Micellar Liquid Chromatography for Determination of Acetaminophen and Chlorpropamide in Human Plasma.

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Master of Science Thesis in Analytical Chemistry

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Author	Miss Siriporn Janttarakeeree
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Abstract

The use of micellar liquid chromatography (MLC) technique for direct determination of acetaminophen and chlorpropamide in human plasma was studied. The effect of mobile phase concentration, type and concentration of organic modifier, pH, and ionic strength on retention time were investigated. Optimum mobile phase for acetaminophen was 0.05 M SDS while chlorpropamide was 0.10 M SDS + 2% propanol (v/v) + 2% sodium chloride (w/v) on C_{18} column. Ultraviolet absorption at wavelength of 245 and 240 nm were used for the detection of acetaminophen and chlorpropamide, respectively. By this technique, after filtration, the plasma was injected into the chromatography without the previous solvent extraction or deproteinization. Acetaminophen and chlorpropamide were eluted at 5.28 min and 6.47 min, respectively with flow rate of 1.0 mL/min. The total elution time for analysis per one injection of each drug was approximately 15 minutes. Minimum detectable concentrations were about 0.52 μg/mL and 0.67 μg/mL for acetaminophen and chlorpropamide, respectively.

ชื่อวิทยานิพนธ์	การวิเคราะห์หา อะเซตามิโนเฟน และ คลอโปรปาไมด์ ในพลาสมามนุษย์ โดยเทคนิคไมเซลลาร์ ลิควิด โครมาโทกราฟี
ผู้เขียน	นางสาวศิริพร จันทรคีรี
สาขาวิชา	เคมีวิเคราะห์
ปีการศึกษา	2539

บทคัดย่อ

ศึกษาการวิเคราะห์อะเซตามิโนเฟนและคลอโปรปาไมด์ ในพลาสมามนุษย์ โดยเทคนิคไมเซลลาร์ ลิควิด โครมาโทกราฟี และศึกษาถึงปัจจัยต่างๆที่มีอิทธิพลต่อ retention time ของยา ได้แก่ ความเข้มข้นของเฟสเคลื่อนที่ ชนิดและความเข้มข้นของสารปรุงแต่ง ความเป็นกรด และความแรงไอออน สภาวะที่เหมาะสมที่สุดในการวิเคราะห์ยาในพลาสมามนุษย์ สำหรับอะเซตามิโนเฟน คือ 0.05 M SDS ที่ความยาวคลื่น 245 nm และคลอโปรปาไมด์ คือ 0.10 M SDS + 2% propanol (v/v) + 2% sodium chloride (w/v) ที่ความยาวคลื่น 240 nm โดยใช้คอลัมน์ C_{18} การวิเคราะห์ยาในพลาสมาด้วยเทคนิคนี้ สามารถทำได้โดยไม่ต้องผ่านการสกัดหรือกำจัดโปรตีนก่อน โดยอะเซตามิโนเฟนและคลอโปรปาไมด์ถูกแยกออกจากคอลัมน์ที่เวลา 5.28 นาที และ 6.47 นาที ตามลำดับ เมื่ออัตราเร็วการเคลื่อนที่ของเฟสเคลื่อนที่คือ 1.0 mL/min การวิเคราะห์ยาแต่ละชนิด ในแต่ละครั้งจะใช้เวลาประมาณ 15 นาที และขีดจำกัดต่ำสุดในการตรวจวัดปริมาณของอะเซตามิโนเฟนและคลอโปรปาไมด์ โดยเทคนิคนี้ คือ 0.52 μg/mL และ 0.67 μg/mL ตามลำดับ

Acknowledgement

Profound gratitude is expressed by the author to those who have unselfishly shared their knowledge and expertise to make this research a reality. First of all, my advisor, Associate Professor Dr. Manop Arunyanart and co-advisor, Associate Professor Malinee Wongnawa. Thesis will not be sucessfull without the kindness, guidance, and assistance from them.

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Siriporn Janttarakeeree

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CHAPTER 1

INTRODUCTION

Drugs always play an important role in the treatment of disease. But the lack of suitable calculation of dose for individual drug treatment, *e.g.*,over-treatment, drugs can give toxic response. For example, *Prescott, et al. (1971 : 519-322)* showed that half-life of acetaminophen in the blood was the most reliable early guide for a diagnosis of liver damage and that liver damage could be expected if the half-life exceeded 4 hours.

It is now recognised that therapeutic response of some drugs correlate better with the concentration in plasma than with the daily dose (*Moffat, et al., 1986 : 3-4*). Thus, a method is needed to develop analytical techniques for measuring drugs that is both enable measurements to be carried out quickly on small samples of blood and specific for a parent compound, whilst producing accurate results with good precision.

Several techniques have been applied to the determination of drugs in serum. Original methods such as colorimetric and ultraviolet spectrophotometric are subject to many interference, time-consuming, non-specific and lack of sensitivity. In gas chromatography procedures, some drugs such as theophylline (*Habel, et al., 1993 :*

1511-1513) and chlorpropamide (*Hill and Crechiolo, 1987 : 165-168*) are thermolabile and derivatization step is required in the analysis. High-performance liquid chromatography (HPLC) become a technique of choice for the separation and quantification of drugs, because HPLC analysis can be operated on a small volume of sample and provides good sensitivity and specificity. However, HPLC procedure has been described using sample pretreatments such as solvent extraction, solid-phase extraction and protein precipitation. By this technique, drugs may be lost during sample pretreatment.

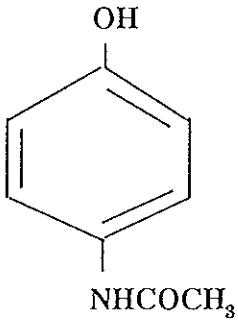
DeLuceia, et al. (1985 : 1564-1568) reported that micellar liquid chromatography (MLC) involves direct injection of untreated serum sample, which uses a surfactant solution of sodium dodecyl sulfate (SDS) above the critical micelle concentration (CMC) as the mobile phase. The unique selectivity of such system is due to the solute-micelle association and the solute-stationary phase interaction. *Haginakai, et al. (1987 : 2732-2734*) stated that serum proteins were solubilized without their precipitation, a drug bound to protein was displaced by this surfactant monomers and/or micelles, and being released for partition to the stationary phase.

MLC has several potential advantages such as capability of simultaneous separation of ionic and nonionic compounds, performing gradient elution without a need for column re-equilibrium (*Dorsey, et al., 1984 : 183-191*), the possibility of determining drugs in biological

fluids without previous separation of proteins, enhancement of fluorescence and phosphorescence detection *(Kord and khaledi, 1992 : 1894-1900)*, reduction in hazardous wastes and inexpensive mobile phase *(Bailey and Cassidy, 1992 : 2277-2282)*.

