solutions. Below pH 2.0 the silyl ether linkage is labile and the functional groups on the surface will begin to cleave. In practice bonded silicas may be used for sorbent extractions in the pH range 1-14 since degradation of the sorbent is a finite process and sorbents are typically exposed to solvents for only short periods of time. Bonded silica sorbents are rigid materials that do not shrink or swell in different solvents, unlike many polystyrene-based resins. For this reason, bonded silicas equilibrate rapidly to new solvent conditions. This allows complex extraction procedures involving many different solvent changes to be performed rapidly.

Sorbent extraction is a physical extraction process that involves a liquid and a solid phase. In sorbent extraction the solid phase has a greater attraction for the isolate than the solvent in which the isolate is dissolved. As the sample solution passes through the sorbent bed, the isolate concentrates on this surface, while the other sample components pass through the bed. Very selective extractions resulting in highly purified and concentrated isolates can be achieved by choosing sorbents with an attraction for the isolate but not for the other sample components.

A silica sorbent resembles a forest of functional groups anchored through a root system of siloxane bridges. These bonds and the silica beneath

them are responsible for two important properties of silica sorbents: the need for solvation of the materials and the potential for secondary interactions with isolate molecules. Therefore solvation of a sorbent is necessary before the sorbent will interact reproducibly with the isolate. Solvation is a wetting of the sorbent creating an environment suitable to the isolate the drug. Retention is accomplished by passing several bed volumes of a suitable solvent. The adsorptive properties of a bonded silica are due principally to the functional groups bonded to the silica substrate. Any unbonded silanols remaining on the surface are a contributory factor to retention. Interaction between the substrate and isolate molecules is called a secondary interaction. It may be the predominantly active property of a sorbent. As a particular sample is passed through a sorbent bed, compounds present in the sample will either pass through the sorbent or be retained on the sorbent due to an attraction that exists between the sorbent and the isolate molecules, causing the isolate to be immobilized; this phenomenon is called retention. The process by which an isolate is removed from a sorbent bed in which it has been retained is called elution.

The selectivity of the sorbent is the ability of the sorbent to discriminate between the isolate and all other matrix components. It is a function of three parameters: the chemical structure of the isolate, the properties of the sorbent and the composition of the sample matrix.

The capacity of a given sorbent is defined as the total mass of a strongly retained isolate that can be retained by a given mass of the sorbent under optimum conditions. Capacity values range from less than 1% to 5% of

sorbent mass that is, 100 mg of sorbent might retain as much as 5 mg of a strongly retained isolate (Horne, 1985).

5.2.2.1 THE CHOICE OF BONDED PHASE

The choice of bonded phase depends on the sample matrix. For aqueous samples such as serum or urine, non-polar extraction is often used because non-polar drugs are easily extracted from an aqueous sample onto a non-polar phase and recovery depends less on pH and salt concentrations. To improve the selectivity of extraction, polar or weak ion exchange phases may be used; however, these phases often require sample pretreatment (e.g. sample dilution or pH adjustment) to efficiently extract a drug from the sample matrix. The lipid solubility and polar characteristics of the drug should first be considered. If the drug is very hydrophobic, the initial trial should include C_2 and C_8 phases, as well as C_{18} for comparison. Alternatively, if the drug has polar substituents, a cyano and/or diol phase may be more selective than C_8 or C_{18} columns.

After conditioning the bonded phase, the sample is added (usually 0.1-1 ml). Serum or plasma samples are usually added directly, but urine may be diluted with water or buffer to improve extraction from very concentrated samples. When extracting whole blood, the haemolysed sample is filtered or centrifuged to remove cellular debris that can clog the frits in the extraction column or physically interfere with extraction (Harkey, 1989). As the sample passes through the extraction column, components will either bind to the bonded phase or pass through the column unretained, depending on the

affinity of the compound for the bonded phase as compared to its solubility in the sample matrix.

After the sample is extracted potential interferences may be removed by washing the column. For most non-polar phases, water can be used to remove many of the polar constituents of serum or urine without eluting drugs. More non-polar contaminants may be removed by adding relatively weak solutions of methanol or acetonitrile in water or buffer.

Finally, the analyte is eluted from the extraction column. The elution solvent for non-polar phases is usually methanol, acetonitrile, or a mixture of organic solvent with water or buffer. Depending on the concentration of the analyte and the method of analysis, this solution may be analysed directly without evaporation and reconstitution. To improve sensitivity the analyte should be eluted in as small a volume as possible, which typically concentrates the samples five to tenfold. However, the volume of eluting solvent must be sufficient to pass through all pores and interstitial spaces in the bonded phase.

Large numbers of publications in recent years have reported the use of bonded silica for benzodiazepine drugs extraction from serum, plasma and urine using C_{18} columns (Wilhelm and Kemper, 1990; Suzuki et al, 1988; Kabra and Uche Nzekwe, 1985; Seno et al, 1991; Carlucci, 1988; Good and Andrews, 1981; Stewart et al, 1984; Rao et al, 1982). Moore and Oliver, 1988 and 1992 - used other commercially bonded phase columns (C_8 and -CN columns) to extract benzodiazepine drugs from greyhound urine. Recoveries in excess of 85% for C_8 columns and above 90%, for nineteen benzodiazepine drugs extracted from CN columns were reported. More selective sorbents

such as ion exchange is (SCX) have been used for the extraction of the benzodiazepines and drugs from human urine (Logan et al 1990). This workers recorded with absolute recoveries ranging from 78 $\pm$ 5.5 to 95.0 $\pm$ 6.4 for diazepam and nordiazepam respectively.

The above-reported benzodiazepine drugs extraction procedure is not applicable to whole post-mortem human blood or tissue samples. Since most of the post-mortem blood which is routinely encountered in forensic toxicology casework is usually haemolysed and often putrefied.

Seno et al (1991) claimed a good recovery but, unfortunately, this paper gave no recovery data for the twelve benzodiazepines extracted from urine, plasma and whole blood. His method was based on converting the benzodiazepines to benzophenones. This method of extraction required sample preparation including treating the biological materials with hydrochloric acid and heating at 100^{o}C for one hour in the case of urine or centrifugation at 35000 rpm for 5 minutes in the case of serum and whole blood. This makes this method time-consuming. Also, the GC chromatograms showed big impurity peaks, which could be due to using a non-selective solid phase sorbent (C_{18}).

A literature review provided no reports on direct extraction of benzodiazepines from human post-mortem blood employing solid phase sorbent systems. A method has been developed to extract benzodiazepines from human post-mortem blood directly and with special sample treatment in the case of human brain tissue extraction.

Solid phase extraction techniques are gaining a wider acceptance as sample clean-up tools in pharmaceutical analysis especially for drugs in biological material.

Four commercially available solid adsorbent materials were evaluated for their applicability as tools for the extraction of five selected benzodiazepine drugs from aqueous, post-mortem human blood and human brain tissue samples. Five benzodiazepine drugs were selected.

5.2.2.2 POLAR INTERACTION (NH_2/CN)

Polar interactions are exhibited by many different sorbents and functional groups on isolates. Polar interactions include hydrogen bonding, dipole/dipole induced dipole/dipole, Pi-Pi, and a variety of other interactions in which the distribution of electrons between individual atoms in the functional groups is unequal, causing positive and negative polarity. This property allows an isolate molecule bearing a polar functional group to interact with a polar group on a sorbent. Groups that exhibit these types of interactions typically include hydroxyls, amines, carbonyls, aromatic rings, sulphydryls, double bonds and groups containing hetero-atoms such as oxygen, nitrogen, sulphur, and phosphorus.

Because of the polar nature of the silica substrate (and especially of unbonded silanol groups), polar interactions are characteristic of all bonded silicas. These secondary polar interactions of the silica are most significant in

non-polar solvents. Amine and hydroxy isolate groups are the most sensitive to polar secondary interactions.

Hydrogen bonding is one of the more significant polar interactions. It occurs between one group that contains hydrogen bonded to an electro-negative atom such as oxygen or nitrogen, and another group bearing an electro-negative atom. Hydroxyl groups and amines are the most common hydrogen bond donors. Functional groups that can interact with hydrogen bond donors (i.e., hydrogen bond acceptors) are other groups containing oxygen, nitrogen, or sulphur atoms.

Polar interactions between polar sorbent groups and polar groups on isolates are facilitated by non-polar solvents. This is because the strong electronic interaction between the sorbent and the isolate cannot be easily disrupted by non-polar solvent molecules. Conversely, polar interactions are most effectively disrupted by polar solvents, because polar isolates are more soluble in polar solvents, and because polar solvents can compete more effectively with the isolate for the sorbent than can non-polar solvents.

High ionic strength also will disrupt polar interactions. Often, polar isolates will be retained on non-polar sorbents through secondary interactions with the silica substrate, but this retention is inhibited by high ionic strength.

NH_2 (AMINOPROPYL) SORBENTS

The NH_2 functional group is capable of exhibiting all possible interactions. For this reason, it is important to pay close attention to the solvent/sample matrix environment, which will dictate the interactions occurring. An NH_2 group imparts polar properties to a sorbent, and strong hydrogen bonder that also can function as an anion-exchanger. Since the pKa of the NH_2 sorbent is 9.8, at any pH below 9.8 NH_2 is positively charged.

It is a weaker anion-exchanger than sorbents like SAX (a quaternary amine sorbent that is always charged), and is therefore a better sorbent choice for retention of very strong anions, such as sulphonic acids which may be retained irreversibly on SAX.

Although NH_2 has been used for non-polar isolations from polar solvents, its extreme polarity makes its non-polar character less significant than its other properties.

CN (CYANOPROPYL) SORBENTS

A medium polarity sorbent, CN is ideal for applications in which extremely non-polar isolates would be irreversibly retained on non-polar sorbents such as C_{18} or C_8. Conversely, CN can be used as a polar sorbent that is less retentive for very polar isolates that might be retained irreversibly on the more polar sorbents, such as silica or diol. The cyano group provides a

unique selectivity, which can be moderated by intelligent use of eluting solvents.

I. Extraction Buffer Preparation

Disodium hydrogen orthophosphate dihydrate (Sorensen's Salt, $Na_2HP0_4.2H_20$, m.wt 177.99) buffer was made up by dissolving 1.78 grams of $Na_2HP0_4.2H_20$ in deionised water and making up to one litre. The resulting buffer concentration of 0.01M was adjusted with a few drops of orthophosphoric acid (H_3P0_4) to pH 7.

II. Standard and Internal Standard Solutions

A stock solution containing the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) was prepared by dissolving 2 mg of each drug in a few drops of methanol and then diluting to 100 ml with deionised water to produce a stock standard solution of 20 ug/ml. Further dilution with deionised water was made to produce a working solution of 9 ug/ml.

The internal standard solution (prazepam) was prepared by dissolving 2 mg in a few drops of HPLC grade methanol and making up to 100 ml with deionised water, the resulting concentration being 20 ug/ml.

III. Sample Preparation

0.9 ml of deionised water was transferred to a 6 ml screw-capped vial. The following solutions were added:

1. 0.1 ml of the working standard mixture of benzodiazepine drugs (9 ug/ml).
2. 0.1 ml internal standard (prazepam 20 ug/ml).
3. 1 ml of 0.01M disodium hydrogen orthophosphate buffer (pH 7).

The vial was mixed well and the samples were extracted as follows:

IV. Extraction Procedure

CN (cyanopropyl) is a medium polarity sorbent, while NH_2 (Aminopropyl) is a very polar sorbent and a strong hydrogen bonder.

Bond Elut^R (CN, NH_2) columns of 3ml capacity were used. They were conditioned with 3 x 1ml of methanol followed by 3 x 1ml deionised water and followed by 3 x 1ml (0.01M, pH 7) disodium hydrogen orthophosphate dihydrate buffer.

V. Addition of Sample

The above mixture (III above) was transferred to pre-conditioned Bond Elut^R CN, NH_2 columns and drawn slowly through under vacuum. The vacuum pressure generated by the water pump was kept between 5-10 PS.I. The cartridge was not allowed to dry out.

The sorbent was washed twice with 1 x 1 ml 0.01M $Na_{2H}P0_4$ (pH 7), and drained by passing air for 30-40 seconds, the column was washed twice with 1 x 1ml of 0.01M Na_2HPO_4 (pH 7).

VI Elution of the Sample

The drugs were eluted from the sorbents using different elution solvents:

a. 1% H_3PO_4 in methanol - the pH of the solution was adjusted to pH4 by adding a few drops of ammonia solution (NH_3 35%).

b. Methanol was adjusted with a few drops of 1N sodium hydroxide (NaOH) to pH 11.

VII. Analysis of the Sample

The eluent was evaporated to dryness by placing the vial on a hot plate at 60^{o}C under a nitrogen gas stream. The extract was reconstituted in mobile phase (100 ul) (Section 5.1.3). 20 ul was injected onto the HPLC system.

VIII. Results and Discussion

The relative recoveries of the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) from spiked deionised water using the two sorbents (CN = cyanopropyl and NH_2 = aminopropyl) were calculated by comparing the peak height ratio of the extracts from deionised water with un-extracted standard solutions prepared in mobile phase at the same concentration and under identical conditions.

Table 29 lists a recovery of above 88% for all benzodiazepine drugs using the acidic eluent and low recoveries using the basic eluent (less than 76%).

Extracting whole post-mortem blood samples with four benzodiazepine drugs with Bond Elut CN columns, of 6ml capacity, using acidic elution. The extracts were susceptible to emulsion formation which account for the recovery and there was considerable sample to sample formation. Peaks were broad and badly tailed.

IX. CONCLUSIONS

In order to improve the efficiency, selectivity and to avoid problems arising from polar sorbent, it was decided that further work should be done using other solvents and changes in columns.

