Determination of Phenobarbital and Pentobarbital in Rat Plasma by Reversed Phase and Micellar Mobile Phase Chromatography

Charuwan Khamkaew

Master of Science Thesis in Analytical Chemistry

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Thesis Title	Determination of Phenobarbital and Pentobarbital in Rat Plasma by Reversed Phase and Micellar Mobile Phase Chromatography
Author	Miss Charuwan Khamkaew
Major Program	Analytical Chemistry

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Dean, Graduate School

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Abstract

Two chromatographic techniques for the determination of phenobarbital and pentobarbital in rat plasma were studied according to different preparation procedures : reversed phase and micellar mobile phase chromatography. The reversed phase chromatography required a removal of plasma proteins by drug extraction and / or protein precipitation prior to chromatographic analysis. In the case of micellar chromatography, the surfactant like SDS at the concentration above its cmc for the mobile phase allowed the direct injection of untreated plasma onto the chromatographic column.

The effects on sample retention time, separation and the determination of drugs in rat plasma such as mobile phase concentration, pH, salt, organic modifier types and mobile phase velocity were studied. The optimum ratio of mobile phase components in reversed phase was 30 : 70 of acetonitrile : phosphate buffer pH 5.0. Phenobarbital and pentobarbital were eluted respectively at 8.29 and 13.36 minutes. The reversed phase chromatography was precised with RSDs ranging from 2.33 to 2.85 %. The recoveries were in the range of 95.52 to 95.67 % with a limit of detection at 1.18 and 3.14 ng for phenobarbital and pentobarbital, respectively.

The appropriate micellar mobile phase was 0.01 M SDS in phosphate buffer pH 5.0 which eluted the phenobarbital at 5.30 minutes and pentobarbital at 9.56 minutes. The precision of the method gave the range of RSDs from 5.64 to 6.79 % and recoveries of the drugs were found ranging from 76 to 88 %. Phenobarbital could be detected as little as 3.33 ng while only 9.80 ng was pentobarbital.

A comparison of the amounts of phenobarbital acquired from the reversed phase and micellar mobile phase chromatography using CRD of ANOVA showed that there was not a significant difference at 95 % CL, whereas on the amount of pentobarbital, there was a significant difference at 99 % CL.

ชื่อวิทยานิพนธ์	การหาปริมาณ ฟีโนบาร์บิทอล และ เพนโทบาร์บิทอลในพลาสมาหนู โดยวิธีรีเวอร์สเฟส และ ไมเซลลาร์โมบายเฟสโครมาโทกราฟี
ผู้เขียน	นางสาวจารุวรรณ คำแก้ว
สาขาวิชา	เคมีวิเคราะห์
ปีการศึกษา	2538

บทคัดย่อ

ศึกษาวิธีวิเคราะห์ ปริมาณ ฟีโนบาร์บิทอล และ เพนโทบาร์บิทอล ในพลาสมาหนู โดยเทคนิคโครมาโทกราฟีที่มีขั้นตอนการเตรียมตัวอย่างที่แตกต่างกันคือ รีเวอร์สเฟส และ ไมเซลลาร์โมบายเฟสโครมาโทกราฟี วิธีรีเวอร์สเฟสโครมาโทกราฟี เป็นเทคนิคที่จะต้องสกัดตัวยาหรือแยกโปรตีนออกจากพลาสมาก่อนนำไปวิเคราะห์หาปริมาณ ส่วนไมเซลลาร์โมบายเฟสโครมาโทกราฟีจะใช้ตัวเคลื่อนที่ เป็นสารละลายเซอร์แฟคแตนท์ เช่น SDS ที่มีความเข้มข้นที่สูงกว่า cmc เป็นตัวชะ ตัวยาในพลาสมา ที่ไม่ผ่านการสกัดหรือแยกโปรตีนออก ก่อนการวิเคราะห์ โดยศึกษาปัจจัยต่างๆที่มีผลต่อ รีเทนชันไทม์ การแยก และการหาปริมาณของตัวยา ในพลาสมาหนูเช่น ความเข้มข้นของตัวเคลื่อนที่ ความเป็นกรด เบส เกลือ ชนิดของตัวทำละลายอินทรีย์ และอัตราเร็วของตัวเคลื่อนที่พบว่า อัตราส่วนที่เหมาะสมของตัวเคลื่อนที่ ที่ใช้ในวิธีรีเวอร์สเฟสคือ 30 : 70 ของ acetonitrile : phosphate buffer pH 5.0 จะให้ค่ารีเทนชันไทม์ ของ ฟีโนบาร์บิทอล และ เพนโทบาร์บิทอลที่ 8.29 และ 13.36 นาที ตามลำดับ ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 2.33-2.85% ประสิทธิภาพการสกัดอยู่ในช่วง 95.52 - 95.67 % ขีดจำกัดต่ำสุดที่วิเคราะห์ได้สำหรับฟีโนบาร์บิทอลคือ 1.18 ng และเพนโทบาร์บิทอลคือ 3.14 ng ส่วนตัวเคลื่อนที่ ที่เหมาะสม ที่ใช้ในวิธีไมเซลลาร์โมบายเฟสโครมาโทกราฟี คือ 0.01 M SDS ใน phosphate buffer pH 5.0 จะให้ค่ารีเทนชันไทม์ของฟีโนบาร์บิทอลและเพนโทบาร์บิทอลที่ 5.30 และ 9.56 นาที ตามลำดับ ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 5.64-6.79 % ประสิทธิภาพของการแยกตัวยาอยู่ในช่วง 76-88 % ขีดจำกัดต่ำสุดที่วิเคราะห์ได้สำหรับฟีโนบาร์บิทอลคือ 3.33 ng และเพนโทบาร์บิทอลคือ 9.80 ng

เมื่อเปรียบเทียบผลการวิเคราะห์หาปริมาณ ฟีโนบาร์บิทอล โดย วิธีรีเวอร์สเฟส และ ไมเซลลาร์โมบายเฟสโครมาโทกราฟี ด้วยการวิเคราะห์ความแปรปรวนแบบ CRD พบว่า ความแตกต่างทางสถิติไม่มีนัยสำคัญที่ระดับความเชื่อมั่น 95 % แต่ปริมาณเพนโทบาร์บิทอล ที่วิคราะห์โดย 2 วิธีข้างต้น จะแตกต่างทางสถิติ อย่างมีนัยสำคัญที่ระดับความเชื่อมั่น 99 %

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Charuwan Khamkeaw

Contents

List of Tables

List of Figures

List of Schemes

Chapter 1

Introduction

It is known that the toxic effects from drug administration is verdosage. The toxic effects, especially of barbiturate overdosage are respiratory depression, profound shock and hypotension, with peripheral vascular collapse, feeble heart-beat, lower body temperature, renal failure, and prolonged coma, with depressed or absent reflexes. Also, alcohol enhances the action of the barbiturates and reduces the margin of safety between the therapeutic and toxic doses. Therefore, the drug administration for patients should be controlled. Also, the determination of drugs in patients is necessary. It will employ the body fluids such as serum, plasma and urine. Proteins in body fluids can be bound with the administered drugs. Thus, the suitable technique for the analysis of drugs is the high performance liquid chromatographic (HPLC) technique. Because the HPLC technique not only can separate the interesting drugs from proteins in body fluids, but also can perform rapidly and give the reliable results. The main limitation of the determination of drugs from body fluids by HPLC is the prevention of column packing bed damage from protein precipitation. Reversed phase and micellar mobile phase chromatography are selected as the analytical techniques to be compared with each of the results. Concentration of phenobarbital and pentobarbital (derivatives of barbiturate) are measured in rat plasma samples.

Literature Review

Phenobarbital

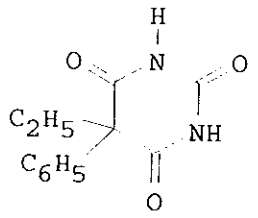


Figure 1. Structural Formula of Phenobarbital

Chemical Name : 5-Ethyl-5-phenylbarbituric acid

Trademark : Phenobarb ; Phenobarbitone ; Luminal

Chemical and Physical Properties

Molecular Weight : 232.2

Dissociation Constant : pKa 7.4 at 25 ^{o}C

Description : An odourless, white, crystalline powder or colourless crystals.

Solubility : Soluble in 100 parts of water and in 10 parts of alcohol at 20 ^{o}C.

Soluble in ether and chloroform.

Melting Point : About 176 ^{o}C.

Proteins Binding : 20 to 40 % bound to plasma proteins.

Pentobarbital

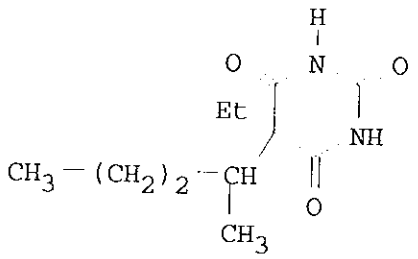


Figure 2. Structural Formula of Pentobarbital

Chemical Name : 5-Ethyl-5-(1-methylbutyl)-barbituric acid

Trademark : Pentobarbital ; Nembutal

Chemical and Physical Properties

Molecular Weight : 226.3

Dissociation Constant : pKa 8.2 at 25 ^{o}C

Description : An odourless, white, crystalline powder or colourless crystals.

Solubility : Very slightly soluble in water.
Freely soluble in absolute ethanol, ether and chloroform.

Melting Point : About 133 ^{o}C.

Proteins Binding : 50 % bound to plasma proteins.

Phenobarbital

Phenobarbital has the actions of long-acting barbiturate drugs. It has been used as a sedative in nervous and anxiety states, chorea, neurasthenia, climacteric disorders, dysmenorrhoea and thyrotoxicosis. It is also used in the treatment of migraine and in epilepsy to diminish the frequency of attacks. The usual dose is 30 to 125 mg 3 times daily.

Phenobarbital cause drowsiness and patients receiving it should not take charge of vehicles or machinery where loss of attention could cause accidents. The concomitant administration of barbiturates and alcohol may produce very serious respiratory depression and lowering of the lethal dose of the barbiturates.

In veterinary usage, phenobarbital has been used as a hypnotic and anticonvulsant in dogs and cats in doses of 6 to 12 mg per kg body-weight.

Pentobarbital

Pentobarbital is an intermediate-acting barbiturate drugs. It has been used as a basal anaesthetic before surgical operations. Its sedative effect on the patient reduces the amount of general anaesthetic required. It is also used as a hypnotic in the treatment of insomnia and as a sedative, and used in the production of obstetric amnesia.

In veterinary usage, pentobarbital is used as a general anaesthetic in dogs and cats. The usual dose by intravenous injection is 20 to 35 mg per kg body-weight. Larger doses are used for euthanasia.

A. Reversed Phase Liquid Chromatography (RPLC)

Reversed phase liquid chromatography for the determination of phenobarbital and pentobarbital from plasma requires the extensive sample preparation before chromatography. The procedure of sample preparation is the protein extraction which employs organic solvent such as acetonitrile and the other for protein precipitation. The extraction procedure not only requires time consumption, but also employs the hazardous organic solvent.

The RPLC employing hydrocarbonaceous-bonded stationary phase has become one of the most widely used modes of liquid chromatography, in part, because of the impressive selectivity available via mobile phase participation in the equilibrium distribution of solute molecules between the stationary phase and mobile phase. The lack of excessively strong solvent-surface interactions facilitates the use of a wider range of mobile phase, which allows for greater flexibility in the control of selectivity as well as more options in the choice of a suitable solvent for a sample. Separation in RPLC is achieved by means of differences in the interactions of the solutes with both the mobile and stationary phase. The mobile phase must be chosen to ensure solubility of the sample solutes. The use of a stationary phase that interacts strongly with solutes relative to solute-mobile phase interactions will result in very long retention time (t_r), a situation which is not analytically useful. Retention in RPLC is dominated by the solute-mobile phase interactions, with solute-stationary phase interactions making important but secondary contribution (*Dorsey, DeEchegaray and Landy , 1983 : 924 - 928*). Thus, the key to selective separations is the ability to control solute-mobile phase interactions by changing the composition of the mobile phase.

Kabra, et al. (1977) measured the phenobarbital and pentobarbital in serum by HPLC with the mobile phase of acetonitrile in phosphate buffer pH 4.4 (95 : 405). They adjusted the retention time of the analytes by adjusting the pH of the mobile phase. They selected to use pH 4.4 because they observed the least amount of interference from other drugs at this pH. The agreement of the result had been able to achieve the same resolution by using an acetate buffer. The retention times of phenobarbital and pentobarbital were 4.3 and 9.3 minutes, respectively, with the flow rate of 3.0 ml/min.

Szabo and Brown (1982) reported that the changes in pH of the mobile phase had the greatest effect on phenobarbital retention time. At pH less than 6.0, phenobarbital would co-elute with 5-(4-methyl phenyl)-5-phenylhydantoin (HPPH). The phenobarbital was eluted with the mobile phase of acetonitrile-methanol-phosphate buffer pH 6.8 (17+28+55, % v/v) and gave the t_r of 9.4 minutes.

Cope and Davidson (1987) reported that the effect of ionic strength on the retention of sodium camphorsulphonate on the application of polymeric (PLRP-S) column. They described that the retention of sodium camphorsulphonate had a little increase with increasing ionic strength by adding concentration of NaCl in the mobile phase.

Lipczynski (1987) reported that buffer in RPLC, an increase in buffer concentration increased the buffering capacity within the chromatograpic band to a limiting value where buffer capacity exceeded the ability of the acidic analytes to modify local pH.

Mira, et al. (1987) developed the method for determination of phenobarbital in serum by HPLC. Serum protein was precipitated with an acetonitrile solution containing HPPH as the internal standard using sample-to-solvent ratio of 1: 1. The drug was eluted from a 5 μm, C-18 reversed phase column at 40 ^{o}C with a mobile phase consisting of an acetonitrile-methanol-phosphate buffer pH 4.8 (22+28+50, % v/v), at a flow rate of 1 ml/min with UV detection at 214 nm. The analysis time required no longer than 12 minutes and the t_r of phenobarbital was 5.0 minutes.

Alila and Heavner (1988) analyzed the pentobarbital and other barbiturates in rabbit serum by a simple extraction technique using sample-to-solvent ratio 1: 2 and then determined by HPLC with UV detection. The chromatographic conditions employed the mobile phase of equal volume of acetonitrile and 0.01 M phosphate-buffered solution pH 7.8, with a flow rate of 0.75 ml/min. The retention time of phenobarbital and pentobarbital were 4.26 and 6.26 minutes, respectively. The sensitivity for pentobarbital was about 1 μg/ml and the recovery was more than 97 %.

B. *Micellar Mobile Phase Chromatography*

Micellar chromatography (MC) is the method which using a micellar solution of surfactant as sodium dodecyl sulfate (SDS) mobile phase allows the direct injection of untreated plasma on a reversed phase column (i.e., C-18 or CN column) for rapid monitoring of drug concentration.

Aqueous solution of surfactants at concentrations above their critical micelle concentration (cmc), where micelle exists along with monomers, dimers, etc., constitute a more complex mobile phase. These micellar solutions are

microscopically heterogeneous, being composed of the amphiphilic micellar aggregate and the bulk surrounding solvent.

The main importance of micelle in the mobile phase is remained in their ability to participate in the partitioning mechanism. The solute can be preferentially solubilized into or onto the micellar assembly, a process which is dynamic and characterized by various rate constants (*Almgren, et al., 1979 : 279-291*). The three equilibria involved in micellar chromatography are schematically represented in Figure 3.

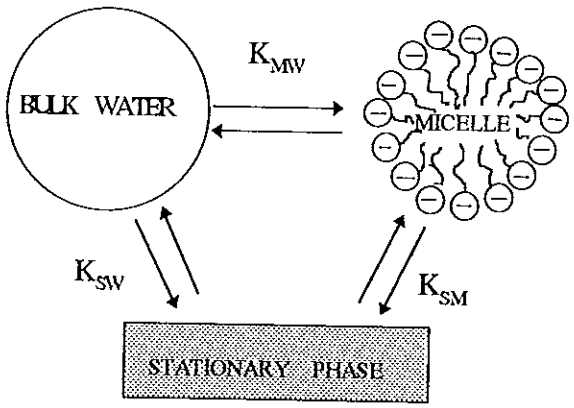


Figure 3. Schematic representation of the three "phase" model for solute partitioning in micellar chromatography. The elution behavior of a solute depends on the combination effects of three partition coefficients :

K_{mw} = partition coefficient between micelle and water

K_{sw} = partition coefficient between stationary phase and water

K_{sm} = partition coefficient between stationary phase and micelle

The partitioning of solutes between micellar and aqueous phases in liquid chromatography (LC) was first treated theoretically by *Herries, et al., (1964).* Since then, other reports have illustrated certain advantages of MC.

Armstrong and Henry (1980) first effectively demonstrated that the micelle could provide a hydrophobic site for interaction with the solute in the mobile phase and could be used in place of traditional organic modifier such as methanol and acetonitrile.

Yarmchuk, et al.,(1982) reported that the selectivity can be enhanced by proper choice of surfactant type and mobile phase concentration. The typical of some surfactants and their cmcs, aggregation numbers were represented in Table 1. (*Cline Love, et al., 1984 : 1134A*).

Table1. Typical surfactants and their cmcs, aggregation numbers

surfactants	cmc (M)	aggregation number
Anionic : SDS	0.0081	62
Cationic : Cetyltrimethylammonium bromide (CTAB)	0.0013	78
Nonionic : Polyoxyethylene(23)dodecanol (Brij-35)	0.0001	40
Zwitterionic : *N*-dodecyl-*N*,*N*-dimethylammonium-3-propane-1-sulfonic acid (SB-12)	0.0030	55

The large number of possible interactions associated with micellar mobile phase separations, i.e., electrostatic, hydrophobic and steric, as well as the modification of the stationary phase by adsorption of monomer surfactants, makes these systems more complicated than conventional RPLC (*Arunyanart and Cline Love, 1984 : 1557*). In addition, *Arunyanart and Cline Love (1984)* proved the parabolic dependence of chromatographic capacity factor (k') on [micelle].

Arunyanart and Cline Love (1985) described that for charged solutes, electrostatic attraction or repulsion with the charged head groups of the micelle and / or with the head groups of surfactant monomers adsorbed on the stationary phase were occured. Also, for ionizable species, the ratio of undissociated-to-dissociated forms was a function of mobile phase conditions, i.e., pH, ionic strength, buffer type, etc. In addition, they described that CN and C-18 columns interacted very differently with surfactant monomers, resulting in a different elution behavior of organic acid and basic as a function of the [micelle] in the mobile phase pH.

The research of this thesis was the determination of phenobarbital and pentobarbital in rat plasma by reversed phase and micellar mobile phase chromatography. Therefore, the study was carried out in the expected purpose and there were :

1. To study the various effects, i.e., mobile phase concentration, pH, salt, organic modifiers, linear velocity (v) and on the k' or optimum t_r for the determination of the two drugs in rat plasma by the two techniques.

2. To study the separation of the two drugs in rat plasma by the two techniques.

3. To analyze drug quantity in rat plasma by the two techniques.

4. To compare the results of the drug measurement in rat plasma from each technique.

Moreover, the extraction and direct injection recovery, detection limit and precision of the two techniques were also evaluated.

Chapter 2

Experiments

Chemicals and Reagents

1. Standard of Analytical Drugs

The standard solutions of phenobarbital 1000 ppm and pentobarbital 980 ppm in methanol were obtained from Sigma Chemical Company, USA.

2. Organic Solvents

Acetonitrile (HPLC grade) and methanol (AR grade) were purchased from Riedel-deHaen, Germany.

1-Propanol and 1-Butanol (AR grade) were supplied by May & Baker, England.

3. Reagents

SDS, sodium hydroxide (NaOH), sodium chloride (NaCl) and ortho-Phosphoric acid 85% (AR grade) were purchased from Fluka (Switzerland), Merck (Germany) and J.T. Baker Chemicals Co., Deventer (Holland), respectively.

4. Samples

Rat plasma samples were obtained from the Department of Phamarcology , Faculty of Science , Prince of Songkla University , Songkla , Thailand.