Literature Review

Acetaminophen (N-acetyl-*p*-aminophenol, paracetamol, *p*-acetamidophenol, APAP), is commonly used as analgesic and antipyretic drugs. Its structural formula is shown in Figure 1.



$C_8H_9NO_2$

M.W. = 151.16

Figure 1 Structural formula of acetaminophen

Physical Properties : white crystals or crystalline powder.

Melting Point : 169 °to 172 °C.

Solubility : one gram dissolves in about 70 mL of water at 25 °C, 25 mL of boiling water, 7 mL of alcohol, 13 mL of acetone, 50 mL of chloroform, 40 mL of glycerine, and 9 mL of propylene glycol. It is insoluble in benzene and ether, and soluble in solution of alkali hydroxides. A saturated solution has a pH about 6 .

Dissociation Constant : pK_a 9.5 (25 °C)

Protein Binding : In plasma, not bound at concentration less than 60 ng/mL. In poisoned subjects, protein binding has been reported to vary between about 8% and 40%.

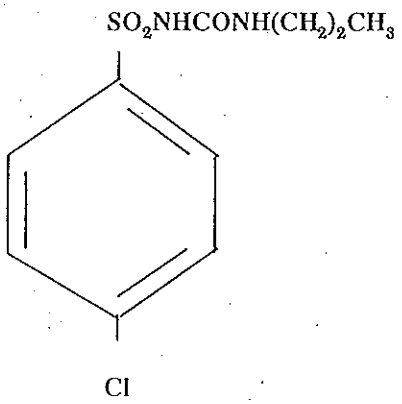
Half-Life : - Normal: 2.0 hours.

- Disease States: Increase in hepatic disease, neonates; fluctuates with uremia.

Therapeutic Concentration : In plasma, usually in the range of 10 to 20 μg/mL

Toxicity : The minimum lethal dose is about 10 g. Symptoms of hepatic damage do not occur for at least 12 hours after overdosage but may not appear until 4 to 6 days later. Plasma concentration have been used to indicate possible hepatic necrosis; at 4 hours, hepatic necrosis is possible at concentration of acetaminophen of 120 to 300 μg/mL *(Moffat, et al., 1986 : 849-850).*

Chlorpropamide, (N-*p*-chlorobenzenesulphonyl-N′-propylurea, CPA) is one of the sulphonylurea derivatives used as oral hypoglycemic agent for the treatment of diabetes mellitus, its structural formula is shown in Figure 2.



$C_{10}H_{13}ClN_2O_3S$

M.W. = 276.7

Figure 2 Structural formula of chlorpropamide

Physical Properties : a white crystalline powder.

Melting Point : 126 °C to 130 °C.

Solubility : Practically insoluble in water ; soluble 1 in 12 of ethanol, 1 in 5 of acetone, 1 in 9 of chloroform, and 1 in 200 of ether.

Dissociation Constant : pK_a 5.0 (20 °C)

Protein Binding : In plasma, 60 to 95% bound.

Half-Life : - Normal: 24 - 42 hours.

- Disease states: Prolonged with impaired renal function.

Therapeutic Concentration : In plasma, usually in the range of 30 to 250 μg/mL.

Toxicity : prolonged hypoglycemic coma has been reported after overdosage but fatalities are comparatively rare, although some instances of fatal blood dyscrasias have been reported. Peak plasma concentration of 200 to 750 μg/mL have been observed in comatose subjects *(Moffat, et al., 1986 : 461-462).*

THEORY

Micellar liquid chromatography, as first described by *Armstrong (1979 : 2160-2163)*, uses surfactant solution above the critical micelle concentration (CMC) as the mobile phase. The ability to control "selectivity" in the mobile phase by the use of micelles results in a high degree of flexibility which is not available from the other methods of chromatography. The importance of micelles in the mobile phase lies in their ability to participate in the partitioning mechanism. The three equilibria involved in micellar chromatography are schematically represented in Figure 3 (*Kirkbright and Mullins, 1984 : 493 - 496*).

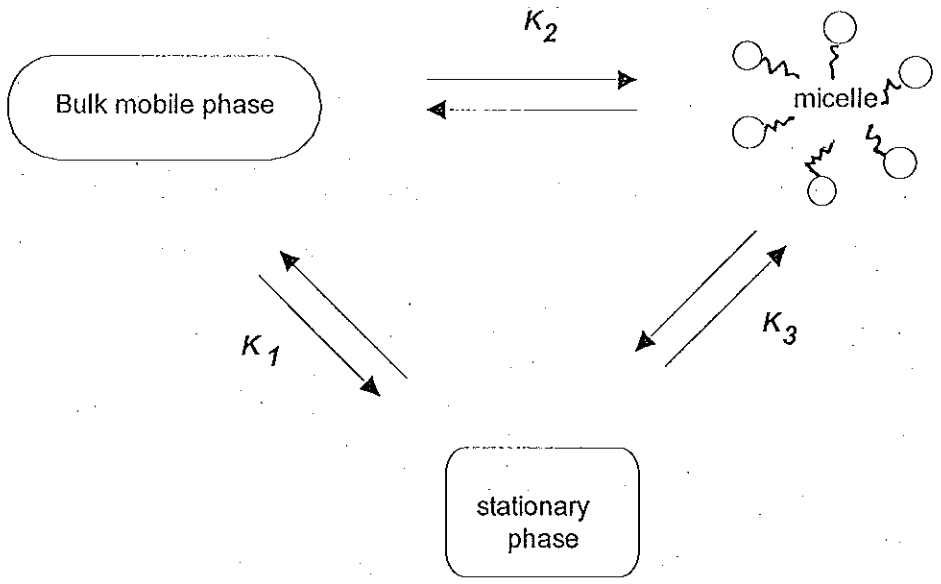


Figure 3. Schematic representation of solute partitioning in micellar chromatography.

Armstrong and Nome (1981 : 1662-1670) proposed a three-phase model (stationary phase, bulk mobile phase and micellar pseudo-phases) to explain the chromatographic behavior of solute eluted with an aqueous micellar mobile phase containing a surfactant. The solute partition not only between water and the stationary phase, but also inside the mobile phase, between water and the micelle. Hence, elution of a solute in MLC depends on three partition coefficients: that between the stationary phase and water, between the stationary phase and the micelle, and between the micelle and water.

In this model the hydrophobic surfactant is assumed to adsorb on to the stationary phase as a primary layer, while a counter ions occupy the secondary layer. Thus, an electric double layer is established. As the mobile phase also contains the hydrophobic salt, a dynamic equilibrium is established between the double layer, hydrophobic salt and the solute.

