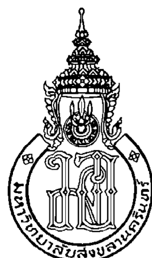

Analysis of 6 Benzodiazepines in Human Serum by High Performance Liquid Chromatography

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Forensic Science

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ชื่อวิทยานิพนธ์	การวิเคราะห์ยานอนหลับ Benzodiazepines 6 ชนิดในซีรัมโดยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง
ผู้เขียน	นางสาวทศพร เหรียญรุ่งโรจน์
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บทคัดย่อ

วิธีการวิเคราะห์โดยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง หลังจากการสกัดด้วยวิธีตกตะกอนโปรตีน ใช้หายานอนหลับ Benzodiazepines 6 ชนิดในซีรัม ประกอบด้วย nitrazepam diazepam lorazepam flunitrazepam midazolam และ triazolam การศึกษาพฤติกรรมการแยก (elution behaviors) ของยานอนหลับในสภาวะที่ใช้เฟสเคลื่อนที่แบบคงที่ (isocratic) ใช้สารผสมของเฟสเคลื่อนที่ 2 และ 3 ชนิด (binary and tertiary mobile phase) จากนั้นศึกษาสภาวะแบบที่มีการเปลี่ยนอัตราส่วนของเฟสเคลื่อนในระหว่างการวิเคราะห์ (linear gradient) โดยใช้คอลัมน์ cyano reverse-phase (Lichrospher, 250 mm × 4 mm, 5 μm) และใช้เฟสเคลื่อนที่เป็นสารผสมของ methanol-acetonitrile-phosphate buffer solution (3.3 mM, pH 2.1) เพื่อหาสภาวะที่เหมาะสมในการวิเคราะห์ ข้อมูลที่ได้จากการศึกษา elution behaviors ในสภาวะแบบ isocratic สามารถใช้เป็นข้อมูลพื้นฐานในการหาสภาวะที่เหมาะสมสำหรับการวิเคราะห์ยานอนหลับในสภาวะ linear gradient ได้ สภาวะที่เหมาะสมในการวิเคราะห์ยานอนหลับ คือ การใช้สัดส่วนของ methanol : phosphate buffer solution (3.3 mM, pH 2.1) : acetonitrile ที่เวลาเริ่มต้นเท่ากับ 27 : 69 : 4 และที่ 30 นาทีเท่ากับ 20 : 80 : 0 ให้ผลการแยกที่ดีโดยใช้เวลาการวิเคราะห์น้อยกว่า 25 นาที เปอร์เซ็นต์การกลับคืนของการสกัดยานอนหลับ benzodiazepines 6 ชนิดในซีรัมอยู่ในช่วงระหว่าง 78.8-87.9%, 81.8-88.2%, และ 82.9-92.5% ที่ความเข้มข้นของยานอนหลับในซีรัมระดับต่ำ กลาง และสูง ตามลำดับ ค่าขีดจำกัดต่ำสุดของการวิเคราะห์ (LOD) และค่าขีดจำกัดต่ำสุดของการหาปริมาณ (LOQ) อยู่ในช่วง 0.027 ถึง 0.084 μg/ml และ 0.091 ถึง 0.28 μg/ml ตามลำดับ ค่าสัมประสิทธิ์ความสัมพันธ์เชิงเส้น (r^2) ของความเป็นเส้นตรง (Linearity) มีค่ามากกว่า 0.990 ค่าความเที่ยง (Precision, %RSD) ทั้งการวิเคราะห์ในวันเดียวกัน (intraday repeatability) และการวิเคราะห์ระหว่างวัน (interday reproducibility) มีค่าน้อยกว่า 5% และ 10% ตามลำดับ ในตัวอย่างซีรัมของอาสาสมัครที่ให้ diazepam ตรวจพบและหาปริมาณ diazepam ได้ค่า 0.22 μg/ml และของอาสาสมัครที่ให้ lorazepam ตรวจพบ lorazepam 0.11 μg/ml วิธีการที่พัฒนาใช้ประยุกต์ในการวิเคราะห์ทางคลินิกและนิติพิษวิทยาได้ทั้งการวิเคราะห์เบื้องต้นและยืนยันผล

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ABSTRACT

An analysis method using High Performance Liquid Chromatography (HPLC) after protein precipitation extraction is described for the determination in human serum of 6 benzodiazepines including nitrazepam, diazepam, lorazepam, flunitrazepam, midazolam and triazolam. HPLC optimization was performed by determining the elution behaviors of drugs in isocratic elution in binary and tertiary mobile phase followed by linear gradient elution of a cyano reversed-phase column (Lichrospher, 250 mm × 4 mm, 5 μm), with a methanol-acetonitrile-phosphate buffer solution (3.3 mM, pH 2.1) eluent. The determination of elution behaviors in isocratic elution gives the base data to approach the optimum condition for 6 benzodiazepines analysis in linear gradient. The optimum condition obtained in linear gradient; methanol : phosphate buffer solution (3.3 mM, pH 2.1) : acetonitrile at 0 minute = 27 : 69 : 4, and 30 minutes: = 20 : 80 : 0 gives well resolution with analysis time less than 25 minutes. The extraction recoveries for 6 benzodiazepines in serum are in ranges between 78.8-87.9%, 81.8-88.2%, and 82.9-92.5% of low, average, and high concentrations, respectively. They are within the limit of acceptance criteria. The limits of detection (LOD, 3SD, n=10) and limits of quantification (LOQ, 10SD, n=10) range from 0.027 to 0.084 μg/ml and 0.091 to 0.28 μg/ml, respectively. The linearity data proved the analytical procedure to be linear (linear correlation coefficient (r^2) was greater than 0.990). The precision data (%RSD); intraday repeatability and interday reproducibility, were precise for all benzodiazepines within the acceptance intervals of 5% and 10%, respectively. Diazepam was detected and quantified in a real serum sample at a concentration of 0.22 μg/ml. Lorazepam was detected in a real serum sample at a concentration of 0.11 μg/ml. The procedure is satisfactory for the application in clinical and forensic toxicological analysis both for screening and confirmatory.

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

In recent years, the world faces with many problems in economy, society, and politics. Owing to these various problems, the numbers of stress people are rapidly increasing. Most of people use benzodiazepines to decrease their stress. The widespread use of these drugs may have contributed towards the high incidents of overdose. Moreover, they are used leading to dependence and abuse, such a case of plying someone with drugs (sexual assaults). Analysis of benzodiazepines may be indicated in a lot of forensic cases such as driving under the influence of drugs, cases of date-rape or violent crime and cases of unknown causes of death (Smink *et al*., 2004). Therefore, the requirement of detection and identification of benzodiazepines in forensic cases is increasing.

Benzodiazepines (BZDs) belong to a group of substances known for their sedative, hypnotic and anticonvulsant properties and are prescribed for the therapy of anxiety and sleep disorders (Bugey and Staub, 2004). Some well-known examples of benzodiazepines are nitrazepam (Mogadon®), diazepam (Valium®), lorazepam (Ativan®), flunitrazepam (Rohypnol®), midazolam (Dormicum®), and triazolam (Halcion®). At high doses or when they are abused, these drugs may cause unconsciousness and death. The severity of the lethality is influenced by dose of ingestion, age, personal health before ingestion, and interaction with co-ingestion drugs (Smith, 1993).

In practice, the analytical samples of clinical and forensic toxicology laboratory are the biological fluids, including urine, blood, gastric content. The analysis of drugs in body fluid is a source of information about which drug has been taken (Kintz *et al*., 2005). The analysis of benzodiazepines in urine is a difficult task,

due to the pharmacokinetics of all drugs producing the metabolites excreted in urine. For this reason, blood and gastric content are better samples for the detection and identification of drugs. However, there is the analytical problem that some drugs will be transformed to their metabolites and widely distributed to react with the target organ, resulting in the present of low concentration after therapeutic doses. The very low concentration in blood is an additional problem to the forensic toxicologist, as a confirmation procedure is necessary.

Several different techniques have been described for the determination of benzodiazepines in biological fluids. Common methods include thin-layer chromatography that has been very long used and immunoassay, which is a useful method for the rapid screening. In contrast, both techniques are lack of specificity, and in the case of immunoanalysis, parent drugs and metabolites cannot be discriminately analyzed. Gas chromatography (GC) is sensitive and specific, but it requires complicated equipment and also requires the derivatization for the analysis of thermally labile drugs, e.g., lorazepam, oxazepam, and chlordiazepoxide (Yokchue, 2004). Hence, high performance liquid chromatography is suitable for the quantitative determination of benzodiazepines, particularly since some of these compounds exhibit thermal instability (Mubhoff and Daldrup, 1992). It has become the most preferred analytical technique for both screening and quantification of sedative-hypnotics in biological samples because of the selectivity and sensitivity inherent of the technique (Tanaka *et al.*, 1997). The high performance liquid chromatographic technique can provides quantitative assay over a wide concentration range from sub-therapeutic to fatal levels. Owing to the variation in HPLC condition for the separation of drugs affect the different retention characteristic, drug analysis by HPLC require the retention database specific for each drug. In the past decade, the HPLC methods reported are often applicable to either some of benzodiazepines but not simultaneously for these 6 benzodiazepines.

Therefore, this study is set up to analyze 6 well-known benzodiazepines in serum with high performance liquid chromatography to provide the optimum condition of the separation of these 6 benzodiazepines for identification and quantification, which can be considered as a useful technique for forensic toxicologist and widely used in forensic toxicology laboratory.

1.2 Review of Literature

1.2.1 Benzodiazepines

1.2.1.1 History

The benzodiazepines were first synthesized in 1933 but did not undergo preclinical and chemical testing until the 1950s. The first benzodiazepines, chlordiazepoxide, was synthesized by Sternbarch and Reeder in the late 1950s and marketed in 1961 (Yokchue, 2004). After this initial success, other pharmaceutical companies began to introduce other benzodiazepine derivatives (Sample, 2005). Following a few years later thousands of benzodiazepine derivatives have been synthesized and tested.

As early as 1972, some authors suggested that nitrazepam to be close to barbiturate in producing dependence. It is not an approved drug in USA but is widely prescribed in Asia and Africa as hypnotic (Prasad *et al.,* 2001). Diazepam is the second benzodiazepine invented by Leo Sternbach of Hoffmann-La Roche, and was approved for use in 1963. Diazepam is also found in nature. Several plants, such as potato and wheat, contain trace amounts of naturally occurring diazepam and other benzodiazepines (Sample, 2005). Kintz *et al.* (2004) reported that lorazepam has been sold in France since January 1, 1973. For flunitrazepam, it needs to be considered because the intractable symptoms such as pain, nausea, confusion or existential suffering are not managed with other benzodiazepines. However, it is not licensed in New Zealand. Use in palliative care for palliative sedation is permissible under Section 29, Medicines Act, 1981 and patient consent is essential (Christchurch Hospital, 2008). Midazolam was first synthesized in 1976 by Fryer and Walser. Hida *et al.* (1997) described that triazolam has been introduced as a short acting hypnotic drug, and is sometimes abused instead of methamphetamine and marijuana in Japan.

1.2.1.2 Chemical structures

The structures of 6 benzodiazepines are shown in Figure 1 and Table 1. The structures of benzodiazepines are based on a chemical structure composed of benzene ring fused to a seven membered diazepine ring. As most of the chemically important benzodiazepines contain a 5-aryl substituent and a 1,4-diazepine, the term has come to mean the 5-aryl-1,4-benzodiazepines. The different types of benzodiapepines are formed by substitution, in various positions in the two rings at R_1, R_2, R_3, R_5, and R_7 (Drummer, 1998).

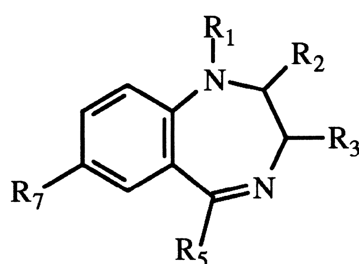
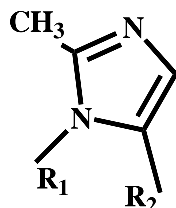


Figure 1. The structures of benzodiazepines (Drummer, 1998).

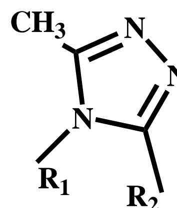
Table 1. The structures information of 6 benzodiazepines.

Benzodiazepines	R_1	R_2	R_3	R_5	R_7
Nitrazepam	H	=O	H	Phenyl	NO_2
Diazepam	CH_3	=O	H	Phenyl	Cl
Lorazepam	H	=O	OH	2-Cl-phenyl	Cl
Flunitrazepam	CH_3	=O	H	2-F-phenyl	NO_2
Midazolam	[Fused triazolo ring][A]		H	2-F-phenyl	Cl
Triazolam	[Fused triazolo ring][B]		H	2-Cl-phenyl	Cl

A



B



Diazepam is the 1,4-benzodiazepine, which is interconvertible, and is all primarily metabolized and excreted as oxazepam and oxazepam gulcuronide in urine. The nitrobenzodiazepines, flunitrazepam and nitrazepam, are substituted 1,4-benzodiazepines with nitro group at R_7 and have a common pathway to a 7-amino metabolite, which is a target metabolite in urine and in postmortem blood specimens. Lorazepam is the structural modification include ring formation on the 5-position as 2-chlorophenyl. Midazolam is the variation of the benzodiazepine structure include a 1,3-diazole (imidazole) annulations on the 1,2-position to form imidazo or diazolo benzodiazepines. Triazolobenzodiazepines, triazolam, has a 1,3,4-triazole ring in place of the diazole ring. Many benzodiazepines in acid solution will hydrolyse, forming the corresponding benzophenone. These benzophenones can be targeted if desired (Prasad *et al.,* 2001).

1.2.1.3 Nomenclatures

IUPAC names and trade names of 6 benzodiazepines are tabulated in Table 2.

Table 2. IUPAC names and trade names of 6 benzodiazepines.

Benzodiazepines	IUPAC name	Trade name
Nitrazepam	7-nitro-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one ($C_{15}H_{11}N_3O_3$)	Mogadon®
Diazepam	7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2-H-1,4-benzodiazepin-2-one ($C_{16}H_{13}ClN_2O$)	Valium ®
Lorazepam	9-chloro-6-(2-chlorophenyl)-4-hydroxy-2,5-diazabicyclo [5.4.0] undeca-5,8,10,12-tetraen-3-one ($C_{15}H_{10}C_{12}N_2$)	Ativan®
Flunitrazepam	5-(2-fluorophenyl)-1-methyl-7-nitro-3H-1,4-benzodiazepin-2-one ($C_{16}H_{12}FN_3O_3$)	Rohypnol®
Midazolam	8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo(1,5-a)-1,4-benzodiazepine ($C_{18}H_{13}N_3ClF$)	Dormicum®
Triazolam	8-chloro-6-(2-chlorophenyl)-1-methyl-4H-triazolo-(4,3-a)-1,4-benzodiazepine ($C_{17}H_{12}Cl_2N_4$)	Halcion®

1.2.1.4 Physical properties

The molecular weight of nitrazepam is 281.27 g/mol (PubChem, 2008). The molecular weight of diazepam is 284.7 g/mol (Xu and Trissel, 1999). The British Pharmacopoeia lists diazepam as being very slightly soluble in water, soluble in alcohol and freely soluble in chloroform. The United States Pharmacopoeia lists diazepam as soluble 1 in 16 of ethyl alcohol, 1 in 2 of chloroform, 1 in 39 of ether, and practically insoluble in water. The pH of diazepam is neutral and it has a pK_a of 3.3 (Munne, 1996). The molecular weight of lorazepam is 321.2 g/mol. Lorazepam is a nearly white powder, which is low lipid soluble. The molecular weight of flunitrazepam is 313.28 g/mol (PubChem, 2008). The molecular weight of midazolam is 325.78 g/mol. Midazolam is water soluble at pH under 4 because the imidazoline ring is opened. However, the slightly alkaline (pH about 7.4) environment of the bloodstream causes the imidazoline ring to close, and it becomes much more lipid soluble. It has a pKa of 6.15 (Schou, 2004). The molecular weight of triazolam is 343.21 g/mol. It is a white crystalline powder, soluble in alcohol and poorly soluble in water (PubChem, 2008).

1.2.1.5 Administration

Benzodiazepines are available in tablet, capsule, ampoule, vial, or syringe form. The most common route of administration is oral and the next is intravenous. Distinctly less common are intramuscular and subcutaneous injections, which are erratic in absorption (Yokchue, 2004). There is the reported that diazepam can be administered orally, intravenously, or intramuscularly. However, it is more reliably absorbed following oral than intramuscular administration which may be due to precipitation in the muscle. The intramuscular injection of diazepam is very painful. Lorazepam can be administered orally, intramuscularly (Gomersall, 2003).

1.2.1.6 Toxicokinetics

1.2.1.6.1 Absorption

All benzodiazepines are well absorbed following oral administration (Trimble, 1983). In detail, nitrazepam is short acting benzodiazepines (Mubhoff and Daldrup, 1992). Diazepam is rapidly absorbed and has a fast onset of action when it is orally administered. When diazepam is administered as an intramuscular injection, absorption is slow, erratic and incomplete (Wishart, 2006). For lorazepam, it is

rapidly and nearly completely absorbed after any mode of administration (oral, sublingual, intramuscular, intravenous). Similar to midazolam, it is rapidly absorbed and has a fast onset of action after the injection.

1.2.1.6.2 Distribution

Following administration of benzodiazepines, there is the usual initial distribution phase to vessel; rich tissues including the central nervous system, kidneys, liver, and heart, from where it is distributed to muscle and later to body fat (Yokchue, 2004). Peak plasma level of nitrazepam is expected around 81 minutes after oral administration. Administration of 10 mg nitrazepam has been reported to be around 82 ng/ml at peak plasma level (Mubhoff and Daldrup, 1992). For diazepam, Peak plasma levels are achieved 30 minutes to 2 hours after oral administration (Wishart, 2006). For lorazepam, because of its relative low lipid solubility, its distribution in the body is mainly the vascular compartment, while midazolam can rapidly uptake into nerve tissue.

1.2.1.6.3 Protein binding

The benzodiazepines and their active metabolites are highly bound to plasma protein. Small changes in this protein binding will produce large alterations to concentrations of available free drugs. In humans, the protein binding of diazepam is around 98.5% (Wishart, 2006). Trimble (1983) reported the bounding of nitrazepam and lorazepam to plasma proteins is 86-87% and 85%, respectively.

1.2.1.6.4 Excretion

Excretion of benzodiazepines and their metabolites usually occurs in urine as the glucoronide conjugate (Yokchue, 2004). The metabolites of diazepam are conjugated with glucuronide, and are excreted primarily in the urine. Most of the drug is metabolized, very little diazepam is excreted unchanged (Wishart, 2006). Similarly, lorazepam glucuronide is excreted in the urine (Wikipedia, 2007).

1.2.1.7 Metabolism

Most benzodiazepines are extensively metabolized by phase I (predominantly dealkylation, aliphatic and aromatic hydroxylation, reduction, and acetylation), and phase II (conjugation) reactions. In most cases the phase I metabolites have some biological activity which may be greater or less than that of the parent, whereas the conjugates possess no significant activity. Conjugates consist

largely of glucuronides. The metabolism of many benzodiazepines is described elsewhere (Mubhoff and Daldrup, 1992).

Nitrazepam has a long half-life of 24-36 hours producing significant withdrawa symptoms following chronic use (Mubhoff and Daldrup, 1992). Diazepam is metabolized by N-dimethylation to the active N-desmethyl-diazepam (also known as nordazepam or nordiazepam) as the major metabolite in plasma (Sadee and Beelen, 1942). Diazepam has a half-life of 20-50 hours, and desmethyldiazepam has a half-life of 30-200 hours. The other active metabolites include temazepam and oxazepam. Lorazepam is metabolized in the liver into lorazepam glucuronide by conjugation. Lorazepam glucuronide has a somewhat longer half-life than lorazepam and therefore remains detectable in blood and urine for longer than unchanged lorazepam (Wikipedia, 2007). Midazolam is metabolized by the hepatic Cytochrome P450 enzyme (CYP3A3/3A4). It is hydroxylated to the active metabolite, 1-hydroxy-midazolam and then glucuronidated before being renally excreted.

1.2.1.8 Overdose

Although it is not usually fatal when taken diazepam alone, overdoses of diazepam with alcohol and/or other depressants may be fatal. The oral LD_{50} (lethal dose in 50% of the population) of diazepam is 720 mg/kg (Thomson, 2007). The duration of action of lorazepam is 6 to 12 hours with therapeutic dose, but a larger dose will cause more marked drug effects and longer duration of action. Whereas lorazepam it self is not usually fatal in overdose, it can cause fatal respiratory depression if taken in overdose with alcohol. In animal models, the oral LD_{50} of midazolam is 825 mg/kg.