Apparatus

The HPLC systems consisted of a constant flow pump (Jasco, model 880-PU, Japan), an ultraviolet (UV) detector (Jasco, model 875-UV, Japan) and a sample injector (Rheodyne, model 7125, USA) with a 20 µl loop. Detection was made with a variable wavelength UV detector set at 215 nm, 0.08 AUFS for reversed phase systems and 0.32 Absorbance Unit of Full Scale (AUFS) for micellar mobile phase systems. Chromatograms were recorded with a one-pen chart recorder(WPA, model CG 95, UK.) at sensitivity 0.5 mV/cm and chart speed 2.0 mm/min. Chromatographic separation was performed on a µBondapack C-18 column for reversed phase systems (Waters Associates, USA), 300 x 3.9 mm i.d.; a µBondapack CN for micellar mobile phase systems (Waters Associates, USA.); 150 x 3.9 mm i.d., and particle size was 10 µm.

Centrifugator : Sigma 201M, B.Braun.

Super-mixer (vortexor) : Lab-Line Instruments, Inc. Melrose park, USA.

Fortunar hypodermic syringe 10.00 ml

Nylon 25 mm membrane filter 0.45 µm

Microsyringe 50.00 µl

Volumetric flasks 10.00, 100.00, 250.00 and 1000.00 ml

Pipettes 1.00, 5.00 and 10.00 ml

Micropipette (adjustable) 100.00 and 1000.00 µl

Cylinder 5.00, 10.00, 50.00 and 100.00 ml

Beaker 10.0, 100.0, 250.0, 600.0 and 1000.0 ml

Appendorf tubes 3 ml

Methods

1. Dose and Drug Administration in Rats

All rats received intraperitoncal a single injection at a dose of 60 mg per kg body-weight per day of phenobarbital for 4 days. In the 5 th day, the dose of 35 mg per kg body-weight of pentobarbital was administered by intraperitoncal injection into these rats. Sleep occured in about 1 to 3 minutes. After awakening, they were decapitated and blood-samples were collected at once. These blood-samples were left standing for 30 minutes, and then centrifuged at 2500 round per minute (rpm) for 10 minutes for plasma collection. The plasma was kept at -4^{o}C until the time of analysis.

2. Analytical Methods

2.1 Preparation of Stock Solutions

2.1.1 Standard Solution of Phenobarbital

The 100 ppm of phenobarbital stock solution was prepared by transfering 1.00 ml of the 1000 ppm standard phenobarbital solution into 10.0 ml volumetric flask and then diluting it to the mark with distilled water. Working standard solutions were prepared by appropriate dilution of the stock standard solution with distilled water.

2.1.2 Standard Solution of Pentobarbital

The 98 ppm of pentobarbital stock solution was prepared by transfering 1.00 ml of the 980 ppm standard pentobarbital solution into 10.0 ml volumetric flask and then diluting it to the mark with distilled water. Working standard solutions were prepared by appropriate dilution of the stock standard solution with distilled water.

2.1.3 Test Solutes

Stock solution of the test solutes were prepared by diluting the appropriate concentration of 100 ppm standard phenobarbital solution to 10.00 ppm and 98 ppm standard pentobarbital solution to 19.60 ppm with distilled water.

2.2 Degassing Systems

The degassing mobile phase is an important step for HPLC systems in order to eliminate the other gasses such as oxygen, etc. that dissolved in mobile phase and interfered the analysis. The degassing systems of mobile phase in this work were performed under vacuum by suction through 4.0 to 5.5 μm of filter glass (Pyrex, USA.). For micellar mobile phase, the vacuum had to be carefully regulated to keep the degassed mobile phase from foaming to such an extent that it entered into the vacuum lines. The degassed mobile phase were kept ready to use in the mobile phase reservoir.

2.3 Equilibration Systems

Before the actual experiments were performed, the pump and detector were turned on to stabilize the light source and equilibrate of the HPLC systems. Equilibration was confirmed by constant retention time obtained from the response of detector for solutes (phenobarbital and pentobarbital). One and a half hours were allowed for reversed phase systems and one hour for micellar mobile phase systems. Retention times were measured manually from the injection point to the peak maxima on the chromatograms. The flow rate was set at 1.0 ml/min for reversed phase systems and 0.5 ml/min for micellar mobile phase systems. The accurate flow rate was measured by collecting the effluent in a 10.00 ml graduated cylinder for a sufficient length of time to collect at least 5.00 ml.

2.4 Optimum Wavelength Determination

The optimum wavelength for these drugs were obtained from UV-Vis spectrophotometer (UV-160 A, Shimadzu, Japan) by scanning wavelength from 200 to 300 nm.

2.5 Determination of Phenobarbital and Pentobarbital by HPLC

The study of various effects, i.e., mobile phase concentration, pH, salt, organic modifiers, mass transfer and linear velocity to select the optimum mobile phase on k' or t_r was described as follows :

A. Reversed Phase Systems

A1. Mobile Phase Concentration Effects

1. Prepare the various concentration of mobile phase as the acetonitrile at 25, 30, 35 and 40 % in distilled water.

2. Degas the mobile-phases before use as described previousely.

3. Employ these mobile-phases to elute the phenobarbital and pentobarbital by HPLC on μBondapack C-18 column.

4. Calculate the k' of each drug.

5. Plot k' and log k' against [acetonitrile].

A2. pH Effects

1. Prepare the desired pH of phosphate buffer by adjusting pH of 5 mM H_3PO_4 with 1.0 M NaOH to the pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4 and 7.8 by pH meter (ion analyzer 255 Corning pH meter and Corning General Purpose Combination electrode).

2. Dilute acetonitrile with the prepared phosphate buffer at the desired pH (from A2.1) for the ratio of 30 : 70 of acetonitrile : phosphate buffer.

3. Measure the t_r to calculate the k' of each drug.

4. Plot k' versus (vs) pH.

A3. Salt Effects

1. Dissolve the appropriate amounts of solid NaCl in acetonitrile solution.

2. Make the concentration of mixture of NaCl and acetonitrile to 0.00, 0.05, 0.10, 0.15, 0.20 M NaCl and 45 % acetonitrile, respectively with distilled water.

3. Calculate the k' of each drug.

4. Plot the k' vs [NaCl].

A4. Effects of Linear Velocity

1. Prepare the ratio of 30 : 70 of acetonitrile : phosphate buffer pH 5.0 as the mobile phase.

2. Adjust the flow rate at 0.50, 0.75, 1.0, 1.2 and 1.5 ml/min to perform with the mobile phase (from A4.1).

3. Measure the t_r and peak width at half height of phenobarbital.

4. Calculate the HETP and mobile phase velocity.

5. Plot the Van Deemter.

A5. Selecting the Optimum Mobile Phase

To study the mobile phase ratio, that was acetonitrile and phosphate buffer at pH 5.0 and 7.8. These ratio were described as follows :

1. The ratio of mobile-phases at pH 5.0, i.e., 30 : 70 and 36 : 64.

2. The ratio of mobile-phases at pH 7.8, i.e., 25 : 75, 30 : 70 and 35 : 65.

The optimum mobile phase for drug determination was 30 : 70 of acetonitrile : phosphate buffer pH 5.0. This mobile phase was prepared in single batch quantities sufficient to determine the phenobarbital and pentobarbital concentration in rat plasma. The flow rate was 1.0 ml/min which gave a pressure of 1100 psi. All analyses were performed at room temperature.

A6. Preparation of Standard drugs in Rat Plasma

The working concentration of standard phenobarbital and pentobarbital in blank rat plasma were 3.00, 6.00, 12.00, 24.00 ppm and 1.96, 3.92, 5.88, 7.84, 9.80 ppm, respectively. The procedure of working concentration was prepared as follows :

1. Dilute the aliquots of phenobarbital and pentobarbital stock solutions with blank rat plasma (call to plasma-based).

2. Mix these plasma-based standard with 180 µl of acetonitrile in appendorf tube for proteins precipitation.

3. Adjust volume of these plasma-based standards to 300 µl with the blank rat plasma.

4. Vortex them for 30 seconds.

5. Centrifuge these mixture at 1000 x g for 10 minutes to separate the precipitated proteins.

6. Inject the 20 µl clear supernatant into HPLC systems (Scheme 1.).

7. Measure the t_r of each concentration of particular drug.

8. Prepare the linear calibration curves by plotting peak height versus standard drug concentration of particular drug.

A7. Determination of Drugs in Rat Plasma

1. Mix rat plasma sample 120 µl with 180 µl acetonitrile in appendorf tube for proteins precipitation.

2. Treat these mixture as follows in A6.4 to A6.7.

3. Determine the drug concentration by extrapolating the linear calibration curves of particular drug.

A8. Recovery

1. Prepare the aqueous solution containing known concentration of phenobarbital and pentobarbital as drug-solutes.

2. Prepare the extract plasma (as follows in A6.1 to A6.5) containing the same concentration of the two drugs in A8.1.

3. Determine the chromatographic peak height of each drug from A8.1 and A8.2 by performing with 30 : 70 of acetonitrile : phosphate buffer pH 5.0 as a mobile phase.

4. Compare the chromatographic peak height of each drug from A8.1 and A8.2.

5. Calculate the percentage of extraction recovery of each drug by using :

$$\frac{\text{peak height (cm) from plasma (A8.2)}}{\text{peak height (cm) from aqueous solution (A8.1)}} \times 100$$

B. Micellar Mobile Phase Systems

Stock solution SDS 0.2 M was prepared by dissolving appropriate amounts of SDS in distilled water on single batch quantities sufficient for various effects.

B1. Surfactant Concentration Effects

1. Prepare the micellar mobile phase by diluting appropriate amounts of 0.2 M. SDS stock solution to 0.010, 0.025, 0.035, 0.050 and 0.075 M SDS with distilled water.

2. Degas the mobile-phases before use.

3. Employ these mobile-phases to elute the phenobarbital and pentobarbital by HPLC on μBondapack CN column.

4. Calculate the k' and 1/k' of each drugs.

5. Plot k' and 1/k' against [SDS].

B2. Micelle Bulk pH Effects

1. Prepare the desired pH of phosphate buffer by adjusting pH of 5 mM H_3PO_4 with 1.0 M NaOH to the pH 4.5, 5.5, 6.5 and 7.5 by pH meter.

2. Prepare the micellar mobile phase by diluting appropriate amounts of 0.2 M SDS stock solution to 0.010, 0.025, 0.050, 0.075 M SDS with the prepared phosphate buffer at desired pH.

3. Calculate the k' of each drugs.

4. Plot the k' vs pH (at various [SDS]) and the k' vs [SDS] (at various pH) of each drug.

B3. Salt Effects

B3.1 *Salt Effects on Capacity Factor*

1. Dissolve the appropriate amounts of solid NaCl in 0.20 M SDS stock solution.

2. Make the concentration of NaCl and SDS to 0.00, 0.05, 0.10, 0.15, 0.20 M NaCl and 0.05 M SDS, respectively with distilled water.

3. Calculate the k' of each drug.

4. Plot the k' vs [NaCl].

5. Compare the k' of each drug at 0.025 and 0.050 M SDS (non-salt) with the 0.15 M NaCl at same concentration of SDS (with salt) by calculating the difference values of k' of each drug between salt and non-salt in SDS mobile phase.

6. Plot the difference values of k' against [SDS].

B3.2 *Salt Effect on Separation Factor (α)*

1. Prepare the micellar mobile phase as in B3.1.1 to B3.1.2.

2. Calculate the separation factor.

B4. Organic Modifiers Effects

1. Prepare the 0.01 M SDS by diluting the appropriate amounts of 0.20 M SDS stock solution with distilled water (without alcohol).

2. Transfer the appropriate amounts of methanol to 0.20 M SDS stock solution.

3. Make the concentration of methanol and SDS to 1, 2, 5 % methanol and 0.01 M SDS, respectively with distilled water.

4. Prepare the same concentration of micellar mobile phase by changing the methanol to 1-propanol and 1-butanol (as the same concentration in methanol).

5. Calculate the k' of each drug.

6. Plot the k' vs [organic modifier] in 0.01 M SDS.

B5. Effects of Mass Transfer

1. Prepare the 0.01 M SDS in phosphate buffer pH 5.0 as the mobile phase.

2. Adjust the flow rate at 0.50, 0.75, 1.0, 1.2 and 1.5 ml/min to perform with the micellar mobile phase (from B5.1).

3. Measure the t_r and peak width at half height of phenobarbital.

4. Calculate the HETP and mobile phase velocity.

5. Compare these results with the results from the effects of linear velocity in A4 (Reversed Phase Systems) by plotting the Van Deemter.

B6. Selecting the Optimum Micellar Mobile Phase

The effects of micellar concentration, pH, NaCl and combination of pH and NaCl were studied under the various conditions and compositions of micellar mobile phase. These effects were described as follows :

1. The concentration of SDS in water, i.e., 0.010, 0.025 and 0.035 M SDS ([micellar] effects).

2. The 0.010 M SDS in phosphate buffer at the pH 4.5, 5.0, 5.5, 6.5, 7.5 and the 0.050 M SDS in phosphate buffer pH 7.5 (pH effects).

3. The 0.20 M NaCl in SDS mobile phase, i.e., 0.015, 0.025 and 0.050 M SDS (salt effects).

4. The 0.50 M NaCl in SDS at pH 5.0, i.e., 0.010 and 0.025 M SDS mobile phase (pH and NaCl effects).

The optimum micellar mobile phase was 0.01M SDS in phosphate buffer pH 5.0. for determination of phenobarbital and pentobarbital concentration in rat plasma. This mobile phase was prepared in single batch quantities sufficient to determine these drugs in rat plasma. The flow rate was 1.0 ml/min which gave a pressure of 800 psi. All analyses were performed at room temperature.

B7. Preparation of Standard Drugs in Rat Plasma

The working concentration of standard phenobarbital was 0.00, 2.50, 5.00, 10.00, 20.00 ppm and 0.00, 2.49, 4.90, 9.80, 19.60 ppm of standard pentobarbital in diluted blank rat plasma. The procedure of working concentration was prepared as follows :

1. Filter the blank rat plasma through a 0.45 μm Nylon 25 mm membrane filter(Whatman, England) with 10 ml Fortuna hypodermic syringe.

2. Dilute plasma filtrate with distilled water to 1:1 ratio in appendorf tube (call to diluted blank rat plasma).

3. Dilute the aliquots of phenobarbital and pentobarbital stock solution with the diluted blank rat plasma (from B7.2 and call to plasma-based).

4. Spike the 100 ppm standard phenobarbital with 20 μl (5.0 ppm) and the 98 ppm standard pentobarbital with 40 μl (9.8 ppm) in plasma-based.

5. Adjust volume of these plasma-based standards to 400 μl with the diluted blank rat plasma.

6. Vortex the mixture for 30 seconds.

7. Inject the 20 µl of these mixture into HPLC systems (Scheme 2.).

8. Measure the t_r of each concentration of particular drug.

9. Prepare the linear calibration curves by plotting the peak height versus standard drug concentration of particular drug.

B8. Determination of Drugs in Rat Plasma

1. Dilute rat plasma samples 170 µl with 170 µl of distilled water in appendorf tube.

2. Spike the 100 ppm standard phenobarbital with 20 µl (5.0 ppm) and 40 µl of the 98 ppm standard pentobarbital (9.8 ppm) in diluted plasma (from B8.1).

3. Treat the plasma samples as follows in B7.6 to B7.8.

4. Determine the drug concentration by extraplolating the linear calibration curves of particular drug.

B9. Recovery

1. Prepare the aqueous solution containing known concentration of phenobarbital and pentobarbital as drug-solutes.

2. Prepare the direct plasma injection containing the same concentration of the two drugs in B9.1.

3. Determine the chromatographic peak height of each drug from B9.1 and B9.2 by performing with 0.010 M SDS in phosphate buffer pH 5.0 as a mobile phase.

4. Compare the chromatographic peak height of each drug from B9.1 and B9.2.

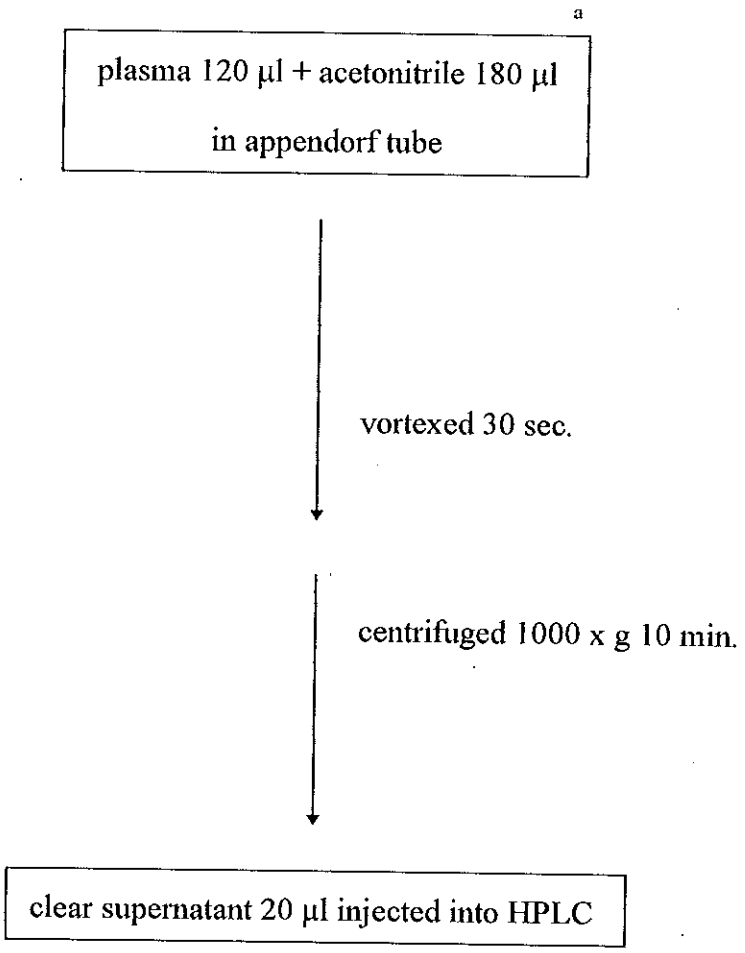
5. Calculate the percentage recovery of each drug by using :

$$\frac{\text{peak height (cm) from direct plasma injection (B9.2)}}{\text{peak height (cm) from aqueous solution (B9.1)}} \times 100$$

2.6 Limits of Detection

The minimum amount of phenobarbital and pentobarbital solution was measured manually from chromatograms. The signal of individual drug-peak from chromatograms was equal to two times of the noise. The noise was measured at the highest sensitivity of detector performance of the particular drug.