Arunyanart and Cline Love (1984 : 1557-1561) proposed the equation to describe the behavior of a solute in micellar chromatography as the micelle concentration is changed. The equation relates capacity factor to micellar mobile phase concentration expressed as :

$$\frac{1}{k'} = \frac{[M_m]K_2}{\phi[L_s]K_1} + \frac{1}{\phi[L_s]K_1}$$

K_1 is the equilibrium constant for the solute between the bulk solvent and the stationary phase.

K_2 is the equilibrium constant for the solute (per monomer of surfactant) between the bulk solvent and the micellar aggregate.

$[M_m]$ is the micelle concentration (the surfactant concentration minus CMC).

$[L_S]$ is the stationary phase sites.

Φ is the chromatographic phase ratio,

$$\Phi = \left(v_S / v_M\right)$$

V_S is the volume of the stationary phase in the column.

V_M is the volume of the mobile phase in the column.

Factors influencing on retention of a solute.

1. The type of interaction of solute with the micelle and the surfactant-modified stationary phase.

Non-polar solute should only be affected by hydrophobic interaction, but for solutes that are charged, two distinct situations can be considered (*Hernandez and Alvarez-Coque, 1992 : 831-837*).

1) The charge on the solute and surfactant has the same sign.

2) The charge on the solute and surfactant has the opposite sign.

The first situation is encountered when an anionic solute is chromatographed with an anionic surfactant or a cationic solute with a cationic surfactant. Electrostatic repulsion from the micelle should not affect retention as the solute would still reside in the bulk mobile phase and, therefore, still move down the column. In contrast, repulsion from the surfactant-modified stationary phase should cause a decrease in retention. Solutes may be eluted in the void volume. However, they may also be retained if hydrophobic intraction with the stationary phase exists.

The second situation appears when a solute is chromatographed with an opposite charged surfactant, where electrostatic attraction occurs between both species. If the electrostatic attraction with the micelle is complemented by a hydrophobic

interaction, the solute will remain in the mobile phase and retention will decrease. However, electrostatic and hydrophobic intraction with the stationary phase may be sufficiently large to offset the increase in micelle attraction and would increase retention.

2. Type of solutes that show properties in micellar liquid chromatrography.

Armstrong and Stine (1983 : 2317-2320) proposed a classification of the solutes according to their chromatographic properties in micellar liquid chromatrography.

1) Solutes binding to micelle, their retention can be decreased when the mobile phase concentration of micelle is increased.

2) Non-binding solutes that do not bind to the micelle, retention can be unaltered by the micelle content of the mobile phase.

3) Antibinding solutes, antibinding results from a compound being strongly excluded or repelled from the micelle, their retention can be increased with increasing micelle concentration.

3. The structure of the surfactants.

Surfactant is an amphiphilic molecules consisting of hydrophobic chain joined to a hydrophilic head group, the hydrophobic part is generally a long chain hydrocarbon, typically 8-18 atoms. Surfactants are classified as

1) Anionic : the surfactant-active portion of the molecule bears a negative charge, for example , $RC(=O)-O^-Na^+$ (soap) , $RC_6H_4SO_3^-Na^+$ (alkylbenzene sulfonate), $CH_3(CH_2)_{11}OSO_3^-Na^+$ (sodium dodecyl sulfate).

2) Cationic : the surfactant-active portion bears a positive charge, for example, $RNH_3^+Cl^-$ (salt of a long-chain amine), $RN(CH_3)_3^+Cl^-$ (quaternary ammonium chloride).

3) Zwitterionic : both positive and negative charges may be present in the surfactant-active portion, for example, $R\overset{+}{N}H_2CH_2COO^-$ (long-chain amino acid), $RN^+(CH_3)CH_2CH_2SO_3^-$ (sulfobetaine).

4) Nonionic : the surfactant-active portion bears no apparent ionic charge , for example, $RCOOCH_2CHOHCH_2OH$ (monoglyceride of long-chain fatty acid), $RC_6H_4(OC_2H_4)_xOH$ (polyoxyethylenated alkylphenol).

Surfactants dissolve in water, form organized molecular assemblies called micelles if the critical micelle concentration (CMC) is exceeded. The structure of the micelle is such that the polar head

groups are in contact with the bulk aqueous solution, while the hydrophobic chains are directed inside the micelles and form the nonpolar core.

Only anionic *(DeLuccia, et al., 1985 : 1564-1568)*, nonionic and zwitterionic (*Habel, et al., 1993 : 1511-1513*) surfactant can be used to determine the drugs in body fluid, because they mix with body fluid without inducing protein precipitation. Attempts to use cationic surfactants were unsuccessful. For example, use of the cationic micelle composed of hexadecyltrimethyl ammonium bromide (CTAB) causes protein precipitation and could not be employed *(DeLuccia, et al., 1985 : 1564-1568)*.

4. Addition of modifier to micellar eluents.

One of the primary cause of band broadening in MLC is poor mass tranfer which solutes are slowly transferred from the micelle to the stationary phase. The addition of the alcohol to the micellar mobile phase and its subsequent adsorption onto stationary phase may result in a decrease in the amount of the adsorbed surfactant. Then, the thickness of the film of surfactant molecules covering the stationary phase is reduced. By this reason, the retention mechanism is also altered by shifting the equilibrium of the solutes from the stationary phase and the micelle toward the bulk mobile phase. The solute-stationary phase diffusion cofficient should increase. This leads

to a reduction in the capacity factors and the enhancement in efficiency.

Another reason why alcohol improve the efficiency in MLC, with ionic surfactant micelles, may be because their presence can reduce the net electric charge density of the ionic micellar surface or the eluent strength increases with increasing organic modifier concentration which results in a decrease in retention.

5. Stationary phase

The amount of adsorbed surfactant on the same stationary phase is constant above the CMC value (because the free surfactant concentration remained constant) but is very different on various polarities stationary phase. The concentration of adsorbed surfactant on C_{18} silica is higher than polar CN silica. The amount of adsorbed surfactant decreases when the polarity of the stationary phase increases. Indeed, the adsorption of an anionic surfactant onto the stationary phase can occurs in at least two ways (*Berthod. et al. 1986 : 1356-1358*).

1) The hydrophobic tail is adsorbed and the ionic head group would then be in contact with the polar solution.

2) If the ionic head group is strongly adsorbed, the stationary phase is more hydrophobic and could subsequently behave as a more hydrophobic surface.