1.2.1.9 Tolerance, Dependence, and Misuse

Diazepam can lead to physiological tolerance, and psychological and/or physical dependence. A particularly high risk for diazepam misuse, abuse, and dependence are patients with chronic pain or other physical disorders and patients with a history of alcohol or drug abuse or dependence (Overclocker, 2005). Lorazepam dependence and withdrawal symptoms are greater than other benzodiazepines because lorazepam binds strongly to the GABA receptor complex and has a short serum half-life. As a benzodiazepine, midazolam shares similar side effects to other members of this drug family. Although it is primarily used in hospital

settings only, there are the cases of sexual assaults using these drugs spiked in food to ply someone with drugs.

In Table 3, the duration of action, half-life, protein binding (%), volume of distribution (Vd, l/kg), therapeutic serum (μg/ml), and toxic serum (μg/ml) of 6 benzodiazepines are concisely classified.

Table 3. Classification of the duration of action of benzodiazepines based on their elimination half-life.

Duration of action	Drugs	Time (hrs)	Protein binding (%)	Vd (l/kg)	Therapeutic serum (μg/ml)	Toxic serum (μg/ml)
Ultra-short (<10 hrs)	Midazolam	2-5	>95	0.8-2.5	0.04-0.1	1
	Triazolam	2-5	89-94	0.8-1.8	0.017	*
Short (10-24 hrs)	Flunitrazepam	9-30	80-90	3.4-5.5	0.001-0.015	0.05
	Lorazepam	8-25	88-92	1-1.3	0.02-0.25	0.3
	Nitrazepam	18-48	2.4-4.8	2.4-4.8	0.03-0.10	0.2
Long (24 hrs)	Diazepam	14-70	98.5	1.1	0.1-1.5	1.5-15

* data not available

1.2.2 Sample preparation

1.2.2.1 Composition of serum

The most readily accessible body fluids are blood, saliva, and urine. The analysis of body fluids is a source of objective information about which drug has been taken (Ruangturakit, 1983). While all of these fluids are utilized for drug assays, measurements of plasma or serum obtained from blood may yield a better correlation between drug concentrations and effects. The analysis of drugs in whole blood should not be encouraged, since the erythrocyte/plasma concentration ratio may impede the interpretation of the results. Blood samples should therefore be centrifuged to obtain either plasma or serum.

Plasma is the liquid component of blood, in which the blood cells are suspended. Plasma specimens can be obtained by preventing blood coagulation with

various agents, including heparin and the Ca^{2+} binding agents, EDTA, citrate, and fluoride (Sadee and Beelen, 1942), and spinning a tube of blood in a centrifuge until the blood cells fall to the bottom of the tube. Serum differs from plasma. It is plasma without fibrinogen or the other clotting factors (Wikipedia, 2009). Serum specimens can be obtained by normal blood coagulation. Some of the major components in normal serum are listed in Table 4.

Table 4. Some normal components of the serum of adults.

Components	Serum	Components	Serum
Water (g/l)	930-955	Uric acid (mg/l)	18-76
Dry subtances (g/l)	80	Total lipids (g/l)	3.5-8.5
Total proteins (g/l)	65-80	Fatty acids (g/l)	
Albumin (% of total protein)	50-65	Total	1-5
Globulin (% of total protein)	35-50	Free	0.1-0.35
Total cations (mEq/l)	149-159	Cholesterol (g/l)	
Sodium (mEq/l)	132-151	Total	1-3
Bicarbonate (mEq/l)	21.3-28.5	Free	0.3-1
Chloride (mEq/l)	99-111	Phosphatides (g/l)	1.5-3.5
Total nitrogen (g/l)	12-14	Triglycerides	0.5-2.2
Non-protein nitrogen (mg/l)	139-307	Bile acids (mg/l)	<10
Urea (mg/l)	230-426	Glucose (mg/l)	750-1170
Creatinine (mg/l)	6.6-18.2	Glucoronic acid (mg/l)	20-44
Free amino acids (mg/l)	28-50	Heparin (mg/l)	1-2.4
Alanine (mg/l)	22-45	Glucoproteins (g/l)	2.7
Lysine (mg/l)	13-31	Succinate (mg/l)	5
Proline (mg/l)	13-51	Citrate (mg/l)	17-31
Catecholamines	ng/ml range	Pyruvate (mg/l)	2.6-10.2
Histamine	ng/ml range	Acetone (mg/l)	2.3-3.5
Serotonine	ng/ml range	Vitamins	Varying
Bilirubin (total) (mg/l)	2.6-14	Ascorbic acid (mg/l)	2-14

Source: Sadee and Beelen (1942)

1.2.2.2 Extraction method

The serum samples are complex mixtures containing numerous endogenous components such as proteins, salts or lipids which can interfere with the analytes during the separation and detection processes (Souverain *et al*., 2004), so the analysis of endogenous substances and drugs in serum samples present special problems. They usually require the preparation of the sample before injection into a liquid chromatographic system. Some of these preparation treatments are protein precipitation, enzymatic digestion, ultra-filtration, dialysis, liquid-liquid extraction, or solid-phase extraction.

In the recent years, solid-phase extraction has been used as an alternative technique to liquid-liquid extraction. An Oasis MCX mixed-mode solid-phase extraction cartridge was used to extract sedative-hypnotics (consisted of 5 barbiturates, 30 benzodiazepine-related drugs, and 11 other sedative-hypnotics) in serum. The extraction efficiencies were sufficient for analysis, although some of the recoveries were relatively low (Miyaguchi *et al.,* 2006). Yokchue (2004) compared liquid-liquid extraction using diethyl ether with solid phase extraction using HLB C_{18} solid-phase cartridge to determine the effective extraction method for benzodiazepines. In the procedure of liquid-liquid extraction, blood samples were treated with Na_2CO_3 and $NaHCO_3$, diethyl ether was then added for the extraction. For solid phase extraction, blood sample was added to an Oasis HLB C_{18} solid-phase cartridge, and then eluted with methanol. Liquid-liquid extraction provided better recovery (64.8-104%) than solid phase extraction.

However, protein precipitation is commonly used for fast sample clean-up and disrupting protein–drug binding. Among the interferences in serum samples, proteins are present in a large amount which can be adsorbed onto the chromatographic support and causes the deterioration of separation efficiency and a rapid column clogging. Polson *et al.* (2003) described that there are many common protein precipitation procedures to remove proteins in biological samples by adding a reagent, e.g., organic solvent, acid, salt or metal ion. After the protein precipitation, the isolation of supernatant is generally performed by centrifugation or filtration.

Mittal and Oroskar (2007) described that the deproteinization of serum samples with organic solvent; acetonitrile is a useful and rapid technique in analysis

of drugs in serum samples. Organic solvent precipitants lower the dielectric constant of the plasma protein solution, which increases the attraction between charged molecules and facilitates electrostatic protein interactions. The organic solvent also displaces the ordered water molecules around the hydrophobic regions on the protein surface. Hydrophobic interactions between proteins are minimized as a result of the surrounding organic solvent, while electrostatic interactions become predominant and lead to protein aggregation (Polson *et al.,* 2003).

In 2009, Catena *et al.* demonstrated the used of a simple deproteinization procedure to human plasma and saliva samples for the quantitative analysis of clindamycin by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI/MS/MS). Methanol was used as the precipitant, and the centrifugation was performed at 10000 g for 10 minutes before injection onto HPLC column. The recovery of clindamycin in plasma was 95.1 ± 2.28 %.

The protein precipitation using organic solvent was also confirmed by Souverain *et al.* (2004) who investigated three different protein precipitation (PP) procedures with acetonitrile (ACN), perchloric acid (PA) and trichloroacetic acid (TCA) for the analysis of a drug cocktail, namely methadone (MTD) and ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine (EDDP), fluoxetine (FLX) and norfluoxetine (NFLX), flunitrazepam (FLZ) and norflunitrazepam (NFLZ) from human plasma samples using a capillary liquid chromatography-electrospray ionisation–mass spectrometry (LC-ESI-MS). The recoveries for ACN precipitation between 80 and 120% were obtained for MTD, EDDP, FLZ, FLX, NFLX and MFLX with CV values inferior to 6.0%. They concluded that among the three selected PP techniques, ACN was the most effective precipitant in terms of recovery and repeatability.

1.2.3 High performance liquid chromatography

Liquid chromatography (LC) is an analytical chromatographic technique in which a mobile phase is a liquid. It is useful for separating ions or molecules that are dissolved in a solvent (Yokchue, 2004). Early liquid chromatography was performed in glass columns having inside diameter of perhaps 10 to 50 mm. The column was packed with 50 to 500 cm lengths of solid particles coated with an adsorbed liquid that performed the stationary phase. Until the late

1960's, the technology was developed for producing and using particles which is as small as 3 to 10 µm diameters. This technology required instruments capable of much high pumping pressures than the simple devices that preceded them. Simultaneously, detectors were developed for continuous monitoring of column effluents. The term high performance liquid chromatography is used to describe this technology. Therefore, high performance liquid chromatography (HPLC) is a form of liquid chromatography in which the mobile phase is mechanically pumped through a column contains the stationary phase.

By the 1980's HPLC was commonly used for the separation of chemical compounds. It has become an indispensable analytical tool in the forensic science laboratories, which often use HPLC in the processing of evidences (Skoog *et al.,* 2004).

HPLC instruments consist of (1) a mobile phase reservoir, (2) a pump to force and control the mobile phase through stationary phase, (3) an injector to introduce the sample into the flowing mobile phase stream, (4) a separation column contains stationary phase to create the separation of mixture, because the different components in the mixture pass through the column at different rates due to their different partitioning behavior between the mobile phase and the stationary phase, (5) a detector to detect the separated compound eluting from the column, which the mobile phase will exit from the detector and can be disposed to waste or collected if desired, and (6) a computer data station to create the chromatogram from the electronic signal provided by the detector (Kazakevich and McNair, 2000). The basic components of High Performance Liquid Chromatographic system are shown in Figure 2.

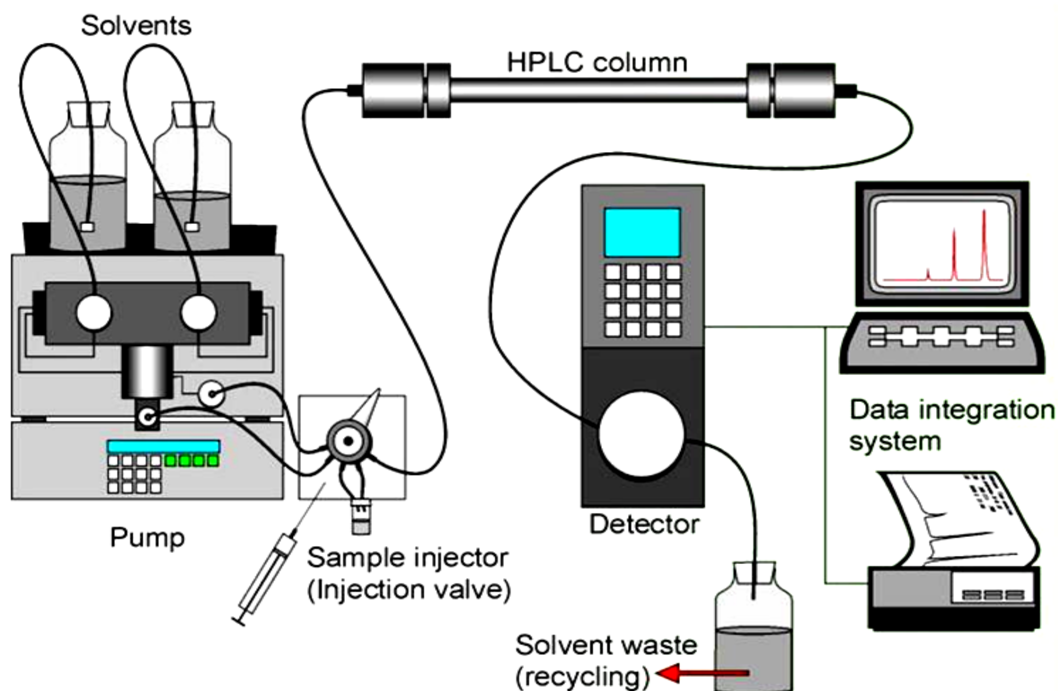


Figure 2. Basic components of High Performance Liquid Chromatographic system.
Source: http://www.uft.unibremen.de/chemie/Chromatography/

Types of liquid chromatography are size exclusion, bioaffinity, ion-exchange, normal-phase, and reverse-phase chromatography.

Size exclusion chromatograph is also known as gel permeation chromatography or gel filtration chromatography. Separations in size exclusion chromatography arise from differenced in molecular size and ability of different molecules to penetrate the pores of the stationary phase to different extents (Lough and Wainer, 1996). The stationary phase consists of porous beads. The larger compounds will be excluded from the interior of the bead and thus will be eluted first.

Bioaffinity chromatography operates by using biochemicals that have a specific affinity to the compound of interest (Lodder, 2007).

Ion exchange chromatography is used almost with ionic or ionizable samples. The retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded.

Normal-phase chromatography uses a polar stationary phase such as alumina, silica gel, or hydroxylapatite and a nonpolar mobile phase such as n-hexane or tetrahydrofuran It is used when the analytes of interest is fairly polar, which are

retained on the polar surface of the column packing longer than less polar materials (Kazakevich and McNair, 2000).

Reversed-phase chromatography is the inverse of this, which consists of a non-polar stationary phase and a moderately polar mobile phase. The majority of reverse-phase methods have been developed on covalently modified silica gel and one common stationary phase is a silica which has been treated with $R(CH_3)_2SiCl$, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention of analytes is determined mainly by the carbon loading of the stationary phase. In addition, it may also be modulated on phenyl-bonded phases by π-π stacking interactions and cyano-bonded phases by dipole-dipole interactions (Lough and Wainer, 1996). The mobile phase is typically water, to which less polar solvent such as methanol or acetonitrile is added. Reversed-phase chromatography operates on the principle of hydrophobic interactions. Here the more non-polar material is, the longer it will be retained (Yokchue, 2004).

High performance liquid chromatography is still widely used for the analysis of drugs. Lo *et al.* (1997) reported that reversed-phase high-performance liquid chromatography with diode array detection (HPLC–DAD) offers a number of advantages for the analysis of acidic and neutral drugs. The protocol was found to be suitable for both clinical toxicology (including emergency toxicology) and postmortem toxicology. In their study, acidic and neutral drugs were analyzed by HPLC–DAD with microbore ODS-Hypersil column in a gradient elution programme of acetonitrile portion and 2 mM phosphate buffer pH 3.2. The retention times of common acidic and neutral drugs relative to that of the internal standard heptabarbitone.

A high-performance liquid chromatographic method for the forensic analysis of 10 barbiturates in human biological samples using a reversed-phase column packed with 2-μm particles was also developed by Tanaka *et al.* (1997). The HPLC method uses a C_{18} reversed-phase column with acetonitrile-8 mM KH_2PO_4 (30:70, v/v) as a mobile phase. The detector was variable-wavelength UV detector monitored at 215 nm. From the results, all drugs eluted within 25 min. The limit of quantification for 6 barbiturates (allobarbital, barbital, hexobarbital, metharbital,

Phenobarbital, and pentobarbital) was 0.05 µg/ml and for amobarbital, cyclobarbital and secobarbital, and thiopental was 0.01 and 0.5 µg/ml, respectively.

Franeta *et al.* (2002) presented a HPLC method for simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in Malophenum tablets. Chromatographic system equipped with UV–Vis detector at 207 nm was achieved using C_{18}, 5 µm, 250×4.6 mm column as well as the further studies. Mixture of acetonitrile-water (25:75 v/v) (pH 2.5) with phosphoric acid was used as a mobile phase. The sensitivity (LOD: 9×10^{-5}-1.7×10^{-4} mg/ml) was found to be satisfactory. They reported that the described method is rapid and sensitive and therefore suitable for routine control of these drugs in dosage form.

For the reasons that HPLC shows greater specificity and sensitivity than any other techniques such as immunoassay technique for the analysis of drugs in routine laboratory, the effective condition of HPLC for separation of 15 benzodiazepines in human blood extracted by liquid-liquid extraction was determined by Yokchue (2004). HPLC analysis was performed using C_{18} column at 30°C with ultraviolet detector at wavelength 254 nm. 60% methanol was used as a mobile phase for detecting alprazolam, clobazam, clorazepate, diazepam, prazepam, and temazepam with the retention time at 4.6, 4.0, 7.0, 7.8, 16 and 5.3 min, respectively. 15 mM KH_2PO_4:methanol:acetonitrile (50:35:15) was used as mobile phase for detecting bromazepam, clonazepam, flurazepam, flunitrazepam, lorezepam, lormetazepam, midazolam, nitrazepam, and triazolam with the retention time of 5.27, 6.9, 13.7, 7.3, 9.02, 13, 17.6, 6.97, and 9.97 min, respectively. She also reported that the detection limits were adequate for clinical and forensic analysis except some benzodiazepines (flunitrazepam, flurazepam, lormetazepam, and triazolam) which have low therapeutic and toxic level.

Kintz *et al.* (2005) also applied HPLC to detect 17 benzodiazepines and hypnotics. The HPLC system with C_{18} Column and mass spectrometry detector was carried out with a gradient elution of 5% acetonitrile, 95%-formic acid, 0.1% to a ratio 80-20% at 10 min. The results showed that the limits of quantification for all benzodiazepines and hypnotics range from 0.1 to 0.2 ng/ml. They concluded that the method is suitable for screening 17 benzodiazepines in oral fluid and detect them at very low concentrations.

Miyaguchi *et al.* (2006) established a single-quadrupole liquid chromatography tandem mass spectrometry for the detection of sedative-hypnotics (consisted of 5 barbiturates, 30 benzodiazepine-related drugs, and 11 other sedative-hypnotics) in serum. C_{18} column (150 mm × 2.1 mm, particle size 3.5 µm) at 35 °C and photodiode array detector for UV measurements were used. Methanol in 10 mmol/l ammonium acetate was used as a mobile phase at a flow rate of 0.2 ml/min by gradient elution: 0-5 min, 10% methanol; 5-45 min, from 10% to 90% linear gradient of methanol in 10 mmol/l ammonium acetate; 45-55 min, 90% methanol; 55-60 min, from 90% to 10% methanol; 60-80 min, equilibration of the column with 10% methanol. The result showed that the non-basic analytes were detectable at concentrations above 1-10 ng/ml except for TCA (50 ng/ml), and the basic analytes were detectable at concentrations above 0.1-1 ng/ml except for lorazepam, lormetazepam, quazepam and zopiclone (5 ng/ml). The developed method was also applied to a case of suicide, which empty packages of non-prescription medicines containing bromisovalum, apronalide and/or diphenhydramine were found at the scene. The screening resulted in the detection of these three compounds. They concluded that the HPLC method is sufficient to permit the screening of a single therapeutic administration of a target drug. Recently, the determination of benzodiazepines in serum with gradient elution of a C_8 reversed-phase column has been published by Pongraveevongsa *et al.* (2007).

Although the C_{18} and C_8 reverse-phase column are commonly used for the analysis of drugs, Sadek (1996) described the use of phenyl column for the analysis of 15 benzodiazepines from postmortem blood. In 2001, Salamone reported that reversed-phase analytical columns with cyano sorbents are frequently used for HPLC analysis of flunitrazepam and metabolites. Furthermore, the characteristics of cyanopropyl (cyano) columns by means of a relationship developed originally for alkyl-silica columns have been studied by Marchand *et al.* (2005). They concluded that cyano columns are much less hydrophobic, less sterically restricted, and have lower hydrogen-bond acidity than type-B alkyl-silica columns (i.e., made from pure silica) because sample retention is generally much weaker on cyano versus other columns (e.g., C_8, C_{18}), a change to a cyano column usually requires a significantly weaker mobile phase.

1.3 Objectives

1.3.1 To optimize the HPLC condition for the separation of 6 benzodiazepines.

1.3.2 To determine the extraction efficiency of extraction method for the analysis of 6 benzodiazepines in human serum.

1.3.3 To apply the proposed method to the real samples and evaluate the quantity of 2 available benzodiazepines in human serum samples.

CHAPTER 2

METHODOLOGY

2.1 Chemicals and reagents

2.1.1 Serum blank supplied by 20 healthy male and female volunteers.

2.1.2 Serum samples contributed by 2 healthy male volunteers orally administrated either 5.0 mg diazepam or 1.0 mg lorazepam.

2.1.3 Standard benzodiazepines, including nitrazepam, diazepam, lorazepam, flunitrazepam, midazolam, and triazolam (Alltech-Applied Science, USA) obtained from Regional Forensic Science sub-Division 41, Office of Police Forensic Science, Royal Thai Police, Thailand.