Table: 29 Percentage Recoveries of Benzodiazepine Drugs (0.9 ug/ml) from Spiked Water Using Polar Sorbents

CYNOPROPYL	Triazolam	Temazepam	Desmethyl-Diazepam	Diazepam
Acidic eluent	97 ± 3.1%	99 ± 2.2%	90 ± 3.8%	89 ± 5.2%
Basic eluent	54 ± 2.8%	75 ± 2.9%	76 ± 4.3%	81 ± 2%
AMINOPROPYL				
Acidic eluent	25 ± 3%	13 ± 2%	19 ± 6.1%	18 ± 3%
Basic eluent	24 ± 2.7%	8 ± 6%	13 ± 3.9%	15 ± 6.2%

Column: Hypersil 5 um C_{18} Universal Cartridge (25 cm x 4.6 mm i.d.)

Mobile Phase: 0.01M disodium hydrogen orthophosphate (pH 8.8):Methanol (70:30)

Detection Wavelength: 230 nm

Flow Rate: 1 ml/minute

5.2.2.3 EXPLORING THE NON-POLAR INTERACTION (C_2,C_8)

Non-polar interactions are those that occur between the carbon-hydrogen bonds of the sorbent functional isolate. These forces are commonly known as "Van der Waals" or "dispersion" forces. Since most organic molecules have some non-polar structure, non-polar interactions are often used to retain isolates on sorbents offering non-polar functional groups on the surface.

Unbonded silica does not exhibit non-polar interactions. Since most sorbent functional groups are bonded to the silica substrate through carbon chains of some length, all of the other commonly available sorbents exhibit some degree of non-polar interactions.

All isolate species have a potential for non-polar interactions. Exceptions include inorganic ions and compounds (carbohydrates, for example) whose structure contains so many polar or ionic groups that the carbon structure of the molecule is masked.

Non-polar interactions between non-polar sorbents and non-polar isolates are facilitated by solvent environments that are very polar in nature. Polar solvent/matrix environments are generally not capable of disrupting the dispersion forces of the non-polar interaction. Even where the isolate has polar groups, any significantly non-polar part of the isolate will interact with non-polar functional groups of the sorbent in a polar environment. The best example of a solvent that facilitates retention due to non-polar interactions is water.

On the other hand, non-polar interactions between the isolate and the sorbent are best disrupted by solvents having some degree of non-polar character. For many isolates, even a solvent as polar as methanol has enough non-polar character to disrupt the non-polar interaction between the isolate and the sorbent, causing elution of the isolate from the sorbent. For more non-polar isolates, a solvent as non-polar as chloroform may be required for elution of the isolate.

C_{18} (OCTADECYL)

C_{18} is the most non-polar sorbent available. It is the most retentive of all sorbents for isolates being retained by a non-polar mechanism.

Extremely non polar compounds are often difficult to elute from C_{18}. C_{18} is generally regarded as the least selective sorbent, since it retains almost everything from aqueous matrices - often a benefit when the isolates vary widely in structure. Because of C_{18}'s low selectivity, final extracts are often not as pure as they are when more selective sorbents are employed. C_{18} is excellent for desalting matrices prior to ion-exchange because salts pass through it unretained.

The potential for polar interactions between isolates and sorbent is less significant with C_{18} than with any other sorbent because of the predominant effect of the long hydrocarbon chain.

C_8 (OCTYL, 8-CARBON STRAIGHT-CHAIN HYDROCARBON)

C_8 is very similar in properties to C_{18}, but not quite as retentive for non-polar isolates, due to its shorter hydrocarbon chain. For this reason, C_8 is a good replacement for C_{18} for isolates that are too strongly retained on C_{18} for effective elution. Almost all other considerations are the same for C_{18} and C_8.

Non-polar isolate retention is facilitated on C_8 by conditioning with organic buffers as opposed to inorganic. In general inorganic buffers at high ionic strenght will promote isolate elution.

The potential of C_8 for polar interactions with isolates is somewhat higher than for C_{18} because the shorter hydrocarbon chain does not mask the silica surface as effectively, but polar interactions are still not a significant property of C_8.

C_2 (ETHYL, 2-CARBON STRAIGHT-CHAIN HYDROCARBON)

Because of the very short chain length of the functional group, leaving the silica surface somewhat exposed, C_2 is a fairly polar sorbent.

C_2 is often used as a replacement for C_{18} and C_8 when isolates are retained too strongly by the latter. C_2's polarity is slightly greater than CN for polar interactions.

A. HUMAN POST-MORTEM BLOOD

Authentic human post-mortem blood tested for the presence of benzodiazepine drugs and shown to be negative for all benzodiazepines (using HPLC and radio-immunoassay) was used to prepare a standard benzodiazepine drugs mixture (triazolam, temazepam, desmethyldiazepam and diazepam).

I. Extraction Buffer Preparation

Disodium hydrogen orthophosphate dihydrate (Sorensen's Salt $Na_2HP0_4.2H_20$, M.wt 177.99) buffer was made up by dissolving 1.78 grams of $Na_2HP0_4.2H_20$ in deionised water, the resulting buffer concentration of 0.01M was adjusted with a few millilitres of 1N sodium hydroxide (NaOH) to pH10.4.

II. Stock Standard and Internal Standard Preparation

A stock solution containing the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) was prepared by dissolving 2 mg of each drug in a few drops of methanol and then diluting to 100 ml with deionised water to produce a stock standard benzodiazepine solution of 20 ug/ml. Further dilution with deionised water was made to produce a working solution of 9 ug/ml.

The internal standard solution (prazepam) was prepared by dissolving 2 mg with a few drops of HPLC grade methanol and making up to 100 ml with deionised water, the resulting concentration of 20 ug/ml.

III. Sample Preparation

0.9 ml of blank human post-mortem blood was transferred to a 6 ml screw-capped vial. The following solutions were added:

1. 0.1 ml of the working standard mixture of benzodaizepine drugs (9 ug/ml).
2. 0.1 ml internal standard (prazepam 20 ug/ml).
3. 1 ml of 0.01M disodium hydrogen orthophosphate buffer (pH 10.4).
4. 1 ml of 2% ammonia solution (NH_3 35%)

Then the tube was mixed well. Six samples of spiked human post-mortem blood were extracted through Bond Elut^R C_8 columns of 3 ml capacity.

IV. Extraction Procedure

Bond Elut^R C_2 or C_8 cartridges (3ml capacity) were attached to a Vac-Elut box, which can hold up to 10 cartridges. Vacuum was applied using a

water pump and the cartridges were conditioned as follows by drawing the following solutions through in succession:

- 3ml x 1ml of HPLC grade methanol
- followed by 3 x 1ml deionised water
- followed by 3 x 1ml 0.01M disodium dihydrogen ortho-phosphate (pH 10.4).

V. Addition of Sample

The prepared sample was transferred to the preconditioned Bond Elut^R C_2 or C_8 column with the aid of a disposable pipette and drawn through under vacuum (the vacuum pressure generated by the water pump was kept between 5-10 PSI and the cartridge was not allowed to dry out between successive applications); then the column was washed twice with 1 ml of 0.01M Na_2HP0_4 (pH10.4) followed by 2 ml HPLC grade hexane.

VI. Elution of the Sample

The sorbent was washed twice with 2ml of 0.01M Na_2HP0_4 (pH10.4) and final elution of the drugs from the sorbent was performed with 1 x 5ml chloroform.

VII. Analysis of the Sample

The extract was evaporated to dryness by placing the vial on a hot plate 60°C under a gentle stream of nitrogen gas. The residue was redissolved in mobile phase (0.1 ml Section 5.1.3.) and complete solution of the extract was achieved by placing in an ultrasonic bath for 5-10 minutes. 20 ul was injected onto the HPLC.

VIII Results and Discussion

Recoveries and Calibration Curves

The relative recoveries of the four benzodiazepines from spiked human post-mortem blood extracted using Bond Elut^{R} C_8 columns of 3 ml capacity, containing C_8 packing material was calculated by comparing the peak height ratios of the extracted samples with un-extracted standards prepared in mobile phase at the same concentration (Tables 30-33).

The calibration curves for the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) were constructed over the concentration range (0.05-2 ug/ml) prepared in blank human post-mortem blood. Six samples at each concentration for each drug were extracted through Bond Elut^{R} C_8 columns. The internal standard (prazepam) was added to all samples. The peak height ratios of drug over internal standard at each concentration were plotted to construct a calibration curve for each benzodiazepine drug. The relation between benzodiazepine drugs (triazolam,

temazepam, desmethyldiazepam and diazepam) concentration and average peak height ratio (std/I.S) of samples analysed by HPLC are highlighted in Tables 34-37. A standard calibration curve was constructed using the peak height ratios (std/I.S) and the benzodiazepine drug concentrations. A linear relation was obtained for each drug as shown in Figure 21-24. A 50 ng/ml of benzodiazepine drugs extracted solution was found to be easily detected at range 0.02, Figure 25.

From the results obtained above it can be seen that the method will detect 50 ng/ml of benzodiazepine, a typical chromatogram for this level is illustrated in Figure 25.

The Reproducibility of theExtraction Method

30 samples of human post-mortem blood spiked with the four benzodiazepine drugs mixture (triazolam, temazepam, desmethyldiazepam and diazepam) in a concentration of 0.9 ug/ml of blood were extracted through Bond Elut^{R} C_8 column 3 ml capacity and analysed over three successive days. The peak height ratio of each drug was used to calculate the within day and day to day variation of the extraction and analysis method of the four benzodiazepines using HPLC system.

Table 38 shows the average recoveries,coefficient of variation and number of samples used each time. Excellent recoveries were obtained for the four benzodiazepine drugs. The recovery was calculated by comparing the peak height of unextracted standard benzodiazepine drugs with extracted samples.

CONCLUSION

The developed method used gives rapid identification and quantitation of the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) most often encountered in Forensic Toxicology Cases.

The developed procedure has the advantages of minimal handling time (the chromatographic run time was less than 14 minutes at a flow rate of 1ml/minute and no peaks eluted after the internal standard, Figure 25

and Table 39), high recovery (even at subtherapeutic levels), and clean sample extracts. The method eliminates the need for expensive equipment such as centrifuges, shakers and glassware. A well-trained technician can obtain the results for twenty samples in less than six hours using the method outlined.

TABLE 30.

The recovery of triazolam from spiked post-mortem blood extracted through a C_8-column and analysed by HPLC.

Concentration µg/ml	Recovery	Number of samples
0.05	101±3.0%	6
0.2	102±4.0%	6
0.9	87±7.5%	6
2.0	95±5.9%	6

TABLE 31.

The recovery of temazepam from spiked post-mortem blood extracted through a C_8-column and analysed by HPLC.

Concentration µg/ml	Recovery	Number of samples
0.05	88±3.5%	6
0.2	98±3.0%	6
0.9	89±3.0%	6
2.0	92±3.0%	6

TABLE 32.

The recovery of desmethyldiazepam from spiked post-mortem blood extracted through a C_8-column and analysed by HPLC.

Concentration µg/ml	Recovery	Number of samples
0.05	86±2.0%	6
0.2	100±2.0%	6
0.9	91±4.0%	6
2.0	92±3.0%	6

TABLE 33

The recovery of diazepam from spiked post-mortem blood extracted through a C_8-column and analysed by HPLC.

Concentration µg/ml	Recovery	Number of samples
0.05	93±4.0%	6
0.2	99±3.0%	6
0.9	94±5.0%	6
2.0	92±4.0%	6

TABLE 34.

Relation between triazolam concentration and the average peak height ratio (std/I.S) of postmortem blood spiked with triazolam extracted through a C_8 column and analysed by HPLC

Concentration µg/ml	Mean peak height ratio n = 6
0.050	0.075
0.200	0.23
0.900	1.13
2.000	2.79

TABLE 35.

Relation between temazepam concentration and the average peak height ratio (std/I.S) of postmorte mblood spiked with temazepam extracted through a C_8 column and analysed by HPLC.

Concentration µg/ml	Mean peak height ratio n = 6
0.050	0.047
0.200	0.185
0.900	0.93
2.000	2.03

TABLE 36.

Relation between desmethyldiazepam concentration and the average peak height ratio (std/I.S) of postmortem blood spiked with desmethyldiazepam extracted through a C_8 column and analysed by HPLC.

Concentration µg/ml	Mean peak height ratio n = 6
0.050	0.0686
0.200	0.30
0.900	1.5
2.000	2.96

TABLE 37.