The C_{18} column is modified by adsorption of SDS monomers with negative head groups in contact with the mobile phase, such that the surface is charged and will repulse anionic species. Cyano packing does not appear to adsorb SDS monomer and generally has the capability to retain various species *(Arunyanart and Cline Love 1985 : 2457-2483).*

6. The pH of mobile phase.

The separation process in micellar system is controlled by a balance of electrostatic and hydrophobic interaction. Variation of pH of the bulk micellar solution affect on the ratio of undissociated-to-dissociated forms. Therefore, the competing partition of molecules to the stationary phase, which is also phase influenced by micellar hydrophobic effects and electrostatic interaction, are difference. These effects can significantly alter the chromatographic retention.

For weak acid using a C_{18} column reveals that the largest k' values occur in acidic solution where the neutral form is present. In more basic solution where the anionic acid form is present (electrostatically repulsed by both the negative micelle and stationary phase), k' is smaller.

For weak base using C_{18} column, at low pH or acidic solution where the protonated species is present and attracted to the negative charged stationary phase, k' is large. The smaller k' values

occur in more basic solution where the neutral, free-base form is present. Adsorption of anionic surfactant monomer on the surface of the C_{18} stationary phase causes protonated organic bases to be retained for a longer period of time than the neutral, free-base form, because of electrostatic attraction.

However, the limit of usefull pH range for column is 2 to 8 since siloxane linkages are cleaved below pH 2 while at pH values above 8 silica may dissolve.

7. Concentration of mobile phase

Generally, the equilibrium constant, K_1, K_2 describing the partitioning of the solute from the bulk aqueous to the column stationary phase and to the micelle, respectively, affect retention. Solutes with large K_2 values exhibit more rapid decrease in k' as the surfactant concentration increase than do solutes with small K_2. Consequently, variation of the micellar mobile phase concentration can profoundly affect the retention of a drug *(Deluecia, et al., 1985 : 1564-1568)*, because increasing micelle concentration, the probability of a solute encountering a micelle increases. However, the stationary phase and bulk aqueous are not affected by the increase in surfactant concentration *(Yarmchuk, et al., 1984 : 47-60)*.

8. Sodium chloride.

The effect of salts e.g. sodium chloride on micelles of ionic surfactant is important. The CMC is greatly decreased, the degree of counterion binding is affected, and the micelle size (and aggregation number) is increased upon the addition of sodium chloride to micellar solution. The CMC of sodium dodecyl sulfate (SDS) is decreased by sodium chloride according to (*Berthod, et al. 1986 : 1362-1367*)

$$\log CMC = (- 0.7 \log \mu) - 3.55$$

The symbol μ is the ionic strength (μ = CMC + [NaCl]).

Armstrong and Stine (1983 : 2317-2320) stated that addition of sodium chloride might be sufficient to change a solute from an antibinding solute to a binding type behavior, because sodium chloride can cause a decrease in the interfacial potential at the micelle surface which will allow stronger solute-micelle interaction (*Arunyanart and Cline Love 1985 : 2457-2483*).

The most noticeable drawback of MLC is slow mass transfer which leads to poor efficiency. *Dorsey, et al. (1983 : 924-928)* reported that the addition of a small amount of 3% propanol and raising the temperature to 40 $^{\circ}$C would improve the column efficiency. *Armstrong, et al. (1986 : 2317-2320)* noted that poor mass transfer from the surfactant-coated stationary phase is the predominant reason for the poor efficiency in MLC. *Yarmchuk, et al. (1984 : 47-60)* reported that the poor efficiency of MLC is due to slow mass tranfer of solute from miclles as well as from stationary phase to the bulk solvent. They suggested that chromatographic efficiency can be improved by increasing the operating temperature and addition of alcohol.

Objective

The aim of this study is to use micellar liquid chromatography for the qualitative and quantitative analysis of acetaminophen and chlorpropamide in human plasma. In this study the optimum condition for separation and the enhancement of efficiency in this chromatography technique for determination of both drugs in human plasma was also investigated.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER 2

EXPERIMENTS

APPARATUS.

1. The high-performance liquid chromatography (HPLC) system consisted of :

- High pressure liquid chromatographic pump (WATER model 501).
- UV/VIS detecter (JASCO model 875).
- Sample injector equipped with 20 μL injector loop (RHEODYNE, model 7125).

2. Chart recorder (WPA model CQ 95) was used to record the chromatogram.

3. μ - BONDAPAK C_{18} column : Water Associates, USA, 300 × 3.9 mm i.d. particle size was 10 μm.

4. μ - BONDAPAK CN column : Water Associates, USA, 150 × 3.9 mm i.d. particle size was 10 μm.

5. pH meter : Corning Benchtop Meter, model 255.

6. Centrifuge : Sigma 201 M,B.Braun.

7. Sintered glass : Pyrex USA.

8. UV spectrophotometer: SHIMADZU, model UV-1601.

9. Ultrasonic bath : BRANSON 2200.
10. Fortunar hypodermic syringe 10 mL.
11. 0.45 μm nylon 25 mm membrane filter : Whatman, (England).

REAGENTS AND STANDARDS.

1. sodium dodecyl sulfate : Fluka (Switzerland).
2. acetaminophen : Sigma Chemical company, USA.
3. chlorpropamide.
4. methanol : Riedel-deHaen, Germany.
5. n- propanol : May & Baker, England.
6. butanol : May & Baker, England.
7. sodium chloride : J.T Baker Chemical Co.
8. orthophosphoric acid 85% : Deventer (Holland).
9. sodium hydroxide : Merck (Germany).
10. heparin : Sigma Chemical company, USA.
11. plasma samples.

All chemicals were analytical reagent grade. Chlorpropamide was obtained from the Goverment Pharmaceutical Organization. Blank plasma samples were obtained from Yala hospital, Had-Yai hospital, and Songklanagarind hospital. Solvent were routinely filtered through filters prior to use in the liquid chromatograph.

PROCEDURE.

1. Preparation of human plasma sample.

1.1 Blank plasma.

Blank plasma was prepared from normal healthy volunteer, taking no drug, by the process shown in Diagram 1.