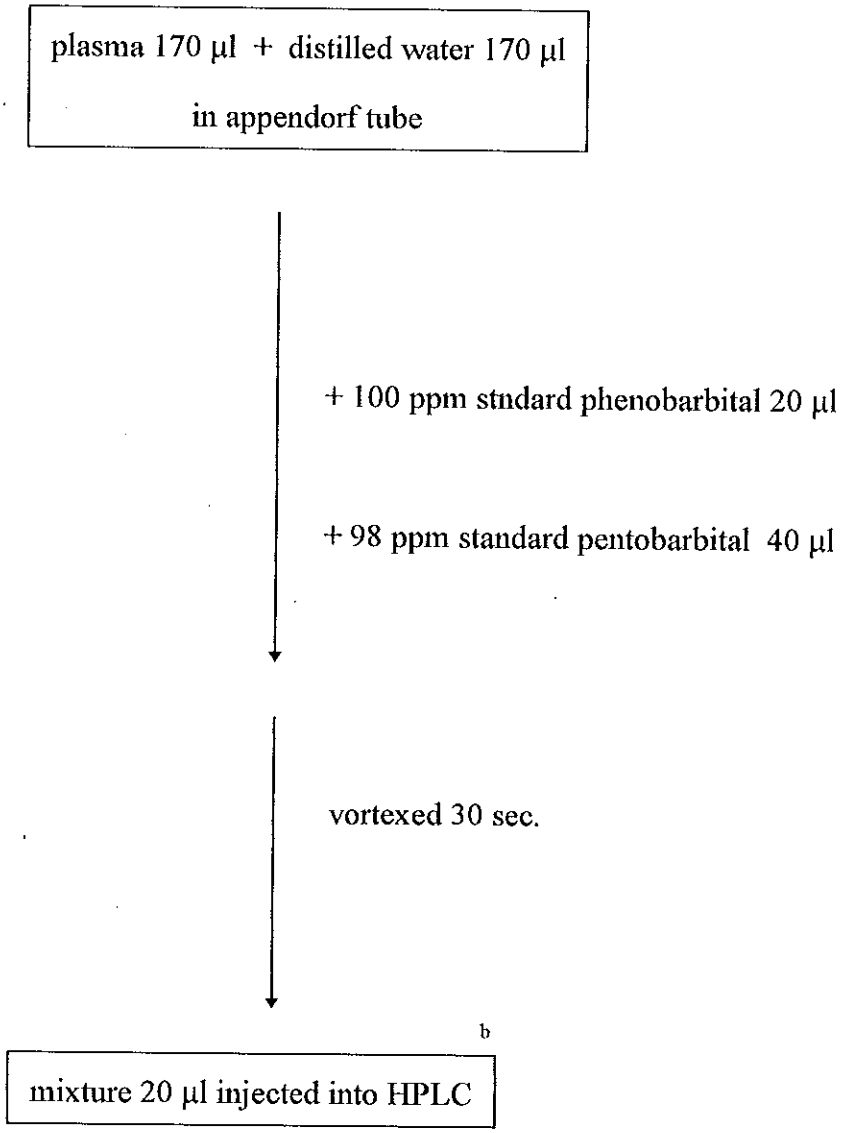
EXTRACTION PROCEDURE



[a] Plasma was diluted with acetonitrile so that dilution factor was 2.5.

Scheme 1. Diagram of plasma extraction procedure for reversed phase systems.

DIRECT INJECTION PROCEDURE



[b] Plasma was diluted with distilled water so that dilution factor was 2.35.

Scheme 2. Diagram of direct plasma injection procedure for micellar mobile phase systems

Chapter 3

Results and Discussions

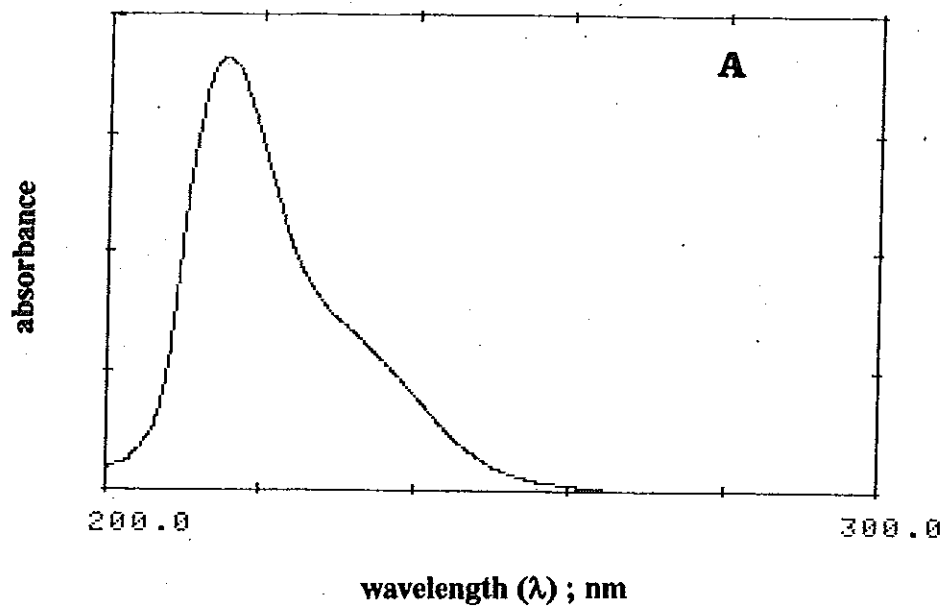
1. Optimum Wavelength Determination

The optimum wavelength of interesting drugs are determined with UV-Vis spectrophotometer by scanning wavelength from 200 to 300 nm. The lists of absorbance data are shown in Table 2. and the UV-spectrum of these drugs are given in Figure 4. (A and B) .

It can be seen that the maximum wavelength of phenobarbital and pentobarbital are 215 nm with the maximum absorbance of 1.817 for phenobarbital and 1.647 for pentobarbital. Thus, the optimum wavelength of 215 nm is chosen in this work.

Table 2. Absorbance of phenobarbital and pentobarbital from UV - Vis spectrophotometer

wavelength (λ) nm	absorbance	
	phenobarbital	pentobarbital
200	0.0945	0.1330
210	1.0990	0.9650
215	1.8170	1.6470
220	1.4635	1.5520
230	0.7200	0.7840
240	0.3995	0.5420
250	0.1015	0.1430
260	0.0195	0.1070
270	-0.0165	0.0620
280	-0.0370	-0.0220
290	-0.0470	-0.0520
300	-0.0160	-0.0600



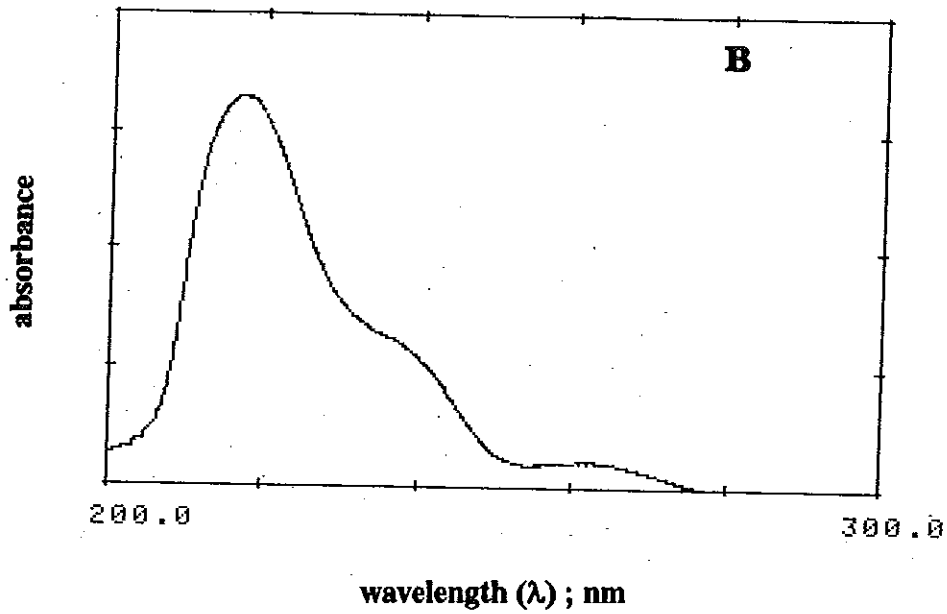


Figure 4. UV- spectrum of phenobarbital (A) and pentobarbital (B) from UV-Vis spectrophotometer

2. Determination of Phenobarbital and Pentobarbital by HPLC

A. Reversed Phase Systems

A1. Mobile Phase Concentration Effects

In reversed phase chromatography, the organic modifier in water as a mobile phase is used as a base solvent, to which varying concentrations of missible organics are added. The elution behavior of solute is dependent on solvent strength. Solvent strength is usually adjusted by varying the composition of solvent mixture, and the capacity factor is changed with changing solvent composition (*Snyder, et al., 1979 : 285*).

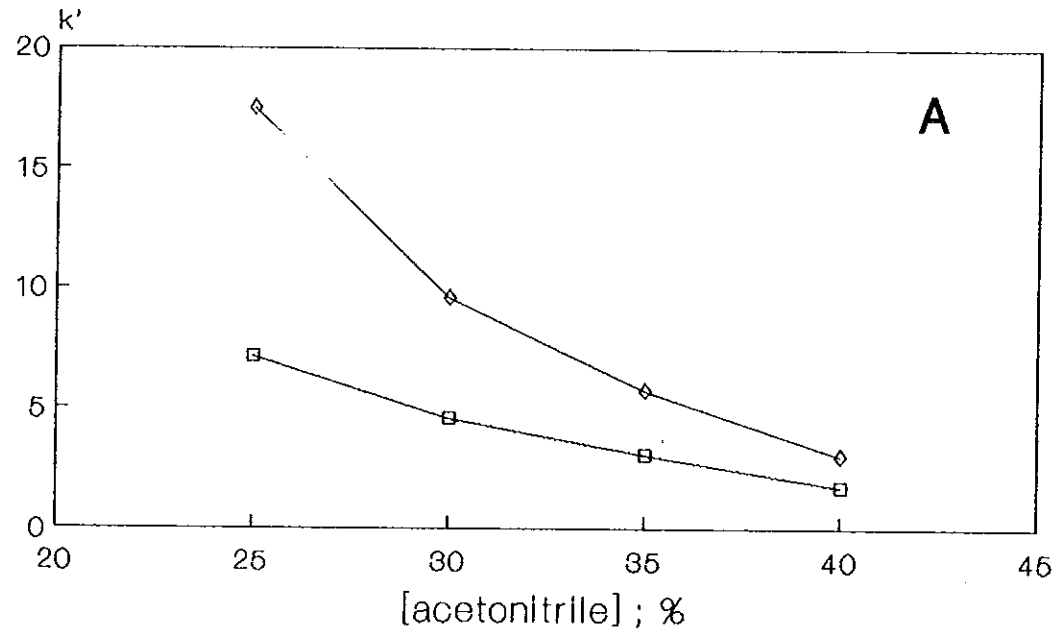
In this work, acetonitrile in water is used as a mobile phase. The t_r of these drugs are measured at four different concentration of acetonitrile in water. The capacity factor for each solute is calculated from the retention data using ;

$$k' = (t_r - t_o) / t_o \qquad 1)$$

where t_r is the retention time of the solute and t_o is the retention time of an unretained components. The experimental capacity factors are tabulated in Table 3. The capacity factors are plotted as a function of acetonitrile concentration, and the resultant graphs are given in Figure 5A. The relationship between the capacity factors and concentration of acetonitrile in the mobile phase is exponential (Figure 5A.) and the plot of log k' against [acetonitrile] is linear (Figure 5B.). The agreement of the view of a 10 % increase in concentration of organic solvent result in a two-to threefold decrease in the k' value of a given solute. (*Horvath, 1980 : 105-106*).

Table 3. Variation of the capacity factors of phenobarbital and pentobarbital as a function of acetonitrile concentration

acetonitrile concentration (%)	capacity factors (k')	
	phenobarbital	pentobarbital
25	7.16	17.51
30	4.55	9.58
35	3.08	5.71
40	1.72	3.03



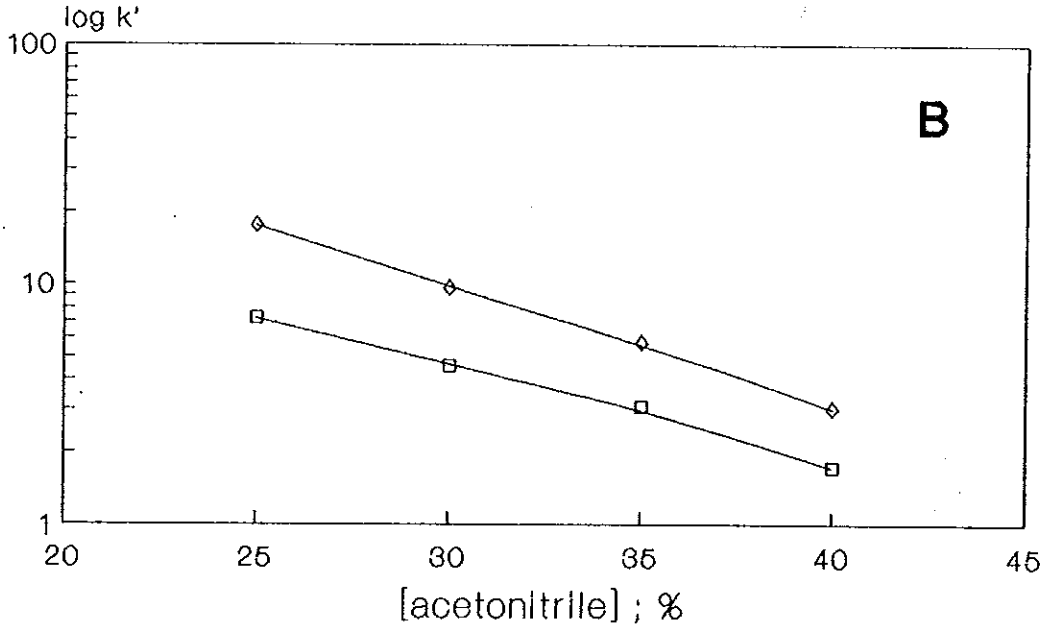


Figure 5. Dependence of k' (A), log k' (B) on [acetonitrile] in the mobile phase for phenobarbital(□) and pentobarbital(◊) : column, 10 μm C-18 μBondapak,300x3.9 mm ; flow rate, 1.0 ml/min ; mobile phase, acetonitrile in water ; t_o, 2.0 min. Each k' is the average of three values.

A2. pH Effects

Table 4. lists capacity factors of phenobarbital and pentobarbital in the presence of acetonitrile as a function of pH using C-18 column. The elution behavior of phenobarbital (pKa = 7.4) and pentobarbital (pKa = 8.2) have a relatively little change in retention with increasing pH until the pH approaches the pKa of these drugs ; then the retention decrease.

This effect is related to the ionization of the drug in a mobile phase as a function of pH causing to partition of nonionized and ionized forms of the drug into the hydrocarbon stationary phase(*Snyder , et al. , 1979 : 286-288*). Therefore, the amount of ionized forms of the drugs at low pH (pH < pKa) are less than at high pH (pH approaches pKa or pH > pKa) ; thus nonionized forms of these drugs at low pH are partitioned into hydrocarbon stationary phase as C-18 bonded silica column and retained at stationary phase, in that way capacity factors of these drugs appeared to increase. On the other hand, ionized form of these drugs at high pH are not retained at stationary phase so that the capacity factors of these drugs become decreasing. The plots of capacity factors versus pH of phosphate buffer in mobile phase for these drugs are shown in Figure 6.

Table 4. Influence of the pH in mobile phase on the capacity factors of phenobarbital and pentobarbital

pH	capacity factors (k')	
	phenobarbital	pentobarbital
5.0	3.15	5.68
5.5	3.52	6.73
6.0	3.52	7.05
6.5	3.62	7.18
7.0	3.15	7.00
7.4	2.65	5.69
7.8	1.80	4.17

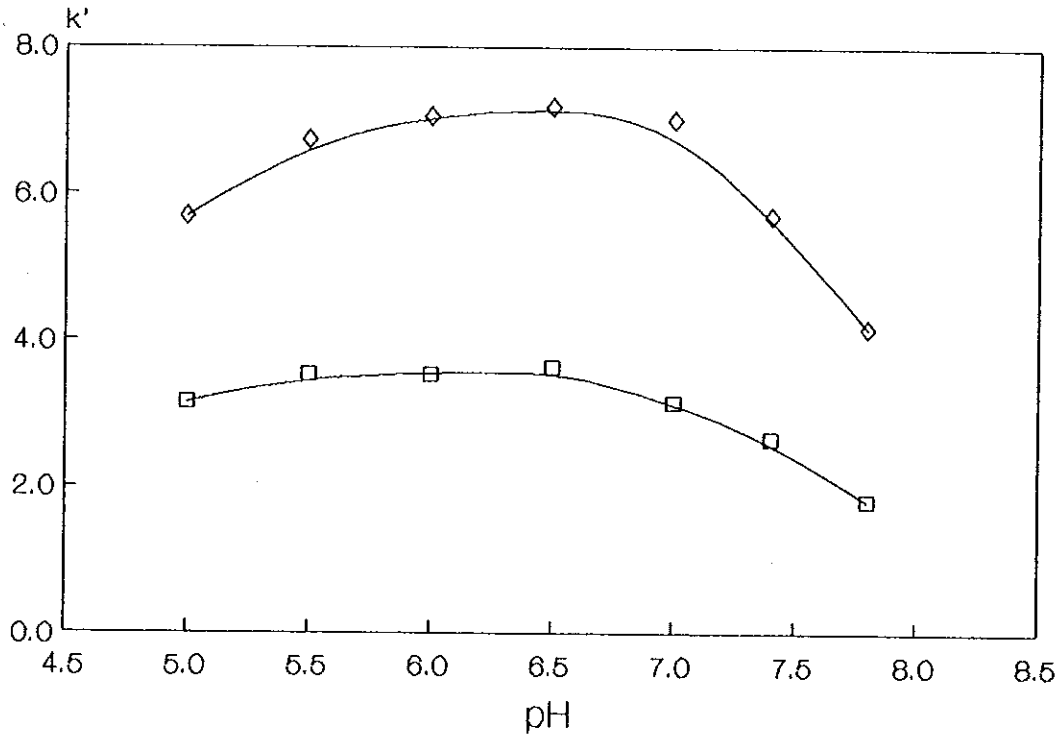


Figure 6. Chromatographic retention variation for phenobarbital (□) and pentobarbital (◊) with different pH of phosphate buffer in mobile phase. Chromatographic conditions are the same as those given in Figure 5. with the exception of mobile phase composition.

A3. Salt Effects

The addition of an electrolyte or neutral salts such as NaCl to the aqueous mobile phase in RPLC can influence chromatographic retention in several ways. It is also worth nothing that added electrolyte will play a significant role in the solubility of weak organic acid in a given solvent, for example, salting-in and salting-out behavior. A strong increase in the solubility of solute occurs with increasing salt concentration (salting-in effect). This increase reaches maximum and then decrease (salting-out effect) (*Voet and Voet, 1990 : 80*).

The data of salt effects on capacity factors are tabulated in Table 5. and chromatographic behavior of phenobarbital and pentobarbital plotted as k' vs [NaCl], are shown in Figure 7. It can be seen that the elution behavior of these drugs have a relatively little increase in retention with increase in NaCl concentration, indicating that the addition of salt decrease the solubility of the solutes in the mobile phase (salting-out effect).

Table 5. Salt effects in 45% acetonitrile as a mobile phase on capacity factors for phenobarbital and pentobarbital

[NaCl] in 45% acetonitrile (M)	capacity factors (k')	
	phenobarbital	pentobarbital
0.00	1.13	1.65
0.05	1.15	1.74
0.10	1.16	1.78
0.15	1.21	1.79
0.20	1.23	2.07

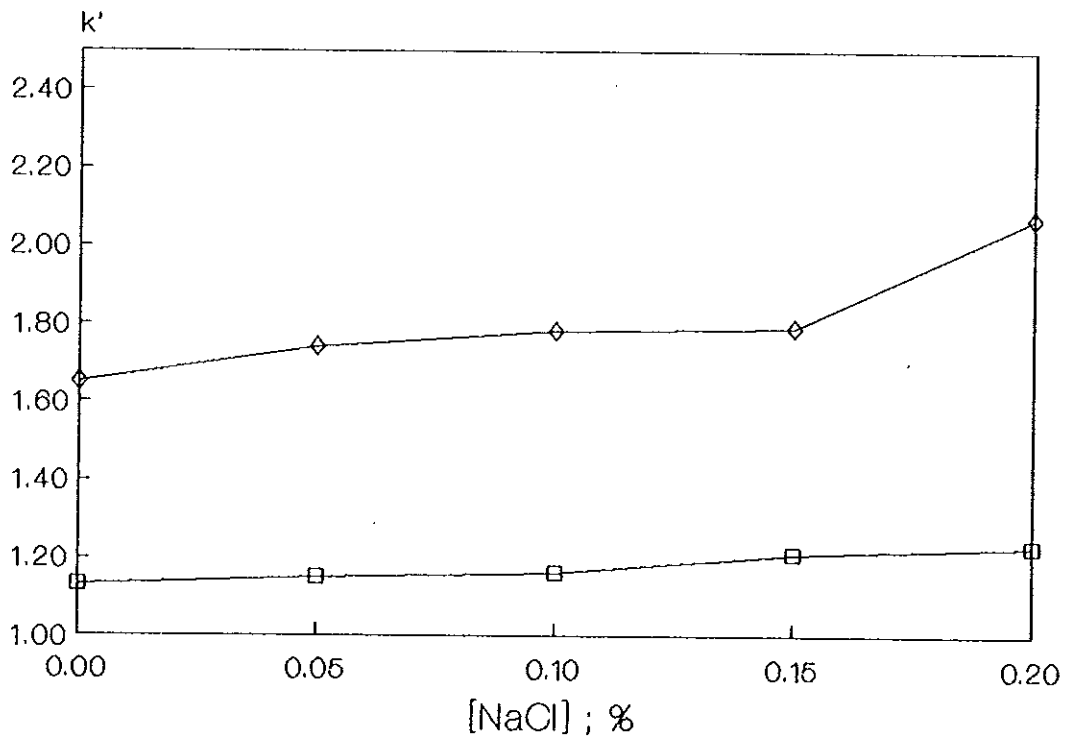


Figure 7. Dependence of capacity factors on [NaCl] in 45% acetonitrile as a mobile phase for phenobarbital (□) and pentobarbital (◊). Chromatographic conditions are the same as those given in Figure 5. with the exception of mobile phase composition.