2.1.4 Acetonitrile (Baker Analyzed® HPLC Solvent, J.T. Baker, USA)

2.1.5 Methanol (Baker Analyzed® HPLC Solvent, J.T. Baker, USA)

2.1.6 Potassium dihydrogen phosphate (Fluka, Switzerland)

2.1.7 Sodium hydroxide (Merck, Germany)

2.1.8 Ortho-phosphoric acid (J.T. Baker, USA)

2.1.9 Ammonium hydroxide (J.T. Baker, USA)

2.1.10 Ultrapure water (Elga Maxima Ultrapure Water Purification System, The Netherlands)

2.2 Instruments and equipments

2.2.1 Instruments and equipments for sample collection, and standard, sample, and mobile phase preparation

2.2.1.1 Electronic balance AB204, 4 digits (Mettler-Toledo, Switzerland)

2.2.1.2 pH meter Denver model 15 (Denver Instrument, USA)

2.2.1.3 Vortex mixer Genie 2 (Scientific Industries, USA)

2.2.1.4 Vacuum Pump Aspirators B169 (Buchi, Japan)

2.2.1.5 Microcentrifuge Denville^{TM} 260D (Denville Scientific, USA)

2.2.1.6 Blood collecting tubes (BD Franklin, USA)

2.2.1.7 Needle syringes and syringes (Terumo, Philippines)

2.2.1.8 Clear homo-polymer microtubes 2.0 ml (Axygen Scientific, USA)

2.2.1.9 Micropipette 10 µl, 100 µl, and 1000 µl, and pipette tips (Eppendorf Research, Germany)

2.2.1.10 Weighing paper 10 cm x 10 cm (P.S. Science Chemical, Thailand)

2.2.1.11 Nylon transfer membrane, 0.45 µm × 47 mm (Magna, USA)

2.2.1.12 Disposable syringe (without needle) 10 ml (Nipro, Thailand)

2.2.1.13 Syringe filter RC-membrane 0.45 µm (Minisart, Germany)

2.2.1.14 Rubber examination gloves, non-sterile, smooth surface, pre-powder, size M (W.A. Rubbermate, Thailand)

2.2.1.15 Laboratory film (Parafilm, USA)

2.2.1.16 Other necessary glasswares; beaker, cylinder, volumetric flask, measuring spoon, ultrapure water bottle, labeled marker

2.2.2 Instruments and equipments for HPLC analysis

2.2.2.1 High Performance Liquid Chromatrography System (HPLC)

- Solvent reservoirs 3 lines; A, B, and C
- Pump Varian 9012 Solvent Delivery System
- Detector Waters 2487 dual λ absorbance detector
- Varian star chromatography workstation

2.2.2.2 Lichrospher 100 mm × 4 mm, 5 μm CN column (Lichrocart® Merck, Germany)

2.2.2.3 Lichrospher 125 mm × 4 mm, 5 μm RP-18 endcapped (Lichrocart® Merck, Germany)

2.2.2.4 Microliter injection syringe 50 μl (Hamilton, Switzerland)

2.2.2.5 Other necessary glasswares; microtube rack, ultrapure water bottle, vials with screw-cap, beaker, labeled marker, etc.

2.3 Method

2.3.1 Preparation of standard solution.

2.3.1.1 Preparation of stock standard solution.

The stock standard solutions of 6 drugs were separately prepared by dissolving each standard drug with methanol to the concentration of 500 μg/ml.

2.3.1.2 Preparation of working standard solution.

The working standard solution was separately prepared by diluting the stock standard solution of 6 drugs in methanol : ultrapure water, ratio of 1 : 1 (v/v) to the concentration of 10 μg/ml before the injection to HPLC system.

2.3.1.3 Preparation of working mixture standard solution.

The working standard solution 10 μg/ml of each drug was mixed by using the same volume of 6 drugs and adjusted to the concentration of 1 μg/ml with ultrapure water.

2.3.2 Preparation of mobile phase.

2.3.2.1 Preparation of methanol, ultrapure water, and acetonitrile.

Methanol, ultrapure water, and acetonitrile were separately filtered through a 0.45 μm nylon membrane filter and degassed. Filtered and degassed methanol, ultrapure water, and acetonitrile were installed to HPLC system prior to use in line A, B, and C of HPLC reservoirs, respectively.

2.3.2.2 Preparation of phosphate buffer solution.

First of all, 10% (w/v) sodium hydroxide was prepared by dissolving the appropriate amounts of sodium hydroxide in ultrapure water. Next, Aqueous phosphate buffer (3.3 mM, pH 2.1) was prepared by dissolving the appropriate amounts of potassium dihydrogen phosphate in ultrapure water and adjusting pH with ortho-phosphoric acid or 10% (w/v) sodium hydroxide previously prepared. Phosphate buffer solution (3.3 mM, pH 2.1) was filtered through a 0.45 µm nylon membrane filter and degassed. Filtered and degassed phosphate buffer solution (3.3 mM, pH 2.1) was installed to HPLC system in line B of HPLC reservoirs alternately with ultrapure water.

2.3.3 Preparation of HPLC instrument.

In all experiments, a flow rate of mobile phase used was 1.0 ml/min. The detector wavelength was set at 254 nm. The injection volume was 20 µl. Before the injection, the CN column was equilibrated on the initial solvent condition until pH before and after column is identical.

2.3.4 Determination of 6 benzodiazepines elution behaviors.

2.3.4.1 The proportions of mobile phase

To determine the elution behaviors of 6 benzodiazepines, each benzodiazepine was separately studied by the injection at each mobile phase composition conditions. The proportions of 3 mobile phase used; methanol, acetonitrile, and phosphate buffer solution (3.3 mM, pH 2.1) were varied to the different conditions both in isocratic and gradient elution.

2.3.4.1.1 Isocratic elution

- First, 2 mobile phases; methanol and phosphate buffer solution were used. The proportions of methanol and phosphate buffer solution were varied to 4 different conditions which are shown in Table 5.

Table 5. The varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Methanol	25	30	35	40
%Buffer	75	70	65	60

- Second, 2 mobile phases; acetonitrile instead of methanol and phosphate buffer solution were used. The proportions of acetonitrile and phosphate buffer solution were varied to 4 different conditions which are shown in Table 6.

Table 6. The varied proportions of acetonitrile and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Acetonitrile	15	20	25	30
%Buffer	85	80	75	70

- Third, All 3 mobile phases; methanol, acetonitrile, and phosphate buffer solution were used. The proportions of methanol and phosphate buffer solution were varied to 3 different conditions which are shown in Table 7.

Table 7. The varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) with 5% acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 μm), flow-rate 1.0 ml/min, 20 μl injection volume.

Mobile phase	Condition		
	I	II	III
%Methanol	20	25	30
%Buffer	75	70	65
%Acetonitrile	5	5	5

- Fourth, All 3 mobile phases; methanol, acetonitrile, and phosphate buffer solution were used. The proportions of methanol and phosphate buffer solution were varied to 3 different conditions which are shown in Table 8.

Table 8. The varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) with 3% fixed acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 μm), flow-rate 1.0 ml/min, 20 μl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Methanol	22	23	24	25
%Buffer	75	74	73	72
%Acetonitrile	3	3	3	3

2.3.4.1.2 Gradient elution

At last, the linear gradient elution was used. The proportions of methanol, acetonitrile, and phosphate buffer solution were varied to 5 different conditions in 30 minutes linear gradient which are shown in Table 9.

Table 9. The varied proportions of methanol, acetonitrile, and phosphate buffer solution (3.3 mM, pH 2.1) in 5 linear gradient conditions.

	Condition									
	I		II		III		IV		V	
	Time (mins)		Time (mins)		Time (mins)		Time (mins)		Time (mins)	
Mobile phase	0	30	0	30	0	30	0	30	0	30
%Methanol	26	20	27	20	28	20	29	20	30	20
%Buffer	70	80	69	80	68	80	67	80	66	80
%Acetonitrile	4	0	4	0	4	0	4	0	4	0

After the optimum condition for the separation of 6 drugs was known from the elution behaviors, the standard mixture of 6 drugs was prepared and applied to HPLC system to perform the result of separation in form of chromatogram.

2.3.5 Determination of the extraction efficiency of the extraction method for the analysis of 6 benzodiazepines in human serum.

2.3.5.1 Preparation of blank serum and drug spiked serum.

Blank serum was prepared from pooled serum of 20 healthy volunteers which was stored at -20°C before analysis. When perform an analysis, blank serum was defrosted into aqueous serum. The aqueous serum obtained was used as blank serum.

Drug spiked serum of 6 benzodiazepines was separately prepared at low, average, and high concentrations. The aqueous serum was transferred to a tube and then spiked with the appropriate aliquots of drug standard solution. The low, average, and high concentrations of 6 benzodiazepines spiked serum are shown in Table 10.

Table 10. The low, average, and high concentrations of 6 benzodiazepines spiked serum.

Benzodiazepines	Concentration (μg/ml)		
	Low	Average	High
Nitrazepam	0.20	0.60	1.00
Diazepam	0.20	0.60	1.00
Lorazepam	0.20	0.60	1.00
Flunitrazepam	0.40	0.80	1.20
Midazolam	0.80	1.20	1.60
Triazolam	0.80	1.20	1.60

2.3.5.2 Extraction procedure.

The protein precipitation extraction was used. To 1.0 ml of blank serum and drug spiked serum in a tube was mixed with 100 μl of ammonia solution, then added 1.0 ml of acetonitrile. After mixing, the tube was centrifuged at 14000 rpm for 10 min. The organic phase was recovered and filtered through the RC membrane filter. Then, extracted serum was injected into the HPLC system, in which 3 replicates injection at each concentration was carried out. The diagram of extraction procedure is shown in Figure 3.

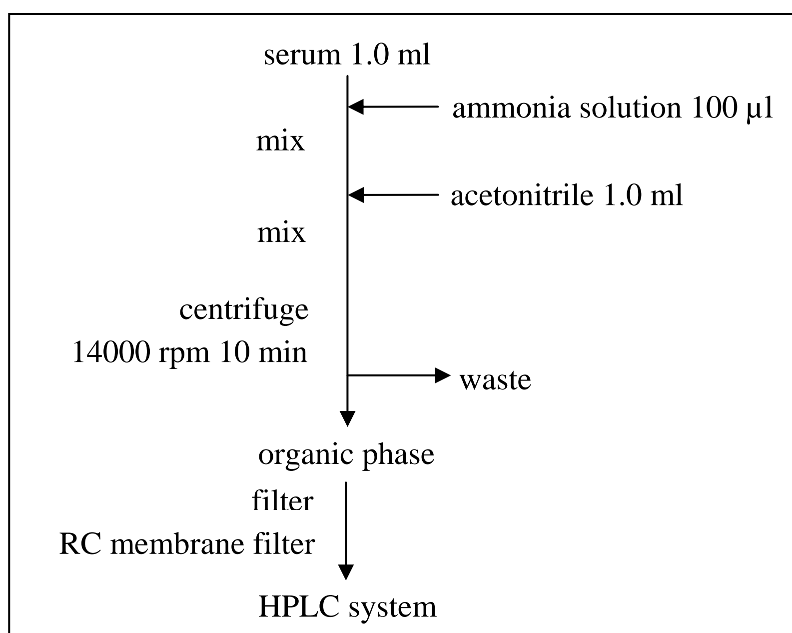


Figure 3. The extraction procedure for the analysis of 6 benzodiazepines in serum.

2.3.5.3 Determination of standard curve.

The standard curve of each benzodiazepine was obtained by plotting the peak area (mV.sec) against the concentration (µg/ml) of the respective drug.

The standard solution of each drug was prepared at low, average, and high concentrations, in which, 3 replicates injection at each concentration were carried out. The concentrations of 6 benzodiazepines used to make standard curve is shown in Table 10.

2.3.5.4 Recovery study

The extraction recovery was evaluated in 3 replicates of low, average, and high concentrations by calculating concentration of drug spiked serum from the standard curve which was obtained by plotting peak area (mV.sec) against the concentration (µg/ml) of benzodiazepines. The calculated concentration was compared to the prepared concentration. In general, recovery should be more than 75% and percent relative standard deviation (%RSD) should be less than 5% (Bugey and Staub, 2004)

2.3.6 Method validation.

2.3.6.1 Linearity

Blank serum and standard drug spiked serum were prepared according to the procedure in section 2.3.5.1, and then extracted according to the extraction method in step 2.3.5.2 or Figure 3. These analytes were applied to the HPLC system. Finally, the calibration curve of each standard drug spiked serum was evaluated by plotting the peak area (mV.sec) against the concentration (µg/ml) of the respective benzodiazepines. The equations and the relative correlation coefficients (r^2) obtained from the calibration curves will give the linearity of results.

2.3.6.2 Limit of detection (LOD) and Limit of quantification (LOQ)

The 1975 International Union of Pure and Applied Chemistry (IUPAC) defined the limit of detection (LOD) and limit of quantification (LOQ) in terms of concentration (C_L) and the signal (X_L) generated by a solution of concentration C_L (Corley, 2002). They defined the value of X_L in terms of the mean blank signal (X_B) and the standard deviation (S_B) of these blank measurements as

$$X_L = X_B + kS_B \qquad (1)$$

where k is a numerical factor chosen in accordance with the confidence level desired. Long and Winefordner further link C_L to X_L as follows:

$$C_L = (X_L - X_B)/m \qquad (2)$$

where m is defined as 'analytical sensitivity' and expressed as the slope of the calibration curve line obtained from the linear regression analysis. By substituting the value of X_L from equation (1) into equation (2), Long and Winefordner define C_L as

$$C_L = kS_B/m \qquad (3)$$

Long and Winefordner along with several other authors agree on a value of k = 3 for the determination of limit of detection (LOD), which allows a confidence level of 99.86% if the values of X_B follow a normal distribution, and 89% if the values of X_B do not follow a normal distribution. On the other hand, a value of k = 10, which was the signal to noise ratio was accepted to determine the limit of quantification (LOQ).

In this study, m was obtained from the linear regression analysis by plotting standard serum curve between responses (mV.sec) and concentrations (µg/ml). Then, blank was analyzed for 10 replicates to determine X_B and S_B.

The limit of detection (LOD) was calculated from the equation:

$$C_L = 3S_B/m \qquad (4)$$

where C_L = Limit of detection (LOD)

3 = signal to noise ratio (numerical factor)

S_B = standard deviation of blank

m = slope of the calibration curve line

The limit of quantification (LOQ) was calculated from equation:

$$C_L = 10S_B/m \qquad (5)$$

where C_L = Limit of quantification (LOQ)

10 = signal to noise ratio (numerical factor)

S_B = standard deviation of blank

m = slope of the calibration curve line

2.3.6.3 Precision of the procedure

The precision of the procedure has been established by calculating the intraday (within-day) repeatability and the interday (between-day) reproducibility. The intraday repeatability was determined over 1 day, with repeated analysis (n = 3) at low, average and high concentrations of each drug (Table 11). The intraday relative standard deviations (R.S.D.) obtained should be lower than 5%. The interday reproducibility was determined with repeated analysis (n = 3) at average concentration, over a period of 3 days. The interday relative standard deviations (RSD) obtained should be lower than 10%.

Table 11. The low, average, and high concentrations of 6 benzodiazepines standard spiked serum for the determination of the intraday (within-day) repeatability and the interday (between-day) reproducibility.

Benzodiazepines	Intraday (n = 3) Concentration (µg/ml)			Interday (n = 9) Average Concentration (µg/ml)
	Low	Average	High	
Nitrazepam	0.20	0.60	1.00	0.60
Diazepam	0.20	0.60	1.00	0.60
Lorazepam	0.20	0.60	1.00	0.60
Flunitrazepam	0.40	0.80	1.20	0.80
Midazolam	0.80	1.20	1.60	1.20
Triazolam	0.80	1.20	1.60	1.20

2.3.7 Application of the method to the real sample.

This study was approved by the Ethical Conduct of Research Involving Humans Committee of the Faculty of Science, Prince of Songkla University. The application of evaluated method in this study was concentrated in 2 available benzodiazepines, which are diazepam and lorazepam.

2.3.7.1 Diazepam administration

A volunteer subject between 20 and 40 years were requested to participate in the study. Subjects having pregnancy or nursing, having associated psychiatric illness and medical illness, having contraindications for use of

benzodiazepines, *e.g.* acute or chronic pulmonary disease, sleep apnea, renal disease, hepatic disease, organic brain disorder etc. were excluded. Contributive blood was collected into blood collecting tube at baseline, and again when reached peak plasma concentration of 2 hours post-administration of 5.0 mg diazepam for 10 ml of each. The collected blood was immediately centrifuged at 14000 rpm for 10 minutes to obtain serum. Blank serum and diazepam serum samples were stored at -20°C before analysis. When perform analysis, blank serum and diazepam serum samples were defrosted into aqueous blood, followed by extracted according to the extraction procedure in section 2.3.5.2 or figure 3, and injected into the HPLC system

The standard diazepam spiked serum curve used to quantify diazepam concentration in diazepam serum samples was obtained from the results in section 2.3.6.1.

2.3.7.2 Lorazepam administration

A volunteer was chosen according to section 2.3.7.1. Contributive blood was collected into blood collecting tube at baseline, and again when reached peak plasma concentration of 1 hours post-administration of 1.0 mg lorazepam for 10 ml of each. The collected blood was immediately centrifuged at 14000 rpm for 10 minutes to obtain serum. Blank serum and lorazepam serum samples were stored at -20°C before analysis. When perform analysis, blank serum and lorazepam serum samples were defrosted into aqueous blood, followed by extracted according to the extraction procedure in section 2.3.5.2 or figure 3, and injected into the HPLC system

The standard lorazepam spiked serum curve used to quantify lorazepam concentration in lorazepam serum samples was obtained from the results in section 2.3.6.1.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of 6 benzodiazepines elution behavior.

The main objective in developing an HPLC method for analysis samples of interest is achieving an acceptable degree of separation for all the components of interest in a reasonable time. To assist in this objective, there must be a systematic approach. The initial step is to select an HPLC method and a column that seem suited to the particular sample (Snyder *et al.,* 1998). After the initial selection of the HPLC method, it will be followed by the mapping of retention of analytes. The retention in liquid chromatography depends on the strength of the solute's interaction with both the mobile and stationary phase. Generally, the retention of particular analytes is usually altered by changing mobile phase polarity or mobile phase strength. Therefore the selectivity of separation may be conveniently adjusted by changing the mobile phase strength or changing type of organic modifier in the mobile phase, usually called mobile phase optimization (Lindsay, 1992).

Mobile phase optimization has been investigated by a number of research groups for about the last ten years (Lindsay, 1992). As a result of this work, a number of strategies for optimization have been developed, some of which are sequential method, predictive method, iterative method, commercial system method, and elution behavior method. The optimum exploitation of these approaches requires both the selection of some minimum set of different solvents, and an efficient way to find the best mixture of this set of solvents. Finally, information gathered during these systematic approaches giving result as an adequate separation time of all analyzes with acceptable peak shape can be used to validate and applied to routine works.

Benzodiazepines are one of major interests in clinical laboratories which can be isolated and determined by reversed-phase HPLC both in qualitative and

quantitative analysis. In reversed-phase HPLC method, there are many kinds of column to be used such as; C_{18}, cyano, or amino. In preliminary study, C_{18} column was used to determine the elution behavior of 6 benzodiazepines by using 30, 40, and 50% of varied acetonitrile in phosphate buffer solution (3.3 mM, pH 2.1) as mobile phase defined as condition I, II, and III, respectively. The preliminary result of 6 drugs was combined and built in one graph which is shown in Figure 4.