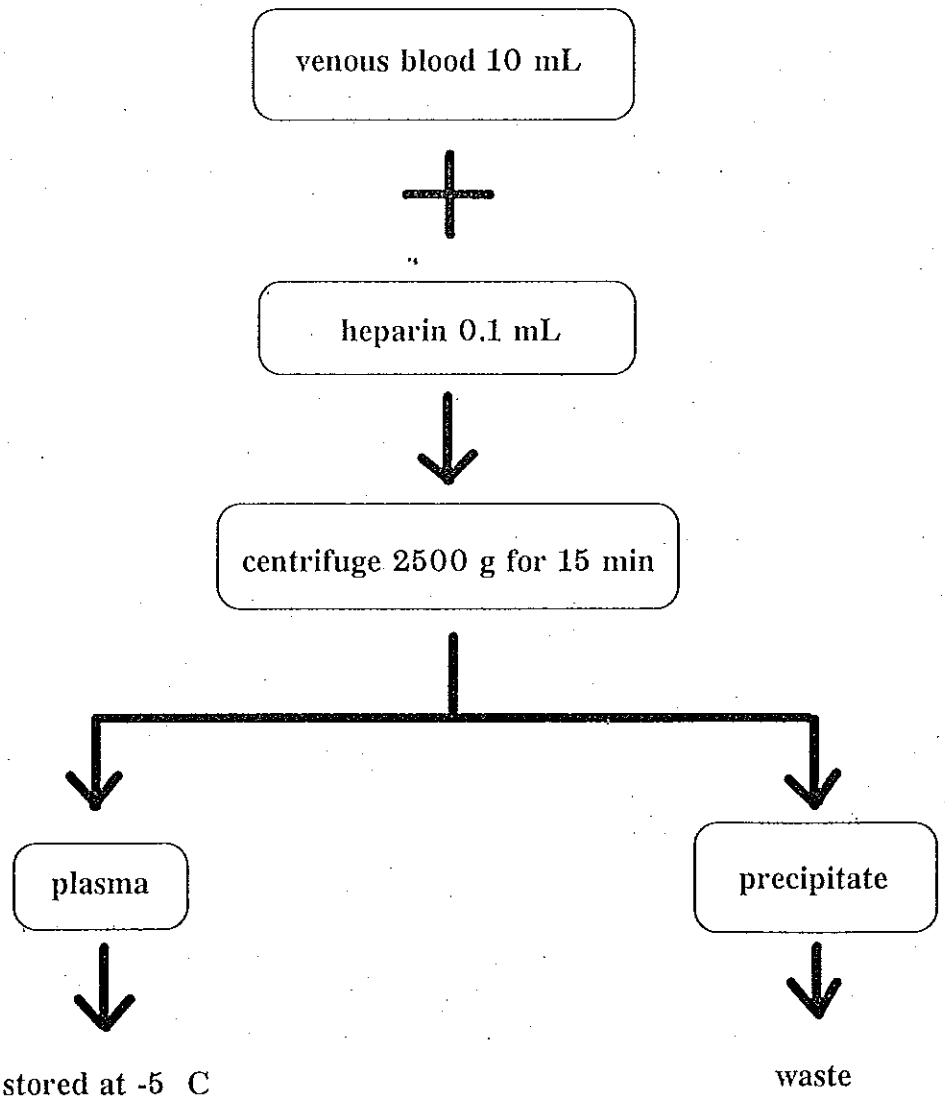


Diagram 1. Preparation of human plasma sample.

1.2 Plasma sample

Volunteers were fasted at least 8 hours prior to the blood collection. Venous blood samples were obtained 3 hours after the oral administration of a single dose of chlorpropamide (125 mg) or acetaminophen (250 mg). Plasma samples were prepared by the process shown in Diagram 1 .

2. Preparation of stock and working standards in aqueous solution.

- Prepared 100 μg/mL stock solution of acetaminophen and chlorpropamide by dissolving in distilled water and 10% methanol, respectively.

- Prepared working standards over the range of 1.0 - 20.0 μg/mL by diluting the stock solution with distilled water.

3. Preparation of stock and working standards in human plasma.

The stock solution (100 μg/mL) was serially diluted with blank plasma to prepare working plasma standards over the range of 1.0 - 20.0 μg/mL. The working plasma standard solutions were filtered through a 0.45 μm nylon membrane filter.

4. Preparation and selection of mobile phases.

Mobile phases were prepared by dissolving the appropriate quantity of surfactant in water and filtering through a sintered glass. Selection of this mobile phase was based on preliminary studies on the effects of concentration of SDS, changes in pH, sodium chloride concentration, type and concentration of modifier on resolution and chromatographic time.

4.1 Concentration of SDS.

Study the effect of SDS, varied concentration from 0.01 to 0.10 M, on retenion time on C_{18} and CN column.

4.2 Change in pH.

Study the effect of various types of buffer used to adjust the pH in SDS micellar mobile phase. The buffers used were phosphate buffer, acetate buffer and phosphoric acid.

Study the change in pH, varied from 2 - 8, of selected buffer in 0.025, 0.05, 0.10 M SDS mobile phase.

4.3 Sodium chloride concentration.

Study the effect of concentration of sodium chloride, varied from 1% to 5% (w/v), in 0.05 and 0.10 M SDS mobile phase.

4.4 Type of modifier.

Study the effect of different alcohols added to the 0.05 M SDS micellar mobile phase. The alcohols used were 1% butanol, 1% propanol and 1% methanol.

Study the various concentration of selected organic modifier, varied from 1% to 5% (v/v), in 0.025, 0.05 and 0.10 M SDS mobile phases.

5. Wavelength optimization.

- Scanned each drug in surfactant solution with spectrophotometer over the wavelength range of 200 - 300 nm, compared with blank surfactant solution to select the optimum wavelength.

- Study the effect of plasma on drug 's absorption spectra.

6. Calibration curve.

Calibration curves were prepared by adding a standard drugs solution to blank plasma so that the final concentration of drug in plasma were 1.0, 5.0, 10.0, 15.0 μg/mL. Plasma standard aliquots were stored at -5 $^{\circ}$C until the time of analysis.

7. Standard addition method.

In this procedure, it allows a calibration to be made up each sample solution which was prepared by adding equal volume of standard solution (varied from 0-30 μg/mL) to plasma sample. After measurement, standard addition give an analytical curve like that in Figure 4. The intercept of this curve (S_C) with the abscissa gives the concentration of drug in plasma sample. In this study, the concentration of drug in plasma sample which was determined by calculating from multiplication of measured concentration (S_C) with dilution factor (2.0).