A4. Effects of Linear Velocity

The column efficiency is normally expressed in terms of the *theoretical plate number* (N) of the column and can be calculated by using the formula :

$$N = 5.54 (t_r / W_{1/2})^2 \qquad 2)$$

where t_r is the retention time of the solute and $W_{1/2}$ is the peak width at the half height. The plate count N is approximately constant for different bands in the chromatogram, for a given set of operating conditions (a particular column and mobile phase, with mobile phase velocity and temperature fixed). The quantity N is proportional to column length (L), so that-other factors being equal-an increase in L result in an increase in N and better separation. This proportionally of N and L can be expressed in terms of the equation :

$$N = L / H \qquad 3)$$

where H is the so-called *height equivalent of a theoretical plate* (plate height) or HETP value. The quantity H (equal to L/N) measures the efficiency of a given column (operated under a specific set of operating conditions) per unit length of column. Small H or large N values mean more efficient columns, to which be usually favored by slow mobile phase velocity (v) with long columns packed with small particles.

A knowledge of controlling for column efficiency is depended on column H values on mobile phase velocity. The relationship of H and v for column efficiency are described by Van Deemter equation :

$$H = A + B/v + Cv \qquad 4)$$

where A, B, and C are constants for a given column, and also depend to some extent on the solutes, mobile phase and separation temperature, and v is the linear velocity. The first term, which describes Eddy diffusion, is determined primarily by packing structure of the column bed and particle diameter of the packing material. The second term is a measurement of longtitudinal or axial diffusion and is proportional to the diffusion rate of the solutes in the mobile phase. In RPLC the solutes diffusion rates are very low and this term is only significant at very low flow -rates. The third term is concerned with mass transfer, both in mobile phase and stationary phase.

In RPLC, the typical Van Deemter plots shows a decrease in efficiency with increasing linear velocity above the optimum. The agreement of experimental results are shown in Table 6. and Van Deemter plots are given in Figure 8.

Table 6. Influence of the linear velocity on the column efficiency for phenobarbital in C-18 column

flow rate (ml/min)	t_o (sec)	linear velocity [c] (cm/sec)	plate counts (N)	HETP
0.50	240	0.13	3000	0.0100
0.75	180	0.17	2400	0.0125
1.00	120	0.25	1800	0.0167
1.20	108	0.28	1300	0.0231
1.50	90	0.33	1100	0.0273

[c] Linear velocity is calculated from L / t_o.

L is referred to the length of column (30 cm).

t_o is referred to the retention time of an unretained components.

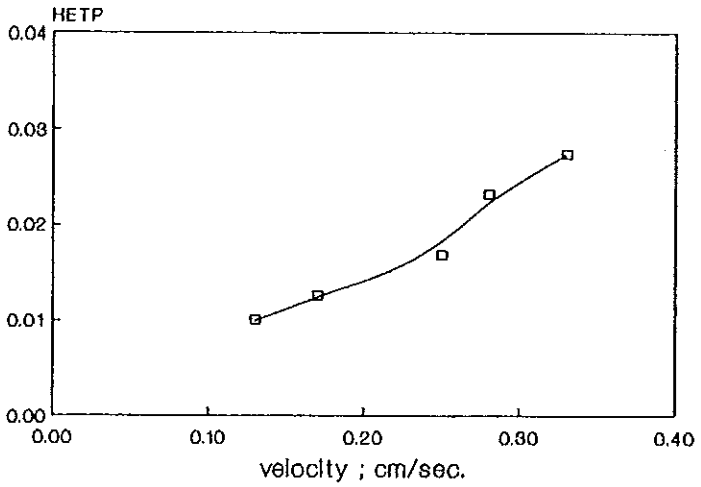


Figure 8. Van Deemter plots for phenobarbital in 30:70 of acetonitrile:phosphate buffer pH 5.0 as a mobile phase on C-18 column

A5. Selecting the Optimum Mobile Phase

Organic solvents such as acetonitrile is added to the mobile phase primarily as a way of conveniently change k' values (i.e., adjusting solvent strength). If the sample components elute at or near t_o, a lower concentration of the organic phase is indicated. Conversely, if the sample is too strongly retained, the organic solvent concentration must be increased. It is indicated that an increase in the concentration of acetonitrile produces a decrease k' values.

Not only does the choice of organic solvent optimizes k' values but pH also plays an important role. The pH control is no less important with valid as in pure aqueous media in order to maintain equilibrium constant within the column and to achieve a good reproducibility from run to run. Thus, a buffer can often exert special effects that contribute significantly to retention beyond simple pH control, since buffer species can interact with the solute and mobile and stationary phase components, changing the equilibrium distribution. It is thus clear that the chioce of particular buffer and its concentration will in practice be the result of the buffer capacity and the special effects on retention beyond pH control. Phosphate buffer is selected for its ability to control or maintain pH at the value selected. Buffer range of the phosphate is 3 to 9 and this is the pH range over which the buffer can be used. The molarity of the buffer in the mobile phase should range from about 0.001 to 0.500 M for most separations. Within this range of concentrations, the k' values of sample can be varied over wide limits by a variation of buffer concentration. The pH of the mobile phase can be varied to adjust selectivity. Selectivity in RPLC can be drastically altered by varying the pH of the mobile phase. If the sample components are acids or bases, the mobile phase pH will depend on the kind of sample to be separated. Thus, the result will

ultimately depend on the properties and concentration of the buffer, solute, and type of mobile and stationary phase. In this experiment, the mobile-phases are acetonitrile in phosphate buffer at pH 5.0 and 7.8. A series of solvent mixture at different pH are shown in Table 7.

It is known that the decrease in retention with increasing acetonitrile concentration or mobile phase pH for acidic solute, and the agreement results appeared. Although, the mobile phase pH 7.8 gives the high sensitivity, but blank plasma producing background response is eluted after 5 minutes, so that the peaks of these solutes are interfered from the peak of background response of blank plasma. For the mobile phase of 25:75 of acetonitrile : phosphate buffer pH 7.8, the retention time is too long to study. The phenobarbital peak with the mobile phase of 36:64 of acetonitrile : phosphate buffer pH 5.0 is interfered from the peak of background response of blank plasma. The mobile phase of 30:70 of acetonitrile : phosphate buffer pH 5.0 gives the appropriate selectivity, retention time of 8.29 and 13.36 minutes for phenobarbital and pentobarbital, respectively. These drug-peaks are uninterfered from background response of blank plasma. Therefore, this mobile phase ratio is the most appropriate for the determination of these drugs in rat plasma.

Table 7. Variation of the retention time and selectivity[d] of phenobarbital and pentobarbital as a function of ratio of acetonitrile and phosphate buffer at different pH in the mobile phase.

ratio of acetonitrile : phosphate buffer	pH	retention time (t_r)		selectivity (α)
		phenobarbital	pentobarbital	
30 : 70	5.0	8.29	13.36	1.80
36 : 64		7.14	11.10	1.77
25 : 75	7.8	8.04	21.20	3.18
30 : 70		5.60	10.34	2.32
35 : 65		5.22	9.20	2.24

[d] Selectivity is $\alpha = (t_2 - t_0) / (t_1 - t_0)$

t_1 is referred to the retention time of phenobarbital.

t_2 is referred to the retention time of pentobarbital.

A6. Determination of Drugs in Rat plasma

The calibration curves of phenobarbital and pentobarbital are constructed by plotting the peak heights (in cm) of particular drug versus known concentrations (in ppm) of each drug by using the data from Table 8. Typical calibration curves are shown in Figure 9 and 10.

It can be seen that linear calibration curves are obtained for phenobarbital and pentobarbital. These calibration curves are linear over most of the concentration range studied, and therefore, accurate results should be obtained using the linear portion of the calibration curve. A scientific calculator is used to calculate the regression line. These allow the subsequent calculation of the concentrations in unknown samples, which will be more accurate and precise than direct reading from a graph. However, the concentration of an unknown must fall in the linear portion of the curve.

Under the conditions of this study, retention times of the phenobarbital and pentobarbital peaks are sufficient to resolve them from each other and from solvent front. Retention times of phenobarbital and pentobarbital are 8.29 and 13.36 minutes, respectively. Figure11. illustrates the chromatogram of a rat plasma sample with a total elution time of 40 minutes per one injection. The blank plasma produces a background response completely eluted after 5 minutes with the exception of small peak occurring at 10 minutes and 39 minutes. Figure 12. illustrates the chromatograms of a rat plasma that are spiked with phenobarbital and pentobarbital. Because the plasma is diluted with acetonitrile for proteins precipitation by extraction procedure and given dilution factor of 2.5 so that the concentrations of drug with determination by calculating from linear regression are multi-

plied by dilution factor. The concentrations of drugs in rat plasma analyzed by RPLC are summarized in Table 10. and 11.

The precision of RPLC method is established by obtaining three replications for ten plasma samples. The relative standard deviations are found to be between ± 0.00 and ± 1.93 percent for phenobarbital and ± 0.00 and ± 6.07 percent for pentobarbital. The relative standard deviations for measurement of these drugs in rat plasma are tabulated in Table 10. and 11.

The reproducibility of RPLC method is established by obtaining three replications for the same standard solutions in each drug. Table 9. shows the relative standard deviations (RSD) for measurement of working concentration of drugs in plasma. The relative standard deviations are found to be between ± 0.17 and $\pm$ 2.04 percent and ± 0.78 and ± 1.48 percent for phenobarbital and pentobarbital, respectively.

Regression analysis by the least-squares method yielded a slope of 1.59 and an intercept of 0.18 ($r = 0.9999$) for phenobarbital and a slope of 3.53 and an intercept of - 0.14 ($r = 0.9998$) for pentobarbital.

Table 8. Working concentration and the percentage recovery of phenobarbital and pentobarbital from extraction plasma

drugs	concentration (ppm)	mean peak height (cm)		recovery (%)
		in aqueous	in plasma	mean ± s.d.
phenobarbital	3.00	1.925	1.775	92.21 ± 0.10
	6.00	3.900	3.675	94.23 ± 1.92
	12.00	7.600	7.450	98.03 ± 1.32
	24.00	15.300	15.025	98.20 ± 0.16
				mean recovery = 95.67 ± 2.34 %
pentobarbital	1.96	0.625	0.605	96.80 ± 0.80
	3.92	1.175	1.160	98.74 ± 1.25
	5.88	1.825	1.675	91.78 ± 0.11
	7.84	2.450	2.275	92.87 ± 0.88
	9.80	2.900	2.825	97.41 ± 0.87
				mean recovery = 95.52 ± 1.94 %

Table 9. Precision for measurement of working concentration of drugs in plasma

drugs	concentration (ppm)	mean measured value (ppm)	RSD (%)
phenobarbital	3.00	2.77	1.44
	6.00	5.65	2.04
	12.00	11.76	1.36
	24.00	23.57	0.17
		mean	± 2.85 %
pentobarbital	1.96	1.90	0.80
	3.92	3.87	0.78
	5.88	5.40	1.48
	7.84	7.28	1.10
	9.80	9.55	0.89
		mean	± 2.33 %

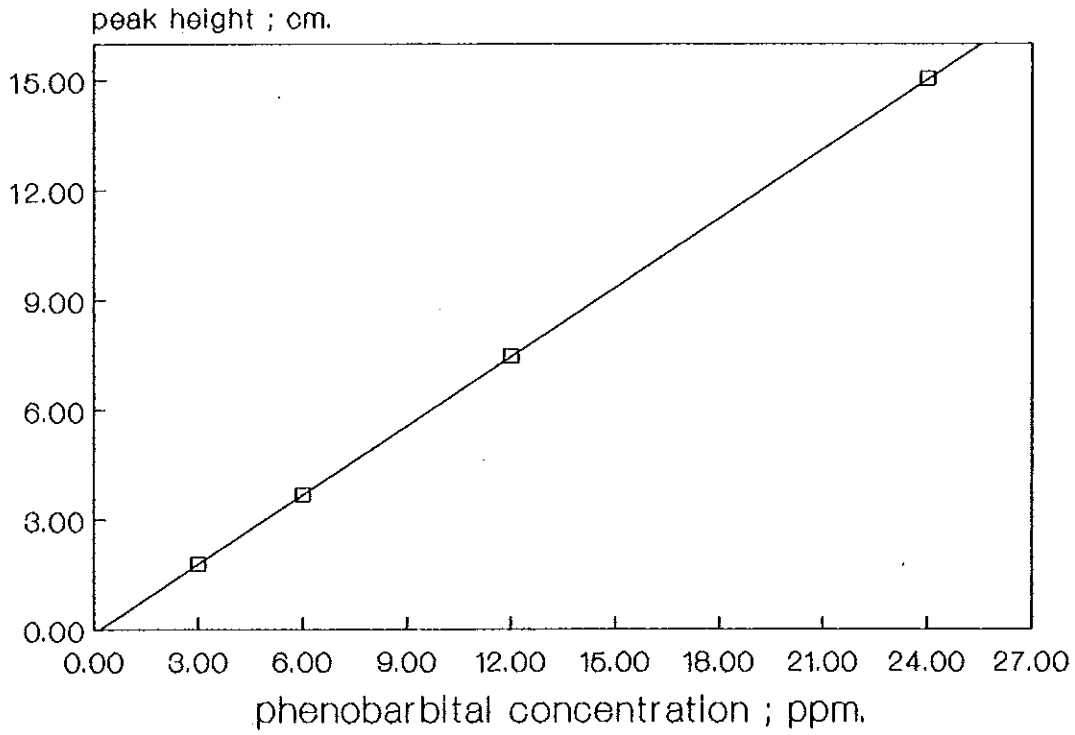


Figure 9. Calibration curve for the determination of phenobarbital from plasma obtained using RPLC. The RSD mean is ± 2.85 %. Regression analysis by the least-squares method yielded a slope of 1.59 and an intercept of 0.18 ($r = 0.9999$)

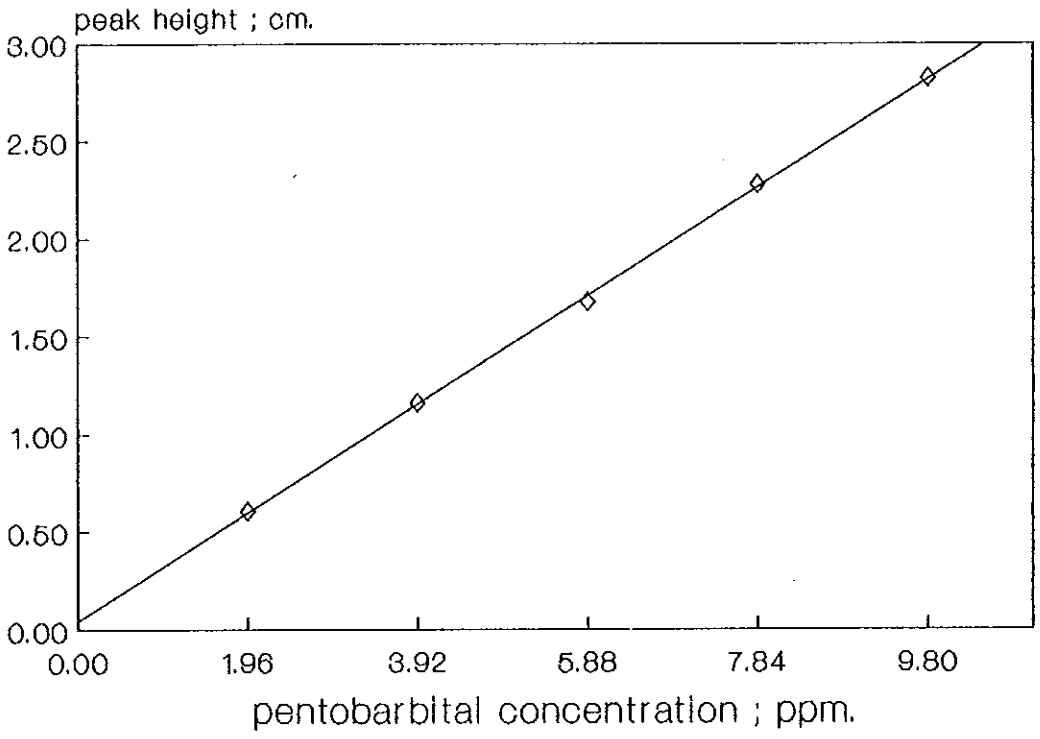


Figure 10. Calibration curve for the determination of pentobarbital from plasma obtained using RPLC. The RSD mean is $\pm$2.33 %. Regression analysis by the least-squares method yielded a slope of 3.53 and an intercept of - 0.14 (r = 0.9998)

Table 10. Mean measured concentration and concentration of phenobarbital from rat plasma by RPLC.

sample no.	mean peak height (cm)	mean measured concentration (ppm)	[e]concentration in rat plasma (ppm)	RSD (%)
MC_4	2.800	4.62	11.55	0.00
MC_8	4.150	6.76	16.90	0.00
MSO_4	5.825	9.42	23.55	1.27
MSO_9	5.550	8.98	22.45	0.89
MSP_5	4.750	7.71	19.28	1.93
MSP_{11}	6.600	10.65	26.63	0.00
FSO_2	13.575	21.70	54.25	0.18
FSO_{12}	12.675	20.28	50.70	0.20
FSP_{10}	4.000	6.52	16.30	0.00
FSP_{13}	9.450	15.16	37.90	0.53

[e] Concentration in rat plasma is calculated from multiplication of mean measured concentration with dilution factor (2.5).

Table 11. Mean measured concentration and concentration of pentobarbital from rat plasma by RPLC.

sample no.	mean peak height (cm)	mean measured concentration (ppm)	[e] concentraion in rat plasma (ppm)	RSD (%)
MC_4	0.800	2.68	6.70	0.00
MC_8	0.900	3.03	7.58	0.00
MSO_4	0.725	2.41	6.03	3.48
MSO_9	0.875	2.94	7.35	3.06
MSP_5	1.000	3.38	8.45	0.00
MSP_{11}	0.900	3.03	7.58	0.00
FSO_2	1.275	4.35	10.88	6.07
FSO_{12}	1.400	4.79	11.98	0.00
FSP_{10}	1.500	5.15	12.88	0.00
FSP_{13}	1.050	3.56	8.90	5.06

[e] Concentration in rat plasma is calculated from multiplication of mean measured concentration with dilution factor (2.5).