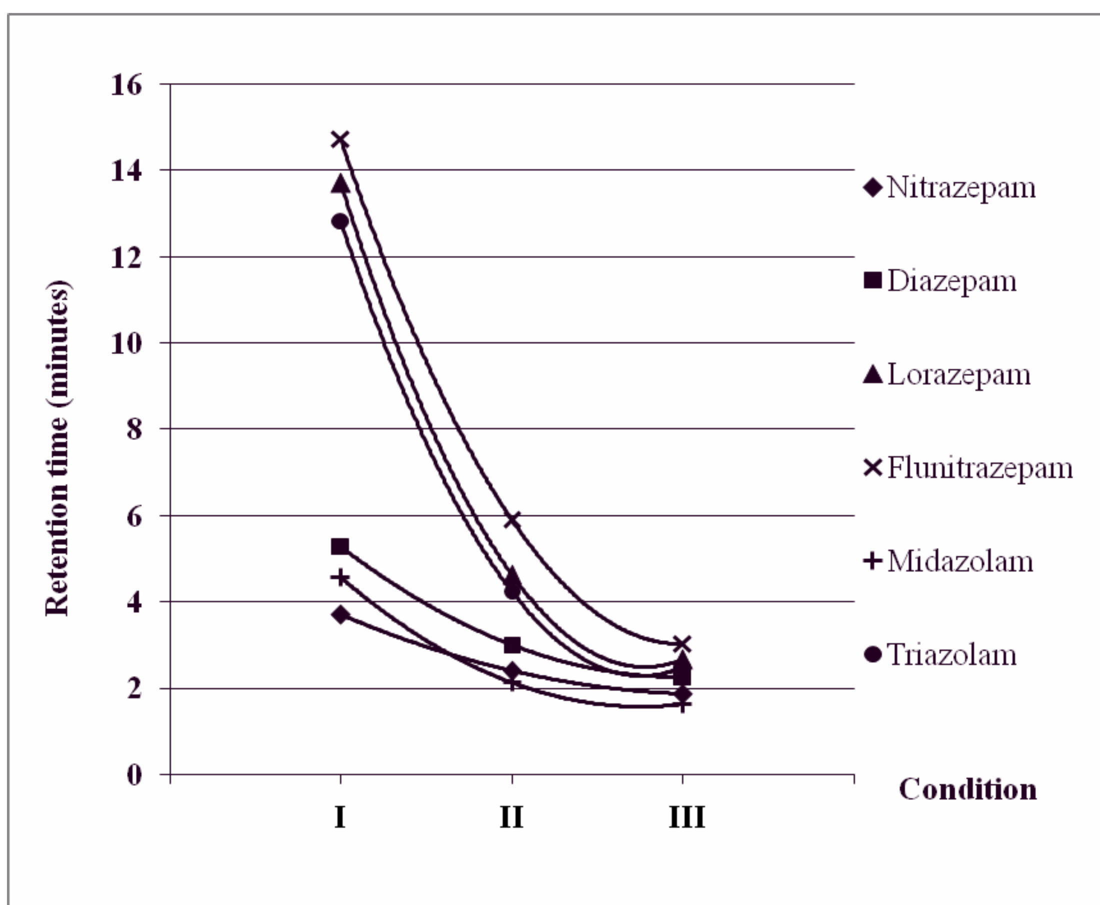


Figure 4. Elution behaviors of 6 drugs obtained by the use of 30, 40, and 50% of varied acetonitrile in phosphate buffer solution (3.3 mM, pH 2.1) as mobile phase defined as condition I, II, and III, respectively, Lichrospher 125 mm × 4 mm, 5 µm RP-18 endcapped, flow-rate 1.0 ml/min, 20 µl injection volume.

From Figure 4, it was shown that there are 2 groups of drugs tend to retain in similar time and cannot be separated because of the less tendency separation

when elution behavior was preliminarily determined by C_{18} column. However, the analysis of 6 drugs using cyano column was also preliminarily studied and the results show the more tendency separation than using C_{18} column. Therefore, in this study, the reversed-phase HPLC method combined with cyano column was chosen.

Next step, for the mobile phase optimization, the elution behavior method was used, which is one of the strategies methods for optimization. The advantages of this method are small number of experiments is required and easy to evaluate.

In case of reversed-phase HPLC, the solvent strength of mobile phase plays a major role in optimizing band spacing (Snyder *et al.,* 1998). The solute retention can be controlled by changing the strength or polar of the mobile phase. For instance, if we make the mobile phase less polar by increasing the ratio of organic solvent to water then we will shift the distribution of solutes towards the mobile phase, and their retention will decrease (Lindsay, 1992).

The requirement that the solvent be miscible with water significantly limits the number of organic modifiers available for reversed-phase liquid chromatography (Lough and Wainer, 1996). The choice of organic solvent used is usually methanol, or acetonitrile mixed with water or buffer solution (Snyder *et al.,* 1998). Benzodiazepines were soluble in methanol differential in each solubility property and exhibit only weakly basic functions that made it eluted in different times (Yokchue, 2004). In addition, the main driving force for retention in reversed-phase liquid chromatography is the hydrophobic effect. These hydrophobic interactions are modulated by specific solute-solvent interactions in the mobile phase and the stationary phase. A monolayer of methanol will adsorb onto the surface of the stationary phase if the concentration of methanol in the mobile phase is greater than 10%. Acetonitrile has low viscosity and it was a strong solvent than methanol (Yokchue, 2004). For such stronger modifiers the surface of the stationary phase will be saturated at mobile phase concentrations of less than 10% (Lough and Wainer, 1996). The different properties between methanol and acetonitrile may differently affect to benzodiazepines and cause them to separate in column.

Besides, if a certain binary solvents do not provide sufficient separation, the mixing of solvent can be used to solve this problem. If we wish to

choose a mobile phase consisting of three solvents, then we want to choose solvents having selectivity differences that are as large as possible, so that we can fully exploit these differences by varying the mobile phase composition. In practice, many HPLC separations can be carried out using simple binary mixtures as mobile phases. However, the use of ternary and quarternary solvent mixtures as mobile phases may give more sufficient separation. For a reverse phase separation we might choose methanol, acetonitrile, with a third solvent (water) added to adjust the polarity to the required range (Lindsay, 1992).

It has been long regarded as a research technique for separating certain kinds of samples which cannot be easily handled by isocratic method (Snyder *et al.,* 1998). If a satisfactory separation cannot be achieved using a binary, a ternary, or a quaternary solvent mixture as mobile phase (Lindsay, 1992), another strong incentive is that isocratic runs can often be replaced by a gradient separation. When the strength of mobile phase must be changed to increase or decrease the solvent strength during the analysis, the gradient elution method is an alternative method can be considered to solve the problem of isocratic elution (Papadoyannis, 1990).

Actually in gradient elution, the composition of the mobile phase changes during a separation, the strength of the mobile phase can be decreased or increased during the separation (Dolan and Snyder, 1989). Gradient elution techniques are divided into two categories for HPLC:

1) Those techniques in which the solvents are mixed at atmospheric pressure and delivered through the column.
2) Those techniques in which the solvents are mixed at high pressure and then delivered through the column.

In the first category, the solvents are mixed at atmospheric pressure and then pressurized by a single high-pressure pump (Papadoyannis, 1990). The best pump for this reason is the reciprocating pump which is the pump used in this study.

Actually, gradient elution can be easier to understand and use than isocratic elution. In any case, once a good understanding of isocratic separation is developed, a corresponding understanding of gradient elution is easily attained. The design of gradient procedures is similar to that for isocratic separations because these

two elution techniques are based on the same fundamental processes of HPLC retention and separation.

Therefore, in this experiment, the individual retention of 6 benzodiazepines were conducted from various analysis conditions originated by adjusting the proportion of 3 mobile phase; methanol, phosphate buffer solution (3.3 mM, pH 2.1), and acetonitrile in both of isocratic and gradient elution modes.

3.1.1 Isocratic elution modes

- First, 4 different conditions from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) demonstrate the results of individual experimental retention time of 6 drugs tabulated in Table 12.

Table 12. Experimental retention times of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Methanol	25	30	35	40
%Buffer	75	70	65	60
Benzodiazepines	Retention times (minutes)			
Nitrazepam	11.04	10.19	9.21	8.34
Diazepam	14.27	11.96	10.32	9.00
Lorazepam	20.68	14.72	10.89	8.18
Flunitrazepam	23.58	18.11	13.48	10.45
Midazolam	26.03	19.26	14.40	11.74
Triazolam	30.19	19.82	13.24	9.34

From the data obtained, the elution behavior of 6 drugs using varied methanol in phosphate buffer solution (3.3 mM, pH 2.1) as mobile phase can be determined by plotting graph between the retention time of analysis drugs and condition numbers. The resultant graph is illustrated in Figure 5.

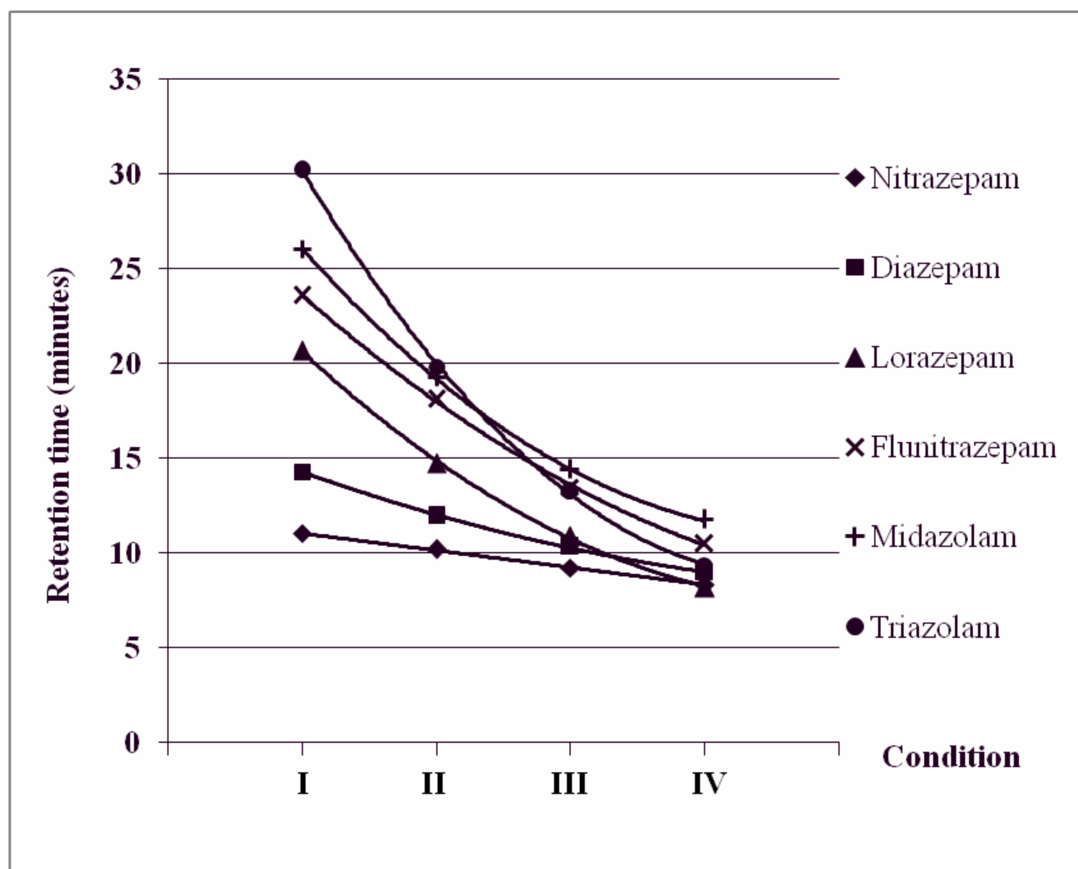


Figure 5. Elution behaviors of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

It was shown that the more proportion of methanol in phosphate buffer solution, the less time analytes retained in column, and the less period of time between each analytes. Moreover, the selectivity of lorazepam and triazolam has changed. In addition, condition II, III, and IV as 30, 35, and 40% of varied methanol in phosphate buffer (3.3 mM, pH 2.1), respectively, was used as mobile phase, 6 drugs cannot be separated. So that, this 3 conditions cannot be used to simultaneously analyze 6 benzodiazepines. On the other hand, 6 drugs can be separated at condition I as 25% methanol in phosphate buffer solution (3.3 mM, pH 2.1) or lower proportion of methanol used. However, condition I is not the good choice to be used because of too much analysis time spent, approximately 30 minutes, per one injection.

- Second, 4 different conditions from the varied proportions of acetonitrile and phosphate buffer solution (3.3 mM, pH 2.1) demonstrate the results of individual experimental retention time of 6 drugs tabulated in Table 13.

Table 13. Experimental retention times of 6 drugs obtained from the varied proportions of acetonirile and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Acetonitrile	15	20	25	30
%Buffer	85	80	75	70
Benzodiazepines	Retention times (minutes)			
Nitrazepam	11.54	9.85	8.66	7.59
Diazepam	14.30	11.92	10.37	9.14
Lorazepam	24.76	16.04	11.10	8.00
Flunitrazepam	25.36	18.47	14.21	10.36
Midazolam	24.96	17.89	13.83	11.30
Triazolam	34.16	20.81	13.63	9.61

From the data obtained, the elution behavior of 6 drugs using varied acetonitrile in phosphate buffer solution (3.3 mM, pH 2.1) as mobile phase can be determined by plotting graph between the retention time of analysis drugs and condition numbers. The resultant graph is shown in Figure 6.

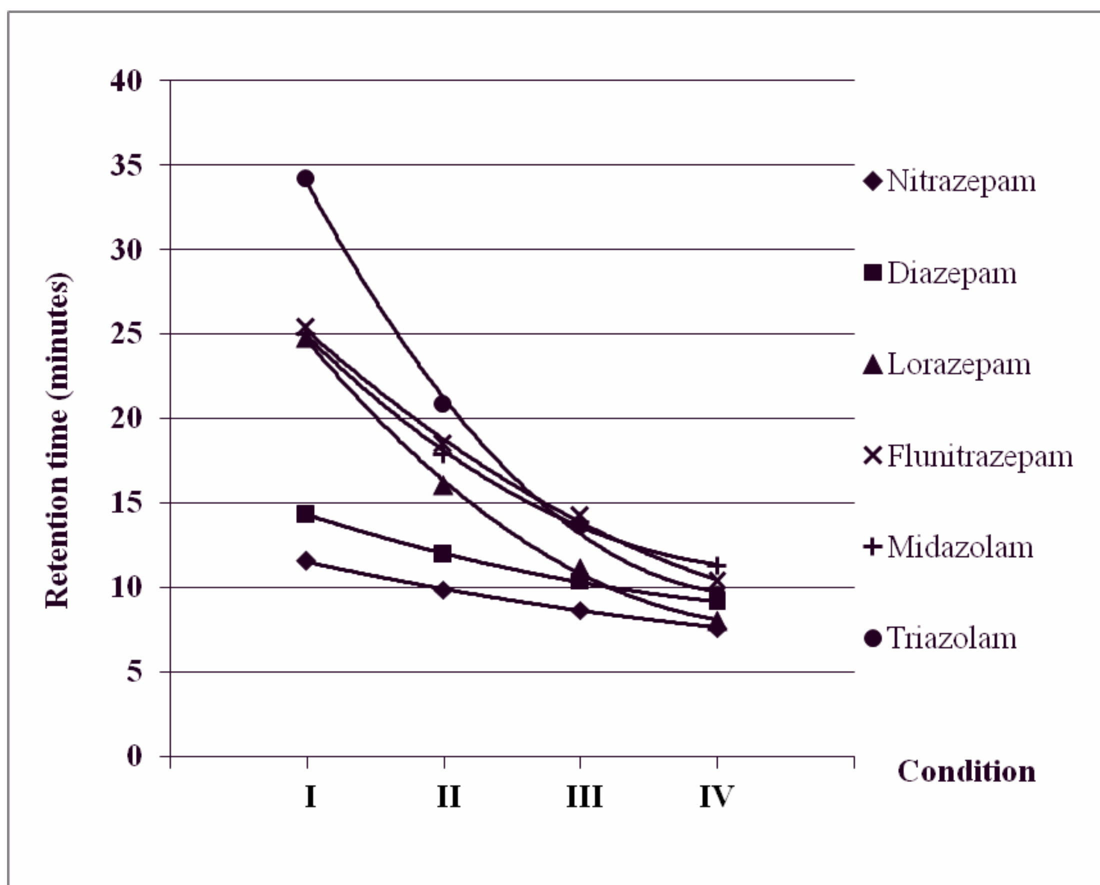


Figure 6. Elution behaviors of 6 drugs obtained from the varied proportions of acetonitrile and phosphate buffer solution (3.3 mM, pH 2.1) in 4 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

General, trend of retentions were the same with single reception. In order to compare methanol with acetonitrile, the more proportion of acetonitrile in phosphate buffer solution (3.3 mM, pH 2.1), also the less time analytes retained in column, and the less resolution between each analytes. Moreover, the selectivity of triazolam and lorazepam has also changed. However, if we compare methanol with acetonitrile at the same proportion such at 25% proportion of both, the analytes tend to retain in column for acetonitrile less than for methanol. Furthermore, for acetonitrile, the less resolution between each analytes can be observed. This can be described that acetonitrile has the solvent strength more than methanol. Although it takes less analysis time when acetonitrile has been used, it should not be used alone

with phosphate buffer solution because there is no condition that drugs can be separate altogether.

In conclusion, the resolution of 6 drugs when using methanol was better than acetonitrile, but it takes more analysis time. On the other hand, acetonitrile gives the opposite effects. The appropriate mixture of both in phosphate buffer solution (3.3 mM, pH 2.1) should be further studied to find out the optimum condition these drugs can be simultaneously separated with a good resolution and a reasonable runtime.

In the next study, 5% acetonitrile was used in all experiments, at which, methanol was varied at more concentration than previous study. The reason is to evaluate if methanol is used as main organic modifier to make 6 drugs separated and acetonitrile is mixed at low concentration to reduce analysis time.

- Third, 3 different conditions from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 5% acetonitrile demonstrate the results of individual experimental retention time of 6 drugs tabulated in Table 14.

Table 14. Experimental retention times of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 5% acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition		
	I	II	III
%Methanol	20	25	30
%Buffer	75	70	65
%Acetonitrile	5	5	5
Benzodiazepines	Retention times (minutes)		
Nitrazepam	10.56	9.78	8.50
Diazepam	12.78	11.34	9.49
Lorazepam	17.94	13.24	9.59
Flunitrazepam	20.34	15.94	11.95
Midazolam	21.48	16.71	13.18
Triazolam	24.48	16.94	12.82

From the data obtained, the elution behavior of 6 drugs using varied methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 5% acetonitrile as mobile phase can be determined by plotting graph between the retention time of analysis drugs and condition numbers. The resultant graph is shown in Figure 7.

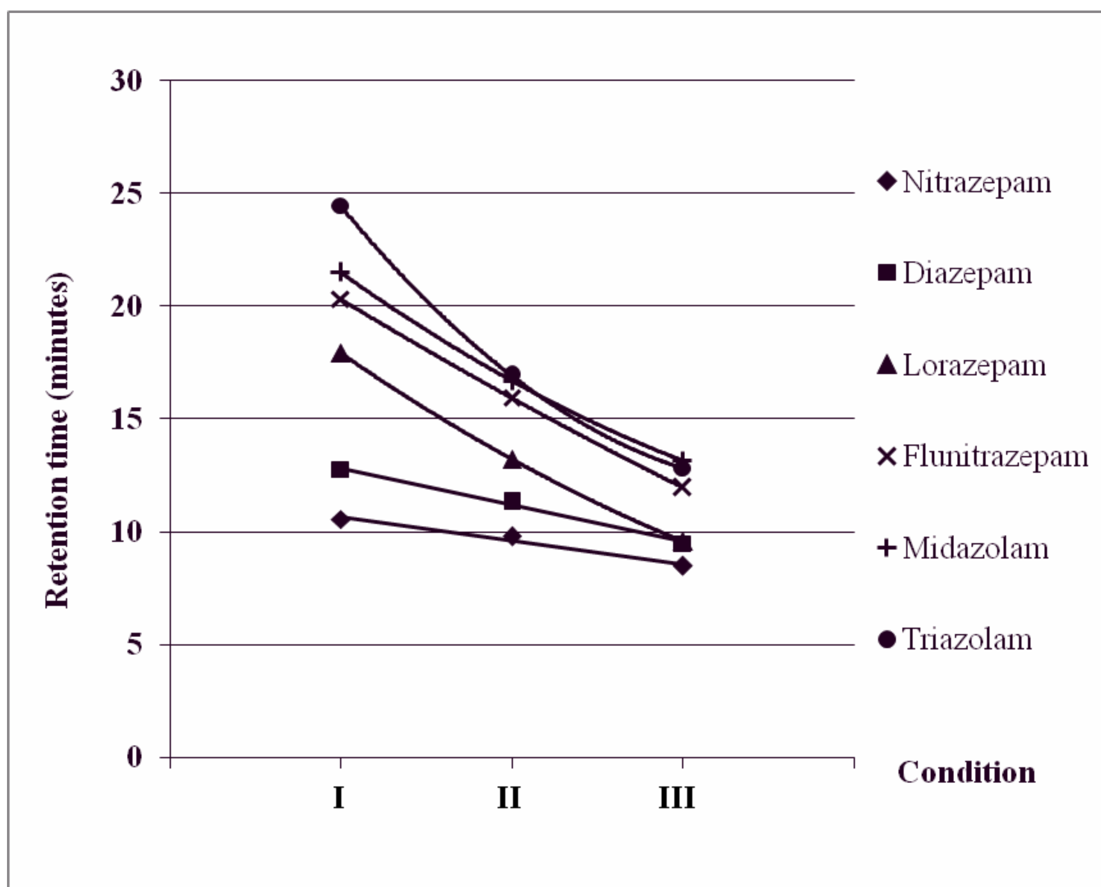


Figure 7. Elution behaviors of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 5% acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

From the study, it can be concluded that the assumption is true. When methanol was mixed with 5% acetonitrile in phosphate buffer solution (3.3 mM, pH 2.1), the last drugs, triazolam, has less time retained in column, but there are 2 groups of drugs, the first group are nitrazepam, diazepam, and lorazepam, and the second are flunitrazepam, midazolam, and triazolam have poor resolution. For example, if we compare the second condition (25 : 70 : 5 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) of this experiment with the condition II of 30% methanol (Figure 5), the analysis time has been reduced from about 20 minutes to 17 minutes, while these 2 groups of drugs have poor resolution. Therefore, to improve resolution and decrease runtime, the proportion of methanol and acetonitrile should be reduced.