Relation between diazepam concentration and the average peak height ratio (std/I.S) of postmortem blood spiked with diazepam extracted through a C_8 column and analysed by HPLC

Concentration µg/ml	Mean peak height ratio n = 6
0.050	0.059
0.200	0.24
0.900	1.20
2.000	2.47

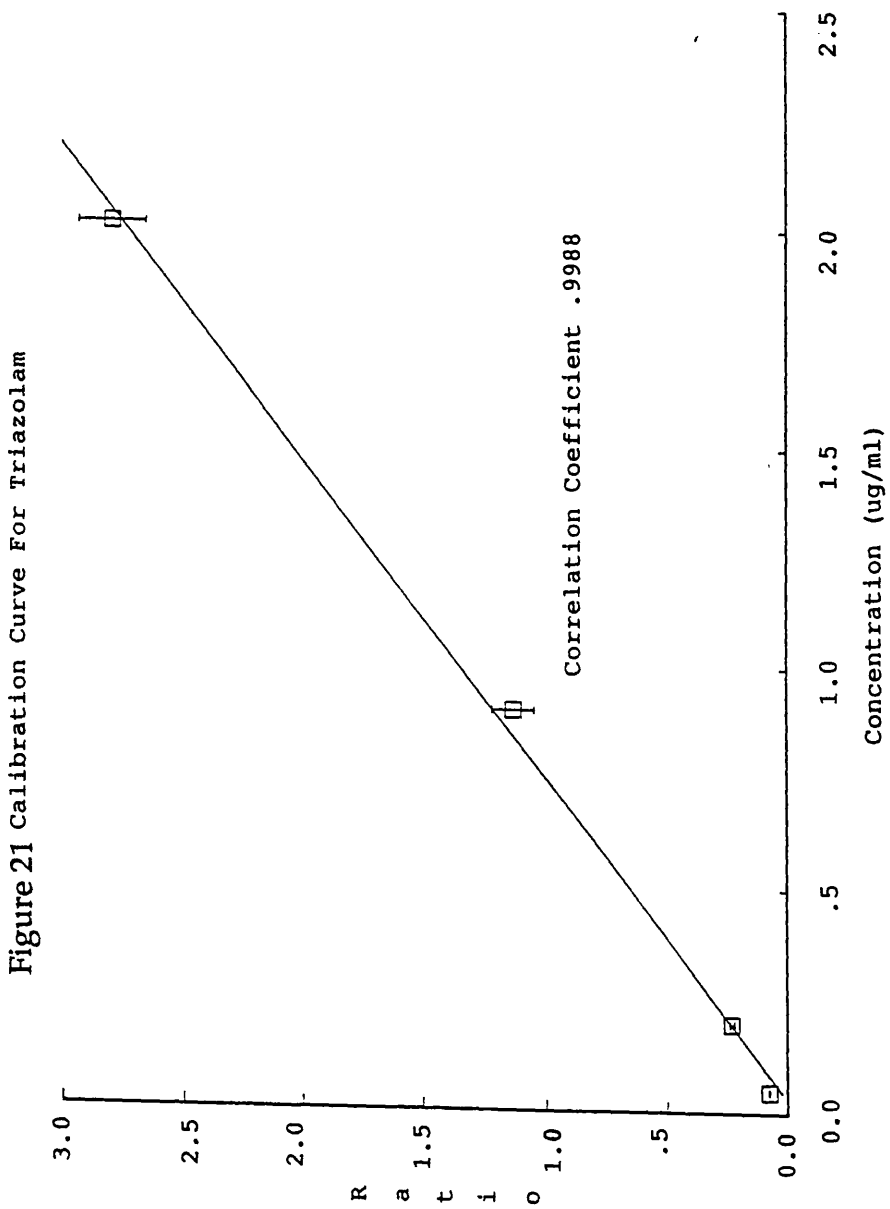


Figure 21 Calibration Curve For Triazolam
Correlation Coefficient .9988
Ratio
3.0
2.5
2.0
1.5
1.0
.5
0.0
0.0
.5
1.0
1.5
2.0
2.5
Concentration (ug/ml)

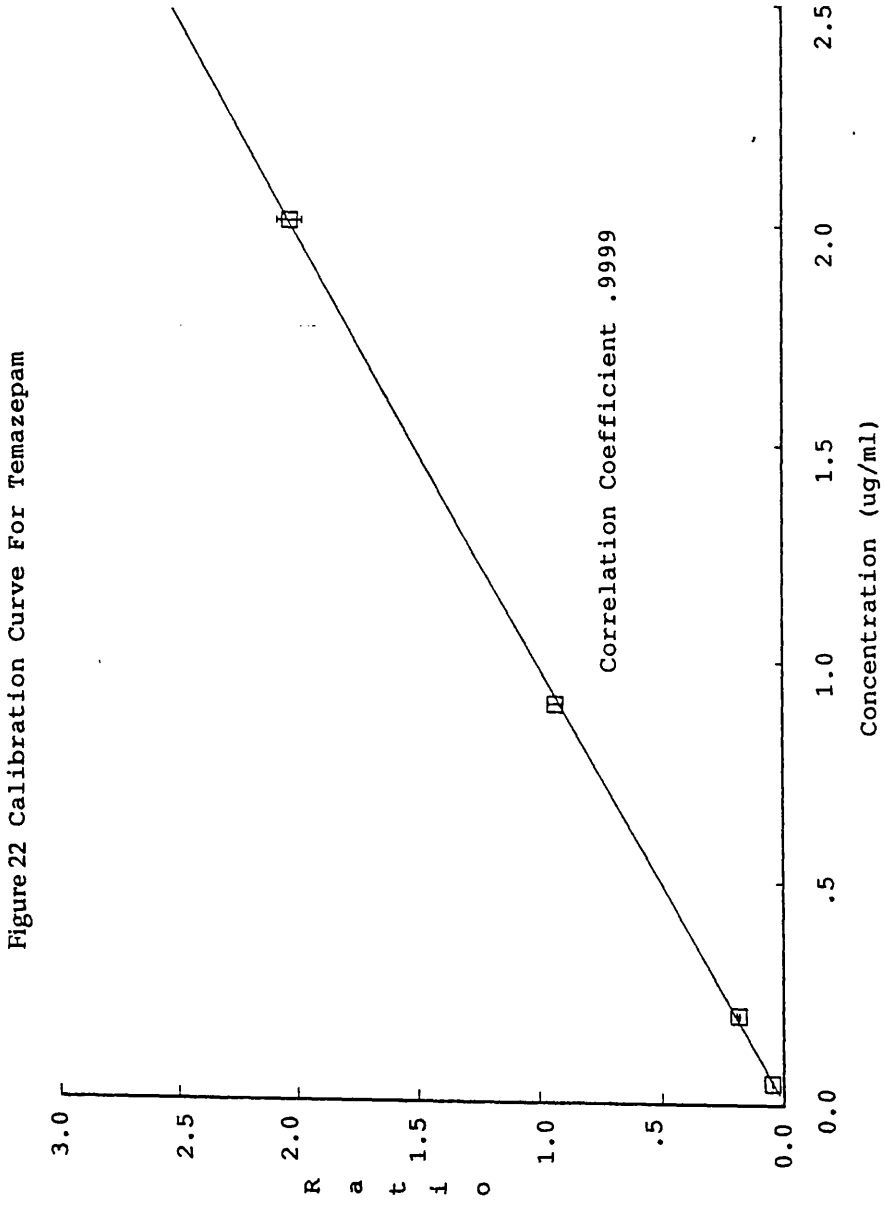


Figure 22 Calibration Curve For Temazepam
Ratio
3.0
2.5
2.0
1.5
1.0
.5
0.0
0.0
.5
1.0
1.5
2.0
2.5
Concentration (ug/ml)
Correlation Coefficient .9999

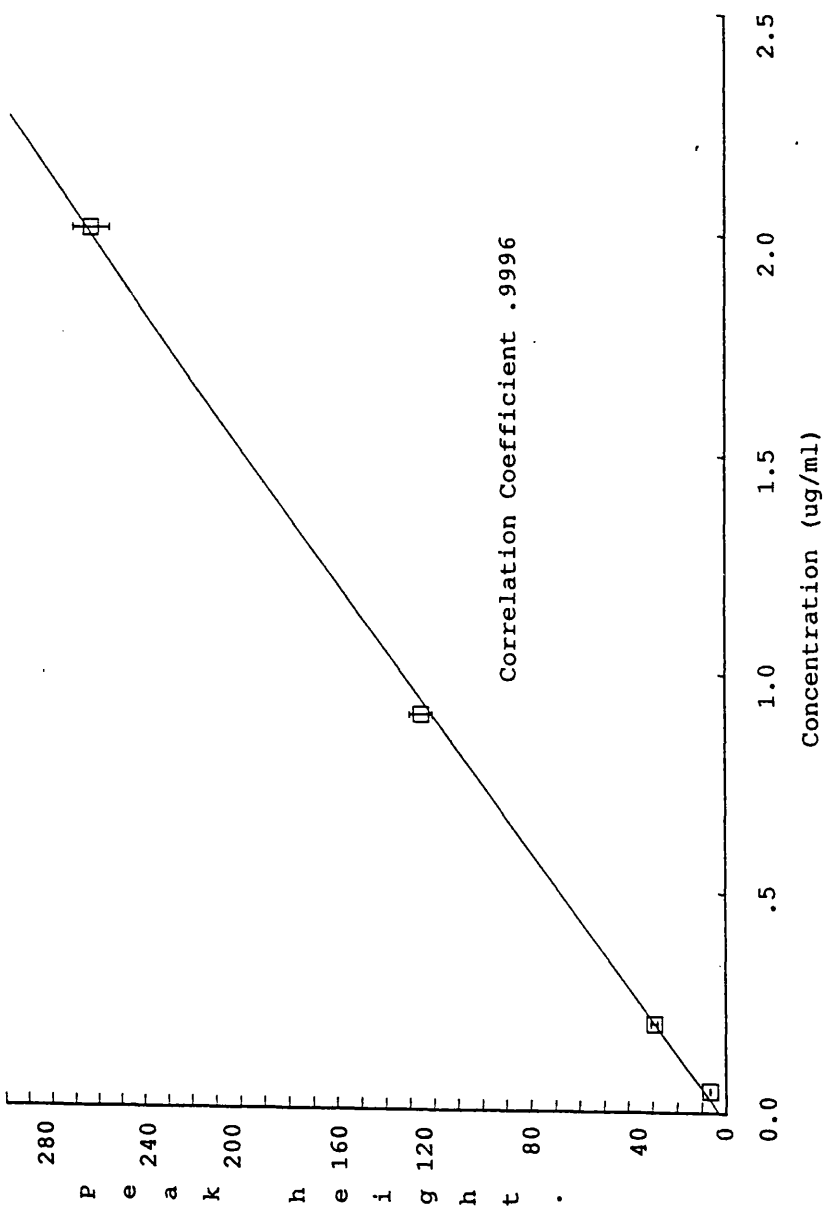


Figure 23 Calibration Curve For Desmethyldiazepam

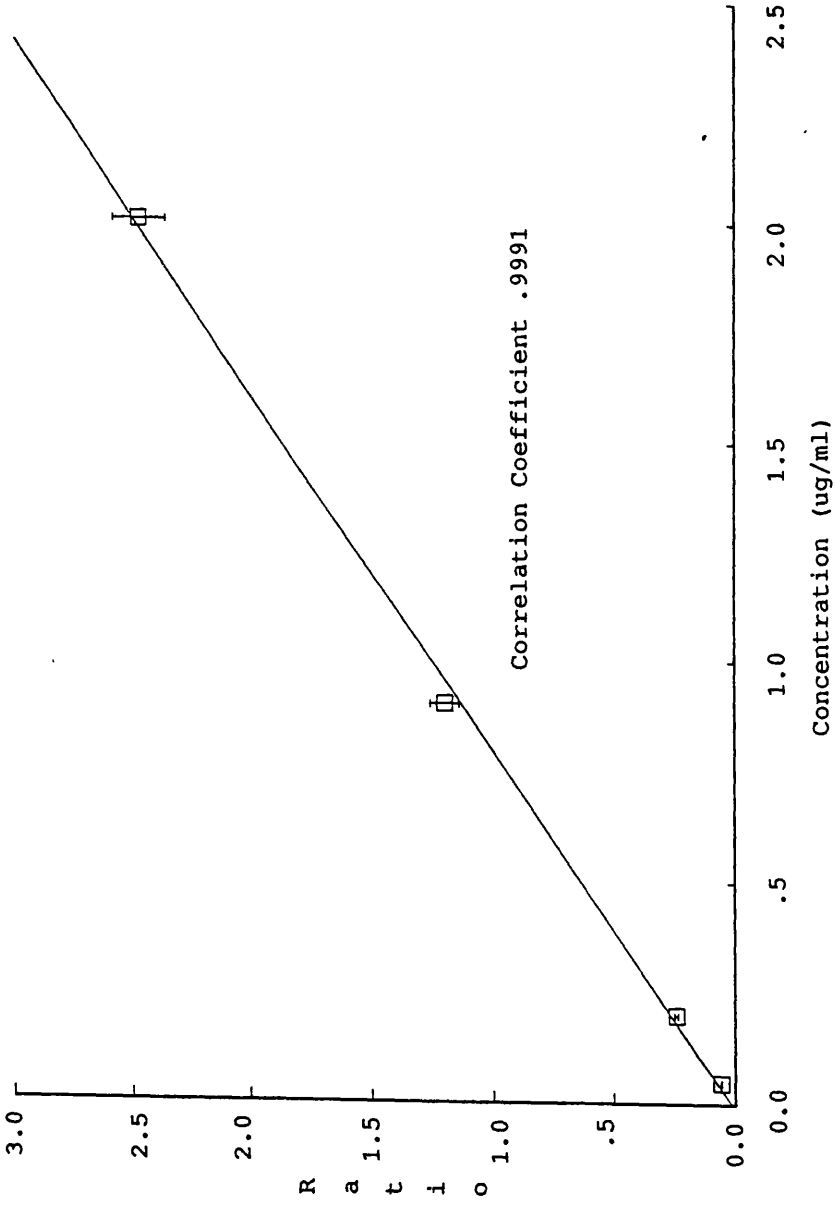
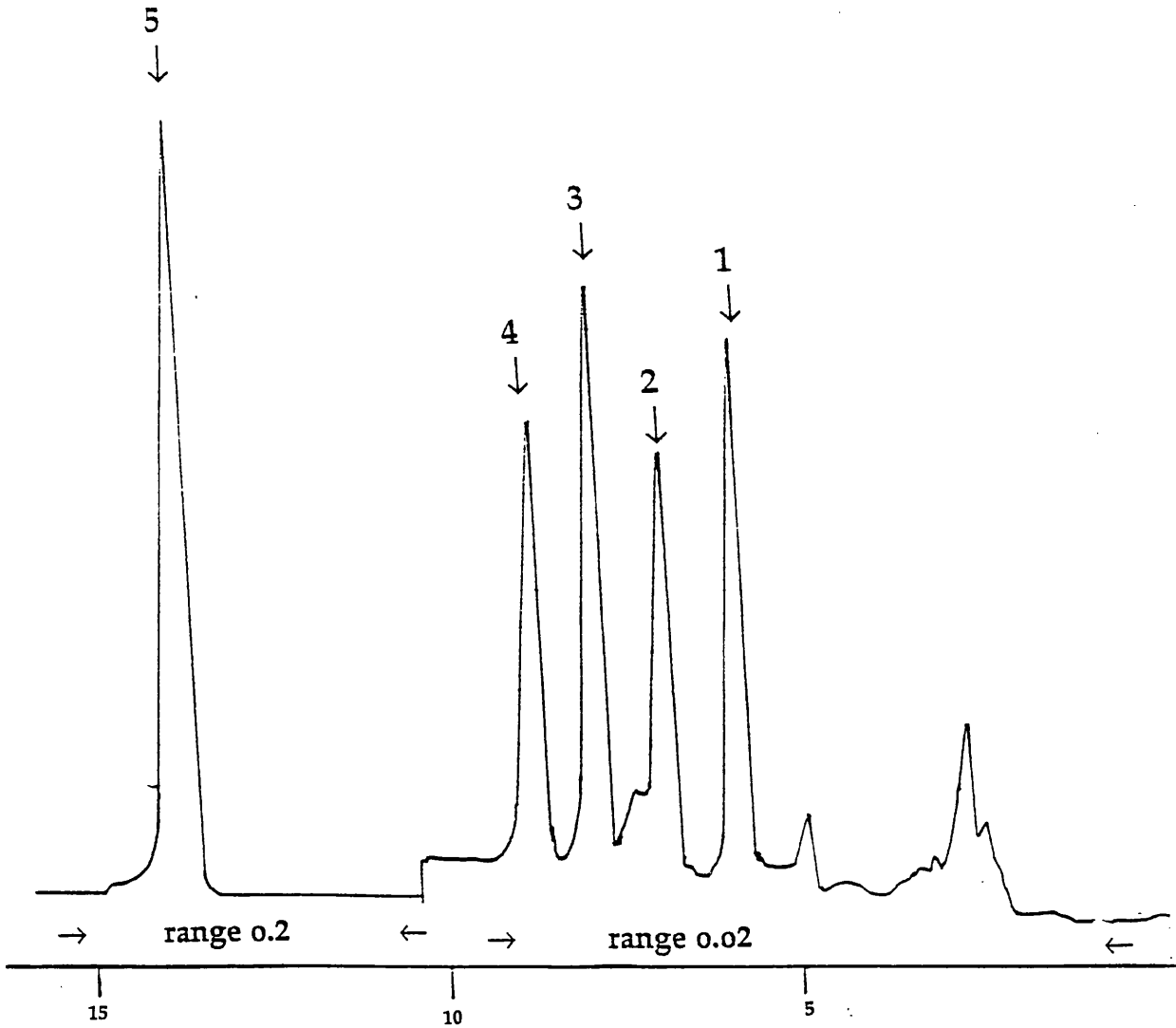