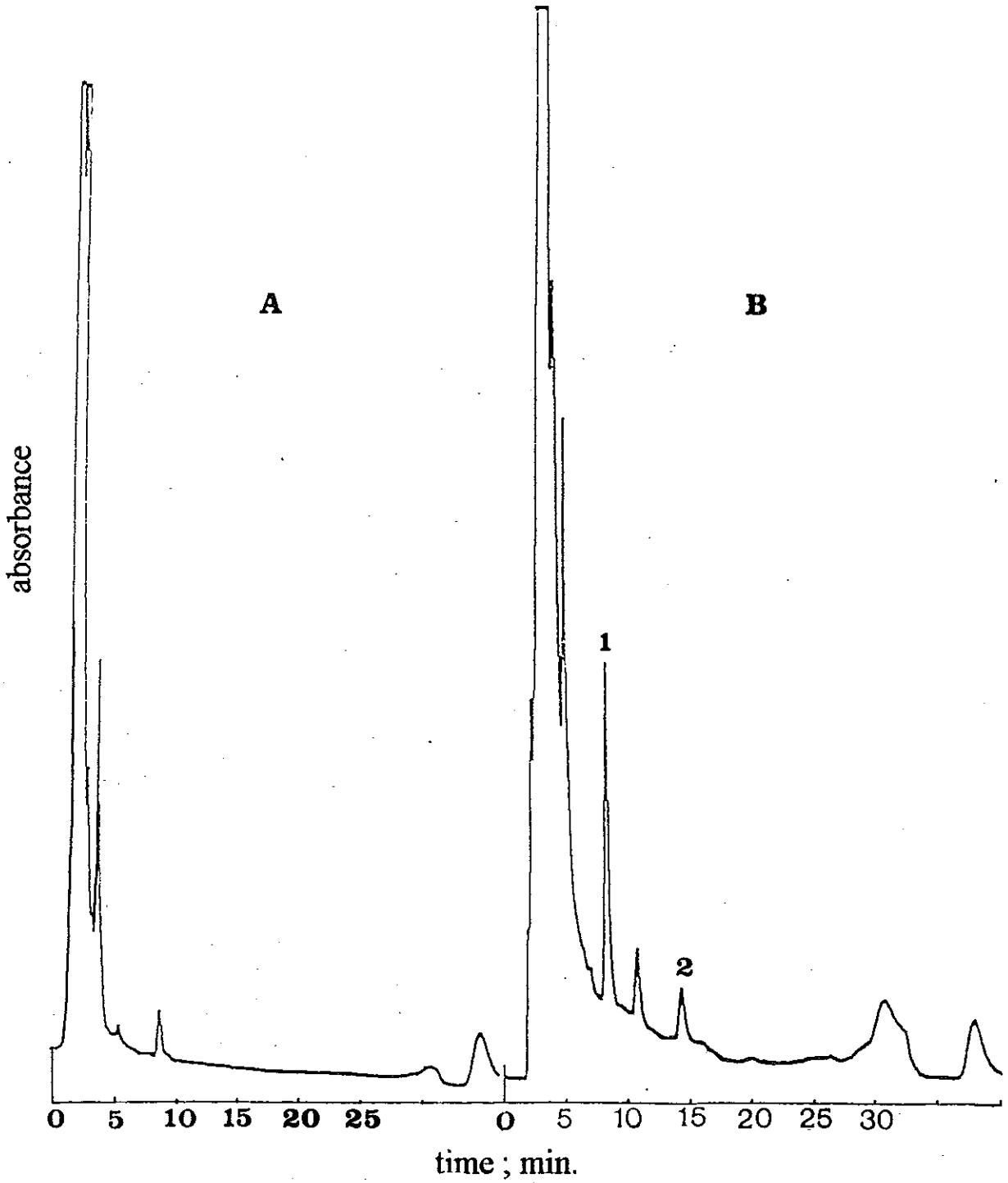


Figure 11. Chromatograms of (A) blank plasma and (B)plasma sample MSO_9 with (1) 8.98 ppm phenobarbital and (2) 2.94 ppm pentobarbital. Chromatographic conditions are as follows:column, C-18μBondapack, 300x3.9 mm.i.d.; mobile phase, 30:70 of acetonitrile : phosphate buffer pH 5.0 ; flow rate, 1.0 ml/min ; chart speed, 2.0 mm/min.

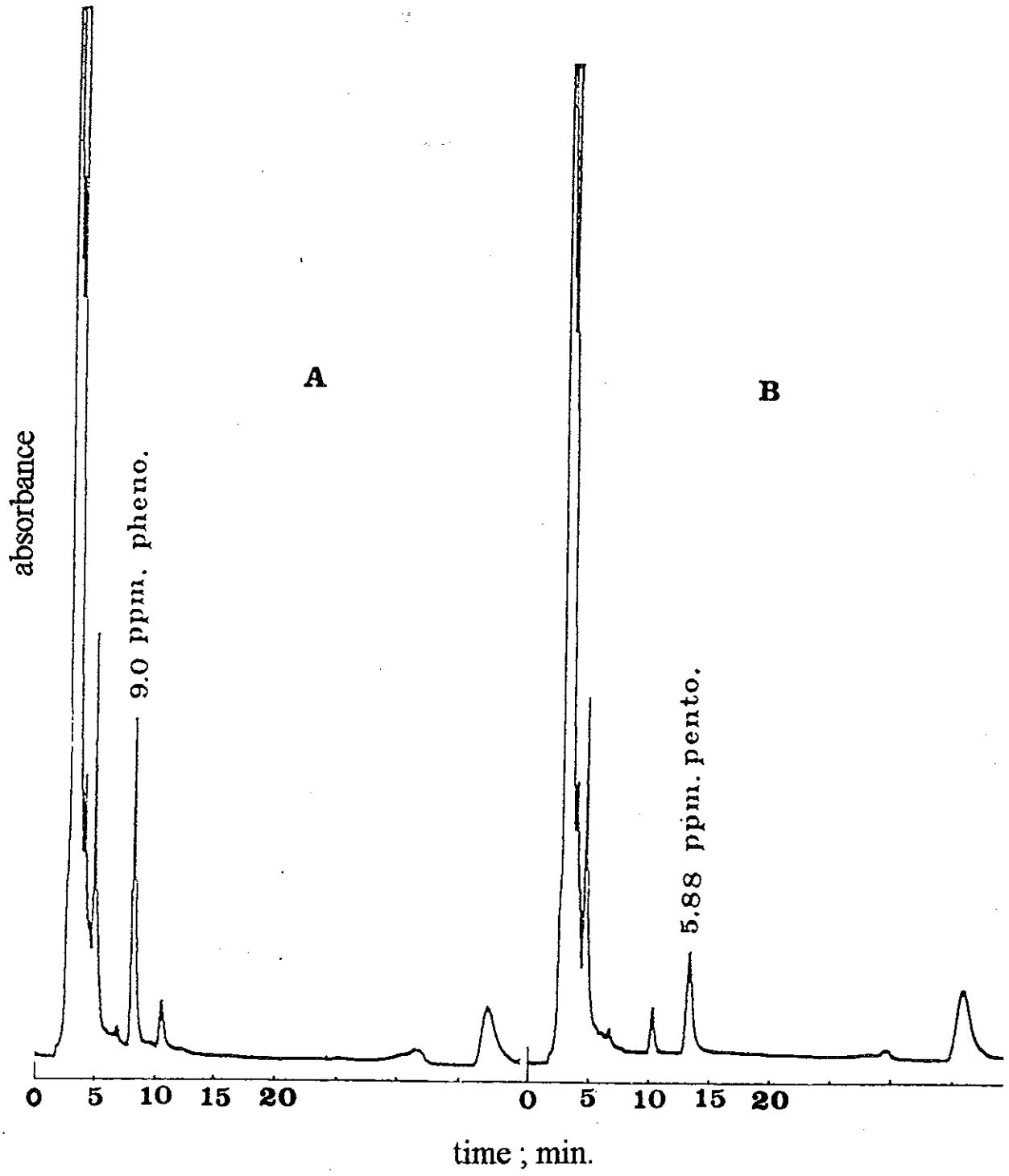


Figure 12. Chromatograms of spiked drugs in blank plasma with (A) 9.00 ppm phenobarbital , (B) 5.88 ppm pentobarbital. Chromatographic conditions are the same as those given in Figure 11.

A7. Recovery

The analytical recovery of drugs are measured by extracting plasma containing known amounts of drug. The peak height of extracted solutions (clear supernatant) is compared with known amounts of drugs in aqueous solutions. Aqueous solutions are obtained from unextracted solutions that contained the same concentration of each compound. The results are given in Table 8. The everage analytical recoveries of phenobarbital and pentobarbital are approximately 95 percent.

Limits of Detection

The minimum amount of a species that can be reliably seen on the chromatogram is the detection limit. Usually detection limits are measured with an individual species in a standard solution. In a real sample, the detection limit for these species will usually be higher due to baseline disturbance or interference from the matrixes or orther species. In this experiment, the detection limits of phenobarbital and pentobarbital are 1.18 and 3.14 ng, respectively. These are measured manually from chromatograms that give individual peak to where signal equals to two times of the baseline noise.

B. Micellar Mobile Phase Systems

B1. Surfactant Concentration Effects

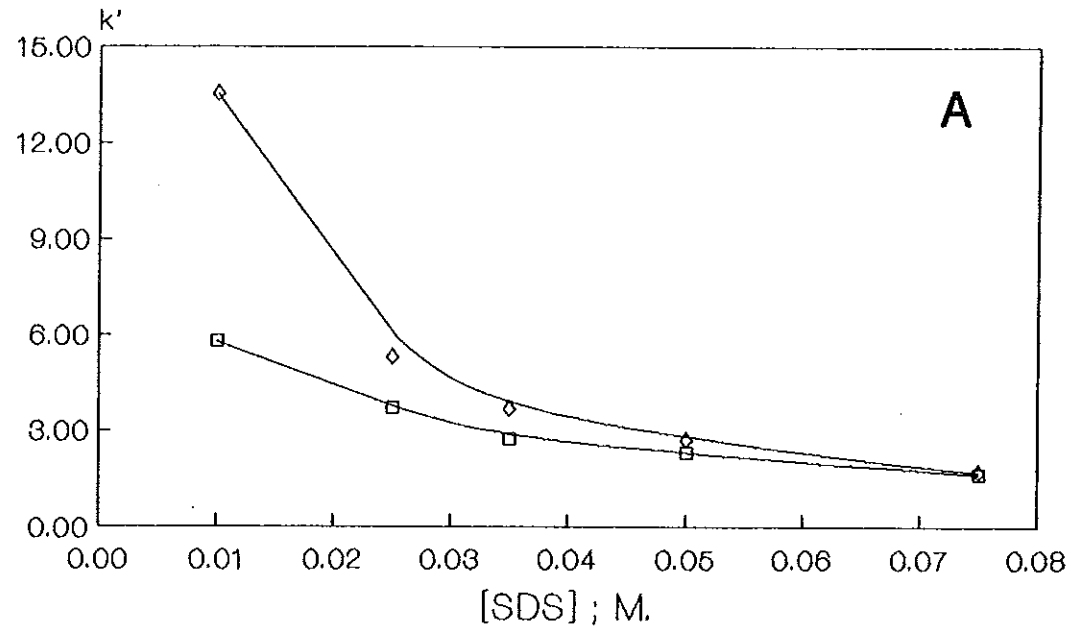
In this work, SDS is used for the surfactant as a micellar solution that dissolved in water. The surfactant concentration in the micelle ($[M_m]$) is defined by :

$$[M_m] = [\text{surfactant}] - \text{cmc} \qquad 5)$$

where [surfactant] is the total concentration of surfactant in solution and cmc is the critical micelle concentration (*Arunyanart and Cline Love, 1984 : 1557-1560*). The cmc for SDS is 8.2×10^{-3} M (*Rosen, 1978 : 94*). The retention times of the test solutes are measured at five different SDS concentration. The capacity factor for each solute is calculated from the retention data using equation 1). If a micellar solution mimics the behavior of a conventional reversed phase mobile phase, then increasing the surfactant concentration should result in a decrease in retention. Indeed, the decrease in retention with increasing micelle concentration is found and shown in Table 12., when capacity factors are plotted against surfactant concentration, as in Figure13A.. It can be seen that the relation-ship between the k'and [SDS] is parabolic (Figure 13A.)and the plot of 1/k' against [SDS] is linear (Figure 13B.). The dependence of capacity factor on micelle concentration is mainly controlled by hydrophobic interactions of the test solutes with the micellar assembly and stationary phase.

Table 12. Variation of the capacity factors of phenobarbital and pentobarbital from a CN-RP column as a function of micelle concentration in the mobile phase

drugs	total [SDS] in the mobile phase (M)				
	0.010	0.025	0.035	0.050	0.075
	[SDS] in the micelle ([M_m]) (M)				
	0.0018	0.0168	0.0268	0.0418	0.0668
	capacity factors (k')				
phenobarbital	5.78	3.69	2.73	2.29	1.63
pentobarbital	13.51	5.29	3.67	2.70	1.69
	reciprocal capacity factors (1/ k')				
phenobarbital	0.1730	0.2710	0.3663	0.4367	0.6135
pentobarbital	0.0740	0.1890	0.2725	0.3704	0.5917



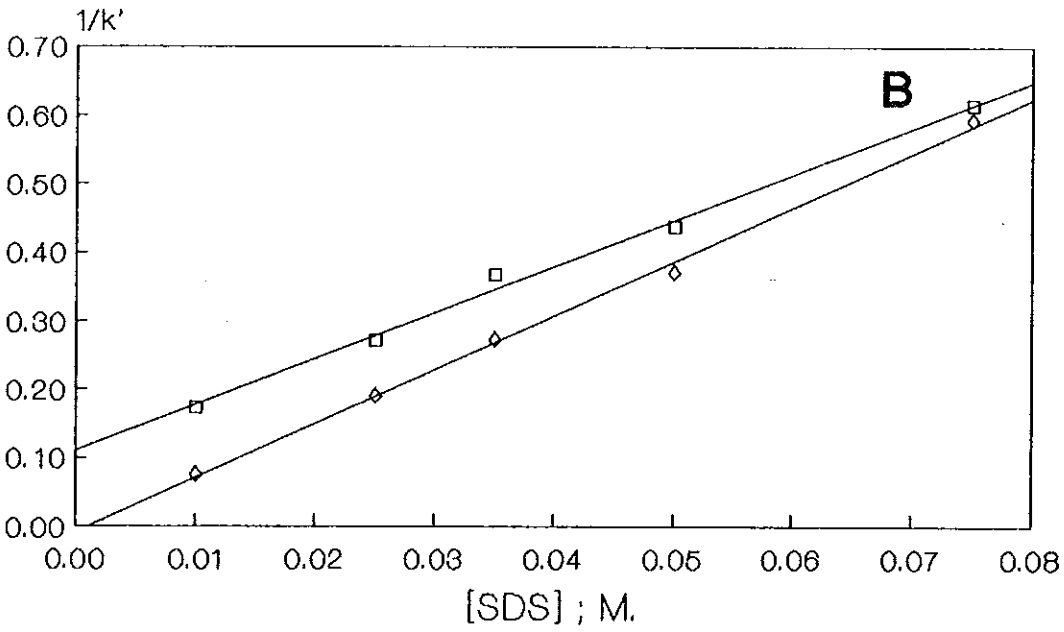


Figure 13. Dependence of k' (A), 1/k' (B) on [SDS] in the mobile phase for phenobarbital (□) and pentobarbital (◊) : column, 10 μm μBondapak CN-RP, 3.9x150 mm ; flow rate 0.5 ml/min ; mobile phase, SDS in water ; t_o 2.0 min. Each k' is the average of the value that obtained from three replicate injection.

B2. Micelle Bulk pH Effects

Influence of the pH in micellar mobile phase on retention of ionizable drugs such as phenobarbital (pKa = 7.4) and pentobarbital (pKa = 8.2) can be studied by using cyano-bonded silica column. Table 13. lists capacity factors of these drugs in various concentration of SDS as a function of pH. At high concentration of SDS, the retention of both drugs have relatively a little change with increasing pH of mobile phase (Figure 14. A and B). At lower SDS concentration (see also Figure 14. A and B), the capacity factor of phenobarbital is clearly decreased when pH approaches pKa s. Because of the aqueous solution pKa value for phenobarbital is within the pH range examined (pH 4.5-7.5) except pentobarbital, so that the changing pH of mobile phase on capacity factor of pentobarbital is not clear.

Figure 15. A and B show the behavior of phenobarbital and pentobarbital on cyano column plotted as k' vs [SDS] at various mobile phase pH. It can be seen that the capacity factors decrease with increasing [SDS] and seeming independent of pH for pentobarbital, in contrast for phenobarbital at low concentration of SDS. Therefore, suitable selectivity and retention times of both drugs should be obtained by manipulation of the mobile phase concentration and pH.

The chromatographic conditions for the experiment provide a neutral form for phenobarbital at low pH, and anionic form at high pH but pentobarbital in its dominant neutral form under this condition. This separation process is mainly controlled by hydrophobic interactions for neutral form, and electrostatic repulsion between negatively charge of micelle head group and anionic species of the drug. Thus phenobarbital is eluted more rapidly than pentobarbital from column at high pH.

Table 13. Influence of the pH in SDS mobile phase on the capacity factors of phenobarbital and pentobarbital from a CN-RP column as a function of SDS concentration

SDS concentration (M)	pH	capacity factors (k')	
		phenobarbital	pentobarbital
0.010	4.5	5.59	11.13
	5.5	5.11	10.30
	6.5	4.78	10.12
	7.5	2.70	9.28
0.025	4.5	4.06	5.21
	5.5	4.00	5.13
	6.5	3.58	4.70
	7.5	2.15	4.69
0.050	4.5	2.63	3.01
	5.5	2.55	2.72
	6.5	2.51	2.72
	7.5	1.63	2.59
0.075	4.5	1.23	1.23
	5.5	1.23	1.23
	6.5	1.22	1.23
	7.5	1.10	1.22

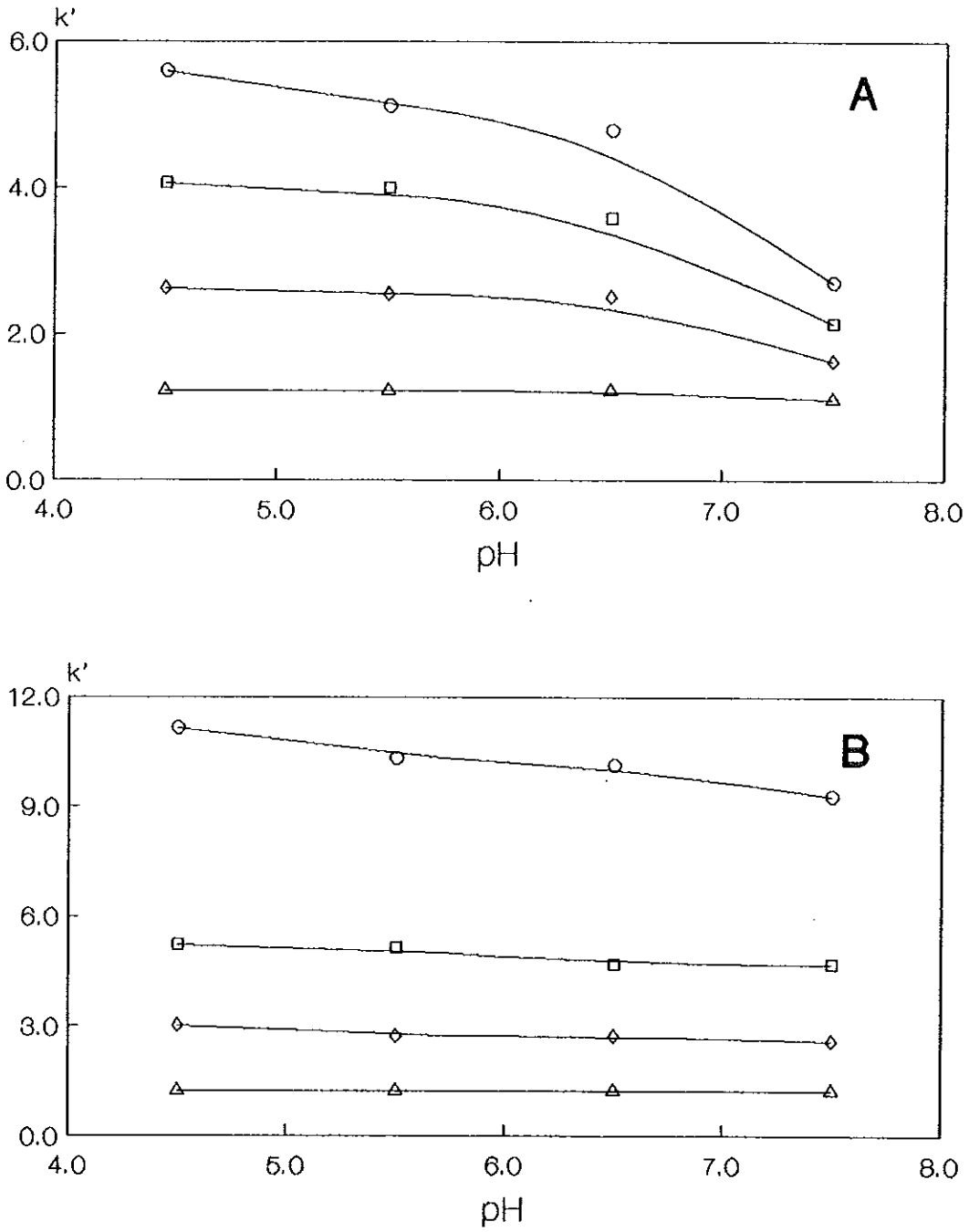


Figure 14. Chromatographic retention variation for phenobarbital (A) and pentobarbital (B) with pH at various concentration of SDS : 0.010 M (o), 0.025 M (□), 0.050 M (◊) and 0.075 M (Δ) ; column, 10 μm μBondapak CN - RP, 3.9 x 150 mm ; flow rate 0.5 ml/min ; t_o = 2.0 min ; SDS in phosphase buffer. Each k' is the average of the value that obtained from three replicate injection.