If we consider condition II (25 : 70 : 5 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) with condition III (30 : 65 : 5 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile), it takes only 10 to 15 minutes to analyze 6 drugs but poor resolution was observed. At condition I (20 : 75 : 5 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) seems to give the most satisfied results, but there are 2 pairs of benzodiazepines that cannot be separated from each other, the first is nitrazepam and diazepam, and the second is flunitrazepam and midazolam.

To compromise the fast analysis and high resolution for the separation of these 6 drugs, both of methanol and acetonitrile should be decreased. Therefore, the next experiment, 3% acetonitrile was used. From the hypothesis that 6 drugs would be separated in a reasonable runtime if methanol and acetonitrile were decreased, the researcher expected to obtain the optimum condition for 6 drugs analysis in the next study.

- Fourth, 4 different conditions from the varied proportions of methanol and phosphate buffer solution in 3% acetonitrile demonstrate the results of individual experimental retention time of 6 drugs tabulated in Table 15.

Table 15. Experimental retention times of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 3% acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Mobile phase	Condition			
	I	II	III	IV
%Methanol	22	23	24	25
%Buffer	75	74	73	72
%Acetonitrile	3	3	3	3
Benzodiazepines	Retention times (minutes)			
Nitrazepam	10.94	10.77	10.50	10.34
Diazepam	13.89	13.58	13.04	12.47
Lorazepam	19.69	18.44	17.36	16.14
Flunitrazepam	22.53	21.40	20.24	19.06
Midazolam	25.09	23.60	22.01	20.59
Triazolam	28.42	26.13	24.08	21.91

From the data obtained, the elution behavior of 6 drugs using varied methanol and phosphate buffer solution in 3% acetonitrile as mobile phase can be determined by plotting graph between the retention time of analysis drugs and condition numbers. The resultant graph is shown in Figure 8.

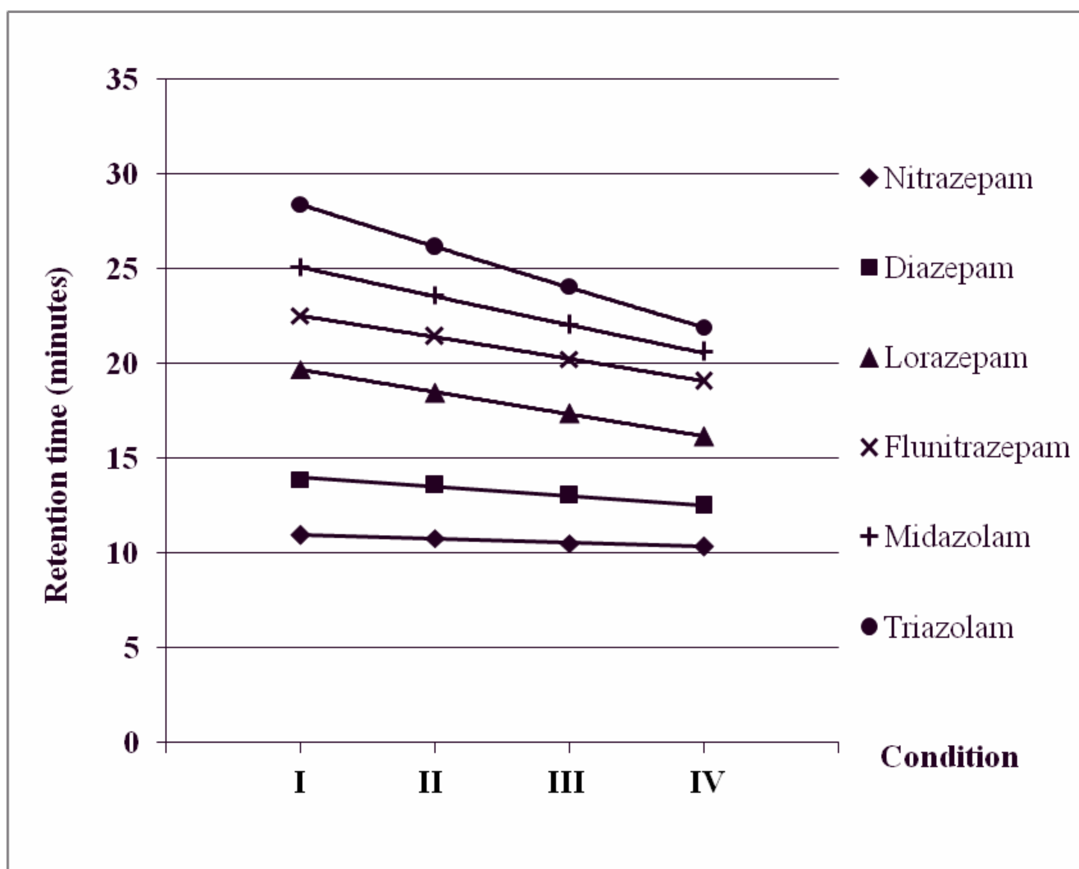


Figure 8. Elution behaviors of 6 drugs obtained from the varied proportions of methanol and phosphate buffer solution (3.3 mM, pH 2.1) in 3% acetonitrile in 3 isocratic conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

From the hypothesis that 6 drugs would be separated in a reasonable runtime if methanol and acetonitrile were decreased, the optimum condition for 6 drugs analysis can be obtained from this study. If we consider condition III (24 : 73 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile), and condition IV (25 : 72 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) 6 drugs cannot be separated. On the other hand, condition I (22 : 75 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) and condition II (23 : 74 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) tend to give the satisfied results. Although 6 drugs can be separated in these 2 conditions, it took more than 25 minutes to analyze 1 sample.

To reduce the time of analysis, the factors affect to retention mechanism of drugs should be considered. The simple way in this study is to increase the solvent strength, which is methanol and acetonitrile, but this could not be done because the previous study indicated to decrease the strength of mobile phase.

Thus, the next experiment is a decision of decreasing methanol and acetonitrile during the analysis to decrease the solvent strength of mobile phase. In case of decreasing methanol and acetonitrile during the analysis, the proportion of both should be more than 25% and 3%, respectively, in the start time and then slowly decreased the proportion until end time of 30 minutes. This assumption might be explained that the stronger strength of mobile phase at start time may affects to all 6 compounds to leave the column faster, and when mobile phase strength is decreasing when time pass these compounds which are moving on the way in HPLC system may have sufficient time to provide more spacing of bands and better resolution, or briefly described that gradient elution was used to reduce the run time while all drugs were still separated. If the assumption is correct, we will obtain the condition for separation 6 drugs with less time of analysis. The result of gradient elution modes is shown in section 3.1.2.

3.1.2 Gradient elution modes

At last, 5 linear gradient elution conditions from the varied proportions of methanol, acetonitrile, and phosphate buffer solution (3.3 mM, pH 2.1) in 30 minutes demonstrate the results of individual experimental retention time of 6 drugs tabulated in Table 16.

Table 16. Retention times of 6 drugs in 5 linear gradient conditions, Lichrospher CN column (250 mm × 4 mm, 5 μm), flow-rate 1.0 ml/min, 20 μl injection volume.

	Condition									
	I		II		III		IV		V	
	Time (mins)		Time (mins)		Time (mins)		Time (mins)		Time (mins)	
Mobile phase	0	30	0	30	0	30	0	30	0	30
%Methanol	26	20	27	20	28	20	29	20	30	20
%Buffer	70	80	69	80	68	80	67	80	66	80
%Acetonitrile	4	0	4	0	4	0	4	0	4	0
Benzodiazepines	Retention times (minutes)									
Nitrazepam	9.98		9.86		9.60		9.57		9.44	
Diazepam	12.09		11.93		11.48		11.34		11.08	
Lorazepam	15.83		15.30		13.67		13.60		12.83	
Flunitrazepam	19.26		18.62		17.14		17.00		16.29	
Midazolam	21.38		20.63		18.75		18.63		17.94	
Triazolam	23.64		22.42		19.36		19.23		17.94	

From the data obtained, the elution behavior of 6 drugs in 5 linear gradient elution conditions from the varied proportions of methanol, acetonitrile, and phosphate buffer solution in 30 minutes can be determined by plotting graph between the retention time of analysis drugs and condition numbers. The resultant graph is shown in Figure 9.

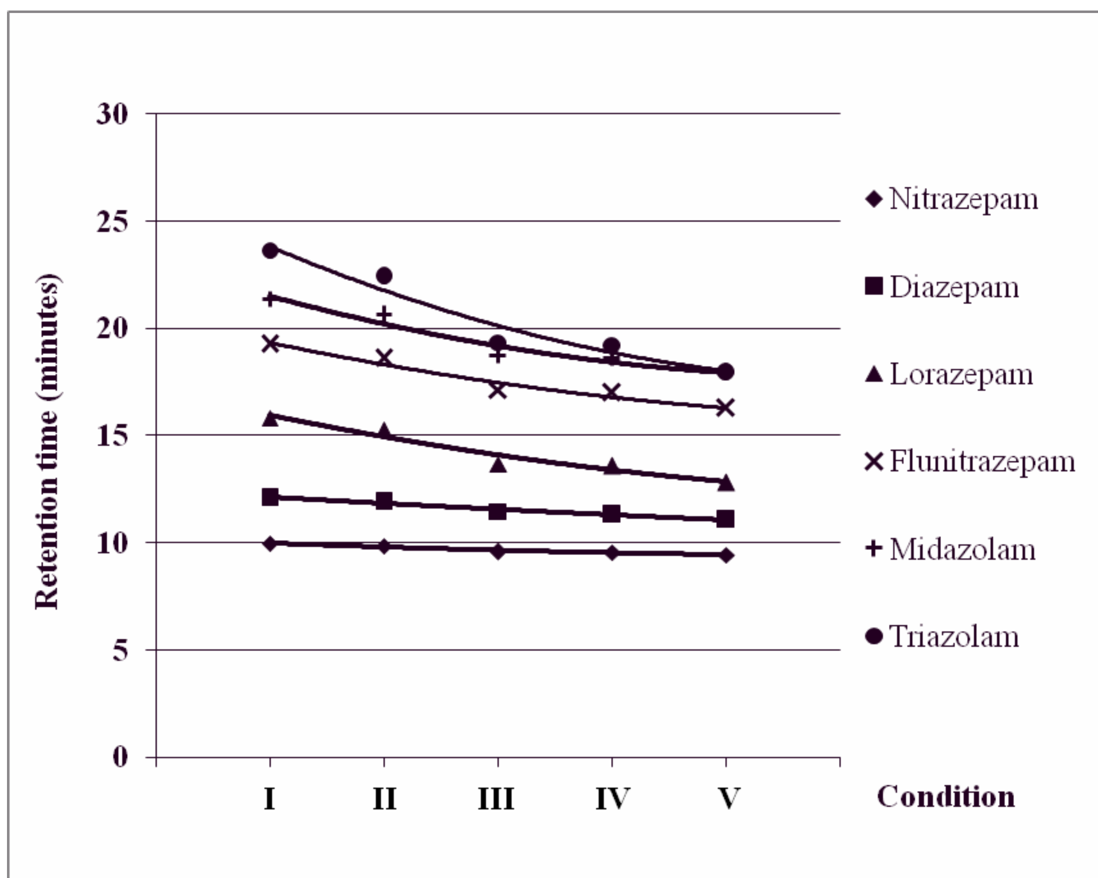


Figure 9. The elution behaviors of 6 drugs in 5 linear gradient conditions, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

Although 6 benzodiazepines can be separated well by condition I (22 : 75 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) and condition II (23 : 74 : 3 of methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile) of isocratic elution modes from Figure 8, the sample also contains late-eluting compounds that can lengthen run time.

From this fact, gradient elution experiment was designed base on the results obtained from isocratic elution experiment. In addition, the results proved the assumption that there are the effects of stronger strength of mobile phase to 6 compounds at start time, and when mobile phase strength is decreasing when time pass these compounds which are moving on the way in HPLC system have sufficient time to provide more spacing of bands and better resolution. Therefore, the optimum condition for 6 drugs analysis could be obtained.

In details, all 5 gradient elution conditions were considered. The condition III (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 28 : 68 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes), condition IV (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 29 : 67 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes), and condition V (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 30 : 66 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes), triazolam and midazolam cannot be separated. Next, the other 2 conditions left; condition I (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 26 : 70 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes) and condition II (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 27 : 69 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes), all component can be well separated. Comparatively, the latter condition (methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 27 : 69 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes) seems to spend less run time while gives the best resolution.

In conclusion, the determination of 6 benzodiazepines elution behavior has been investigated. Its results can be used as based data to approach the optimum condition for 6 benzodiazepines analysis. Among the optimization, it can be concluded that the gradient condition II using methanol : phosphate buffer (3.3 mM, pH 2.1) : acetonitrile = 27 : 69 : 4 at 0 minute to 20 : 80 : 0 at 30 minutes is the optimum condition for analysis of 6 benzodiazepines.

For further analysis, we must apply the optimum condition to forensic samples. Previously, the optimum condition was obtained from the elution behavior of 6 drugs individually determined. To prove that the method can be used to detect mixed drugs in samples when perform an analysis, the standard working mixture contains 6 drugs was prepared and applied to HPLC system. The Chromatogram obtained from the injection in Figure 10 was shown that the optimum condition studied is a new method developed for the analysis of 6 benzodiazepines in both single and mixture samples.

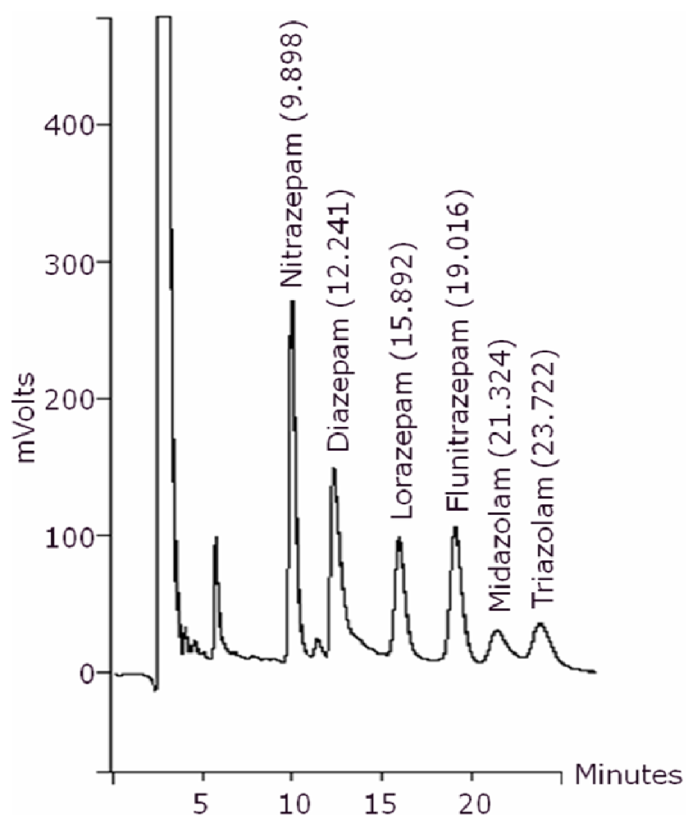


Figure 10. Chromatogram for the separation of 6 drugs by HPLC optimum condition in linear gradient; methanol : phosphate buffer solution (3.3 mM, pH 2.1) : acetonitrile at 0 minute = 27 : 69 : 4, and 30 minutes: = 20 : 80 : 0, Lichrospher CN column (250 mm × 4 mm, 5 µm), flow-rate 1.0 ml/min, 20 µl injection volume.

3.2 Determination of the efficiency of benzodiazepines extraction from human serum.

The analysis of drugs in biological fluids, particularly plasma or serum is one of the most common uses of HPLC. Proteins and fats present in blood, plasma, or serum samples are known to have a deleterious effect on HPLC columns. The minimum pretreatment for such sample is to denature and precipitate the proteins present. This can be achieved by a variety of methods such as heating or treatment with acids, bases, water-miscible organic solvents such as methanol or acetonitrile or by the addition of chaotropic reagents. These methods will cause the proteins to precipitate such that the supernatant can be removed after centrifugation. The supernatant after centrifugation is thus protein-free (Lough and Wainer, 1996).

Actually, protein precipitation methods are simple and rapid. Moreover, acetonitrile is the organic solvents used in protein precipitation method which yields a protein precipitate that can be readily centrifuged into a small pellet (Sadee and Beelen, 1942). Therefore, sample preparation of human serum in this study was carried out using protein precipitation method by the treatment with ammonia solution and acetonitrile.

Of the extraction recovery study, the results were divided by the kinds of 6 drugs which are shown in section 3.2.1.

3.2.1 Extraction recovery study of nitrazepam

3.2.1.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for nitrazepam prepared at 3 standard concentrations; low, average, and high, were evaluated for 3 replicates which are tabulated in Table 17. The standard curve of nitrazepam obtained by plotting the peak area (mV.sec) against the concentration of the nitrazepam (μg/ml) is shown in Figure 11.

Table 17. The responses in the form of peak area (mV.sec) for 3 concentrations of nitrazepam standard curve (n=3).

Concentration (µg/ml)		0.20 (low)	0.60 (average)	1.00 (high)
Response (mV.sec)	1	787	1897	3069
	2	738	2012	2905
	3	765	1907	3156
	Mean	763	1939	2987
	%RSD	3.22	3.29	3.88

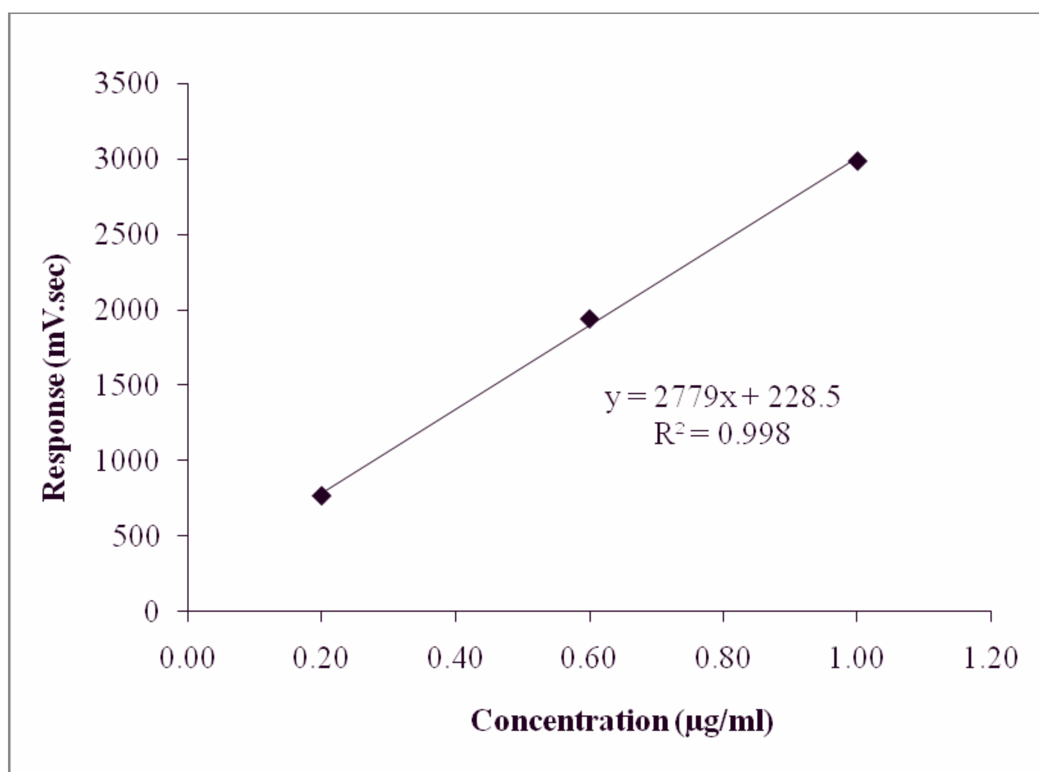


Figure 11. The standard curve of nitrazepam obtained by plotting the peak area (mV.sec) against the concentration of the nitrazepam (µg/ml).

The equation from the standard curve of nitrazepam is y=2779x+228.5 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.1.2 Extraction recovery in low concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for nitrazepam spiked serum prepared at low concentration (0.20 µg/ml) and evaluated for 3 replicates are tabulated in Table 18.