Figure 24 Calibration Curve For Diazepam
Correlation Coefficient .9991
Ratio
3.0
2.5
2.0
1.5
1.0
.5
0.0
0.0
.5
1.0
1.5
2.0
2.5
Concentration (ug/ml)



Injectio

Figure 25 Separation Of Benzodiazepine Drugs From Spikedwhole Post-mortem Blood (50ng)By HPLC System.

1-Triazolam. 2-Temazepam. 3-Desmethyldiazepam.
4-Diazepam. 5-Prazepam.

TABLE 38 Reproducibility of recovery of benzodiazepine drugs over a 3 day period using C_8 columns for extraction.

Drug *	Days	Average Recoveries	Coefficient Variation	No.of Samples Extracted
Triazolam	1	88%	4.6%	10
	2	87%	5.0%	10
	3	88%	4.0 %	10
Temazepam	1	89%	3.6%	10
	2	89%	2.8%	10
	3	89%	4.0%	10
Desmethyldiazepam	1	90%	5.1%	10
	2	91%	3.4%	10
	3	91%	4.2%	10
Diazepam	1	94%	4.8%	10
	2	94%	3.7%	10
	3	94%	3.4%	10

*Drug concentration 0.9ug/ml.

Table 39 Retention Times Of Benzodiazepine Drugs Analysed By HPLC.

Drug	Retention Times (minutes)
Triazolam	5.8
Temazepam	6.8
Desmethyldiazepam	7.9
Diazepam	8.8
Prazepam	13.8

Column: Hypersil 5μm C18 Universal Cartridge (25cmx4.6 i.d).
Mobile phase: 0.01M Di-sodium hydrogen orthophosphate(PH 8.8):methanol (30:70)
Detection Wavelength: 230 nm.
Flow rate: 1ml/minute.

IX. Applications of the Developed Method to Authentic Blood Samples And a Comparison of the Concentration of Benzodiazepine Drugs from Four Different Sites.

Introduction

Having established that levels of benzodiazepine drugs in post-mortem human blood can be detected at concentrations as low as 50 ng/ml following extraction on a C_8 column, of 3ml capacity, the developed method applied to twenty-four human post-mortem blood samples from 6 cases. The aim was to compare the concentration of the benzodiazepine drugs from four different sites (jugular vein, axillary vein, femoral vein and the heart). Post-mortem blood samples were collected from the fatalities. The characteristics of these cases with respect to age and binding site concentration in which triazolam, temazepam, desmethyldiazepam or diazepam were detected are summarised in Table 28.

Samples (1 ml whole human post-mortem blood) were extracted using non-polar interaction columns (Bond Elut^R C_8, 3 ml capacity) for the analysis of the benzodiazepine drugs (5.2.2.3). Spiked whole human post-mortem blood and blank (whole post-mortem blood) were extracted in parallel each time, and an internal standard was used as before for purposes of quantification. Each extraction was performed in duplicate. A single point calibration was used since the linearity of the procedure has been established above (pp 161-166).

Instrumental Conditions

The extraction procedure was carried out as described in 5.2.2.3.A-IV. The residue was reconstituted in 100 ul of the mobile phase and 20 ul were injected into HPLC system. HPLC conditions and the mobile phase were as described in 5.12 and 5.13. Detection by UV monitoring at wavelength of 230 nm.

Results and Discussion

The chromatograms produced from extracts of spiked whole human post-mortem blood sample were good. Examples for extracts of spiked whole human post-mortem blood, blank autopsy and authentic human autopsy blood samples are shown in Figures 26-28.

Table 40 lists the average concentrations of benzodiazepine drugs investigated in twenty-four post-mortem human blood samples. This study shows that there is a good correlation between the benzodiazepine drugs concentration taken from the heart and that taken from the other sites.

Conclusion

It can be concluded from the above results that the concentrations of benzodiazepine drugs in the heart, jugular vein, axillary vein and femoral vein are comparable. No significant differences could be detected.

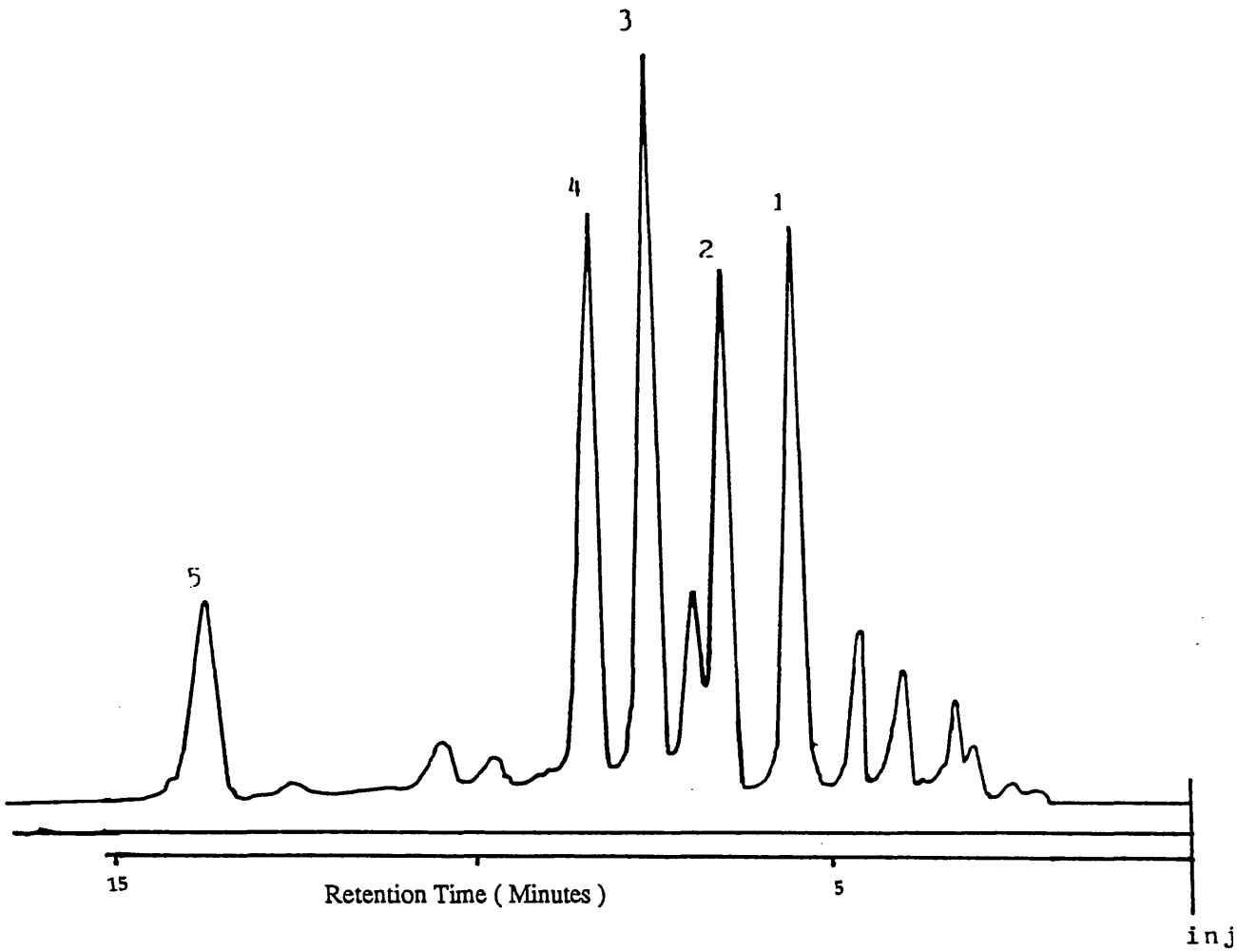


Figure 26 **The chromatogram of the four benzodiazepine analysed be HPLC system.**

1. Triazolam 2. Temazepam 3.Desmethydiazepam

4. Diazepam 5.Prazepam(Internal standard)

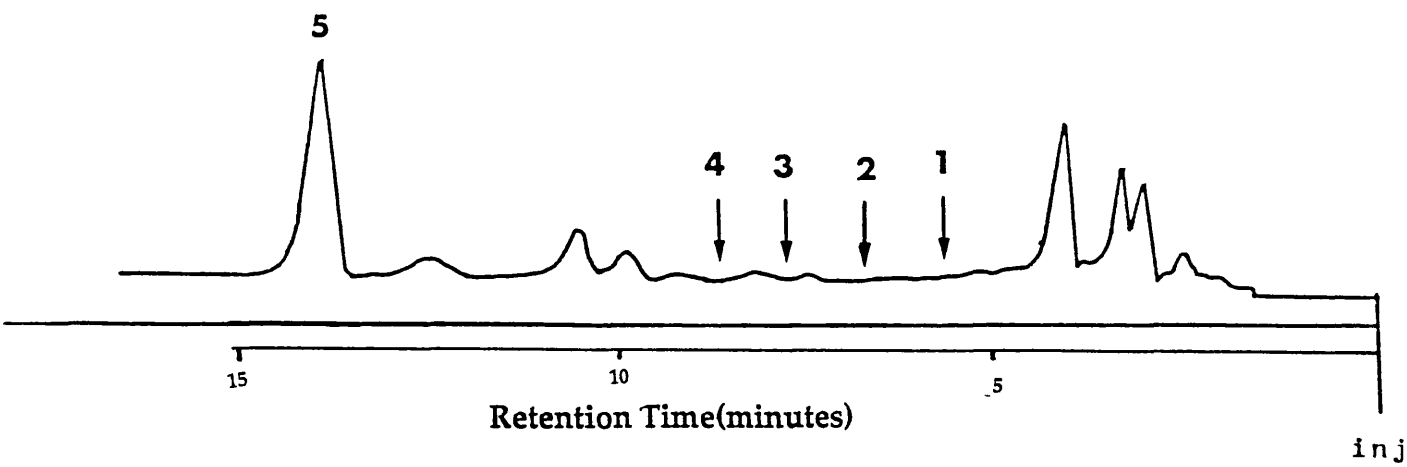


Figure 27 The chromatogram of negative (Blank)human post-mortem blood analysed by HPLC system.

1. Traizolam 2. Temazepam 3. Desmethyldiazepam

4. Diazepam 5. Internal standard (Prazepam)

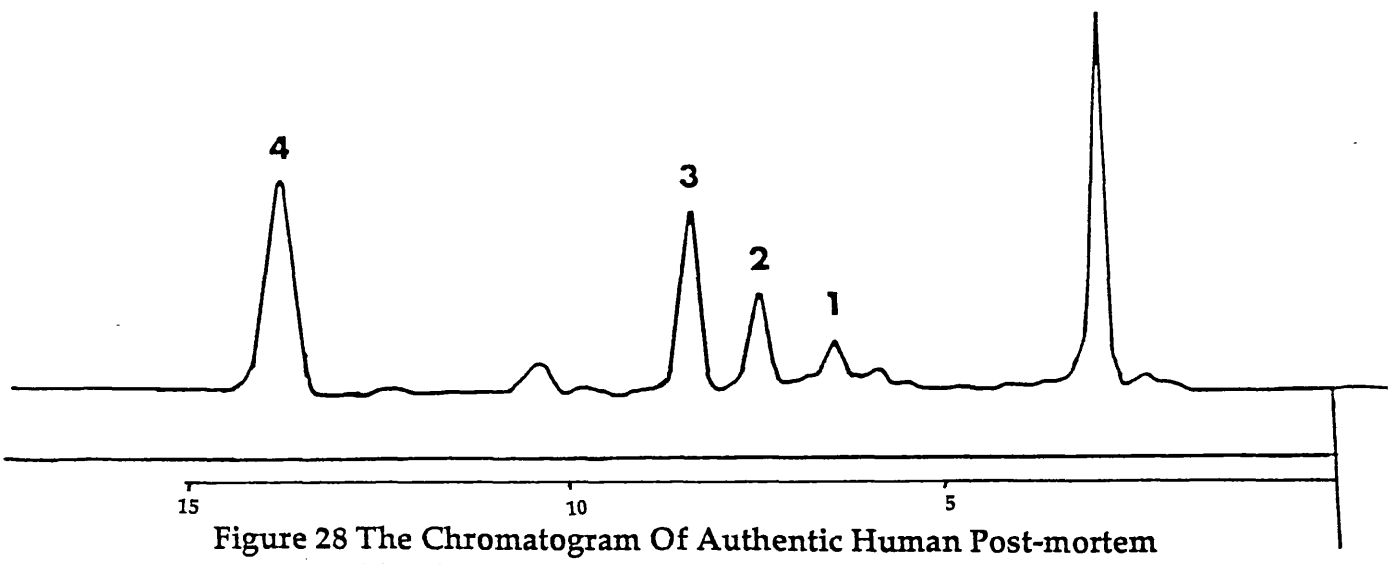


Figure 28 The Chromatogram Of Authentic Human Post-mortem blood Sample Analysed By HPLC System.