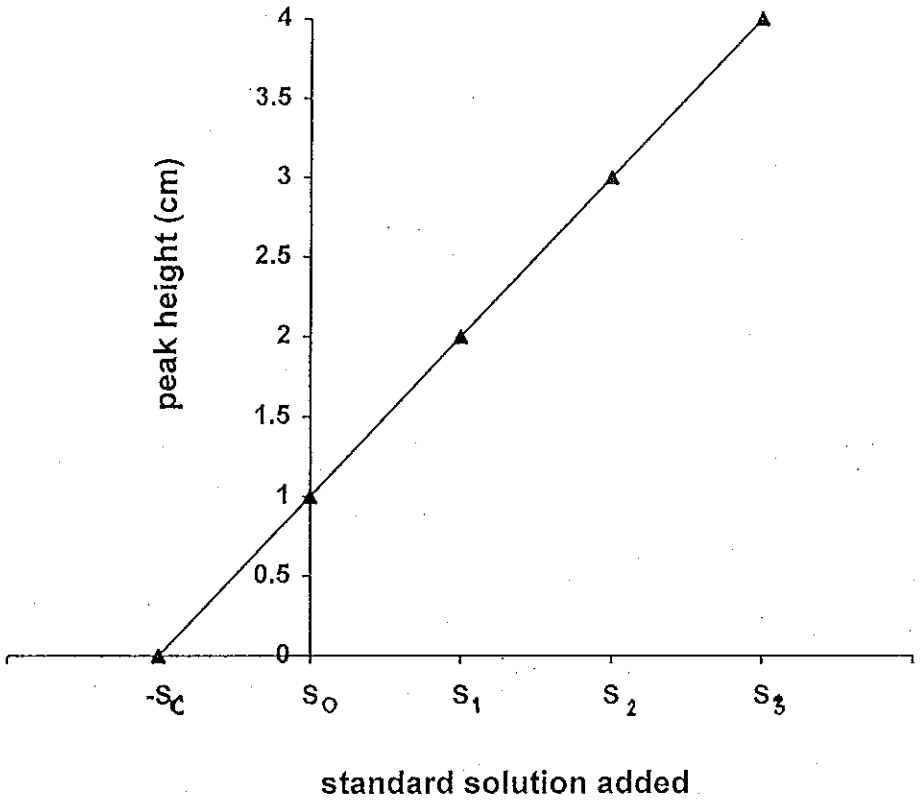


Figure 4. A representation standard addition plot.

8. Linear range.

The linear response was determined by injection of each drug, ranging from 1.0 to 15.0 μg/mL, and measured the resulting peak height at optimum wavelength.

9. Limit of detection.

The limit of detection (LOD) was determined by the analyses of eight replicates of drugs in aqueous solution (range setting = 0.01) and run under the same condition as working condition. The standard deviation (S_n) of the baseline noise was used to calculate the LOD as follows :

$$LOD = 3S_n \text{ / slope of calibration curve}$$

10. Precision.

Within-day precision was determined using 10 replication of each drug in blank plasma at concentration of 10.0 μg/mL and run under working condition. In this study, the useful measure of precision are coefficient of variation (CV) and the calculation of CV will involve the following expression :

$$CV = \frac{S}{\bar{X}} \times 100$$

S is the standard deviation,

$$S = \sqrt{\frac{\sum_i \left(X_i - \bar{X}\right)^2}{n - 1}}$$

n is replicate measurements, X_1, X_2,..., X_i

$\bar{X}$ is mean of this population,

$$\bar{X} = \frac{\sum_i X_i}{n}$$

11. Analytical recovery.

The percentage of analytical recovery of drugs was determined by triple injection of working standard in aqueous solution over the range of 1.0-15.0 μg/mL compared with working standard in plasma. Calculate the percentage recovery of each drug by using

$$\text{recovery (\%)} = \frac{\text{peak height (cm) from working standard in plasma}}{\text{peak height (cm) from working standard in aqueous solution}} \times 100$$

12. Chromatographic condition.

Mobile phases for acetaminophen and chlorpropamide were 0.05 M SDS and 0.10 M SDS + 2% sodium chloride (w/v) + 2% propanol (v/v), respectively. The operating temperature was ambient and the flow rate was 1.0 mL/min, resulting in a pressure of 1000 psi. Detector output was recorded at 0.5 mV and a paper speed was 2.0 mm/min. The column effluent was monitored at 245 nm and 240 nm for acetaminophen and chlorpropamide, respectively. Peak heights were used as a response parameter.

13. Equilibration of column.

Column was equilibrated by flowing the mobile phase through the column until the constant base line and retention time were obtained. In case the mobile phases were adjusted to the desired pH, the column was equilibrated by purging with the mobile phase until the pH of the inlet and the outlet was the same.

14. The dead time.

The dead time (t_m) of system was measured from the injection point at flow rate of 1.0 mL/min of distilled water at 245 nm to the first deviation of the base line and found to be 2.43 min and 0.53 min, for C_{18} and CN column, respectively.

15. The capacity factor.

The capacity factor (k') is used as a measure of the degree of retention of the solutes, is defined as :

$$k' = \frac{t_R - t_m}{t_m}$$

t_R is the elution time of the solute.

t_m is the elution time of unretained solute.

Each k' is the average of the value that obtained from three replicate injection.

16. Efficiency Study.

The problem in MLC is that the peak shape of many solutes deviated from the ideal Gaussian shape by the appearance of a tail. Efficiency was calculated as theoretical plates, according to the equation of *Foley and Dorsey (1983 : 730-737)* who described a simple yet accurate equation for plate count which corrects for the asymmetry of skewed peaks. The equation used to calculate the efficiency in this work was

$$N = \frac{41.7(t_R / W_{0.1})^2}{(B / A) + 1.25}$$

Where $W_{0.1}$ is the peak width at 10 % peak height.

B/A is the peak asymmetry factor measured by the ratio of the back (B) and front (A) half portion of the peak (A+B = $W_{0.1}$).

CHAPTER 3

RESULTS AND DISCUSSION

Retention of solute in MLC can be controlled by the bulk mobile phase, micellar pseudophase and stationary phase. The key to separation is the ability to change the bulk mobile phase-stationary phase or bulk mobile phase-micellar pseudophase interaction in such a way as to shift the retention of some overlapping compounds. In some cases, the selectivity of the chromatographic system can be modified by simply changing concentration of surfactant, type or concentration of organic modifier and salt concentration or pH of the mobile phase. In this study, the results will be devided into 2 section as follow :

Section A. The study of acetaminophen 's and chlorpropamide 's behavior eluted with an aqueous micellar mobile phase containing a surfactant (SDS) in various conditions.

Section B. The quantitative determination of acetaminophen and chlorpropamide in human plasma.

Section A. The study of acetaminophen 's and chlorpropamide 's behavior eluted with an aqueous micellar mobile phase containing a surfactant (SDS) in various conditions.

1. Effect of SDS concentration on capacity factor in different stationary phases.

The effect of SDS concentration on retention time is controlled by the bulk mobile phase-stationary phase interaction (K_1) and bulk mobile phase-micellar pseudophase interaction (K_2). (*Yarmchuk, et al. : 1982 : 2233-2238*). Hence, stationary-phase polarity has effect on K_1 and retention time of solute.

Cyano-bonded and ODS-bonded silica columns interact very differently with surfactant monomer resulting in different elution behavior of organic acids and bases as a function of micellar mobile phase concentration and pH. In this study, both acetaminophen and chlorpropamide were found to have a different elution behavior on CN and C_{18} column as in the following details :

1.1 On C_{18} column.

The packing meterial of C_{18} column is octadecyl-sillica, containing C_{18} chains, which produces a non-polar surface. Hence, drugs with different polarity will show the different retention behavior on C_{18} column.