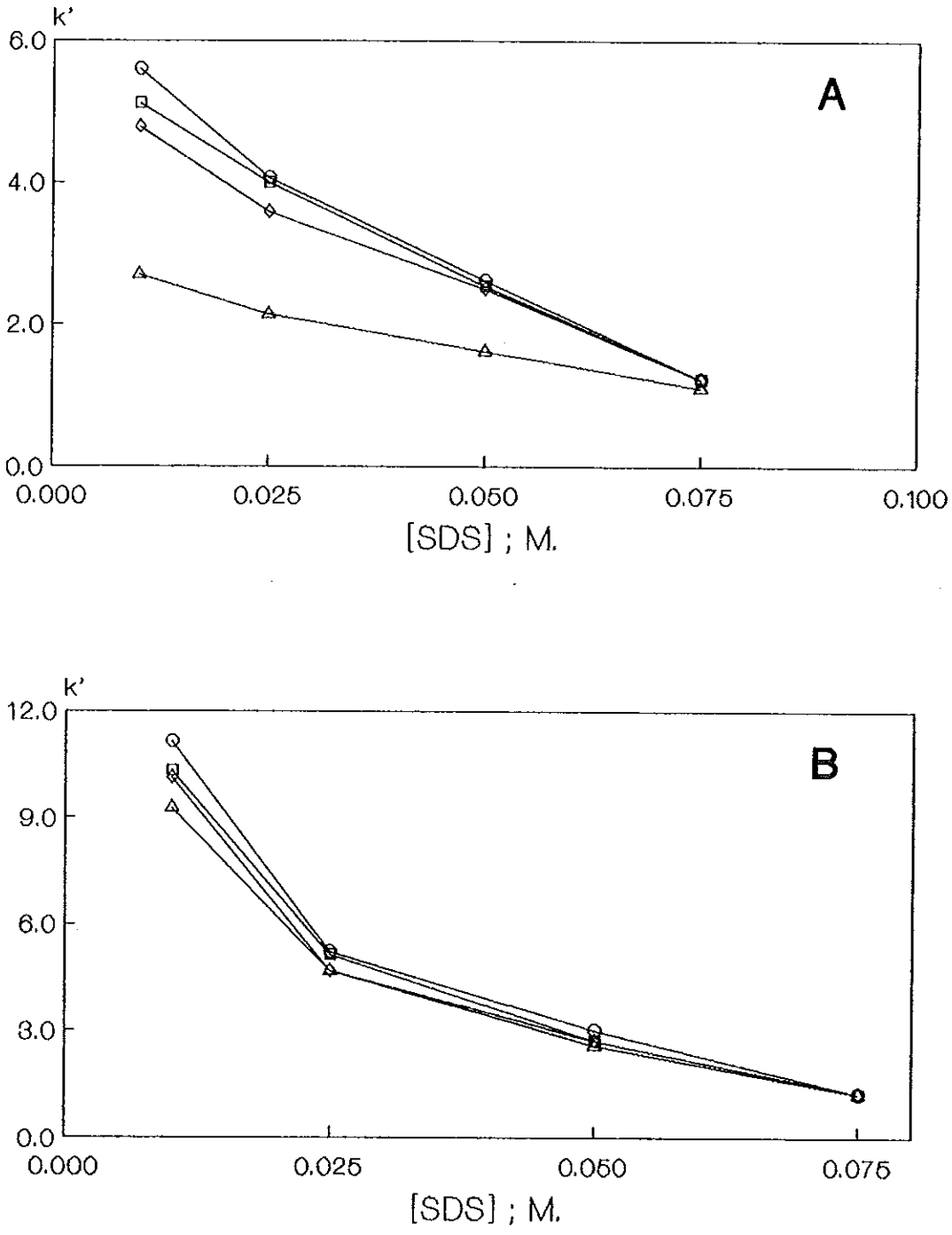


Figure 15. Chromatographic retention variation for phenobarbital (A) and pentobarbital (B) with concentration of SDS at various mobile phase pH : pH 4.5 (o) , pH 5.5 (□) , pH 6.5 (◊) and pH 7.5 (Δ). Chromatographic condition are the same as those given in Figure 14.

B3. Salt Effects

B3.1 *Salt Effects on Capacity Factor*

In micellar systems, with added salt such as sodium chloride to the micellar mobile phase. The term "salting-in" and "salting-out" apply to the solute becoming more or less soluble in the bulk aqueous phase, respectively.

The results of salt effects on capacity factor are tabulated in Table 14. and chromatographic behavior of phenobarbital and pentobarbital plotted as capacity factors versus sodium chloride concentration are shown in Figure 16. It can be seen that the elution behavior of these drugs have a relatively increasing in retention with an increase in concentration of NaCl, indicating that these solutes can be less soluble in the bulk aqueous phase with addition of salt to micellar mobile phase (salting-out effects).

Table 14. Influence of NaCl in SDS mobile phase on the capacity factors of phenobarbital and pentobarbital from a CN column as a function of SDS concentration

[NaC]	capacity factors (k')	
in 0.05 M SDS (M)	phenobarbital	pentobarbital
0.00	2.29	2.70
0.05	3.11	3.71
0.10	3.28	4.27
0.15	3.29	4.56
0.20	3.61	5.05

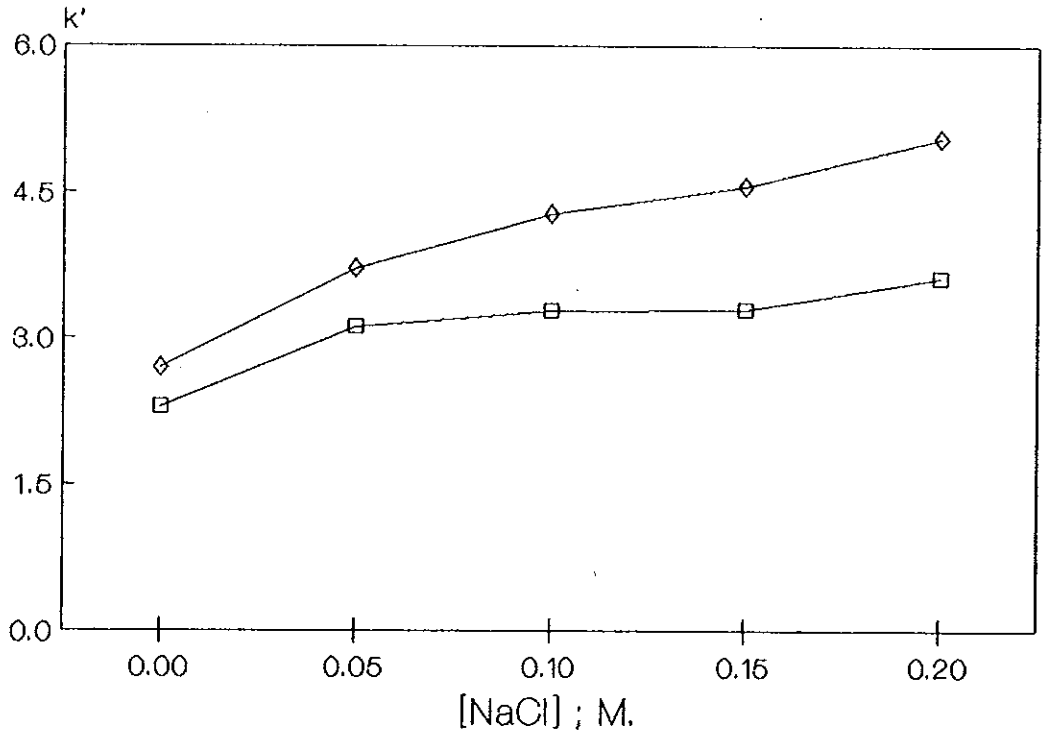


Figure 16. Dependence of capacity factors on [NaCl] in SDS mobile phase for phenobarbital (□) and pentobarbital (◊) : column,10 μm μBondapak CN-RP, 3.9x150 mm, flow rate 0.5 ml/min, NaCl in 0.05 M SDS. Each k' is the average of the values obtained from three replicate injections.

The effects of salt on capacity factor of phenobarbital and pentobarbital are studied. The results obtained from these studies are compared with non-salt in the same concentration of SDS mobile phase for these drugs. Table 15. show the results of different values of capacity factor of phenobarbital and pentobarbital with salt and non-salt in SDS mobile phase. The graphs correlate to these results are depicted in Figure 17.

It is found that the different values of capacity factor of pentobarbital are more than phenobarbital. This means that the addition of NaCl to SDS mobile phase affects pentobarbital more than phenobarbital. Thus, pentobarbital can be separated from phenobarbital by addition of NaCl to SDS mobile phase.

Table 15. Salt effect on phenobarbital and pentobarbital in SDS mobile phase.

drugs	capacity factors (k')				[f]different values of k' at the same [SDS]	
	[SDS] ; M		0.15M NaCl in [SDS]			
	0.025	0.050	0.025	0.050		
phenobarbital	3.69	2.29	4.74	3.29	1.05	1.00
pentobarbital	5.29	2.70	7.05	4.56	1.76	1.86

[f] Different values of capacity factors are defined by :

(capacity factors on SDS with NaCl) - (capacity factors on SDS without NaCl)

at the same concentration of SDS.

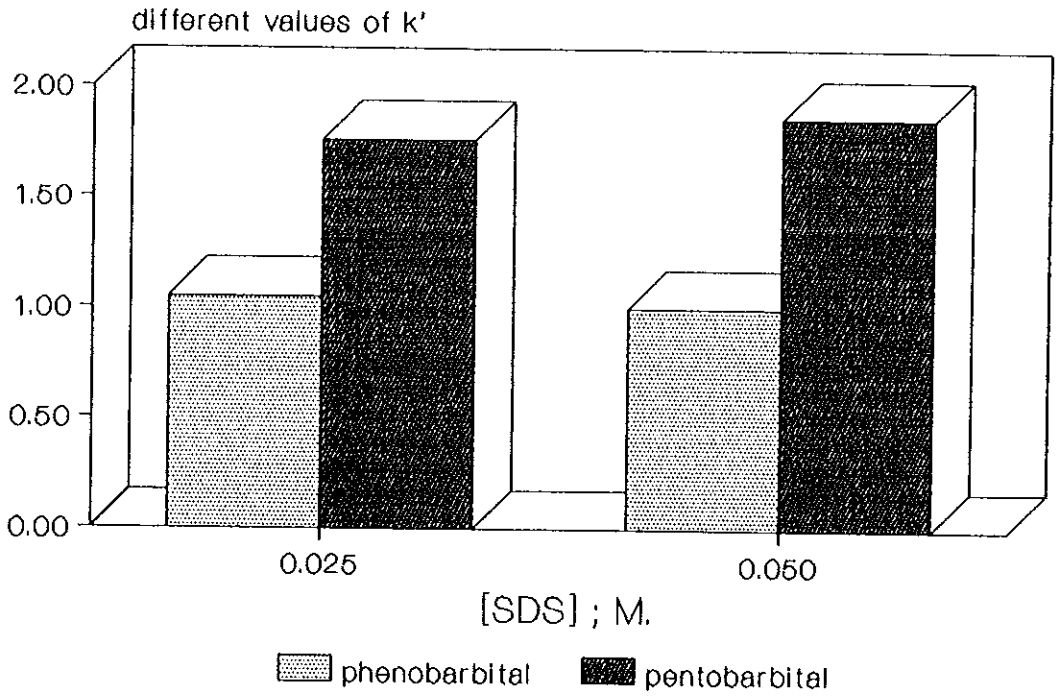


Figure 17. The effect of NaCl in SDS mobile phase for phenobarbital and pentobarbital on different values of capacity factor between salt (NaCl) and non-salt in the SDS mobile phase

B3.2 *Salt Effect on Separation Factor (∝)*

The results obtained from the studies of salt effects on separation factor are compared with non-salt in the same concentration of SDS mobile phase, as can be seen in Table 16. The correlation of these results and chromatograms of phenobarbital and pentobarbital are shown in Figure 18. A to E.

It is found that separation is improved by addition of various amount of NaCl to SDS mobile phase (Figure 18 B. to E). Thus, increasing in NaCl results in an increase in separation factor.

Table 16. Salt effects on separation factor (∝)

[NaCl] in 0.05 M SDS (M)	[g] V_1 of phenobarbital	[h] V_2 of pentobarbital	[i] separation factor (∝)
0.00	3.29	3.70	1.17
0.05	4.11	4.71	1.19
0.10	4.28	5.27	1.30
0.15	4.29	5.56	1.38
0.20	4.61	6.01	1.39

[g] V_1 referred to the elution volume of phenobarbital.

[h] V_2 referred to the elution volume of pentobarbital.

[i] ∝ referred to separation factor is calculated by $\propto = (V_2 - V_0) / (V_1 - V_0)$

where V_0 referred to void volume.

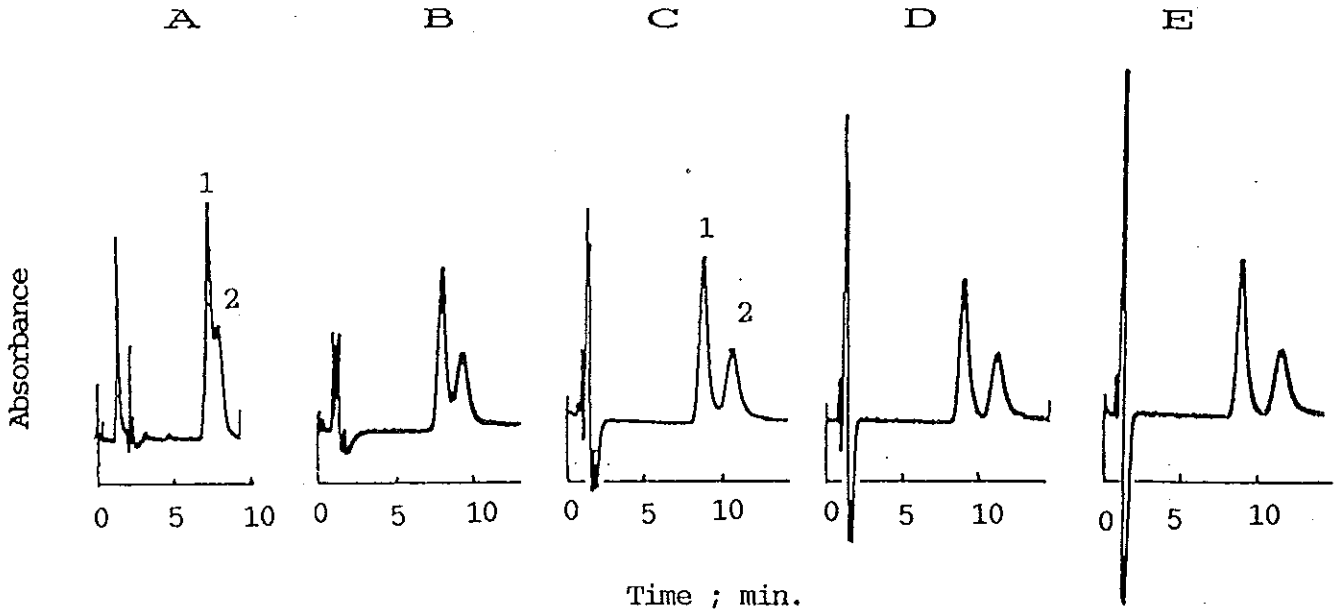


Figure 18. Separation of (1) phenobarbital and (2) pentobarbital via micellar chromatography. Chromatographic conditions are as follows : column, CN-RP μBondapak, 150x3.9 mm. i.d. ; mobile phase, (A) 0.05 M. SDS, (B to E) same concentration of SDS addition of 0.05, 0.10, 0.15 and 0.20 M. NaCl, respectively ; flow rate, 0.5 ml/min. ; t_o = 2.0 min.

B4. Organic Modifier Effects

The addition of short-chain alcohols (C1-C4) to the micellar mobile phase alters the retention mechanism by shifting the equilibria of the solutes from the stationary phase and the micelle toward the bulk aqueous phase. This leads to a reduction in the capacity factor (*Domingo, et al., 1992 : 845*).

A comparative study is performed to observe the effect of different alcohols added to the SDS micellar mobile phase, on the retention of phenobarbital and pentobarbital. For the preparation of these mobile phase a 0.01M SDS solution is selected. The alcohols used are methanol, 1-propanol and butanol at different concentration, i.e., 1%, 2% and 5% of each alcohol. Table17. gives the results of the studies. The graphs correlated to these results are showh in Figure 19.

It can be seen that the presence of alcohols in the SDS micellar mobile phase produce a decreasing in capacity factor ; all are lower than with a 0.01M SDS mobile phase without modifiers. The higher concentration of alcohol in the micellar mobile phase is also more decreased in retention, and addition of butanol produces the smallest capacity factors as a compare with 1-propanol and methanol.

It is known that the elution behavior of solute depends on solvent strength (*Snyder, et al, 1979 : 285*). Because of butanol has a highest solvent strength compared with the other alcohols in this study, the least retentions of phenobarbital and pentobarbital are appeared.