1-Temazepam 2-Desmethyldiazepam
2-Diazepam 4-Prazepam(internal standard)

Table 40 Average Recoveries Of Benzodiazepine Drugs From human Post-mortem Blood Samples From Different Sites Using Solid Phase Extraction.

Blood Site	Triazolam	Temazepam	Desmethyl diazepam	Diazepam
Jugular vein				
1	-	0.12	0.33	0.65
2	-	0.32	0.11	0.87
3	-	0.13	-	0.57
4	-	2.54	-	-
5	0.45	-	0.80	0.24
6	0.31	-	0.43	0.23
Axillary vein				
	-	0.12	0.32	0.64
	-	0.31	0.11	0.87
	-	0.12	-	0.56
	-	2.53	-	-
	0.45	-	0.80	0.24
	0.33	-	0.41	0.22
Heart				
	-	0.12	0.33	0.64
	-	0.30	0.10	0.86
	-	0.12	-	0.56
	-	2.53	-	-
	0.45	-	0.78	0.22
	0.33	-	0.42	0.22
Femoral vein				
	-	0.12	0.33	0.64
	-	0.32	0.11	0.87
	-	0.13	-	0.56
	-	2.55	-	-
	0.44	-	0.80	0.24
	0.32	-	0.41	0.23

X. Comparison of Extraction Efficiency of the Developed (Solid-Phase Extraction) Method with Liquid-liquid Extraction

Human Post-Mortem Blood Samples

Twenty-four post-mortem blood samples were extracted by a conventional solvent extraction method (Eppel, 1980) in parallel with the developed non-polar solid-phase extraction procedure.

Liquid-Liquid Extraction

1. Extraction Buffer Preparation

Disodium hydrogen phosphate dihydrate (M.wt. 177.99) buffer was made up by dissolving 1.78 grams of Na_2HP0_4 in deionised water and making up to one litre, producing a buffer concentration of 0.01M with a pH of 7.4.

2. Standard Solution Preparation

A standard solution of benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam, diazepam) was made up as before (5.2.2.2.II)

3. Extraction Procedure

Samples (1 ml whole human post-mortem blood) were extracted using liquid-liquid extraction (Eppel, 1980) for analysis for benzodiazepine drugs. Spiked and blank whole post-mortem blood were extracted in duplicate each time.

0.1 ml of the internal standard solution (prazepam 20 ug/ml) and 1 ml of the phosphate buffer (0.01M, pH 7.4) were transferred into a ground-glass stoppered test tube. 1 ml of sample and blank blood were added to the tube. In the case of standards, 0.9 ml of blank blood followed by 0.1 ml of standard benzodiazepine mixture (20 ug/ml) were added then the solutions were extracted using 5 ml of dichloromethane in ground glass stoppered test tubes placed on a (rock and roll) machine for twenty minutes. The supernatant layer was removed by Pasteur pipette. The dichloromethane was filtered through phase separation paper into a screw-capped glass vial. The dichloromethane was evaporated to dryness at 60^{o}C under a nitrogen gas stream. The extracts were redissolved by adding 100 ul of mobile phase and 20 ul was injected onto the HPLC.

4. Instrumental Conditions

Both extraction procedures (liquid-liquid extraction and solid-phase) were carried out as described in 5.2.2.3. HPLC conditions and the mobile phase were as described in 5.1.2.

5. Results and Discussion

The results of the comparison are given in Table 41. The C_8 column extractions were more efficient than the liquid-liquid extraction. Solid-Phase extraction, had the advantage of being extremely clean compared to the conventional solvent extraction method.

The developed method has a number of advantages over conventional liquid-liquid extraction. The use of bonded silica phase and a solvent eliminates emulsion formation between the two immiscible solvents, common in liquid-liquid extraction, which results in loss of analyte. The large volume of organic solvent utilised by liquid-liquid extraction is often hundreds of millilitres compared to the few millilitres used in solid-phase, single-step extraction compared to the multiple back extractions often required by liquid-liquid extraction. The ability of solid phase extraction to allow separation of a drug and its metabolites which cannot often be achieved successfully by liquid-liquid extraction, successful automation. Hence solid-phase extraction is more efficient than liquid-liquid extraction, in terms of increased amount of analyte extracted from the endogenous material and reduced analysis time and cost.

Table 41 Comparison of solid-phase and liquid-liquid extraction of benzodiazepines from human blood samples.

No.	Triazolam (ug/ml)		Temazepam (ug/ml)		Diazepam (ug/ml)		Desmethyl-diazepam (ug/ml)	
Extraction	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid
1	-	-	0.12	0.09	0.64	0.60	0.33	0.28
2	-	-	2.54	2.31	-	-	-	-
3	0.45	0.41	-	-	0.22	0.17	0.78	0.68
4	-	-	0.32	0.23	0.86	0.76	0.10	0.09
5	-	-	0.12	0.08	0.56	0.42	-	-
6	0.33	0.26	-	-	0.22	0.18	0.42	0.36

B. HUMAN BRAIN TISSUE EXTRACTION

I. Introduction

The distribution of benzodiazepine receptors have been investigated in post-mortem human brain tissue (4.6) using the binding method described in 4.5.5. Results indicate a high density in the cortex of the precentral area (frontal lobe cortex).

II. Human Brain Tissue

Human post-mortem brain tissue sample was obtained at autopsy from a male aged twenty-one years, who died from hanging and had no treatment with any benzodiazepine drug at the time of death. In confirmation of the above information, a blood sample was taken from the deceased and analysed for the presence of benzodiazepines using radio-immunoassay and HPLC and was found to be negative by both methods.

The brain was removed at the time of the autopsy (as described in 4.5.3) and the grey matter (from precentral area) was freshly dissected without thawing and pulverized on dry ice. Dissected tissue (grey matter from the precentral cortical area) was stored at -20^{o}C until used.

III. Extraction Buffer Preparation

0.01M (pH10.4) disodium hydrogen orthophosphate dihydrate ($Na_2HP0_4.2H_20$, M.wt 177.99) and 2% ammonia solution (NH_3 35%) were prepared as described in 5.2.2.1.

IV. Standard And Internal Standard Solutions

A stock standard solution 20 ug/ml containing the four benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) and the internal standard (prazepam 20 ug/ml) were prepared in water as described in 5.2.2.2.II. These stock solutions were diluted with deionised water to yield concentrations of 1, 2, 9 and 2 ug/ml.

V. Sample Preparation

One gram of pulverised control human brain tissue (from the precentral cortex area) was transferred to 15 ml screw-capped vial. The following solutions were added:

1. - 0.1 ml benzodiazepine drugs standard mixture 20 ug/ml (triazolam, temazepam, desmethyldiazepam and diazepam).
2. - 0.1 ml internal standard (prazepam 20 ug/ml).
3. - 2.9 ml of 0.01 M Na_2HP0_4 (pH10.4).
4. - 1.9 ml of 2% ammonia solution (35% NH_3).

5. 1 mg of subtilisin Carlsberg (crystalline bacterial alkaline protease).

Then the tube was placed in a water bath for 30 minutes at 50-60°C with continuous magnetic stirring (to liberate benzodiazepine drugs from the tissue). The resulting solution was cooled to room temperature and the tube was centrifuged for 10 minutes at 5.000 rpm using a Beckman (J2-21) single head centrifuge.

The supernatant was transferred, using a Pasteur pipette, into a 6 ml screw capped glass vial.

VI Column Conditioning

Bond Elut^{R} C_8 column with a capacity of 6 ml were positioned in a Vac-Elut system. Vacuum pressure generated by the water pump was kept between 50-10 mmHg and the cartridge was not allowed to dry out. The cartridges were conditioned by successive application of the following solutions:

- 2 x 4 ml HPLC grade methanol
- followed by deionised water 2 x 4 ml.
- followed by 2 x 4 ml 0.01M Na_2HP0_4(pH 10.4)

VII. Addition Of Sample

The supernatant (V. above) was transferred to a preconditioned Bond Elut^{R} C_8 column, 6 ml capacity and drawn through under vacuum pressure. The column was washed with two millilitres of 0.01M disodium hydrogen orthophosphate buffer (pH10.4) followed by five millilitres of hexane.

VIII. Elution Of The Sample

The drug mixture was then eluted with 6 x 1 ml chloroform.

IX. Analysis Of The Sample

The eluate was evaporated under a gentle nitrogen stream on a hot plate at 60^{o}C, then the residue was redissolved in 100 ul of the mobile phase. Twenty microlitres were injected onto the HPLC system.

X. Results and Discussion

Recoveries Of The Four Benzodiazepines

A set of spiked human brain tissue samples containing the four benzodiazepine drugs mixture were prepared in blank human brain tissue to produce the following concentrations of 0.1, 0.2, 0.9 and 2 ug/gram of human brain frontal cortex. Internal standard (prazepam) was added to the above samples to produce a concentration of 2 ug/gram brain tissue.

Six samples of each concentration set were extracted through Bond Elut^{R} C_8 column, 6 ml calculated by comparing the peak height ratio of the extracted sample with un-extracted standard prepared in mobile phase at the same concentration.

The chromatograms produced from the human brain tissue (frontal cortex) were good. An example is shown in Figure 33.

Tables 42-45 list the concentrations, average recoveries and number of samples used each time. Excellent recoveries were obtained for the four benzodiazepine drugs.

TABLE 42.

The recovery of triazolam from spiked human brain tissue extracted through C_8 column-column and analysed by HPLC.

Concentration µg/g	Recovery	Number of samples
0.1	101±2.9%	6
0.2	83±5.0%	6
0.9	97±6.2%	6
2.0	92±7.2%	6

TABLE 43.

The recovery of temazepam from spiked human brain tissue extracted through C_8-column and analysed by HPLC.

Concentration µg/g	Recovery	Number of samples
0.1	84±7.0%	6
0.2	100±4.7%	6
0.9	96±3.3%	6
2.0	100±7.0%	6

TABLE 44.

The recovery of desmethyldiazepam from spiked human brain tissue extracted through C_8-column and analysed by HPLC.

Concentration µg/g	Recovery	Number of samples
0.1	95±8.2%	6
0.2	104±6.2%	6
0.9	101±2.2%	6
2.0	99±7.3%	6

TABLE 45.

The recovery of diazepam from spiked human brain tissue extracted through C_8-column and analysed by HPLC.

Concentration µg/g	Recovery	Number of samples
0.1	95±7.5%	6
0.2	97±3.6%	6
0.9	98±1.4%	6
2.0	99±7.1%	6

Calibration Curves (Linearity)

The previous experiments were used to find optimum conditions for the extraction and separation of the drugs. To enable the developed procedure to be used for quantitation, it was necessary to demonstrate a linear response for the procedure to enable quantitative analysis for each drug from low (0.1 ug/gram), sub-therapeutic (0.2 ug/gram), therapeutic (0.9 ug/gram) and the level expected from fatal ingestion (2 ug/gram).

Standard mixtures were prepared with concentrations ranging from 0.01 ug to 2 ug/gram to find the detection limit for each drug and the cleanness of human brain extracts using a solid phase extraction, C_8 Bond Elut^R column. The above concentration ranges included the subtherapeutic, therapeutic, toxic and fatal levels.

Six samples of each concentration set were extracted and analysed by the HPLC system. The peak height ratio against the concentration was plotted to construct a calibration curve for each drug over the concentration range of 0.1-2 ug/gram of human brain tissue (frontal cortex area).

The relationshps between the concentration of benzodiazepine drug concentrations (triazolam, temazepam, desmethyldiazepam and diazepam) and the average peak height ratios (std./I.S) of samples analysed by HPLC are illustrated in Tables 46-49. The standard curves were constructed for each drug using the peak height ratios (std.I.S), and the benzodiazepine drugs concentrations. A linear relation was obtained for each drug as expressed in Figure 29-32.

TABLE 46.

Relation between triazolam concentration in human brain and average peak height ratio (std/I.S.) following extraction through a C_8-column and analysis by HPLC .

Concentration µg/g	Mean peak height ratio n= 6
0.100	0.130
0.200	0.240
0.900	1.126
2.000	2.420

TABLE 47.

Relation between temazepam concentration in human brain and average peak height ratio (std/I.S.) following extraction through a C_8-column and analysis by HPLC.

Concentration µg/g	Mean peak height ratio n= 6
0.100	0.11
0.200	0.19
0.900	0.99
2.000	2.08

TABLE 48.

Relation between desmethydiazepam concentration in human brain and average peak height ratio (std/I.S.) following extraction through a C_8-column and analysis by HPLC.

Concentration µg/ml	Mean peak height ratio n= 6
0.100	0.17
0.200	0.314
0.900	1.45
2.000	3.2

TABLE 49.

Relation between diazepam concentration in human brain and average peak height ratio (std/I.S.) following extraction through a C_8-column and analysis by HPLC.