Retention behaviors of aectaminophen and chlorpropamide were shown in Table 1, and the plot of k' versus SDS concentration for acetaminophen and chlorpropamide were shown in Figure 5 and 6, respectively. From this data, the capacity factor of both acetaminophen and chlorpropamide decreased with the increase in micelle concentration. However, k' of acetaminophen is lower than chlorpropamide at any SDS concentration. Acetaminophen is a polar compound and has less hydrophobic interaction. Then, it is eluted quickly on C_{18} column because of the repulsion from both the micelle and negative charged modified stationary phase. Chlorpropamide is a less polar compound, therefore it should only be affected by hydrophobic interaction. This effect may be sufficiently for chlorpropamide to retain in the column for a longer period of time.

Table 1. The capacity factor ($k' = (t_R - t_m)/t_m$) of acetaminophen and chlorpropamide on C_{18} column with various SDS concentrations in the mobile phase.

[SDS], M	capacity factor (k')	
	acetaminophen	chlorpropamide
0.01	2.12	9.86
0.025	1.60	9.11
0.05	1.26	7.86
0.10	0.88	7.02

* t_m = 2.43 min.

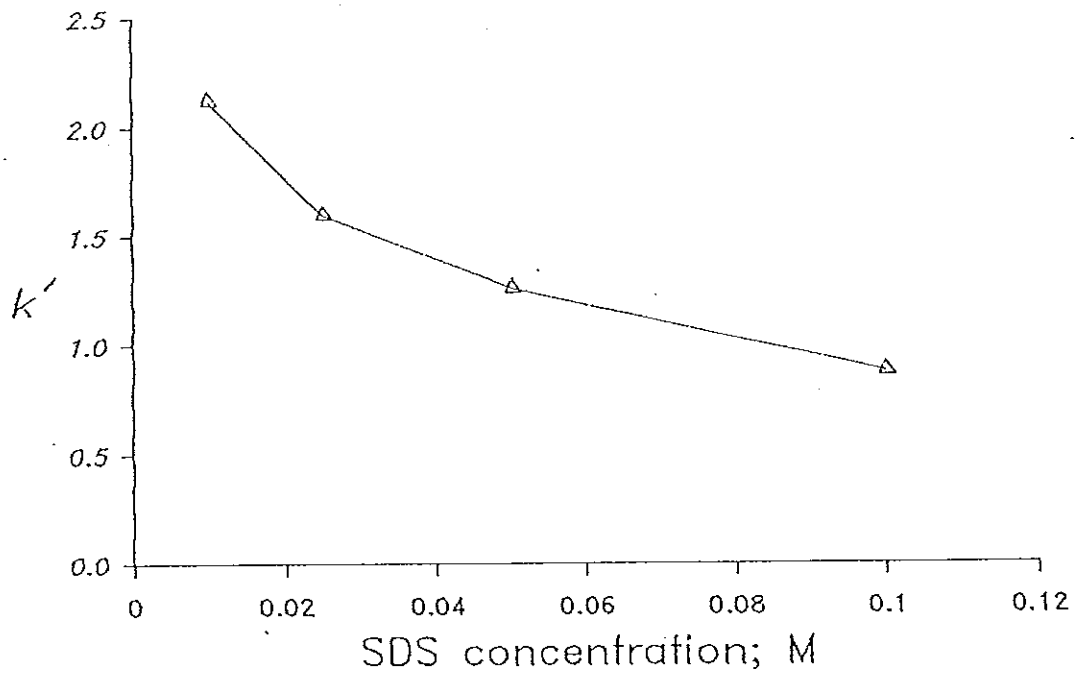


Figure 5. Dependence of k' on various SDS concentrations of acetaminophen on C_{18} column with the flow rate of 1.0 mL/min.

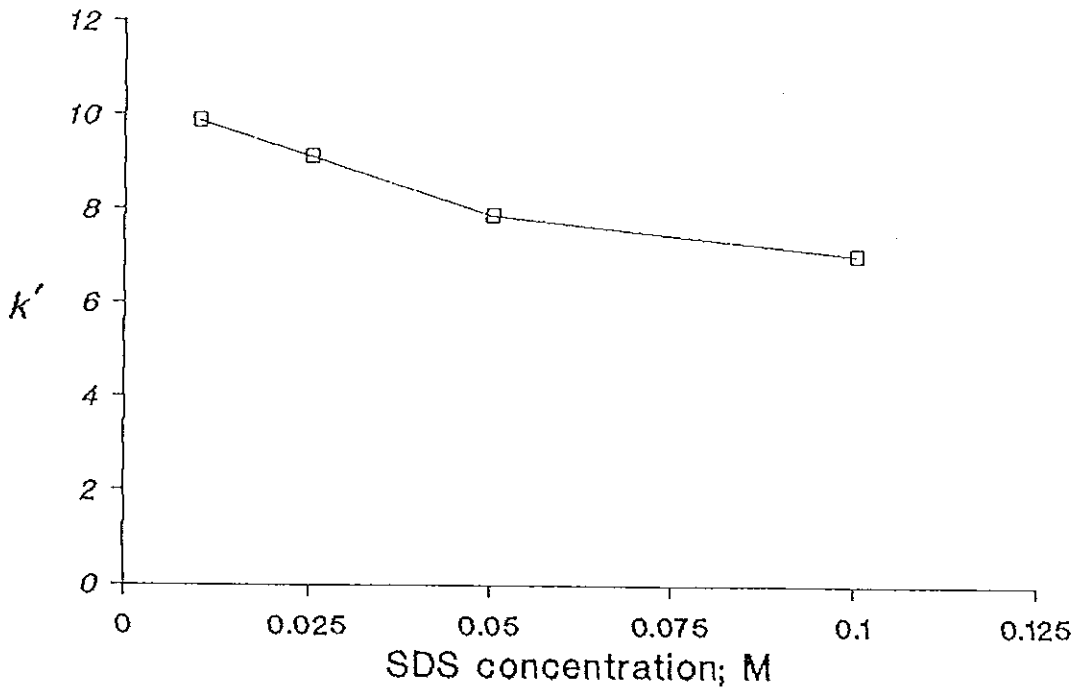


Figure 6. Dependence of k' on various SDS concentrations of chlorpropamide on C_{18} column with the flow rate of 1.0 mL/min.

1.2 On cyano column.

On cyano (CN) column, retention behavior of acetaminophen and chlorpropamide are shown in Table 2, and the plot of capacity factor (k') versus SDS concentration where k' decrease with increasing SDS concentration from 0.025 to 0.10 M are illustrated in Figure 7 and 8 for acetaminophen and chlorpropamide, respectively.