Table 18. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for nitrazepam spiked serum prepared at low concentration (0.20 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	698	0.169
2	706	0.172
3	674	0.160
Mean		0.167
%RSD		3.59
%Recovery		83.5

From the results, recovery yields of nitrazepam spiked serum prepared at low concentration (0.20 µg/ml) was 83.5%, and percent relative standard deviation (%RSD) was 3.59%.

3.2.1.3 Extraction recovery in average concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for nitrazepam spiked serum prepared at average concentration (0.60 µg/ml) and evaluated for 3 replicates are tabulated in Table 19.

Table 19. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for nitrazepam spiked serum prepared at average concentration (0.60 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	1710	0.533
2	1634	0.506
3	1607	0.496
Mean		0.512
%RSD		3.76
%Recovery		85.3

From the results, recovery yields of nitrazepam spiked serum prepared at average concentration (0.60 µg/ml) was 85.3% and percent relative standard deviation (%RSD) was 3.76%.

3.2.1.4 Extraction recovery in high concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for nitrazepam spiked serum prepared at high concentration (1.00 µg/ml) and evaluated for 3 replicates are tabulated in Table 20.

Table 20. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), the correlation coefficient, and %recovery for nitrazepam spiked serum prepared at high concentration (1.00 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	2804	0.927
2	2606	0.856
3	2599	0.853
Mean		0.88
%RSD		4.77
%Recovery		87.8

From the results, recovery yields of nitrazepam spiked serum prepared at high concentration (1.00 µg/ml) was 87.8% and percent relative standard deviation (%RSD) was 4.77%.

Recovery yields of nitrazepam spiked serum prepared at 3 concentrations (low, average, and high concentrations) were higher than 75% and percent relative standard deviation (%RSD) was less than 5%.

3.2.2 Extraction recovery of diazepam

3.2.2.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for diazepam prepared at 3 standard concentrations; low, average, and high, and evaluated for 3 replicates are tabulated in Table 21. The standard curve of diazepam obtained by plotting the peak area (mV.sec) against the concentration (µg/ml) of the diazepam is shown in Figure 12.

Table 21. The response in the form of peak area (mV.sec) for 3 concentration of diazepam standard curve (n=3).

Concentration (μg/ml)		0.20 (low)	0.60 (average)	1.00 (high)
Response (mV.sec)	1	787	1897	3069
	2	738	2012	2905
	3	765	1907	3156
	Mean	398	1152	1798
	%RSD	4.86	3.74	2.12

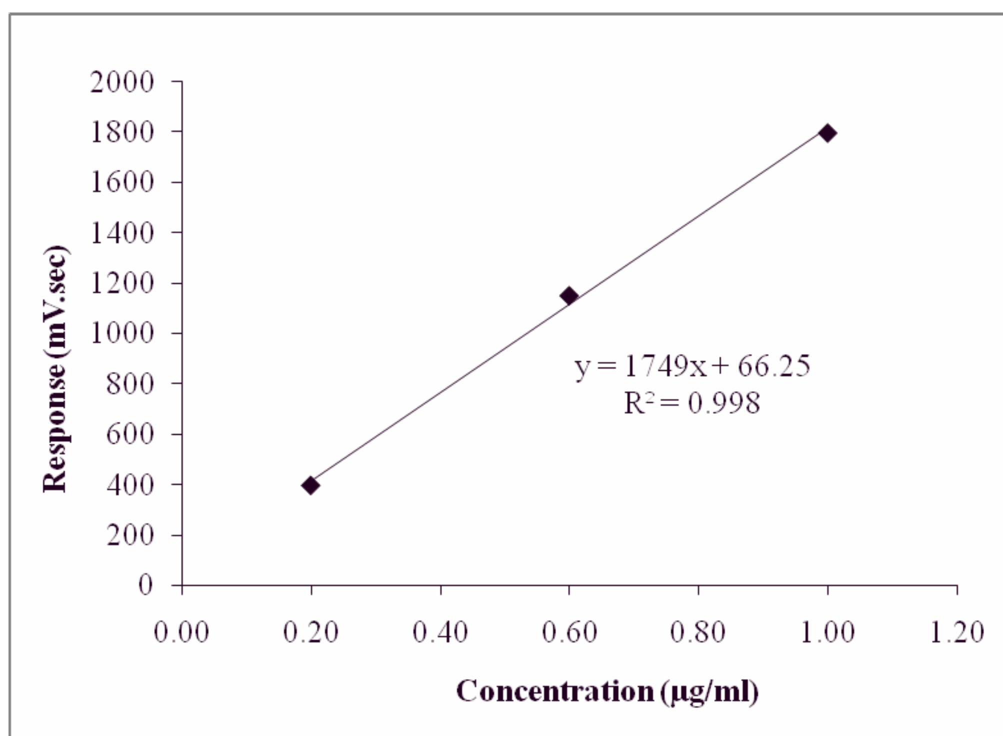


Figure 12. The standard curve of diazepam obtained by plotting the peak area (mV.sec) against the concentration of the diazepam.

The equation from the standard curve of diazepam is y=1749x+66.25 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.2.2 Extraction recovery in low concentration

The responses in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard

deviation (%RSD), and recovery yields for diazepam spiked serum prepared at low concentration (0.20 µg/ml) and evaluated for 3 replicates are tabulated in Table 22.

Table 22. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for diazepam spiked serum prepared at low concentration (0.20 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	363	0.170
2	348	0.161
3	342	0.158
Mean		0.163
%RSD		3.80
%Recovery		81.4

From the results, recovery yields of diazepam spiked serum prepared at low concentration (0.20 µg/ml) was 81.4%, and percent relative standard deviation (%RSD) was 3.80%.

3.2.2.3 Extraction recovery in average concentration

The responses in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for diazepam spiked serum prepared at average concentration (0.60 µg/ml) and evaluated for 3 replicates are tabulated in Table 23.

Table 23. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for diazepam spiked serum prepared at average concentration (0.60 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	930	0.494
2	957	0.509
3	887	0.469
Mean		0.491
%RSD		4.11
%Recovery		81.8

From the results, recovery yields of diazepam spiked serum prepared at average concentration (0.60 μg/ml) was 81.8%, and percent relative standard deviation (%RSD) was 4.11%.

3.2.2.4 Extraction recovery in high concentration

The responses in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for diazepam spiked serum prepared at high concentration (1.00 μg/ml) and evaluated for 3 replicates are tabulated in Table 24.

Table 24. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for diazepam spiked serum prepared at high concentration (1.00 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	1496	0.817
2	1555	0.851
3	1497	0.818
Mean		0.829
%RSD		2.33
%Recovery		82.9

From the results, recovery yields of diazepam spiked serum prepared at high concentration (1.00 µg/ml) was 82.9%, and percent relative standard deviation (%RSD) was 2.33%.

Recovery yields of diazepam spiked serum prepared at 3 concentrations (low, average, and high concentrations) were higher than 75% and percent relative standard deviation (%RSD) was less than 5%.

3.2.3 Extraction recovery of lorazepam

3.2.3.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for lorazepam prepared at 3 standard concentrations; low, average, and high, and evaluated for 3 replicates are tabulated in Table 25. The standard curve of lorazepam obtained by plotting the peak area (mV.sce) against the concentration of the lorazepam is shown in Figure 13.

Table 25. The response in the form of peak area (mV.sec) for 3 concentration of lorazepam standard curve (n=3).

Concentration (µg/ml)		0.20 (low)	0.60 (Average)	1.00 (High)
Response (mV.sec)	1	186	692	1194
	2	198	711	1257
	3	192	647	1166
	Mean	195	683	1206
	%RSD	2.18	4.83	3.87

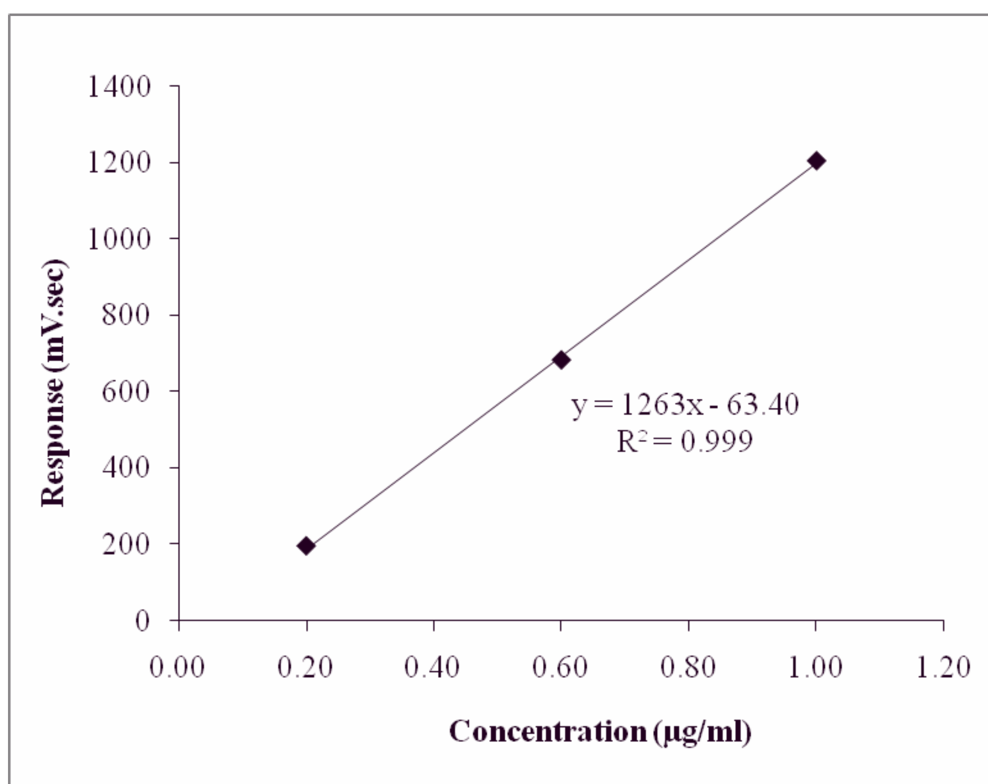


Figure 13. The standard curve of lorazepam obtained by plotting the peak area (mV.sec) against the concentration of the lorazepam.

The equation from the standard curve of lorazepam is y=1263x-63.40 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.3.2 Extraction recovery in low concentration

The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at low concentration (0.20 μg/ml) and evaluated for 3 replicates are tabulated in Table 26.

Table 26. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at low concentration (0.20 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	158	0.175
2	164	0.180
3	154	0.172
Mean		0.176
%RSD		2.29
%Recovery		87.9

From the results, recovery yields of lorazepam spiked serum prepared at low concentration (0.20 μg/ml) was 87.9%, and percent relative standard deviation (%RSD) was 2.29%.

3.2.3.3 Extraction recovery in average concentration

The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at average concentration (0.60 μg/ml) and evaluated for 3 replicates are tabulated in Table 27.

Table 27. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at average concentration (0.60 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	629	0.548
2	604	0.528
3	583	0.512
Mean		0.529
%RSD		3.44
%Recovery		88.3

From the results, recovery yields of lorazepam spiked serum prepared at average concentration (0.60 μg/ml) was 88.3%, and percent relative standard deviation (%RSD) was 3.44%.

3.2.3.4 Extraction recovery in high concentration

The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at high concentration (1.00 μg/ml) and evaluated for 3 replicates are tabulated in Table 28.

Table 28. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for lorazepam spiked serum prepared at high concentration (1.00 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	1089	0.912
2	1130	0.945
3	1096	0.918
Mean		0.925
%RSD		1.88
%Recovery		92.5

From the results, recovery yields of lorazepam spiked serum prepared at high concentration (1.00 μg/ml) was 92.5%, and percent relative standard deviation (%RSD) was 1.88%.

Recovery yields of lorazepam spiked serum prepared at 3 concentrations (low, average, and high concentrations) were higher than 75% and percent relative standard deviation (%RSD) was less than 5%.

3.2.4 Extraction recovery of flunitrazepam

3.2.4.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for flunitrazepam prepared at 3 standard concentrations; low, average, and high, and evaluated for 3 replicates are tabulated in Table 29. The standard curve of flunitrazepam obtained by plotting the peak area (mV.sec) against the concentration of the flunitrazepam is shown in Figure 14.

Table 29. The response in the form of peak area (mV.sec) for 3 concentration of flunitrazepam standard curve (n=3).

Concentration (µg/ml)		0.40 (low)	0.80 (Average)	1.20 (High)
Response (mV.sec)	1	967	1516	2330
	2	932	1601	2116
	3	973	1561	2197
	Mean	953	1559	2214
	%RSD	3.05	2.73	4.88

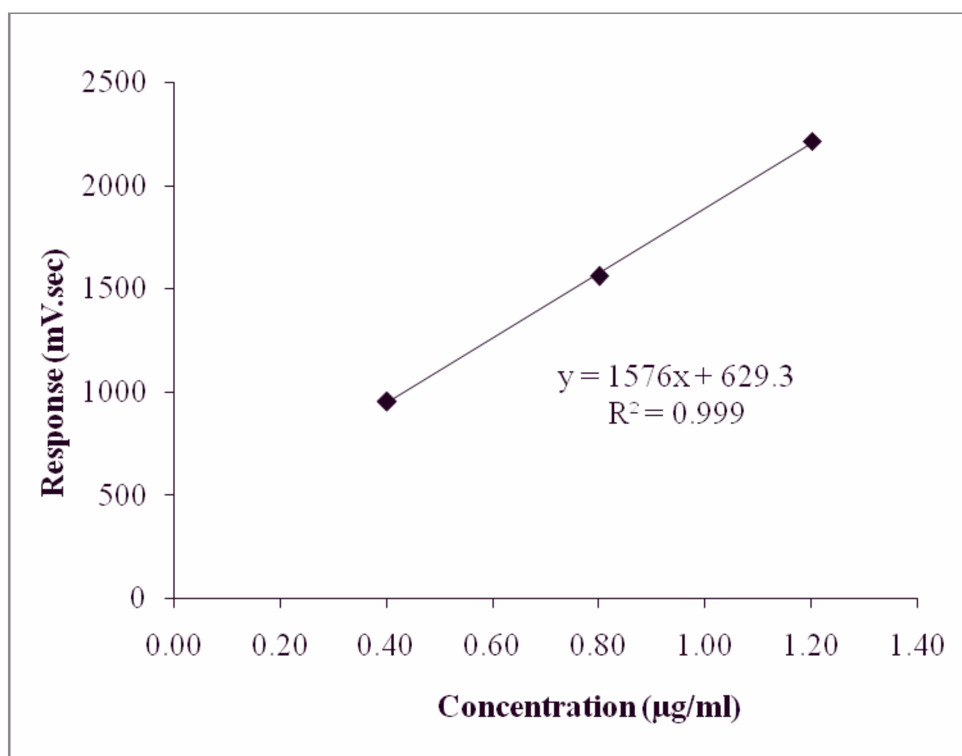


Figure 14. The standard curve of flunitrazepam obtained by plotting the peak area (mV.sec) against the concentration of the flunitrazepam

The equation from the standard curve of flunitrazepam is y=1576x+629.3 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.4.2 Extraction recovery in low concentration

The responses in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard

deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at low concentration (0.40 µg/ml) and evaluated for 3 replicates are tabulated in Table 30.

Table 30. The responses in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at low concentration (0.40 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	795	0.305
2	814	0.317
3	824	0.324
Mean		0.315
%RSD		2.96
%Recovery		78.8

From the results, recovery yields of flunitrazepam spiked serum prepared at low concentration (0.40 µg/ml) was 78.8%, and percent relative standard deviation (%RSD) was 2.96%.

3.2.4.3 Extraction recovery in average concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at average concentration (0.80 µg/ml) and evaluated for 3 replicates are tabulated in Table 31.

Table 31. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at average concentration (0.80 µg/ml) (n=3).

Replicates	Response (mV)	Calculated concentration (µg/ml)
1	1352	0.659
2	1398	0.688
3	1364	0.666
Mean		0.671
%RSD		2.26
%Recovery		83.9

From the results, recovery yields of flunitrazepam spiked serum prepared at average concentration (0.80 µg/ml) was 83.9%, and percent relative standard deviation (%RSD) was 2.26%.

3.2.4.4 Extraction recovery in high concentration

The responses in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at high concentration (1.20 µg/ml) and evaluated for 3 replicates are tabulated in Table 32.

Table 32. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for flunitrazepam spiked serum prepared at high concentration (1.20 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	2001	1.070
2	1910	1.013
3	1973	1.053
Mean		1.045
%RSD		2.83
%Recovery		87.1

From the results, recovery yields of flunitrazepam spiked serum prepared at high concentration (1.20 µg/ml) was 87.1%, and percent relative standard deviation (%RSD) was 2.83%.

Percent recovery yields of flunitrazepam spiked serum prepared at 3 concentrations (low, average, and high concentrations were higher than 75% and percent relative standard deviation (%RSD) was less than 5%.

3.2.5 Extraction recovery of midazolam

3.2.5.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for midazolam prepared at 3 standard concentrations; low, average, and high, and evaluated for 3 replicates are tabulated in Table 33. The standard curve of midazolam obtained by plotting the peak area (mV.sec) against the concentration of the midazolam is shown in Figure 15.

Table 33. The response in the form of peak area (mV.sec) for 3 concentration of midazolam standard curve (n=3).

Concentration (µg/ml)		0.80 (Low)	1.20 (Average)	1.60 (High)
Response (mV.sec)	1	331	471	642
	2	300	458	621
	3	314	470	650
	Mean	307	466	638
	%RSD	3.24	1.51	2.35

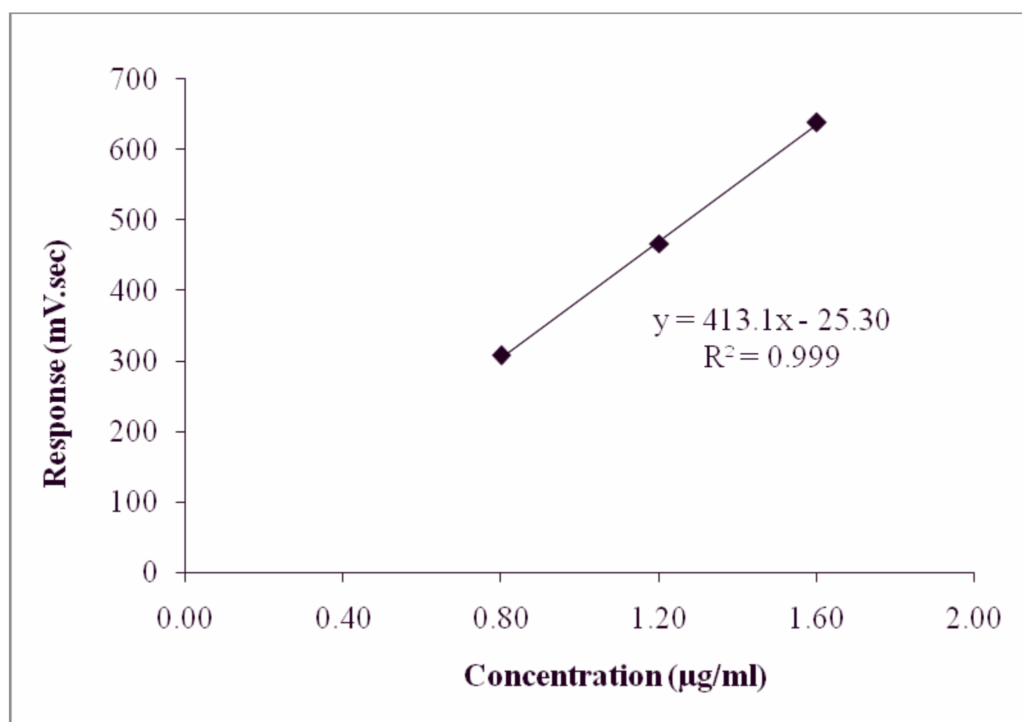


Figure 15. The standard curve of midazolam obtained by plotting the peak area (mV.sec) against the concentration of the midazolam

The equation from the standard curve of midazolam is y=413.1x–25.30 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.5.2 Extraction recovery in low concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard

deviation (%RSD), and recovery yields for midazolam spiked serum prepared at low concentration (0.80 µg/ml) and evaluated for 3 replicates are tabulated in Table 34.

Table 34. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for midazolam spiked serum prepared at low concentration (0.80 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	236	0.633
2	251	0.669
3	231	0.620
Mean		0.641
%RSD		3.93
%Recovery		80.1

From the results, recovery yields of midazolam spiked serum prepared at low concentration (0.80 µg/ml) was 80.1%, and percent relative standard deviation (%RSD) was 3.93%.

3.2.5.3 Extraction recovery in average concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for midazolam spiked serum prepared at average concentration (1.20 µg/ml) and evaluated for 3 replicates are tabulated in Table 35.