Table 17. Influence of the organic modifiers in the SDS micellar mobile phase on the capacity factors of phenobarbital and pentobarbital from a CN-RP column

type of organic modifier	[modifier] in 0.01M SDS (%)	capacity factors (k')	
		phenobarbital	pentobarbital
-	0.0	4.70	8.50
methanol	1.0	4.06	7.09
	2.0	3.75	6.55
	5.0	3.20	5.29
1-propanol	1.0	3.70	5.64
	2.0	3.56	5.20
	5.0	2.72	3.62
butanol	1.0	3.27	4.74
	2.0	2.74	3.70
	5.0	2.59	3.25

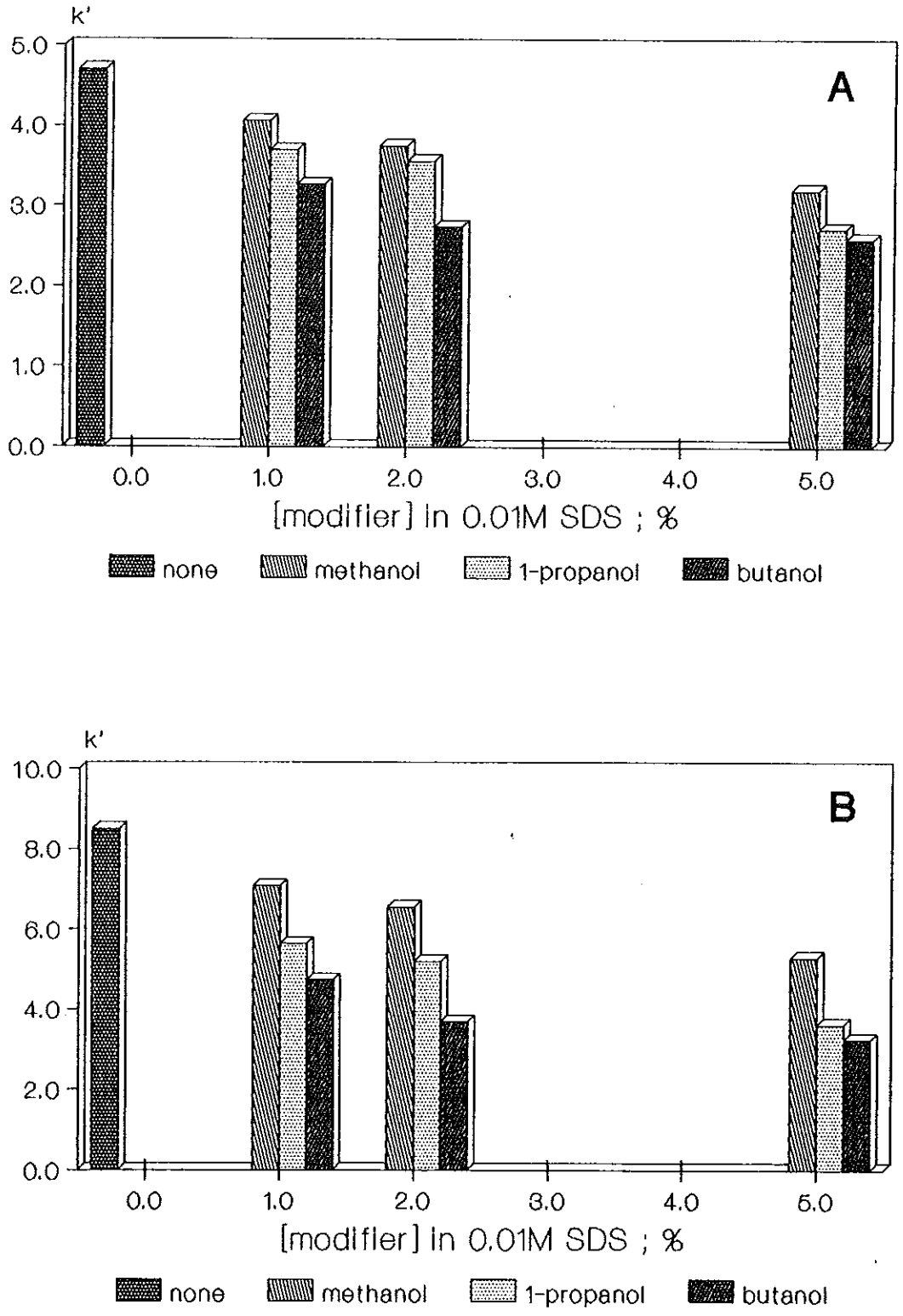


Figure 19. Dependence of capacity factors on organic modifiers in SDS mobile phase for (**A**) phenobarbital and (**B**) pentobarbital

B5. Effects of Mass Transfer

In conventional RPLC the typical Van Deemter plot shows a decrease in efficiency with increasing linear velocity above the optimum (*Yarmchuk, et.al., 1984 : 54-55*). A similar but enhanced effect is seen with micellar mobile phase as shown in Figure 20.and is further evidence of mass transfer effects. The HETP obtained by using a conventional RPLC is less than a micellar mobile phase. This is also shown in Table18. where the number of plate per column, N, drops to low values for micellar system. Unfortunately, mass transfer for this solute in micellar system are slow when compared with conventional RPLC.

Table 18. Comparison of mass transfer for phenobarbital by conventional RPLC (30:70 of acetonitrile : phosphate buffer pH 5.0) and micellar chromatography (0.01 M SDS in phosphate buffer pH 5.0).

flow rate (ml/min)	method							
	micellar mobile phase				[j] conventional reversed phase			
	[k] t_o (sec)	[l] v (cm/sec)	[m] N	[n] HETP	t_o (sec)	v (cm/sec)	N	HETP
0.50	120	0.13	890	0.0169	240	0.13	3000	0.0100
0.75	90	0.17	860	0.0174	180	0.17	2400	0.0125
1.00	60	0.25	700	0.0214	120	0.25	1800	0.0167
1.20	54	0.28	550	0.0273	108	0.28	1300	0.0231
1.50	45	0.33	470	0.0319	90	0.33	1100	0.0273

[j] Conventional reversed phase results are employed as shown in Table 6.

[k] t_o is referred to the retention time of an unretained components.

[l] v is referred to linear velocity to be calculated from L / t_o

[m] N is referred to plate counts per column where using equation 2).

[n] HETP is referred to chromatographic efficiency where using equation 3).

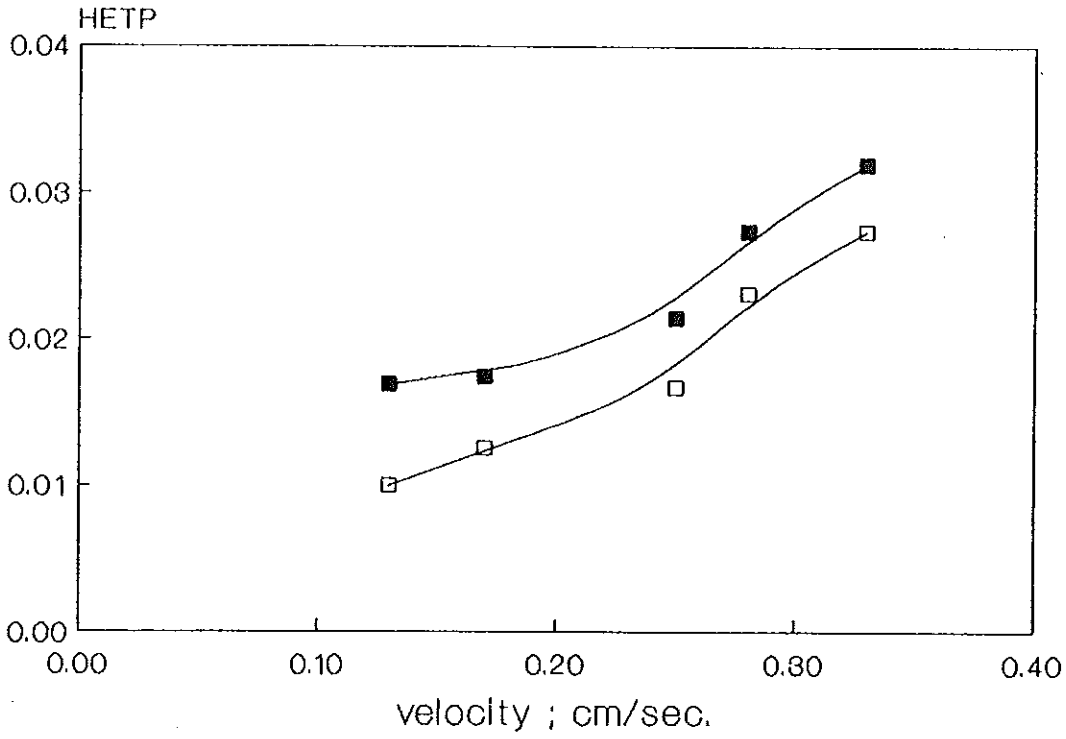


Figure 20. Van Deemter plots for phenobarbital in 0.010 M SDS in phosphate buffer pH 5.0 (closed symbols) and 30 % CH_3CN in phosphate buffer pH 5.0 (opened symbols).

B6. Selecting the Optimum Micellar Mobile Phase

Selection of micellar mobile phase for optimum retention time for the determination of phenobarbital and pentobarbital by MC are found by trial and error about mobile phase compositions. The various conditions and compositions of micellar mobile phase are studied, such as the effects of micelle concentration, pH, [NaCl], and the combination of pH and [NaCl] on retention times. The results of these studies are shown in Table 19.

The micellar mobile phase in studies of SDS concentration is unappropriate for use. This because blank plasma producing background response is eluted after 5 to 6 minutes for these micellar mobile-phases studies, so that the peaks of phenobarbital are interfered by the peak of background response of blank plasma.

The elution behavior of these drugs have a relatively increase in retention with increasing [NaCl] (see also in B3.), and these results are agreement. Although, the micellar mobile phase of 0.05 M SDS with 0.20 M NaCl gives the appropriate retention time, but the sensitivity of the drug-peaks are unpredictable. Thus, micellar mobile-phases with the addition of NaCl are not appropriate to use.

The elution behavior of both drugs have a relatively a little decrease in retention with increasing pH. Because blank plasma producing background response is eluted after 4 minutes, and does not interfere the drug-peaks. The micellar mobile phase of 0.01 M SDS in phosphate buffer pH 5.0 gives the appropriate retention time as 5.30 and 9.56 minutes for phenobarbital and pentobarbital, respectively, and the sensitivity of the drug-peaks are fair. Therefore, the micellar mobile phase of 0.01 M SDS in phosphate buffer pH 5.0 found to be the most appropriate for the determination of these drugs in rat plasma.

The combination of pH and NaCl effects in micellar mobile phase are unsatisfied results. The apparent retention time is too long for the determination of the drugs when these mobile-phases are employed.

Table 19. Variation of the retention time of phenobarbital and pentobarbital as a function of various conditions of micellar mobile phase with the flow rate of 1.0 ml/min.

micellar mobile phase	micellar mobile phase compositions	retention time (min.)	
		phenobarbital	pentobarbital
[micelle] (in aqueous)	0.010M SDS	6.52	13.16
	0.025M SDS	5.59	7.15
	0.035M SDS	4.28	5.35
addition of NaCl	0.015M SDS + 0.2M NaCl	10.17	18.42
	0.025M SDS + 0.2M NaCl	8.45	13.13
	0.050M SDS + 0.2M NaCl	6.58	8.19
bulk pH (phosphate buffer)	0.010 M SDS / pH 4.5	6.02	10.45
	0.010 M SDS / pH 5.0	5.30	9.56
	0.010 M SDS / pH 5.5	5.00	7.30
	0.010 M SDS / pH 6.5	4.30	6.56
	0.010 M SDS / pH 7.5	4.17	5.54
	0.050 M SDS / pH 7.5	4.02	5.33
combination of pH and NaCl	0.010 M SDS / pH 5.0 + 0.5 M NaCl	16.56	24.00
	0.025 M SDS / pH 5.0 + 0.5 M NaCl	13.26	20.00

B7. Determination of Drugs in Rat plasma

The calibration curves of phenobarbital and pentobarbital are constructed by plotting the peak heights (in cm) of particular drug versus known concentrations (in ppm) of each drug by using the data from Table 20. Typical calibration curves are shown in Figure 21. and 22.

Under the conditions of this study, retention times of phenobarbital and pentobarbital peaks are sufficient to separate them from each other and from background response of blank plasma. Retention times of phenobarbital and pentobarbital are 5.30 and 9.56 minutes, respectively. Figure 23. illustrates the chromatograms of (A) blank plasma and (B) the spiked blank plasma with standard phenobarbital and pentobarbital. The blank plasma produces a background response completely eluted after 4 minutes with a total elution time of 15 minutes per one injection. Figure 24. illustrates the chromatograms of a rat plasma sample ; (A)non-spiked the standard drugs and (B) the spiked plasma sample with standard drugs of 5.00 and 9.80 ppm of phenobarbital and pentobarbital, respectively. It can be seen that the small signals of non-spiked drugs in plasma sample (Figure 24.A) appear and are enhanced by spiking the standard drugs to plasma sample. These signals must be substracted from the signals of spiked blank plasma with the same amount of each drugs. Because plasma is diluted with distilled water and given dilution factor of 2.35, the concentrations of drug obtained by calculating from linear regression are multiplied with this dilution factor. The concentrations of drugs in rat plasma analyzed by micellar chromatography are summarized in Table 22. and 23.

The precision of micellar chromatography is established by obtaining three replications for ten plasma samples. The relative standard deviations are found to be between ± 0.59 and ± 5.23 percent for phenobarbital and ± 0.00 and

13.99 percent for pentobarbital. The relative standard deviations for measurement of these drugs in rats plasma are tabulated in Table 22. and 23.

The reproducibility of micellar chromatography is established by obtaining three replications for the same standard solutions in each drug. Table 21. shows the relative standard deviations for measurement of working concentration of drugs in plasma. The relative standard deviations are found to be between $\pm$ 0.63 and $\pm$ 4.94 percent and $\pm$ 1.19 and $\pm$ 5.68 percent for phenobarbital and pentobarbital, respectively.

Regression analysis by the least-squares method yielded a slope of 13.30 and an intercept of - 0.44 (r = 0.9995) for phenobarbital and a slope of 23.73 and an intercept of - 0.21 (r = 0.9996) for pentobarbital.

Table 20. Working concentration and the percentage recovery of phenobarbital and pentobarbital from direct plasma injection

drugs	concentration (ppm)	mean peak height (cm)		recovery (%) mean ± s.d.
		in aqueous	in plasma	
phenobarbital	2.50	0.260	0.200	76.92 ± 0.01
	5.00	0.560	0.430	76.79 ± 0.01
	10.00	1.050	0.790	75.24 ± 0.01
	20.00	2.030	1.530	75.37 ± 0.01
mean recovery = 76.08 ± 0.02 %				
pentobarbital	2.49	0.110	0.103	90.91 ± 0.01
	4.90	0.250	0.223	88.00 ± 0.01
	9.80	0.490	0.430	87.76 ± 0.01
	19.60	0.970	0.830	85.57 ± 0.01
mean recovery = 88.06 ± 0.02 %				

Table 21. Precision for measurement of working concentration of drugs in plasma

drugs	concentration (ppm)	mean measured value (ppm)	RSD (%)
phenobarbital	2.50	1.92	4.94
	5.00	3.84	2.34
	10.00	7.52	1.26
	20.00	15.07	0.63
			mean ± 5.64 %
pentobarbital	2.49	2.34	5.68
	4.90	4.38	2.64
	9.80	8.60	2.33
	19.60	16.77	1.19
			mean ± 6.79 %

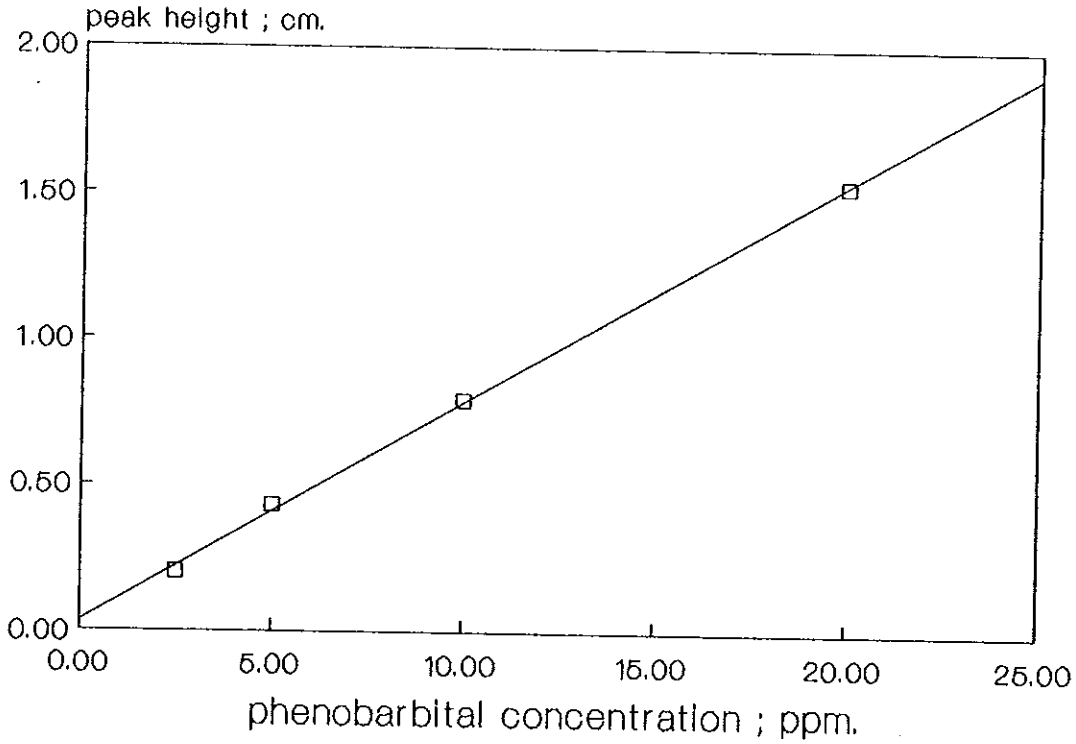


Figure 21. Calibration curve for the determination of phenobarbital from plasma obtained using micellar chromatography. The RSD mean is $\pm$ 5.64 %. Regression analysis by the least-squares method yielded a slope of 13.30 and an intercept of -0.44 (r = 0.9995) .

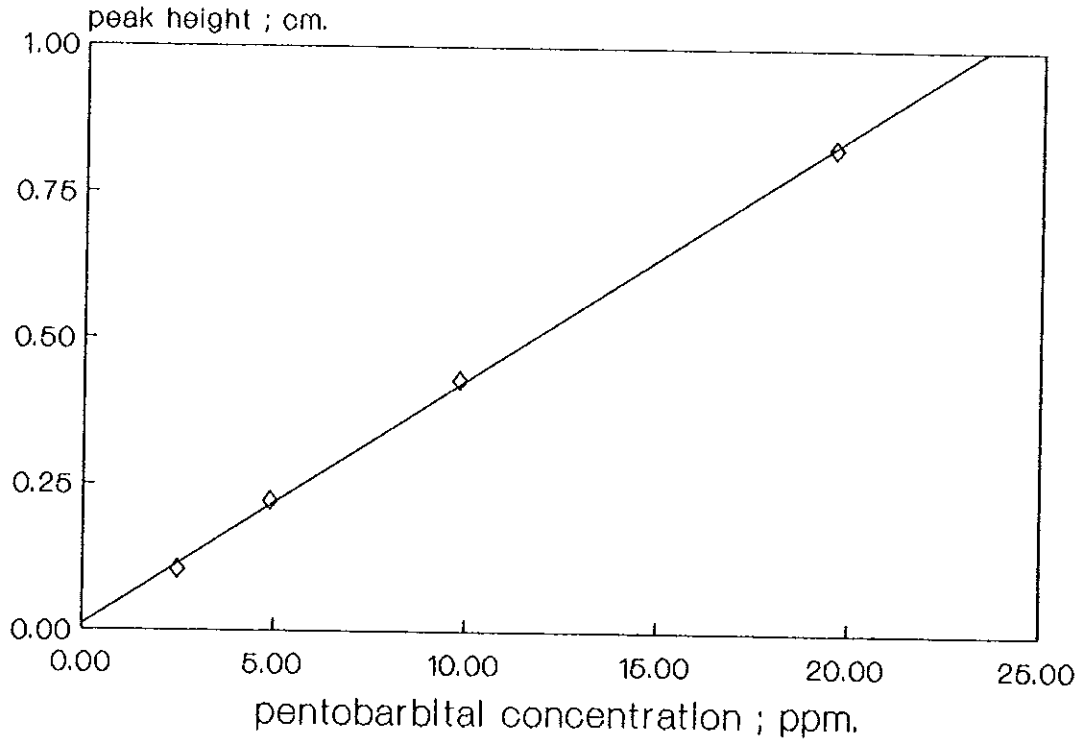


Figure 22. Calibration curve for the determination of pentobarbital from plasma obtained using micellar chromatography. The RSD mean is $\pm$ 6.79 %. Regression analysis by the least-squares method yielded a slope of 23.73 and an intercept of -0.21 (r = 0.9996) .