Concentration µg/g	Mean peak height ratio n = 6
0.100	0.137
0.200	0.27
0.900	1.16
2.000	2.63

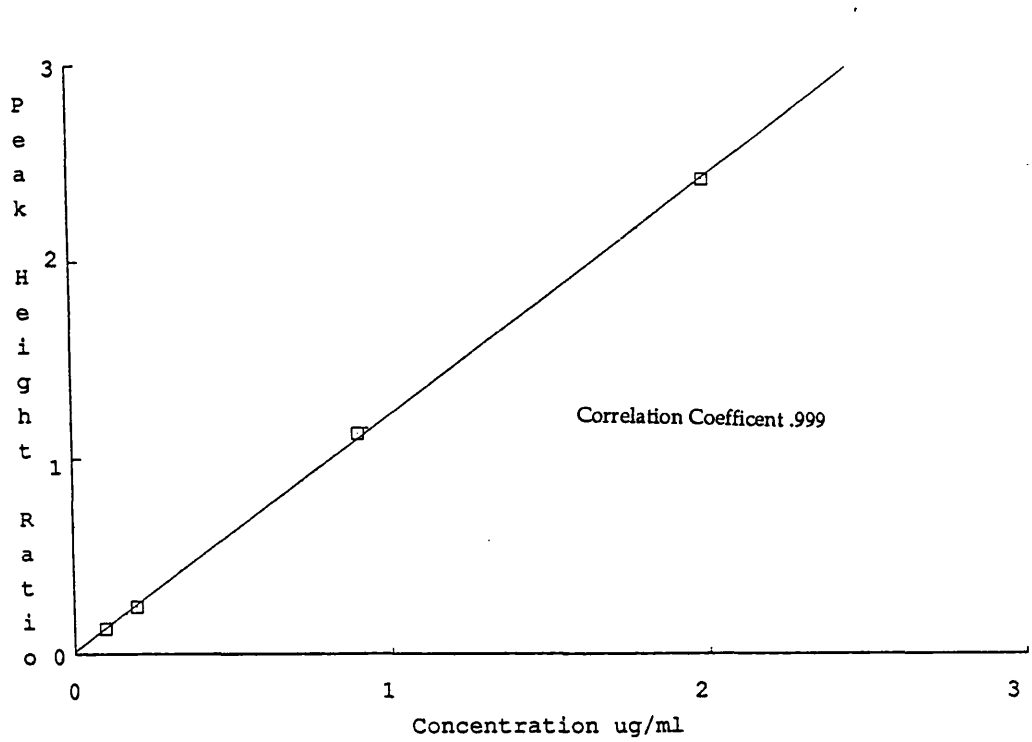


Figure 29 Calibration Curve For Triazolam.

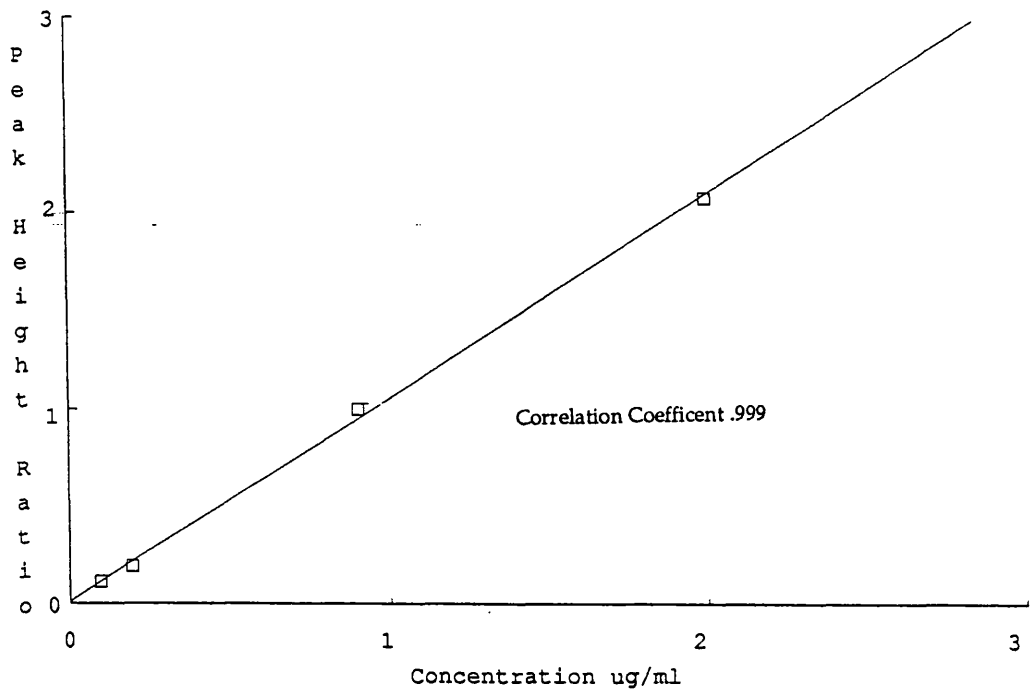


Figure 30 Calibration Curve For Temazepam.

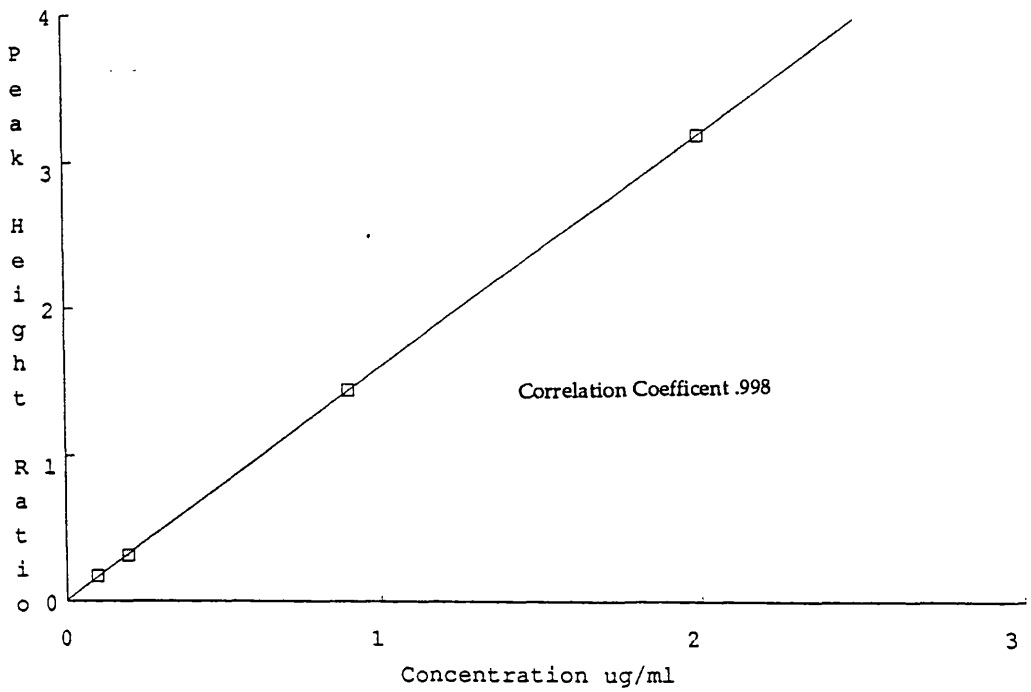


Figure 31 Calibration Curve For Desmethyldiazepam.

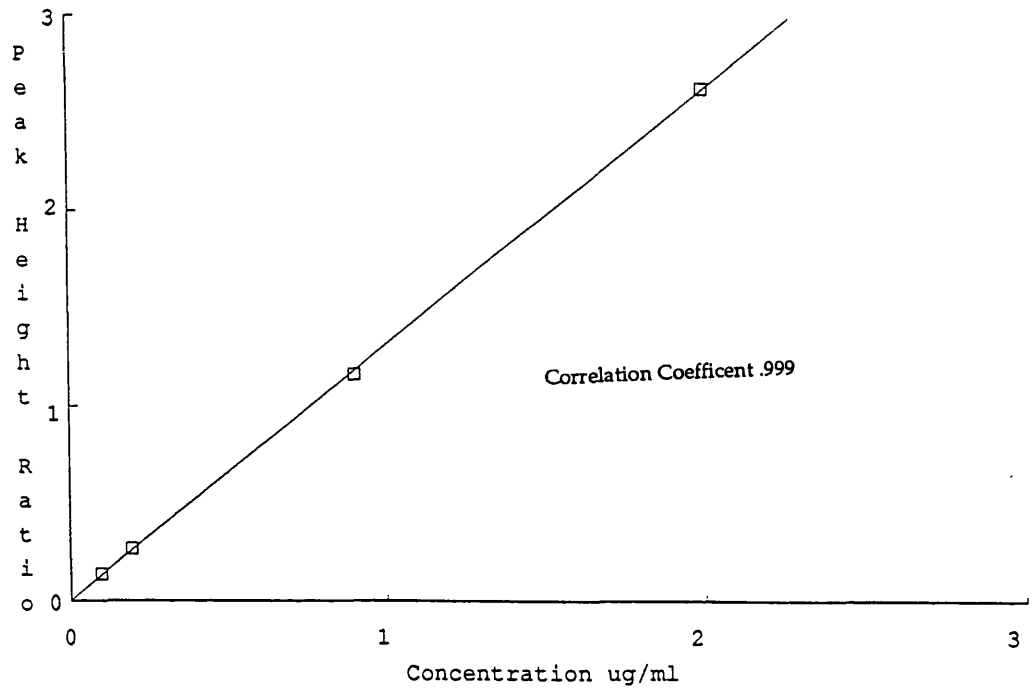


Figure 32 Calibration Curve For Diazepam

The Reproducibility Of The Extraction Method

Twenty-four samples of human post-mortem brain tissue spiked with the four benzodiazepine drugs mixture (triazolam, temazepam, desmethyldiazepam and diazepam) at a concentration of 0.2 ug/gram of human brain tissue were extracted through a Bond Elut^{R} C_8 six millilitres capacity column and analysed by an HPLC system over three successive days.

The ratio of the peak height of each drug over the peak height of internal standard prazepam was used to calculated the within day and day-to-day variations of the extraction and analysis method of the four benzodiazepines in human brain tissue (precentral cortex).

Table 50 outlines the average recoveries, coefficients of variation and numbers of samples used each time. Excellent recoveries were obtained for benzodiazepine drugs samples.

Conclusion

The developed method for extraction of benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) from brain tissue offered a very good sensitivity with a very stable baseline and very little interference. The reproducibility, recoveries and calibration curves were excellent. The chromatographic run time was less than 14 minutes at a flow rate of 1 ml/minute and no peaks eluted after the internal standard.

Figure 50 Reproducibility Of Analysis based on SPE with a C-8 Column For Benzodiazepine Drugs Over Three Days At Concentration Of 0.2μg\ml.

Drug	Days	Average Recoveries	Coeffcient Variation	NO.Of Samples Extracted
Triazolam	1	84.2%	2.5%	8
	2	84.0%	3.7%	8
	3	85.0%	2.0 %	8
Temazepam	1	100%	4.2%	8
	2	99.5%	6.1%	8
	3	100%	4.1%	8
Desmethyediazepam	1	103%	2.2%	8
	2	102%	3.6%	8
	3	103%	2.9%	8
Diazepam	1	97.1%	5.0%	8
	2	98.0%	3.8%	8
	3	97.5%	4.7%	8

XI Application and Comparison of Benzodiazepine Drug Recoveries from Human Post-Mortem Blood Samples and Human Brain Tissue Samples using Solid-Phase Extraction

Introduction

Brain tissue may not be routinely analysed in the toxicology laboratory, however it is of interest because of the high concentration of benzodiazepine receptors localized in the cortex, primarily in the pre-central cortex.

Since most of the benzodiazepine receptors are located in the brain and it is the site where most, if not all, of the effects of the drug originate, it is of interest to determine the concentration of benzodiazepine drugs in the pre-central area and relate it to the blood concentration.

Having established that levels of benzodiazepine drugs in post-mortem human brain tissue as low as 100 ng/gram, can be detected, the developed method was applied to six human post-mortem brain tissue samples. They were obtained from fatalities in which benzodiazepine drugs (triazolam, temazepam, desmethyldiazepam and diazepam) had been detected in their blood samples.

Method

Samples (1 gram of post-mortem human brain tissue) were extracted using non-polar interaction columns (Bond Elut$^{R}C_8$, 6 ml) for analysis for benzodiazepine drugs (5.2.2.3.b). Spiked human post-mortem brain tissue and

blank post-mortem brain tissue were extracted in parallel, and an internal standard (prazepam 2 ug/gram) was used for purposes of quantification. Each extraction was performed in duplicate and the average is given.

The extraction procedures were carried out as described in 5.2.2.3 for both blood and human post-mortem brain tissue samples. Extractions were performed in duplicate.

Results and Discussion

The main objective of this research was to determine the benzodiazepine receptor site concentrations in post-mortem human brain tissue samples from subjects known to have taken benzodiazepine drugs prior to death. The results were compared to a control group and a marked drop in benzodiazepine receptor site concentrations was observed in the presence of the benzodiazepine drug at death as shown in Table 28.

This is confirmed by further evidence using the developed methods of solid-phase extraction followed by HPLC on human brain tissue and human post-mortem blood samples of the same subjects.

Benzodiazepine drugs were measured in both brain and blood samples. The results are presented in Table 51 and the chromatograms produced for spiked, blank and human post-mortem brain tissue samples are given in Figures 33-35.

Conclusions

Detection of benzodiazepine drugs in human brain tissue by using the developed analytical techniques (binding study and solid-phase extract HPLC) provide unquestionable evidence of their use. These methods can be used for routine screening and subsequent confirmation of benzodiazepine drugs in blood and human brain tissue samples.

It has now become possible to study the receptor occupancy in the human brain tissue, which is the critical determinant for biological response. This will help to determine to what extent blood level measurements are a predictor for receptor occupancy in the brain tissue.