Table 35. The responses in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for midazolam spiked serum prepared at average concentration (1.20 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	380	0.981
2	368	0.952
3	395	1.017
Mean		0.984
%RSD		3.33
%Recovery		82.0

From the results, recovery yields of midazolam spiked serum prepared at average concentration (1.20 µg/ml) was 82.0%, and percent relative standard deviation (%RSD) was 3.33%.

3.2.5.4 Extraction recovery in high concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for midazolam spiked serum prepared at high concentration (1.60 µg/ml) and evaluated for 3 replicates are tabulated in Table 36.

Table 36. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for midazolam spiked serum prepared at high concentration (1.60 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	528	1.338
2	547	1.385
3	512	1.301
Mean		1.342
%RSD		3.11
%Recovery		83.8

From the results, recovery yields of midazolam spiked serum prepared at high concentration (1.60 μg/ml) was 83.8%, and percent relative standard deviation (%RSD) was 3.11%.

Recovery yields of midazolam spiked serum prepared at 3 concentrations (low, average, and high concentrations) were higher than 75%) and percent relative standard deviation (%RSD) was less than 5%.

3.2.6 Extraction recovery of triazolam

3.2.6.1 Determination of standard curve

The responses in the form of peak area (mV.sec) for triazolam prepared at 3 standard concentrations; low, average, and high, and evaluated for 3 replicates are tabulated in Table 37. The standard curve of triazolam obtained by plotting the peak area (mV.sec) against the concentration of the triazolam is shown in Figure 16.

Table 37. The response in the form of peak area (mV.sec) for 3 concentration of triazolam standard curve (n=3).

Concentration (µg/ml)		0.80 (Low)	1.20 (Average)	1.60 (High)
Response (mV.sec)	1	233	433	655
	2	227	431	627
	3	240	425	613
	Mean	234	430	632
	%RSD	4.06	0.93	3.40

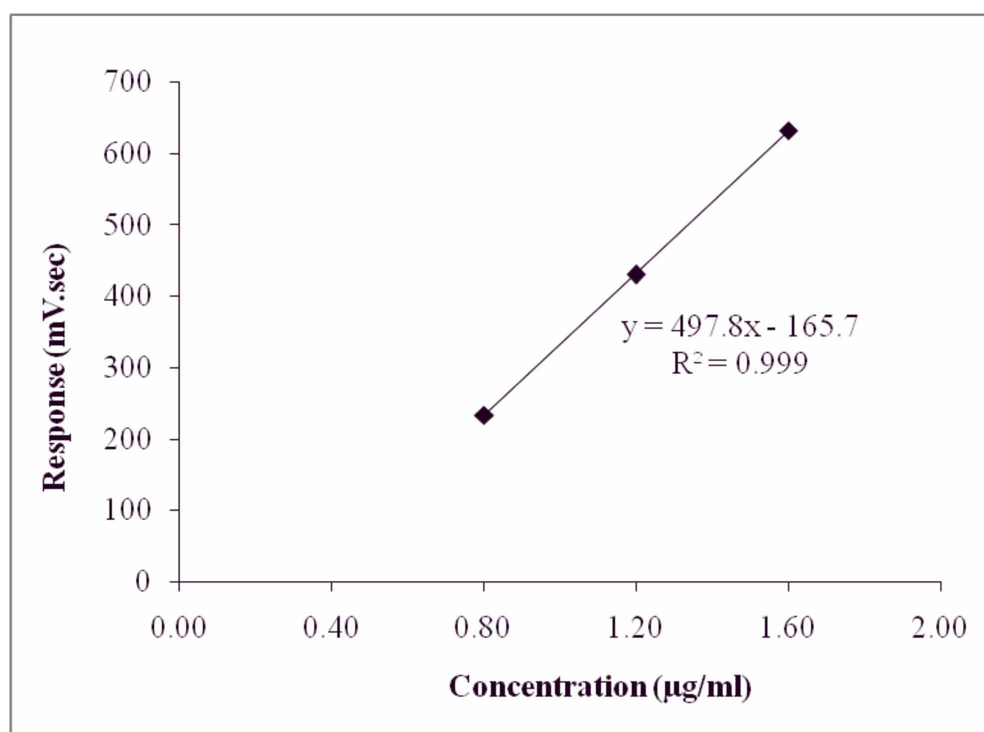


Figure 16. The standard curve of triazolam obtained by plotting the peak area (mV.sec) against the concentration of the triazolam.

The equation from the standard curve of triazolam is y=497.8x–165.7 with a linear correlation coefficient (r^2) greater than 0.990.

3.2.6.2 Extraction recovery in low concentration

The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for triazolam spiked serum prepared at low concentration (0.80 μg/ml) and evaluated for 3 replicates are tabulated in Table 38.

Table 38. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for triazolam spiked serum prepared at low concentration (0.80 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	146	0.626
2	160	0.654
3	158	0.651
Mean		0.644
%RSD		2.39
%Recovery		80.5

From the results, recovery yields of triazolam spiked serum prepared at low concentration (0.80 μg/ml) was 80.5%, and percent relative standard deviation (%RSD) was 2.39%.

3.2.6.3 Extraction recovery in average concentration

The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and recovery yields for triazolam spiked serum prepared at average concentration (1.20 μg/ml) and evaluated for 3 replicates are tabulated in Table 39.

Table 39. The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for triazolam spiked serum prepared at average concentration (1.20 µg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (µg/ml)
1	345	1.026
2	335	1.006
3	317	0.970
Mean		1.000
%RSD		2.85
%Recovery		83.4

From the results, recovery yields of triazolam spiked serum prepared at average concentration (1.20 µg/ml) was 83.4%, and percent relative standard deviation (%RSD) was 2.85%.

3.2.6.4 Extraction recovery in high concentration

The response in the form of peak area (mV.sec), the calculated concentration (µg/ml), the mean concentration (µg/ml), percent relative standard deviation (%RSD), and recovery yields for triazolam spiked serum prepared at high concentration (1.60 µg/ml) and evaluated for 3 replicates are tabulated in Table 40.

Table 40. The response in the form of peak area (mV.sec), the calculated concentration (μg/ml), the mean concentration (μg/ml), percent relative standard deviation (%RSD), and yields for triazolam spiked serum prepared at high concentration (1.60 μg/ml) (n=3).

Replicates	Response (mV.sec)	Calculated concentration (μg/ml)
1	478	1.293
2	526	1.390
3	510	1.357
Mean		1.347
%RSD		3.65
%Recovery		84.2

From the results, recovery yields of triazolam spiked serum prepared at high concentration (1.60 μg/ml) was 84.2%, and percent relative standard deviation (%RSD) was 3.65%.

Recovery yields of triazolam spiked serum prepared at all concentrations (low, average, and high concentrations) were higher than 75% and percent relative standard deviation (%RSD) was less than 5%.

In conclusion, recovery yields and percent relative standard deviation (%RSD) of 6 benzodiazepines spiked serum prepared at low, average, and high concentration are summarized in Table 41.

Table 41. Recovery yields and percent relative standard deviation (%RSD) of 6 drug spiked serum prepared at low, average, and high concentrations.

Benzodiazepines	% Recovery (±%RSD)		
	Low concentration (µg/ml)	Average concentration (µg/ml)	High concentration (µg/ml)
Nitrazepam	83.5±3.59	85.3±3.76	87.8±4.77
Diazepam	81.2±3.80	81.8±4.11	82.9±2.33
Lorazepam	87.9±2.29	88.2±3.44	92.5±1.88
Flunitrazepam	78.8±2.96	83.9±2.26	87.1±2.83
Midazolam	80.1±3.93	82.0±3.33	83.8±3.11
Triazolam	80.5±2.39	83.4±2.85	84.2±3.65

It can be seen that the concentration of benzodiazepines must be differently prepared because the different sensitivity of each benzodiazepine can be detected by the detector. For example, nitrazepam spiked serum prepared at low concentration was 0.20 µg/ml, but triazolam's was 0.80 µg/ml because the detector has less sensitivity for detection triazolam than nitrazepam. Moreover, the higher prepared concentration of benzodiazepines spiked serum, the more percent recovery yields when extraction has been determined. This may be because of the effects of protein binding of drug to protein in serum. When the amounts of benzodiazepines were spiked in serum, some would bind with proteins in serum. After the precipitation has been carried out these drug binding proteins would also precipitated and cannot be determined. From this facts, the more amounts of drugs were spiked in serum, the more free protein-drugs left which could be detected better and yielded higher percent recovery.

In conclusion, percent recovery yields of all 6 benzodiazepines spiked serum prepared at low, average, and high concentrations were more than 75% with percent relative standard deviation (%RSD) less than 5%. Therefore, the protein precipitation method used in sample preparation can be used in routine analysis because of its simple and rapid with recovery yields more than 75%.

3.3 Method validation

3.3.1 Linearity

3.3.1.1 Linearity of nitrazepam

The response in the form of peak area (mV.sec) for each prepared concentration of standard nitrazepam spiked serum is shown in Table 42. The calibration curve of standard nitrazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (µg/ml) of nitrazepam is shown in Figure 17.

Table 42. The response in the form of peak area (mV.sec) for each prepared concentration of standard nitrazepam spiked serum.

Concentration (µg/ml)	Response (mV.sec) ± %RSD
0.20	693 ± 2.40
0.60	1650 ± 3.24
1.00	2670 ± 4.36

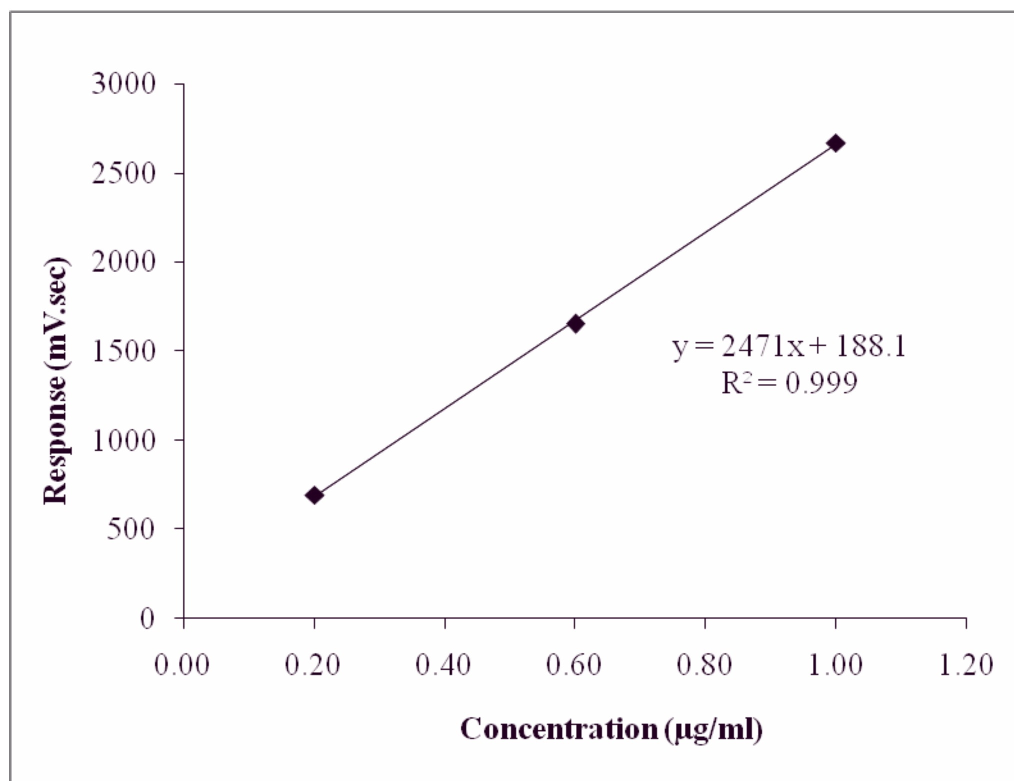


Figure 17. The calibration curve of standard nitrazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration of nitrazepam (µg/ml).

An equation is y = 2471x + 188.1 with a linear correlation coefficient (r^2) 0.999.

3.3.1.2 Linearity of diazepam

The response in the form of peak area (mV.sec) for each prepared concentration of standard diazepam spiked serum is shown in Table 43. The calibration curve of standard diazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration of diazepam is shown in Figure 18.

Table 43. The response in the form of peak area (mV.sec) for each prepared concentration of standard diazepam spiked serum.

Concentration (µg/ml)	Response (mV.sec) ± %RSD
0.20	351 ± 3.08
0.60	925 ± 3.82
1.00	1516 ± 2.23

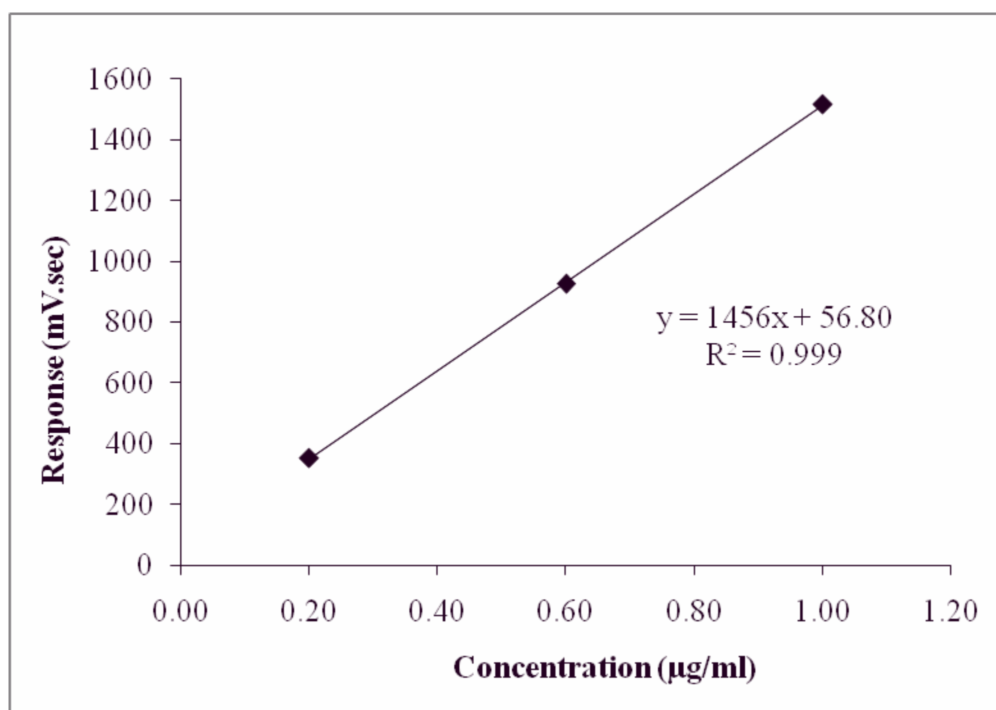


Figure 18. The calibration curve of standard diazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (µg/ml) of diazepam.

An equation is y = 1456x + 56.80 with a linear correlation coefficient (r^2) 0.999.

3.3.1.3 Linearity of lorazepam

The response in the form of peak area (mV.sec) for each prepared concentration of standard lorazepam spiked serum is shown in Table 44. The calibration curve of standard lorazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration of lorazepam is shown in Figure 19.

Table 44. The response in the form of peak area (mV.sec) for each prepared concentration of standard lorazepam spiked serum.

Concentration (μg/ml)	Response (mV.sec) ± %RSD
0.20	159 ± 3.20
0.60	605 ± 3.80
1.00	1105 ± 1.98

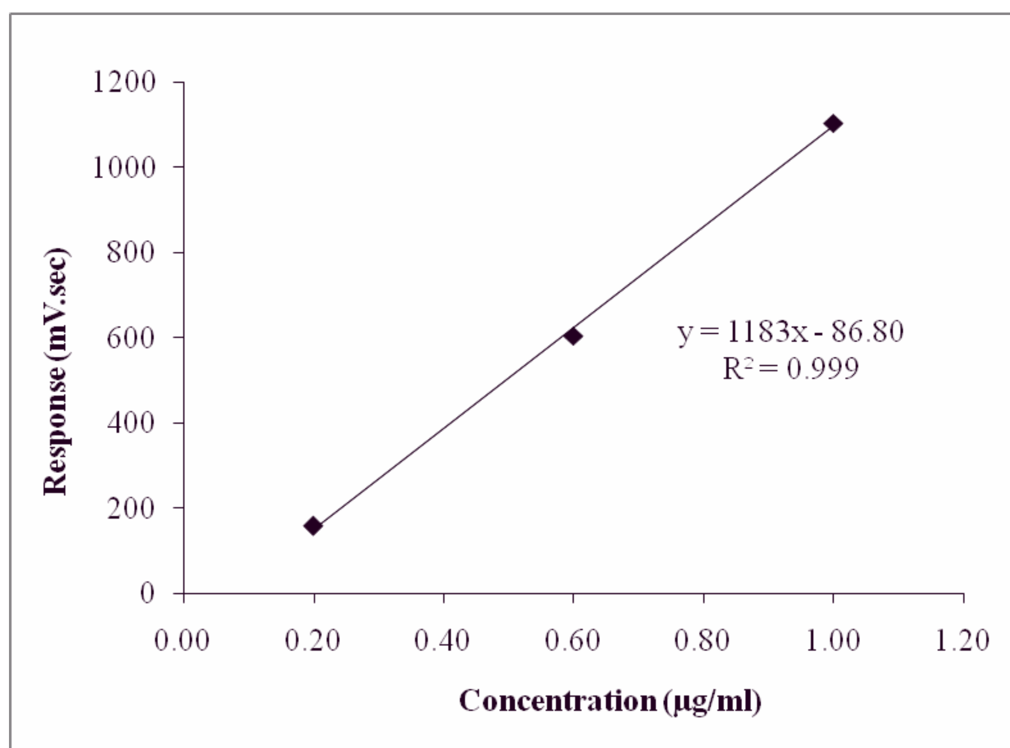


Figure 19. The calibration curve of standard lorazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (μg/ml) of lorazepam.

An equation is y = 1183x – 86.80 with a linear correlation coefficient (r^2) 0.999.

3.3.1.4 Linearity of flunitrazepam

The response in the form of peak area (mV.sec) for each prepared concentration of standard flunitrazepam spiked serum is shown in Table 45. The calibration curve of standard flunitrazepam spiked serum evaluated by plotting the

response in the form of peak area (mV.sec) against the concentration of flunitrazepam is shown in Figure 20.

Table 45. The response in the form of peak area (mV.sec) for each prepared concentration of standard flunitrazepam spiked serum.

Concentration (µg/ml)	Response (mV.sec) ± %RSD
0.40	811 ± 1.82
0.80	1371 ± 1.74
1.20	1961 ± 2.38

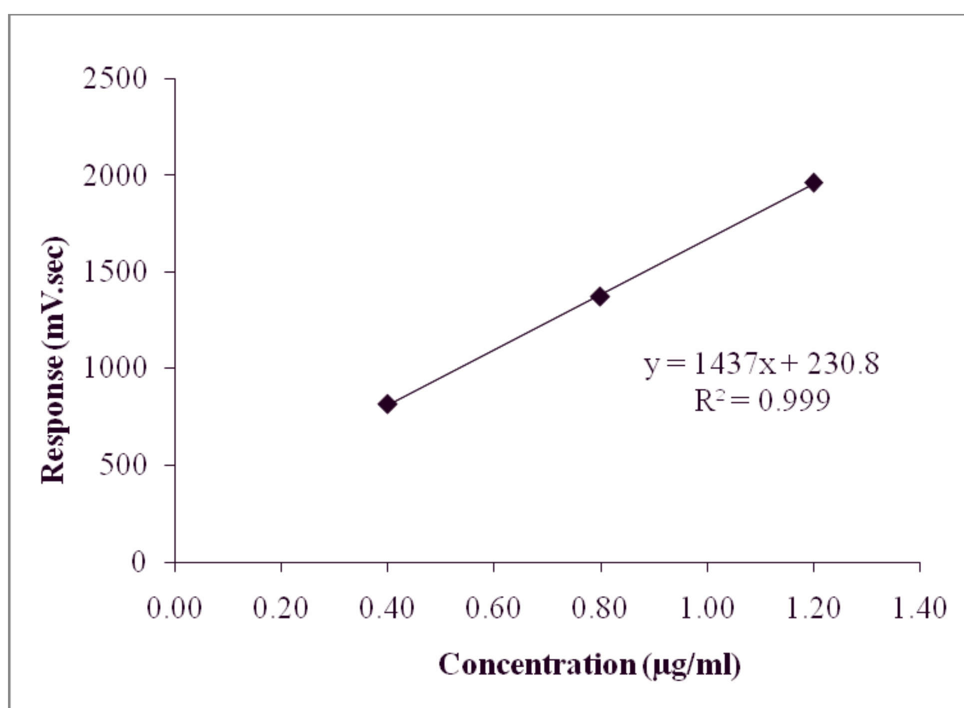


Figure 20. The calibration curve of standard flunitrazepam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (µg/ml) of flunitrazepam.