Table 22. Mean measured concentration and concentration of phenobarbital from rat plasma by MC.

sample no.	mean peak height (cm)	mean measured concentration (ppm)	°concentration in rat plasma (ppm)	RSD (%)
MC_4	0.643	8.12	19.09	1.91
MC_8	0.677	8.57	20.14	3.90
MSO_4	0.997	12.83	30.14	0.59
MSO_9	0.893	11.44	26.89	1.33
MSP_5	0.633	7.98	18.76	2.55
MSP_{11}	0.860	11.00	25.86	1.39
FSO_2	1.743	22.75	53.47	0.68
FSO_{12}	1.680	21.93	51.50	1.75
FSP_{10}	0.580	7.28	17.11	5.23
FSP_{13}	1.230	15.93	37.43	2.40

° Concentration in rat plasma is calculated from multiplication of mean measured concentration with dilution factor (2.35).

Table 23. Mean measured concentration and concentration of pentobarbital from rat plasma by MC.

sample no.	mean peak height (cm)	mean measured concentration (ppm)	° concentration in rat plasma (ppm)	RSD (%)
MC_4	0.120	2.64	6.20	0.00
MC_8	0.073	1.52	3.58	8.99
MSO_4	0.063	1.28	3.02	10.65
MSO_9	0.080	1.69	3.97	13.99
MSP_5	0.073	1.52	3.58	8.99
MSP_{11}	0.077	1.62	3.80	8.55
FSO_2	0.163	3.66	8.59	7.44
FSO_{12}	0.173	3.89	9.15	3.53
FSP_{10}	0.183	4.13	9.71	3.88
FSP_{13}	0.110	2.40	5.64	11.10

° Concentration in rat plasma is calculated from multiplication of mean measured concentration with dilution factor (2.35).

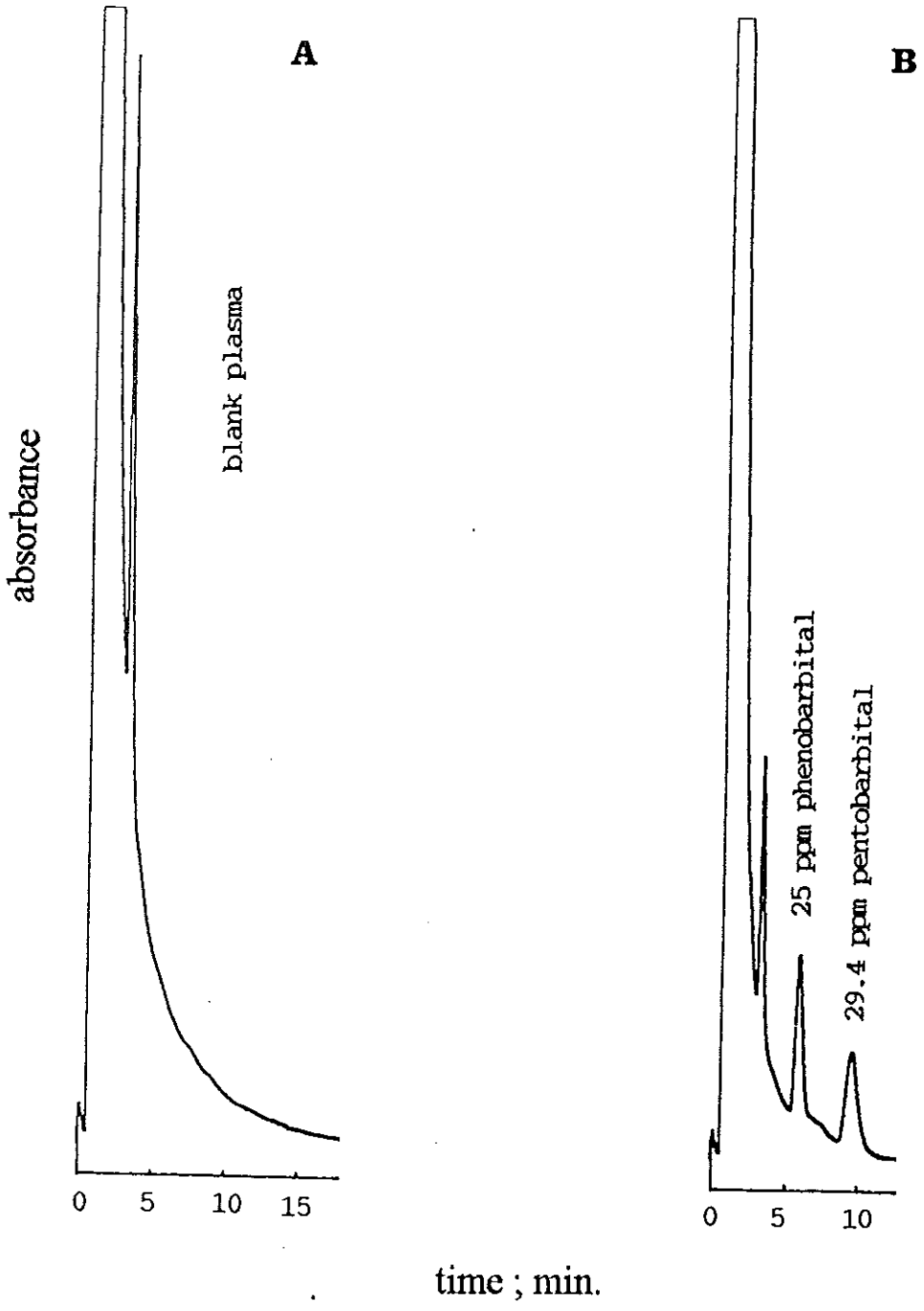


Figure 23. Micellar chromatograms of (A) blank plasma and (B) blank plasma spiked with (1)25.00 ppm phenobarbital and (2) 29.40 ppm pentobarbital. Chromatographic conditions are as follws : column, CN-RP μBondapak, 150x3.9 mm.i.d.; mobile phase, 0.01MSDS in phosphate buffer pH 5.0 ; flow rate, 1.0 ml/min ; chart speed, 2.0 mm/min.

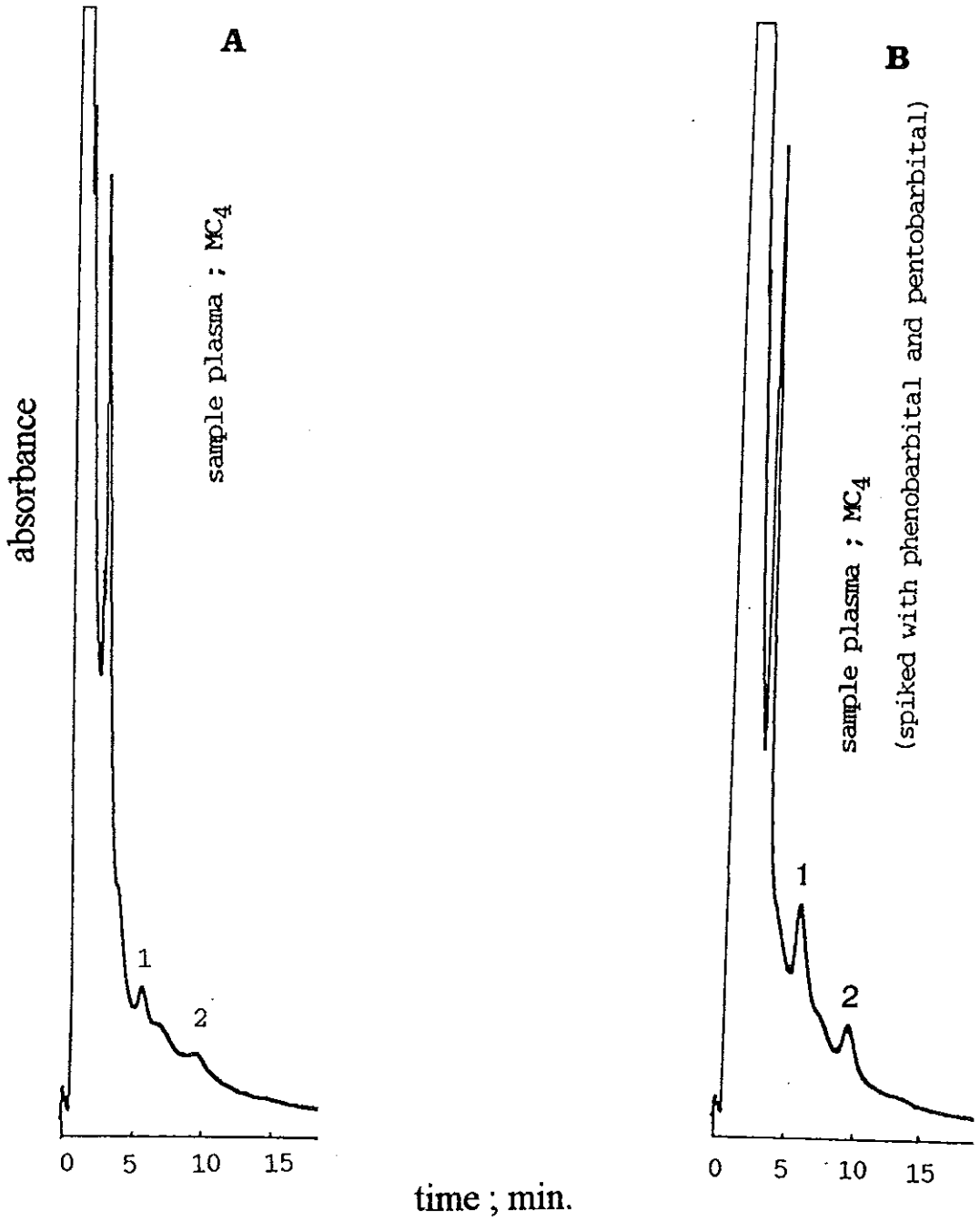


Figure 24. Micellar chromatograms of plasma sample MC_4 : (A) non-spiked standard drugs, (B) spiked with 5.00 ppm phenobarbital (1) and 9.80 ppm pentobarbital (2). Chromatographic conditions are the same as those given in Figure 23.

B8. Recovery

The analytical recovery of phenobarbital and pentobarbital are measured by comparing chromatographic peak height of each drug from direct plasma injection containing known amounts of drug with aqueous solution containing the same concentration of each compound. The results are given in Table 20. The average analytical recoveries of phenobarbital and pentobarbital are approximately 76 and 88 percent, respectively

Limits of Detection

The minimum amount of a species that can be reliably seen on the chromatogram is the detection limit. Usually detection limits are measured with an individual species in a standard solution. In a real sample, the detection limit for these species will usually be higher due to baseline disturbance or interference from the matrixes or orther species. In this experiment, the detection limits of phenobarbital and pentobarbital are 3.33 and 9.80 ng, respectively. These are measured manually from chromatograms that give individual peak to where signal equals to two times of the baseline noise.

3. Comparison of Results

The goal of this study is to compare RPLC and MC for measurement of phenobarbital and pentobarbital in rat plasma samples. A series of ten typical rat plasma samples are chosen, the analyses performed and the results obtained are compared using the paired comparison method (*Sokal and Rohlf, 1969 : 328*). The concentration of the two drugs in each of the ten samples are given in Table24. Paired comparison between RPLC and MC techniques using data obtained from the phenobarbital and pentobarbital analyses are given in Tables 25. and 26., respectively. Both ANOVA tables indicate that there are highly significant sample-to-sample variation. This is expected, since the amounts of these drugs find in various plasma samples depending on condition of rat-bodies such as age, sex, rat-sample treament before drug administration and time period of drug administration. Fs for the comparison of RPLC and MC using data obtained for phenobarbital (Table 25.) is not significant at 95 % confidence level, indicating that the amounts of phenobarbital obtained from these two techniques are in good agreement. Thus, there is no results are different for the RPLC compared with MC. Fs between these two techniques using data obtained for pentobarbital (Table 26.) is highly significant at 99 % confidence level, indicating that the amounts of pentobarbital for MC are not comparable to those obtained by RPLC. The plasma sample matrixes appears to give some difficulties in the determination of pentobarbital by these two techniques.

The mean concentrations of phenobarbital and pentobarbital found in the rat plasma samples tested are presented in Table 27. The results of this investigation indicate that there is generally unagreement between methods. However, the higher of mean RSD and detection limit and the lower recovery of phenobarbi-

tal results obtained by MC are compared with RPLC, but the analysis time for the determination of phenobarbital by MC is shorter than RPLC technique. Therefore, based on the phenobarbital results of this study the MC analysis technique is most attractive because it has some the features required for routine analysis of large number of samples including speed and ease of analysis. No sample pretreatment is necessary for MC.

Although, the pentobarbital results by MC is generally the tendency of phenobarbital results for the same method. The high mean of RSD, detection limit and Fs significant (Table 26.) of the pentobarbital results appear. Therefore, based on the pentobarbital results of this study the RPLC analysis technique is attractive because it has all the features required for the more precision analysis of plasma samples except the longer analysis time for RPLC is unfortunate. Because the protein precipitation steps of plasma are necessary for the determination of drugs from plasma by RPLC.

Table 24. Comparison of phenobarbital and pentobarbital concentration in rat plasma by RPLC and MC

sample no.	concentration in rat plasma (ppm) ± RSD (%)			
	RPLC		MC	
	phenobarbital	pentobarbital	phenobarbital	pentobarbital
MC_4	11.55 ± 0.00	6.70 ± 0.00	19.09 ± 1.91	6.20 ± 0.00
MC_8	16.90 ± 0.00	7.58 ± 0.00	20.14 ± 3.90	3.58 ± 8.99
MSO_4	23.55 ± 1.27	6.03 ± 3.48	30.14 ± 0.59	3.02 ± 10.65
MSO_9	22.45 ± 0.89	7.35 ± 3.06	26.89 ± 1.33	3.97 ± 13.99
MSP_5	19.28 ± 1.93	8.45 ± 0.00	18.76 ± 2.55	3.58 ± 8.99
MSP_{11}	26.63 ± 0.00	7.58 ± 0.00	25.86 ± 1.39	3.80 ± 8.55
FSO_2	54.25 ± 0.18	10.88 ± 6.07	53.47 ± 0.68	8.59 ± 7.44
FSO_{12}	50.70 ± 0.20	11.98 ± 0.00	51.50 ± 1.75	9.15 ± 3.53
FSP_{10}	16.30 ± 0.00	12.88 ± 0.00	17.11 ± 5.23	9.71 ± 3.88
FSP_{13}	37.90 ± 0.53	8.90 ± 5.06	37.43 ± 2.40	5.64 ± 11.10
mean ± RSD	27.95 ± 2.55	8.83 ± 9.16	30.04 ± 8.13	5.72 ± 27.39

Table 25. Comparison of RPLC and MC using data for phenobarbital

ANOVA TABLE FOR PAIRED COMPARISON

Source of Variation	df	SS	MS	Fs
Between Techniques	1	21.7987	21.7987	4.3654 ^{ns}
Among Samples	9	3520.4695	391.1633	78.3345 **
Remainder	9	44.9411	4.9935	
Total	19	3587.2093		
$F_{0.01\ (1,9)} = 10.56$				$F_{0.05\ (1,9)} = 5.12$

Table 26. Comparison of RPLC and MC using data for pentobarbital

ANOVA TABLE FOR PAIRED COMPARISON

Source of Variation	df	SS	MS	Fs
Between Techniques	1	48.3294	48.3294	72.4313 **
Among Samples	9	101.7204	11.3023	16.9388 **
Remainder	9	6.0052	0.6672	
Total	19	156.0550		
$F_{0.01\ (1,9)} = 10.56$				$F_{0.05\ (1,9)} = 5.12$

Notation in ANOVA Tables (24 and 25) ;

df = degree of freedom ; Fs = experimental F ratio

SS = Sum of Squares ; ns = not significant at 95 and 99 %

MS = Mean Squares = SS / df ; ** = significant at 95 and 99 %

Table 27. Comparison of methods

	RPLC		MC	
	[p]phenobarb	[q]pentobarb.	phenobarb.	pentobarb.
Mean Concentrations Found in Rat Plasma Samples Tested (ppm)	27.95	8.83	30.04	5.72
Precision ; mean RSD(%)	± 2.85	± 2.33	± 5.64	± 6.79
Detection Limit (ng)	1.18	3.14	3.33	9.80
Recovery (%)	95.67	95.52	76.08	88.06
[r]Analysis Time. ; man-hour / 10 samples	30		13	

[p]phenobarb. is referred to phenobarbital.

[q]pentobarb. is referred to pentobarbital.

[r]Analysis time is including all steps from standard and sample preparation through data calculation.

Chapter 4

Conclusion

According to the study of various effects on the elution behavior of the sedative drugs as the weak organic acid, i.e., phenobarbital and pentobarbital investigated by RPLC and MC techniques, it has been shown that :

1. The relationship between the capacity factors and acetonitrile concentration in the mobile phase (RPLC) is exponential whereas the k' and [SDS] in the micellar mobile phase is parabolic. That are the agreement of the increase in concentration of mobile phase and result in a decrease in the k'.

2. The elution behavior of these drugs in RPLC have a relatively little change in retention with increase in pH until the pH approaches pKa's, then the retention decreases. In MC system, the elution behavior of phenobarbital at low [SDS] agreees with RPLC whereas the k' of pentobarbital is not altered significantly by changing the mobile phase pH.

3. The addition of NaCl to the mobile phase in RPLC appears to have a little change in retention, in the event of MC system, addition of NaCl not only can enhance retention and separation factor, but also can affect for pentobarbital more than phenobarbital retention.

4. The mass transfer for phenobarbital in micellar mobile phase is slow over RPLC system.

5. The increasing in linear velocity above the optimum results in a decrease in efficiency.

6. The use of additional organic modifiers such as alcohols to SDS micellar mobile phase produces a decreasing in k'. The highest solvent strength of alcohol in micellar mobile phase is less retained than the smaller solvent strength.

The results of this investigation can be considered as the suitable mobile phase condition for the determination of phenobarbital and pentobarbital in rat plasma by RPLC and MC techniques. The optimum mobile phase for use in RPLC is 30 : 70 of acetonitrile : phosphate buffer pH 5.0 and the micellar mobile phase of 0.01M SDS in phosphate buffer pH 5.0 should be the most appropriate for measurement.

The methods presented in this study have been carefully tested with respect to their suitability for routine analytical work. The precision of measurements for phenobarbital is $\pm$2.85 and $\pm$5.64 % (RSD) for RPLC and MC, respectively (Table 27.). Fs for comparison of RPLC and MC is not significant at 95 % confidence level (Table 25.) using paired comparison ANOVA test. The determination of pentobarbital by RPLC and MC is a highly different significant (Table 26.) variation among methods. The precision, given by the % RSD of pentobarbital in typical ten rat plasma samples is $\pm$2.33 and $\pm$6.79 % for RPLC and MC, respectively (Table 27.).

From the results of the studies, it can be concluded that the RPLC technique can give the advantageous in terms of the low detection limit, high precision and recovery but the analysis time is inconvenience. In the event of MC technique, it can offer several advantages, i.e., the elimination of protein precipitation steps prior to analyze, thus significantly reducing analysis time. Also, the hazard and toxic of reagent are less than many organic solvents in conventional HPLC.

The main limitation of employing micellar mobile phase is the high biological fluids background signal.

The choice of technique should therefore depends on other factors, such as detection limit, speed of analysis and hazard. There is no single best technique for all circumstances, but micellar chromatography seems to be the most advantageous in terms of convenience, speed of analysis and hazard for a large number of sample determination.

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Vitae

Name Miss Charuwan Khamkaew

Birth Date October 25, 1966

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. (Chemistry)	Ramkhamheang University	1989

Scholarships Awards During Enrolment

The Prince of Songkla University, Pattani Campus Scholarship Award (Education Supporting for Graduate Students in 1993. and Researches Supporting in 1994.).

Work-Position and Address

Work-Position Scientist

Address Chemistry Division, Department of Science, Faculty of Science and Technology, Prince of Songkla University Pattani Campus, A. Mueng, Pattani, 94010.