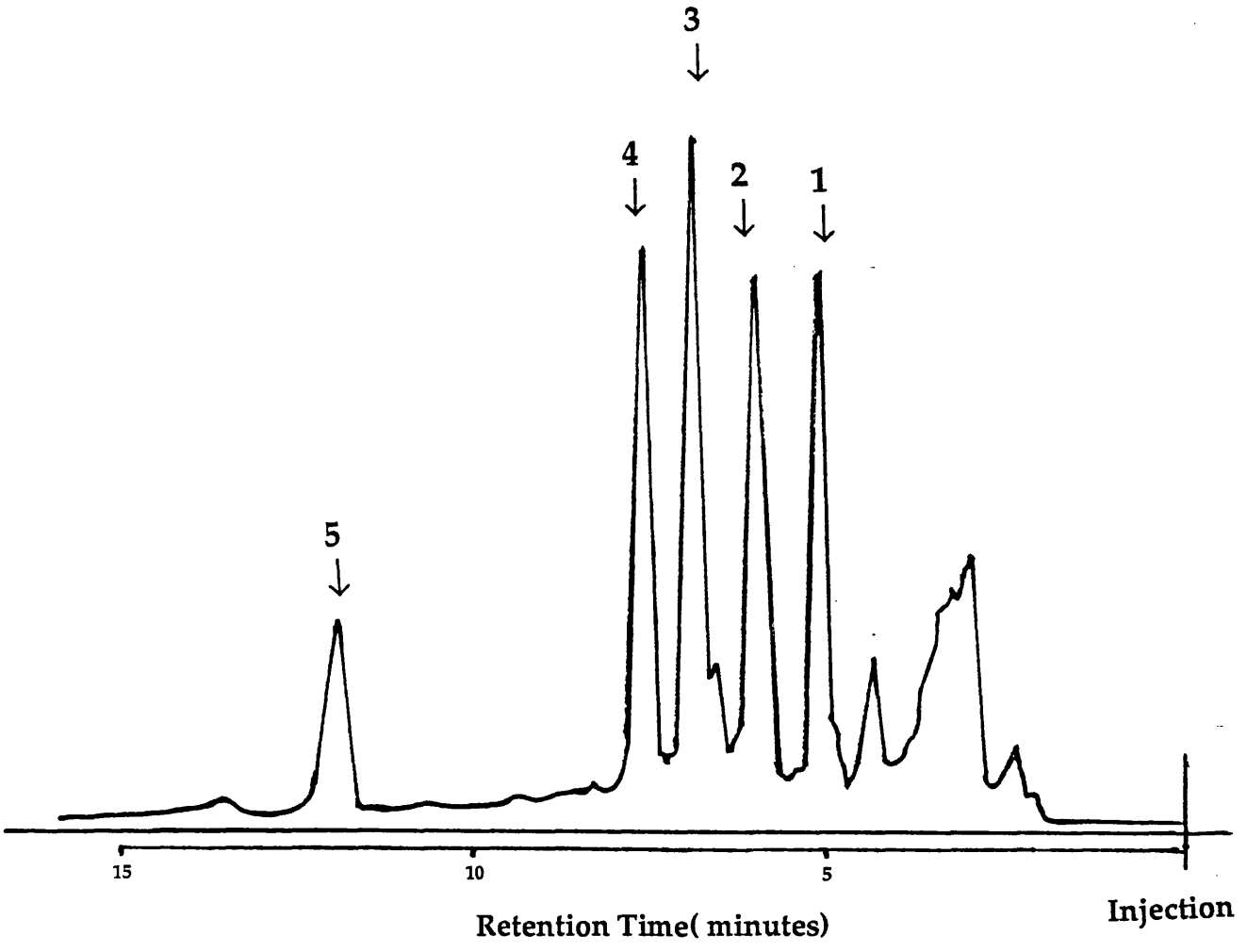


Figure 33 Separation Of Benzodiazepine Drugs By HPLC System.

1-Triazolam 2-Temazepam 3-Desmethyldiazepam

4-Diazepam 5-Prazepam(Internal standard)

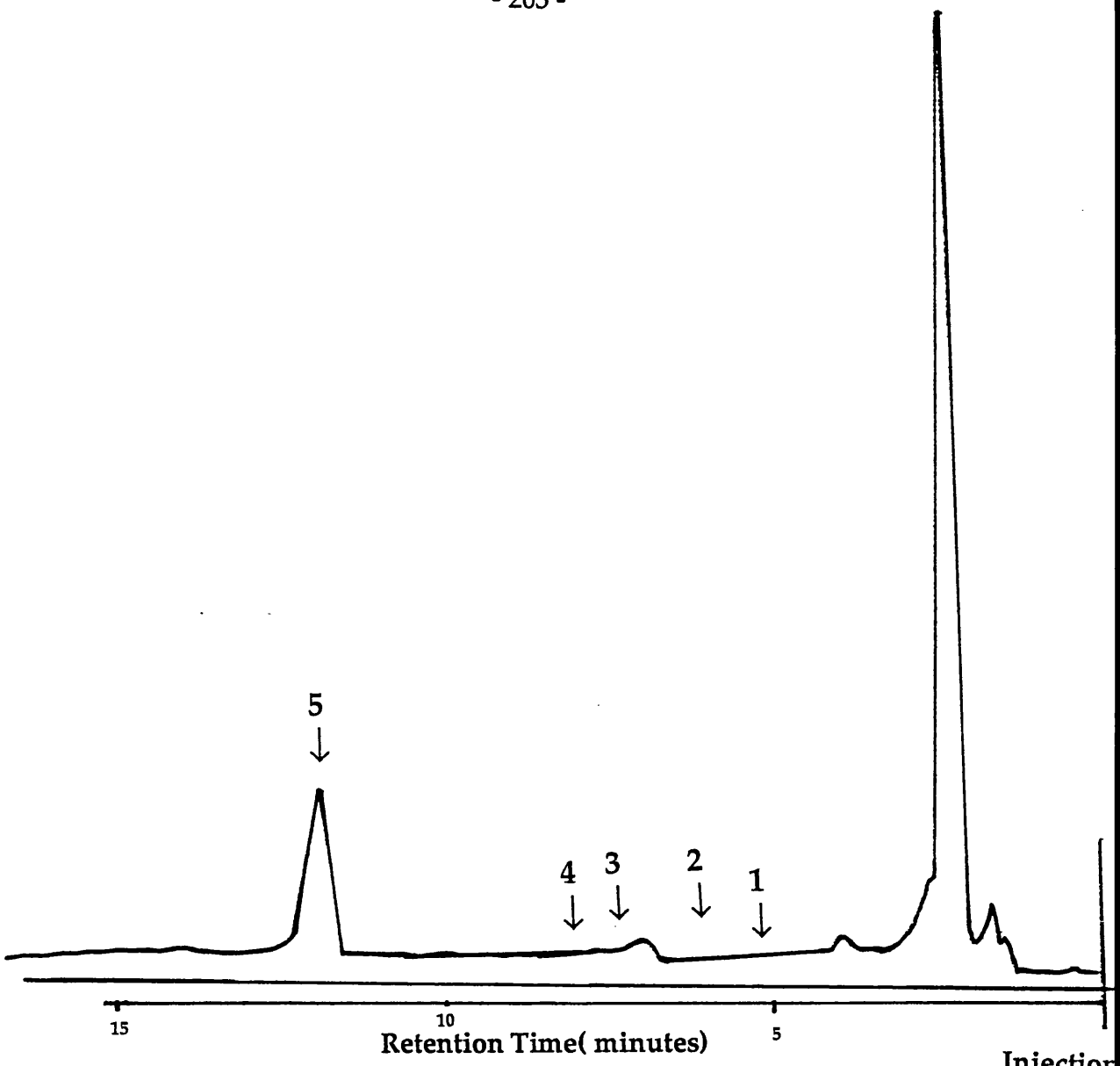


Figure 34 The Chromatogram Of Negative(Blank)Human Post-mortem Brain Tissue Analysed By HPLC System.

1-Triazolam 2-Temazepam 3-Desmethyldiazepam
4-Diazepam 5-Prazepam(Internal standard)

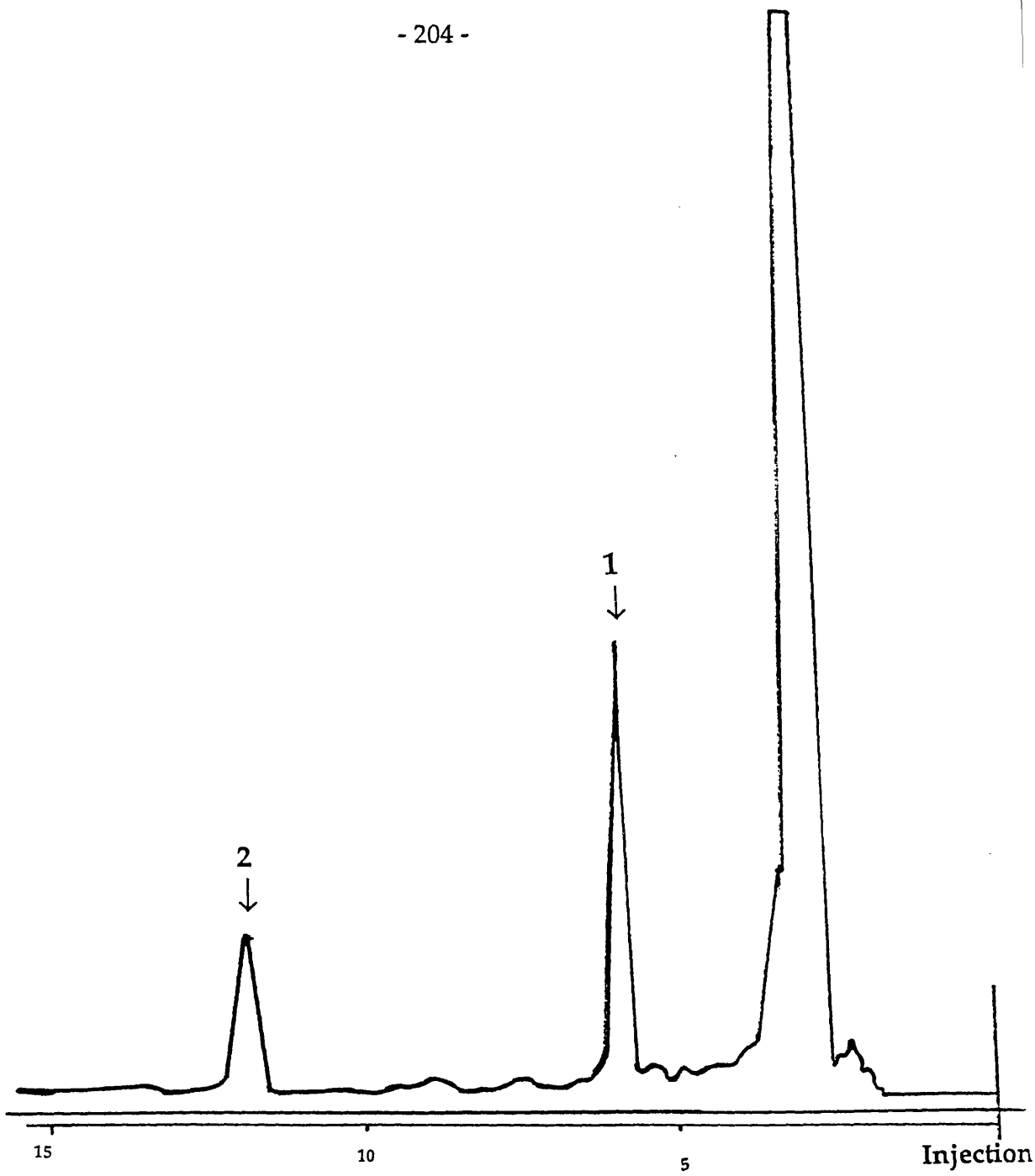


Figure 35 The Chromatogram Of Authentic Human Post-mortem Brain Tissue Sample Analysed By HPLC System.

1-Temazepam 2-Prazepam(internal standard)

Table 51 Analysis Of Benzodiazepine Drugs In Human Post-Mortem Blood and Human Brain Tissue Samples Using Solid-phase Extraction.

No.	Triazolam		Temazepam		Diazepam		Desmethyldiazepam	
Extraction	Blood (ug/ml	Brain (ug/g)	Blood (ug/ml	Brain (ug/g)	Blood (ug/ml	Brain (ug/g)	Blood (ug/ml	Brain (ug/g)
1	-	-	0.12	0.08	0.64	0.57	0.33	0.25
2	-	-	2.54	2.20	-	-	-	-
3	0.45	0.35	-	-	0.22	0.12	0.78	0.66
4	-	-	0.32	0.21	0.86	0.71	0.10	0.07
5	-	-	0.12	0.06	0.56	0.44	-	-
6	0.33	0.23	-	-	0.22	0.14	0.42	0.32

6. CONCLUSIONS

Benzodiazepine drugs are the most frequently encountered drugs in toxicological investigations in the U.K. after paracetamol (acetaminophen). These drugs have been prescribed for over 20 years, primarily as tranquillisers and anti-anxiety agents. Additionally, they have been used as both muscle relaxants and anticonvulsant agents. Temazepam has become one of the most common drugs by injection among drug abusers.

They evoke their pharmacological effects by binding to specific sites in brain tissue (receptors).

Little has been published on the routine screening and subsequent confirmatory analysis of the drugs in whole human post-mortem blood and brain tissue samples. Possibly this is as a result of problems, including co-extracted lipid and other materials which can give rise to interferences. Therefore, it can be seen that recovery of benzodiazepine drugs from post-mortem materials presents a challenging problem for the toxicologist.

Highly modern, sophisticated analytical and instrumental techniques such as binding studies and solid-phase extraction have been used in this research to overcome the problems of co-extracted materials. These techniques provide a quick clean-up procedure, give both high drug recoveries and complete removal of interfering materials, and decreased sample preparation time.

A. Binding Studies

From binding study techniques several conclusions are apparent. The first of these obtained from the saturation curve, is that the optimal incubation time for benzodiazepine receptors and ^{3}-flunitrazepam is between 30 minutes minimum and 60 minutes maximum. Secondly, the highest densities of binding sites were found to be in the frontal lobe cortex (grey matter) area. Intermediate densities were found in other areas (post-central cortex, occipital cortex and hippocampus). The lowest densities were observed in the cerebellum and temporal cortex.

Finally a considerable drop in the receptor binding site concentrations was noted in subjects known to have taken benzodiazepine drugs prior to death as compared to the control group. The drug presence was confirmed by High Liquid Chromatography. Variations in the cortical synaptosomal binding site concentrations were shown to be slightly affected by age and sex factors.