An equation is y = 1437x + 230.8 with a linear correlation coefficient (r^2) 0.999.

3.3.1.5 Linearity of midazolam

The response in the form of peak area (mV.sec) for each prepared concentration of standard midazolam spiked serum is shown in Table 46. The

calibration curve of standard midazolam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration of midazolam is shown in Figure 21.

Table 46. The response in the form of peak area (mV.sec) for each prepared concentration of standard midazolam spiked serum.

Concentration (µg/ml)	Response (mV.sec) ± %RSD
0.80	239 ± 4.35
1.20	381 ± 3.55
1.60	529 ± 3.26

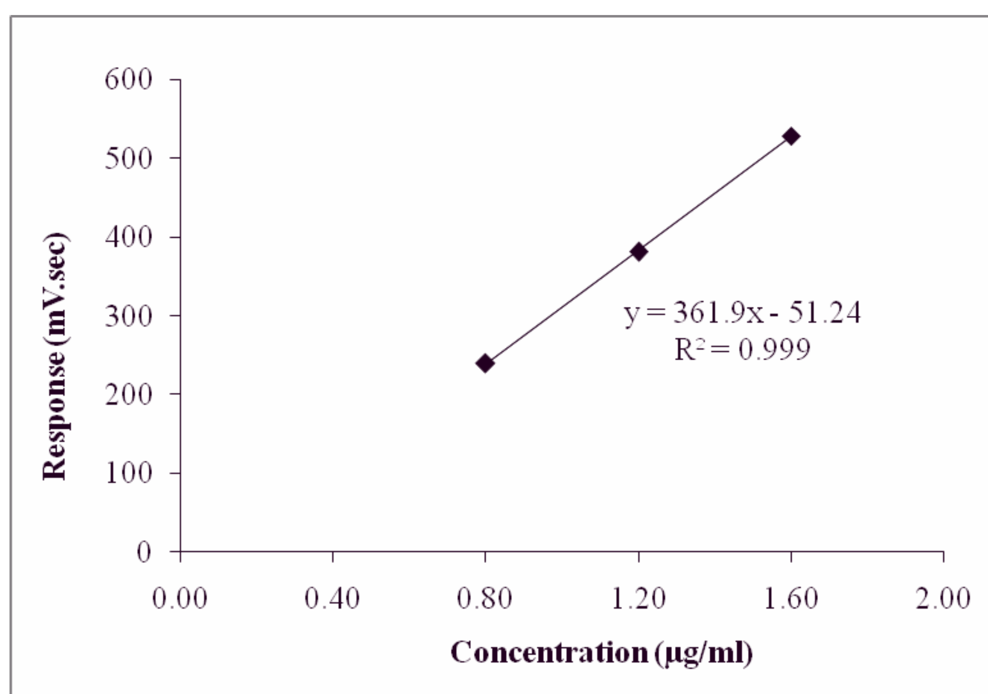


Figure 21. The calibration curve of standard midazolam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (µg/ml) of midazolam.

An equation is $y = 361.9x - 51.24$ with a linear correlation coefficient (r^2) 0.999.

3.3.1.6 Linearity of triazolam

The response in the form of peak area (mV.sec) for each prepared concentration of standard triazolam spiked serum is shown in Table 47. The calibration curve of standard triazolam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration of triazolam is shown in Figure 22.

Table 47. The response in the form of peak area (mV.sec) for each prepared concentration of standard triazolam spiked serum.

Concentration (µg/ml)	Response (mV.sec) ± %RSD
0.80	155 ± 4.95
1.20	332 ± 4.27
1.60	505 ± 4.84

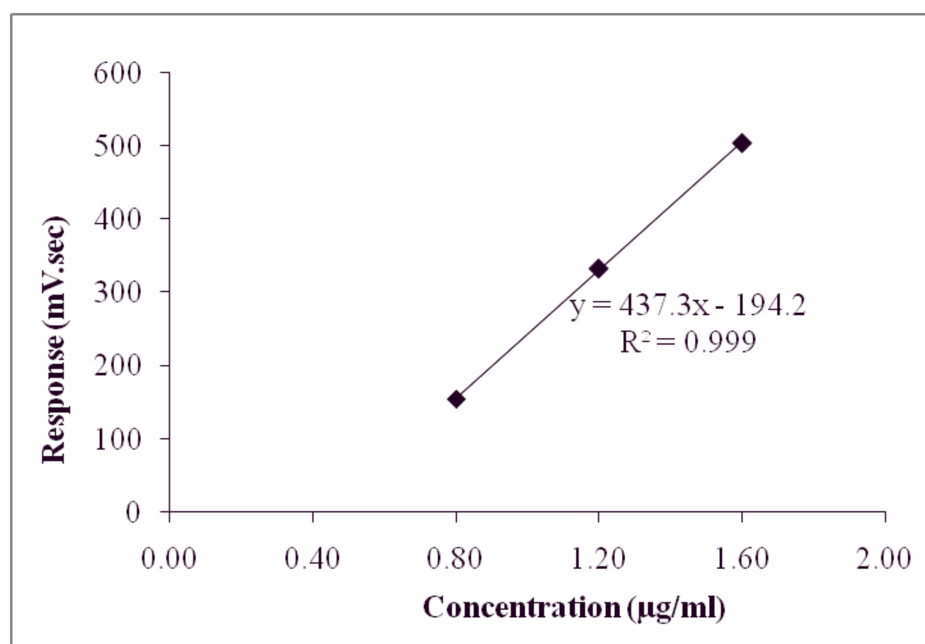


Figure 22. The calibration curve of standard triazolam spiked serum evaluated by plotting the response in the form of peak area (mV.sec) against the concentration (µg/ml) of triazolam.

An equation is $y = 437.3x - 194.2$ with a linear correlation coefficient (r^2) 0.999.

In conclusion, the linearity of 6 benzodiazepines is summarized in Table 48.

Table 48. The linearity of 6 benzodiazepines.

Benzodiazepines	Concentration (μg/ml)			$y = a + bx$	r^2
	low	average	high		
Nitrazepam	0.20	0.60	1.00	$y = 2471x + 188.1$	0.999
Diazepam	0.20	0.60	1.00	$y = 1456x + 56.80$	0.999
Lorazepam	0.20	0.60	1.00	$y = 1183x – 86.80$	0.999
Flunitrazepam	0.40	0.80	1.20	$y = 1437x + 230.8$	0.999
Midazolam	0.80	1.20	1.60	$y = 361.9x – 51.24$	0.999
Triazolam	0.80	1.20	1.60	$y = 437.3x – 194.2$	0.999

The linearity of nitrazepam, diazepam, and lorazepam (0.20, 0.60, and 1.00 μg/ml), flunitrazepam (0.40, 0.80, and 1.20 μg/ml), midazolam and triazolam (0.80, 1.20, and 1.60 μg/ml) have been proved using analytical procedure with linear (linear correlation coefficient (r^2) greater than 0.990).

3.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

From the linearity of 6 benzodiazepines discussed above, the slope of each benzodiazepines calibration curve line (m) can be used to determine limit of detection (LOD) and limit of quantification (LOQ) which is summarized in Table 49.

Table 49. The equation and slope of benzodiazepines calibration curve lines.

Benzodiazepines	Equation	Slope (m)
Nitrazepam	$y = 2471x + 188.1$	2471
Diazepam	$y = 1456x + 56.80$	1456
Lorazepam	$y = 1183x – 86.80$	1183
Flunitrazepam	$y = 1437x + 230.8$	1437
Midazolam	$y = 361.9x – 51.24$	361.9
Triazolam	$y = 437.3x – 194.2$	437.3

From blank analysis of 6 benzodiazepines for 10 replicates, mean blank (X_B) and standard deviation of blank (S_B) are shown in Table 50.

Table 50. Mean blank response (X_B) (mV) and standard deviation of blank (S_B) of 6 benzodiazepines.

Benzodiazepines	Mean Response ($X_{B)}$ (mV.sec)	S_B
Nitrazepam	111.8	22.4
Diazepam	108.8	26.0
Lorazepam	118.6	32.6
Flunitrazepam	46.27	37.8
Midazolam	49.97	7.43
Triazolam	63.30	8.65

3.3.2.1 Limit of detection (LOD) and limit of quantification (LOQ) of nitrazepam

Limit of detection (LOD) of nitrazepam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \times 22.4/2471 \ \mu g/ml$$

$$C_L = 0.027 \ \mu g/ml$$

Limit of quantification (LOQ) of nitrazepam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \times 22.4/2471 \ \mu g/ml$$

$$C_L = 0.091 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of nitrazepam were 0.027 µg/ml and 0.091 µg/ml, respectively.

3.3.2.2 Limit of detection (LOD) and limit of quantification (LOQ) of diazepam

Limit of detection (LOD) of diazepam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \text{ x } 26.0/1456 \ \mu g/ml$$

$$C_L = 0.054 \ \mu g/ml$$

Limit of detection (LOQ) of diazepam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \text{ x } 26.0/1456 \ \mu g/ml$$

$$C_L = 0.18 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of diazepam were 0.054 µg/ml and 0.18 µg/ml, respectively.

3.3.2.3 Limit of detection (LOD) and limit of quantification (LOQ) of lorazepam

Limit of detection (LOD) of lorazepam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \text{ x } 32.6/1183 \ \mu g/ml$$

$$C_L = 0.084 \ \mu g/ml$$

Limit of detection (LOD) of lorazepam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \text{ x } 32.6/1183 \ \mu g/ml$$

$$C_L = 0.28 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of lorazepam were 0.084 µg/ml and 0.28 µg/ml, respectively.

3.3.2.4 Limit of detection (LOD) and limit of quantification (LOQ) of flunitrazepam

Limit of detection (LOD) of flunitrazepam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \times 37.8/1437 \ \mu g/ml$$

$$C_L = 0.079 \ \mu g/ml$$

Limit of detection (LOD) of flunitrazepam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \times 37.8/1437 \ \mu g/ml$$

$$C_L = 0.26 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of flunitrazepam were 0.079 µg/ml and 0.26 µg/ml, respectively.

3.3.2.5 Limit of detection (LOD) and limit of quantification (LOQ) of midazolam

Limit of detection (LOD) of midazolam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \times 7.43/361.9 \ \mu g/ml$$

$$C_L = 0.062 \ \mu g/ml$$

Limit of detection (LOD) of midazolam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \times 7.43/361.9 \ \mu g/ml$$

$$C_L = 0.21 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of midazolam were 0.062 µg/ml and 0.21 µg/ml, respectively.

3.3.2.6 Limit of detection (LOD) and limit of quantification (LOQ) of triazolam

Limit of detection (LOD) of triazolam can be determined by the equation

$$C_L = 3S_B/m$$

$$C_L = 3 \times 8.65/437.3 \ \mu g/ml$$

$$C_L = 0.059 \ \mu g/ml$$

Limit of detection (LOD) of triazolam can be determined by the equation

$$C_L = 10S_B/m$$

$$C_L = 10 \times 8.65/437.3 \ \mu g/ml$$

$$C_L = 0.20 \ \mu g/ml$$

Therefore, limit of detection (LOD) and limit of quantification (LOQ) of triazolam were 0.059 µg/ml and 0.20 µg/ml, respectively.

3.3.3 Precision of the procedure

The intraday repeatability determined, over 1 day, with repeated analysis (n=3) at 3 concentrations (low, average and high), and the interday reproducibility determined with repeated analysis (n=3) at the same concentration, over a period of 3 days by their relative standard deviations (RSD) are established in Table 51.

Table 51. The intraday (within-day) repeatability and the interday (between-day) reproducibility of the low, average, and high concentrations of 6 benzodiazepines standard spiked serum.

Benzodiazepines	% intraday RSD (n = 3)			% interday RSD (n = 9)
	Low	Average	High	Average
Nitrazepam	3.79	2.22	4.69	7.28
Diazepam	2.23	3.22	2.04	7.44
Lorazepam	3.77	3.84	1.84	9.97
Flunitrazepam	2.25	2.09	2.69	6.53
Midazolam	3.88	3.24	3.19	9.92
Triazolam	4.39	4.95	3.74	7.67

From the results of all 6 benzodiazepines, the intraday relative standard deviations (intraday RSD) obtained were lower than 5% and the interday relative standard deviations (interday RSD) obtained were lower than 10%. It can be concluded that the within run and between run data were precise for all benzodiazepines within the acceptance interval of 5% and 10%, respectively.

3.4 Application of method to the real sample.

3.4.1 Diazepam administration

The standard diazepam spiked serum curve was obtained from the results of linearity section 3.3.1.2 in Figure 18 giving an equation $y = 1456x + 56.80$ with a linear correlation coefficient (r^2) 0.999.

Chromatograms of blank serum and diazepam serum samples are shown in Figure 23 and 24, respectively.

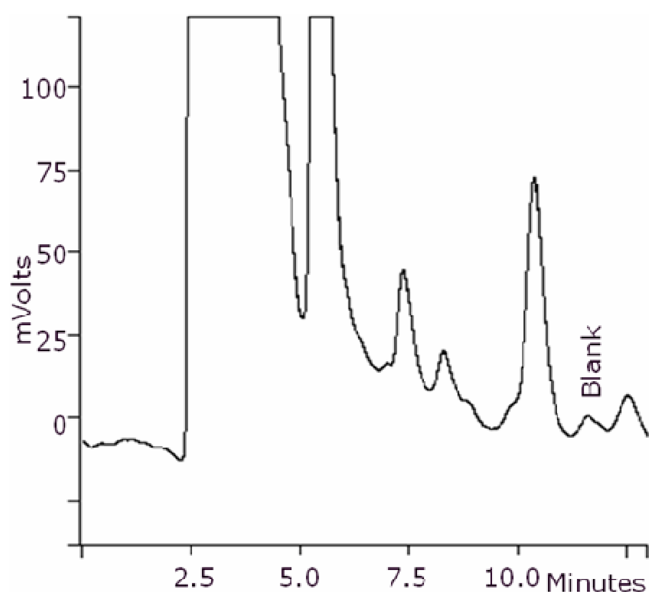


Figure 23. Chromatogram of blank serum.

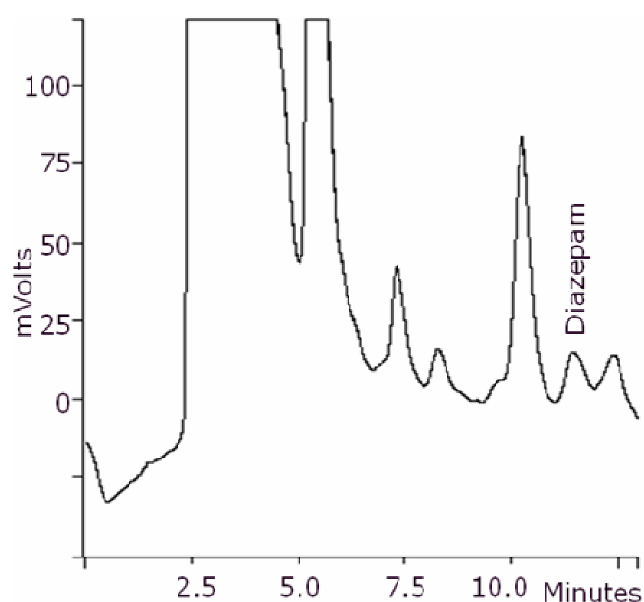


Figure 24. Chromatogram of diazepam serum samples.

The response in the form of peak area (mV.sec) of diazepam serum samples was 379 mV.sec. The concentration of diazepam in serum samples was quantified by the equation y = 1456x + 56.80 from standard diazepam spiked serum curve. The calculated concentration was 0.22 µg/ml.

From section 3.3.2.2, limit of detection (LOD) and limit of quantification (LOQ) of diazepam were 0.054 µg/ml and 0.18 µg/ml, respectively. It

can be seen that the concentration of diazepam serum samples was higher than the limit of detection (LOD) and limit of quantification (LOQ). In addition, it was shown that the calculated concentration of 0.22 µg/ml was in diazepam therapeutic serum range of 0.1-1.5 µg/ml (Table 3).

Therefore, the method in this study can be used to detect and quantify the concentration of diazepam in samples obtained from persons who orally administered diazepam both in therapeutic range and toxic range.

3.4.2 Lorazepam administration

The standard lorazepam spiked serum curve was obtained from the results of linearity section 3.3.1.3 in Figure 19 giving an equation $y = 1183x - 86.80$ with a linear correlation coefficient (r^2) 0.999.

Chromatograms of blank serum and lorazepam post-administrative serum are shown in Figure 25 and 26, respectively.

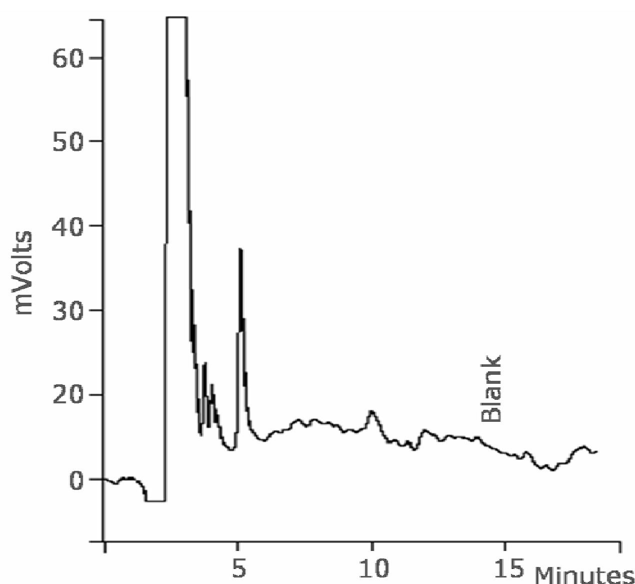


Figure 25. Chromatogram of blank serum.

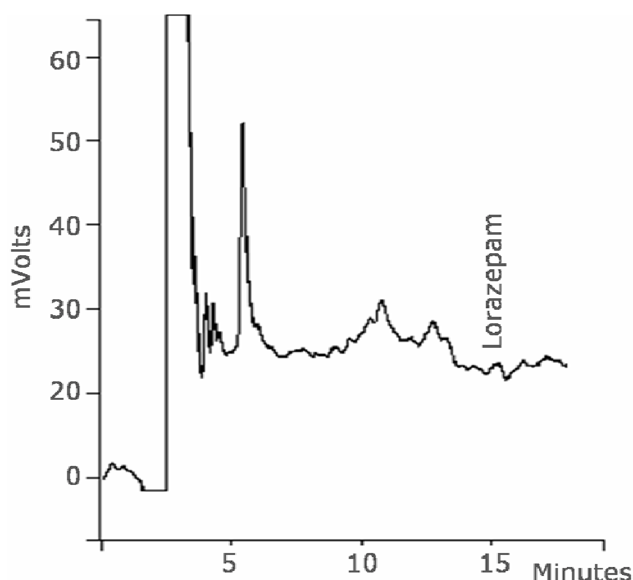


Figure 26. Chromatogram of lorazepam serum samples.

The response in the form of peak area (mV.sec) of lorazepam serum samples was 42 mV.sec. The concentration of lorazepam in serum samples was quantified by the equation y = 1183x – 86.80 from standard lorazepam spiked serum curve. The calculated concentration was 0.11 µg/ml.

From section 3.3.2.3, limit of detection (LOD) and limit of quantification (LOQ) of lorazepam were 0.084 µg/ml and 0.28 µg/ml, respectively. It can be seen that the concentration of lorazepam in serum samples was higher than the limit of detection (LOD), but lower than the limit of quantification (LOQ).

Therefore, the method in this study can be used to detect lorazepam presents in samples, but cannot be used to quantify the concentration of lorazepam in samples obtained from persons who orally administered lorazepam in therapeutic range. Although, the quantification of lorazepam in therapeutic range cannot be performed in this study, it may be quantified in toxic range of 0.3 µg/ml (Table 3).

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The mobile phase optimization by the elution behavior method determined in isocratic elution and gradient elution can be used as based data to approach the optimum condition for 6 benzodiazepines analysis. The optimum condition for these 6 benzodiazepines analysis can be obtained by the used of tertiary mobile phase (methanol-acetonitrile-phasphate buffer solution; 3.3 mM, pH 2.1). The protein precipitation extraction method is suitable for routine works because of its simple and rapid with recovery yields more than 75%. The study method can be used to detect and quantify the concentration of diazepam in samples obtained from persons who orally administered diazepam both in therapeutic and toxic range. However, it can be used to detect lorazepam presents in sample and quantify the concentration of lorazepam obtained from person who orally aministered lorazepam in toxic range.

For further study, the optimization by the elution behavior method based on the study of pH is recommended. In addition, cyano reverse phase column was used in this study, although there are few literatures described. Therefore, it is interesting to study on the used of cyano reverse phase column for benzodiazepines analysis.

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