B. Solid-Phase Extraction of Benzodiazepine Drugs from Post-mortem Blood and Human Brain Tissue Samples.

1. Human Post-Mortem Blood

A highly efficient and sensitive solid phase-extraction method for the dilution and measurement of benzodiazepine drugs in whole human post-mortem blood samples was developed. The method compared well with conventional solvent extraction being more efficient and allowing ready identification and quantitation of the benzodiazepine drugs studied The developed procedure has advantages of minimal handling time, high recovery (even at subtherapeutic levels) clean sample extracts and eliminates the need for expensive equipment such as centrifuges, shakers and glassware.

Blood was also analysed from four different anatomical sites (jugular vein, axillary vein, femoral vein and the heart) and the concentrations of benzodiazepine drugs at each site were measured by HPLC. No significant variations in the drug concentrations were found between these sites.

2. Human Brain Tissue

As shown there is a major diminution in the number of benzodiazepine receptors measured is the presence of benzodiazepine drugs at death. In order to confirm this finding a solid phase extraction procedure for benzodiazepine drugs in human brain tissue (frontal lobe cortex) samples was developed and applied to the same subjects. This method proved to be highly efficient and sensitive. The method was used to confirm the presence of the drugs in the

brain tissues where saturation curve analysis had shown a drop in the number of benzodiazepine receptors.

Finally, a comparison study was made between the concentrations of benzodiazepine drugs extracted from human brain tissue and human post-mortem blood samples. It was observed that drugs were present in both samples from the same subjects. Higher benzodiazepine concentrations were obtained from blood compared to brain samples.

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THE EFFECT OF BENZODIAZEPINE DRUGS ON THE BINDING SITE CONCENTRATION OF BENZODIAZEPINE RECEPTORS

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The toxicological investigation of death by poisoning relies primarily on the analysis of a biological sample for the presence of the suspected drug. As a result, a level can be measured which can be compared with findings from previous cases. The finding will lie within the "fatal", "toxic", "therapeutic", or "sub-therapeutic" range. Increased sophistication on this interpretation has been reported at previous T.I.A.F.T. conferences where reported use has been made of the comparison of drug levels measured in samples taken from different sites in the body. This study presents the results of a preliminary investigation of the potential use of drug receptors as a means of diagnosing death by poisoning.

After paracetamol (acetaminophen), benzodiazepine drugs are the most frequently encountered in toxicological investigations in the West of Scotland. These drugs have been prescribed for over 20 years, primarily as tranquillisers and anti-anxiety agents. Additionally, they have been used as both muscle relaxants and anticonvulsant agents(1,2,3). One of them, Temazepam, has, by injection become one of the most commonly encountered drugs with drug abusers.

They evoke their pharmacological effects by binding to specific sites in brain tissue, receptors (4-10).

Following optimisation of the method, the regional distribution of benzo-diazepine receptor sites in the brain was investigated. As a result, the site with the highest population of receptors was studied to determine the possible effect on the receptor population of age, sex cause of death and time interval between death and autopsy. The results were compared with the apparent binding site concentration measured in subjects shown to be on benzodiazepine drugs at death.

MATERIALS AND METHOD

Clonazepam was obtained from Wyeth Laboratories. The tritiated flunitrazepam ([3H]FNZ), specific activity 85 Ci/mmol was purchased from Amersham International plc. All other chemicals were of analytical quality and were obtained from local suppliers.

Human post mortem tissue samples were collected at the City Mortuary, packed in ice, and transported to the laboratory for immediate processing. For all cases, blood samples were analysed by high pressure liquid chromatography and by radioimmunoassay for the presence of benzodiazepine drugs.

Benzodiazepine receptors were harvested by homogenising approximately 1 gramme of brain tissue in 10 millilitres of ice-cold 0.32mol sucrose solution using 20 strokes of a teflon-glass homogeniser at 1200 rpm.

The homogenate was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was removed and was further centrifuged at 15,000 rpm for 20 minutes at 4°C. The pellet formed was weighed, resuspended in 10 millilitres of 25mmol sodium phosphate buffer (pH 7.4) and stored at -20°C until analysed.

On the day of the investigation, homogenates were thawed. The protein concentration of the preparation was measured by the colourimetric method of Lowrey et al (11) whereby the protein-copper-Folin reagent complex can be measured in a spectrometer at a wavelength of 540nm.

The binding site concentration was measured using a modification of the method described by Horton et al (12,13). The protein concentration of the homogenate was adjusted by dilution to between 0.1 and 0.3mg per millilitre.

In duplicate, aliquots of homogenate (0.8ml) were mixed thoroughly with six serial dilutions of [3H]FNZ (0.1ml of 0.3 - 0.009nmol/ml). For total binding, 0.1ml of assay buffer (25mmol sodium dihydrogen phosphate pH7.4) was added, for non-specific binding, the later was replaced with 0.1ml of Clonazepam (2umol/ml in assay buffer). Each tube was mixed well and incubated for 60 minutes at room temperature (20°C). The bound and free fractions were separated by filtration under low vacuum through Whatman GF/B glass fibre filters. The filters were rinsed under vacuum with 5ml ice-cold assay buffer. The filters were then placed into P_3 Pico vials (Packard), 4ml of scintillant fluid (Ecoscint, National Diagnostics) was added and the vials were shaken vigorously. The bound radiolabel was measured by liquid scintillation counting for one minute (Packard 2200 CA).

The DPM of total added label was determined by addition of 0.1ml of each radioligand concentration directly to 4ml of scintillant fluid which was counted for 1 minute.

All data was processed using the Packard Combicept Program.

RESULTS AND DISCUSSION

1. Effect of Incubation Period

Human post-mortem brain tissue samples were obtained at autopsy from a female aged 18 years, who died from chest and abdominal injuries(RTA). A blood sample was taken from the deceased and analysed for the presence of benzodiazepines using radioimmunoassay and HPLC with negative results. The grey matter from the frontal cortex area was freshly dissected taking care to avoid contamination by white matter.

In order to determine a suitable period of the receptor/ligand equilibrium, 24 LP3 tubes were set up in pairs, enabling duplicates to be filtered at (0,30,60,120 and 150 minutes). Binding site concentration was determined as above.

The results are shown in Table 1. From the chosen time intervals, it can be seen that an incubation of between 30 and 60 minutes is sufficient to allow an equilibrium to be achieved between the receptors and the radioligand. At 120 and 150 minutes, there is clear evidence of breakdown of the receptor-

radioligand bond, perhaps due to denaturation or proteolysis of the receptor protein. A period of 60 minutes was selected for the remaining experiments.

2. Regional distribution of benzodiazepine receptors in human brain tissue

Brain tissue was obtained at autopsy from four females and two males. Analysis for the presence of benzodiazepine drugs in each case gave a negative result.

Table 2 shows the regional distribution of benzodiazepine receptors in the six areas studied. The highest levels of specific ^{3}H-flunitrazepam binding was found in the frontal cortex and the lowest densities were found in the temporal cortex. This study agrees with other investigators.

3. Influence of Age, Sex, Causes of Death and Time Interval between Death and Autopsy

The benzodiazepine receptor concentration in frontal cortex was determined for 36 post-mortem cases which had been found to be negative for benzodiazepine drugs.

Table 3 summarizes the characteristics of these cases with respect to age and sex. There were variations in binding site concentration between the deceased according to age, especially in the male. For the group of age <40 years, the number of benzodiazepine receptors in the frontal cortex was measured as 1305 +/- 92. For group 2 (40-60) and group 3 (>60) a decrease in

the number of benzodiazepine receptors (1267 +/- 9 and 1166 +/- 57 respectively) was found.

In females the 3 age groups showed no variations in binding sites in all three groups of age.

Tables 4-7 lists the binding site concentrations measured in males and females with respect to causes of death and the time interval between death and autopsy.

With respect to the delay between time of death and post-mortem, the results in the table show no significant effect. This is possibly due to storage of the bodies in refrigerator prior to autopsy. Also, the cause of death shows no significant change in the binding site concentrations.

4. The Effect of Benzodiazepine Drugs

The apparent receptor binding site concentration in six subjects who died from a variety of causes but who were shown to have taken benzodiazepine drugs prior to death were measured. The results in table 8 show a considerable drop in the apparent binding site concentrations when compared to the control group.

CONCLUSION

This study has shown the highest concentration of benzodiazepine drug receptors to be found in the Frontal Cortex. A possible diminution of numbers is to be found with age. A larger amount of data will have to be collected to confirm both this and a possible sex difference. The major diminution in the number of benzodiazepine receptors measured is the presence of a benzodiazepine drug at death. Further study is required to quantitate this difference between the apparent and the true and perhaps as a result increase our interpretive powers in cases of drug death.

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Table 1: Effects of incubation time against binding site concentration

Time (min)	Binding site concentration (fmol/mg protein)	Mean
0	800 750	775
30	1253 1238	1245.5
60	1286 1276	1283
120	1138 1162	1150
150	1067 900	983.5

Table 2: Regional distribution of benzodiazepine receptors in human brain tissue

Brain	Maximum specific binding site (fmol/mg protein)						statistics
Frontal cortex	1341	1216	1325	1362	1126	1300	=1278.4 SD=90.2 cv=7.05%
Post-central cortex	1050	981	1062	1170	1098	998	=1059.8 SD=68.89 cv=6.50%
Occipital cortex	1016	1092	927	1060	1090	960	=1024.2 SD=69.05 cv=6.74%
Hippocampus	841	910	807	980	896	900	=889 SD=6 cv=0.7%
Cerebellum	662	542	613	590	642	660	=618.2 SD=46.6 cv=7.5%
Temporal cortex	638	700	590	610	561	531	=605 SD=59.6 cv=9.8%

Table 3: Binding site concentration of benzodiazepine receptors in the human frontal cortex area against sex and age group.

Binding site concentration (fmol/mg protein)					
Age group	Case No.	Male		Female	
< 40 years	n1	1328	1305 SD92 cv7%	1281	1268 SD 30.7 cv2.4%
	n2	1375		1250	
	n3	1270		1305	
	n4	1210		1220	
	n5	1230		1290	
	n6	1217		1260	
40-60 years	n1	1272	1267 SD90 cv7%	1215	1250 SD 6.1 cv4.9%
	n2	1157		1210	
	n3	1212		1320	
	n4	1193		1185	
	n5	1230		1236	
	n6	1290		1333	
> 60 years	n1	1275	1166 SD90 cv4.5%	1281	1268 SD 47 cv3.9%
	n2	1133		1250	
	n3	1168		1305	
	n4	1172		1220	
	n5	1232		1290	
	n6	1208		1260	

Table 4: Effects of age and cause of death on binding site concentration in females

Age	Time interval (death & autopsy)	Binding site Concentration
18	Chest & Abdominal injuries (RTA)	1220
31	Myocarditis	1305
33	Phenobarbitone intoxication	1260
36	Acute Alcohol Intoxication (RTA)	1250
37	Spontaneous subarachnoid haemrrhage due to rupture of cerebral artery aneurysm	1290
38	Acute peritonitis due to chronic pancreatitis	1281
46	Myocardial Infarction	1333
47	Scalding due to hot water	1215
47	Coronary thrombosis	1185
48	Acute myocardial infarction	1236
53	Ischaemic heart disease	1320
59	Hypertension & ischaemic heart disease	1216
62	Ischaemic heart disease	1210
68	Lobar pneumonia	1190
68	Acute myocardial infarction	1330
68	Haemopericardium due to myocardial infarction	1157
78	Ischaemic heart disease. Coronary artery atheroma	1212
85	Metastatic large bowel. Carcinoma & pulmonary thromboembolism	1180

Table 5: Effects of time interval (death & autopsy) on binding site concentration in females

Age	Time interval (death & autopsy)	Binding site Concentration
46	12 hours	1333
62	12 hours	1210
53	14 hours	1320
68	17 hours	1330
68	19 hours	1190
85	24 hours	1181
31	24 hours	1305
18	34 hours	1220
33	34 hours	1260
38	34 hours	1281
47	52 hours	1185
37	54 hours	1290
78	73 hours	1212
47	74 hours	1215
48	76 hours	1236
59	80 hours	1216
36	86 hours	1250
68	88 hours	1157

Table 6: Effects of age and cause of death on binding site concentration in eighteen males

Age	Time interval (death & autopsy)	Binding site Concentration
19	Multiple injuries, Road Traffic Accident	1341
19	Acute alcohol intoxication	1362
37	Acute pancreatitis	1300
37	Coronary artery atheroma	1328
37	Chest & abdominal, Road traffic accident	1375
38	Hanging	1126
46	Cerebral abscess	1223
48	Hanging	1272
48	Acute artery atheroma	1121
49	Acute myocardial infarction	1272
55	Coronary artery atheroma,Bronchitis & emphysema	1325
58	Choking	1157
64	Acute myocardial infraction	1263
68	Ischaemic heart disease	1168
73	Ruptured aortic aneurysm	1119
74	Pulmonary thrombo-embolism	1113
75	Acute broncho-pneumonia	1194
76	Haemopericadium due to myocardial infarction	1136

Table 7: Effects of time interval (death & autopsy) on binding site concentration in males

Age	Time interval (death & autopsy)	Binding site Concentration
49	18 hours	1272
68	23 hours	1168
19	30 hours	1341
64	36 hours	1263
58	38 hours	1157
48	49 hours	1272
19	72hours	1362
55	72 hours	1325
76	72 hours	1136
38	82 hours	1126
37	84 hours	1300
74	84 hours	1113
75	84 hours	1194
37	86 hours	1375
73	95 hours	1119
37	97 hours	1328
46	102 hours	1223
48	141 hours	1121

Table 8: Changes in the density of the benzodiazepine receptors in the post-mortem brain samples of six patients on benzodiazepine treatment.

Age (years)	Sex	Binding Site Concentration (fmol/mg protein)	Blood sample analysis (HPLC & RIA)
26	male	833	Temazepam,D.M.D. & Diazepam
33	male	772	Temazepam & Diazepam
58	female	610	Temazepam
24	male	844	Temazepam,D.M.D. & Diazepam
24	male	976	Triazolam,D.M.D. & Diazepam
20	female	658	Temazepam,D.M.D. & Diazepam