It can be seen that the capacity factor of acetaminophen on cyano column is larger than on C_{18} column. Because the concentration of SDS adsorbed on C_{18} silica is higher than on CN silica *(Berthod, et al., 1986 : 1356-1362)*. Thus, the stationary phase of cyano column trend to have less negative charge than on C_{18} column. Therefore, on cyano column, acetaminophen is less repulsed and longer retained when compare with C_{18} column.

The capacity factor of chlorpropamide on cyano column is less than on C_{18} column. Because chlorpropamide is less polar, it likes to dissolve in non-polar phase (C_{18}-silica) more than polar phase (cyano silica). Thus, it has more hydrophobic interaction and longer retained on C_{18} column than cyano column.

Table 2. The capacity factor ($k' = (t_R - t_m)/t_m$) of acetaminophen and chlorpropamide on CN column with verious SDS concentrations in the mobile phase.

[SDS], M	capacity factor (k')	
	acetaminophen	chlorpropamide
0.025	4.96	5.15
0.050	3.77	3.87
0.075	3.57	2.81
0.100	3.43	2.79

* t_m = 0.53 min.

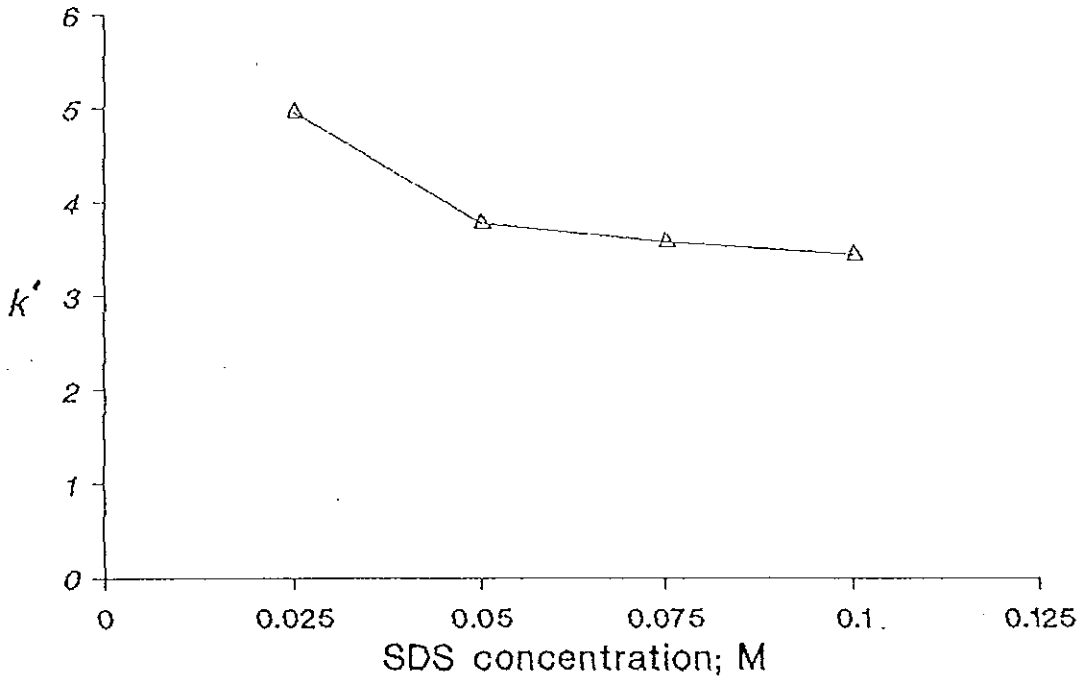


Figure 7. Dependence of k' on various SDS concentrations of acetaminophen on CN column with the flow rate of 1.0 mL/min.

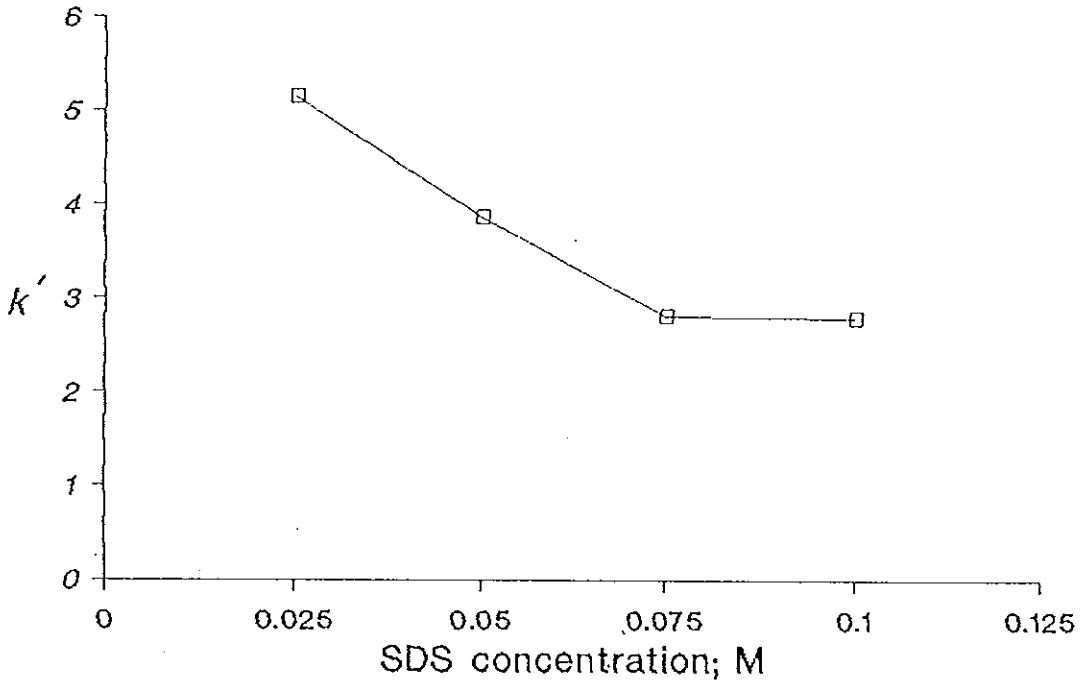


Figure 8. Dependence of k' on various SDS concentrations of chlorpropamide on CN column with the flow rate of 1.0 mL/min.

Another important observation is the elution behaviour of both drugs. When the capacity factor of both drugs are plotted against surfactant concentration, the resulting plots are not parallel but intersect one another, which leads to reversal in the elution. This point is amplified in Figure 9 with less than 0.05 M SDS the elution order is acetaminophen and chlorpropamide, while with more than 0.05 M SDS, the elution order is reversed.

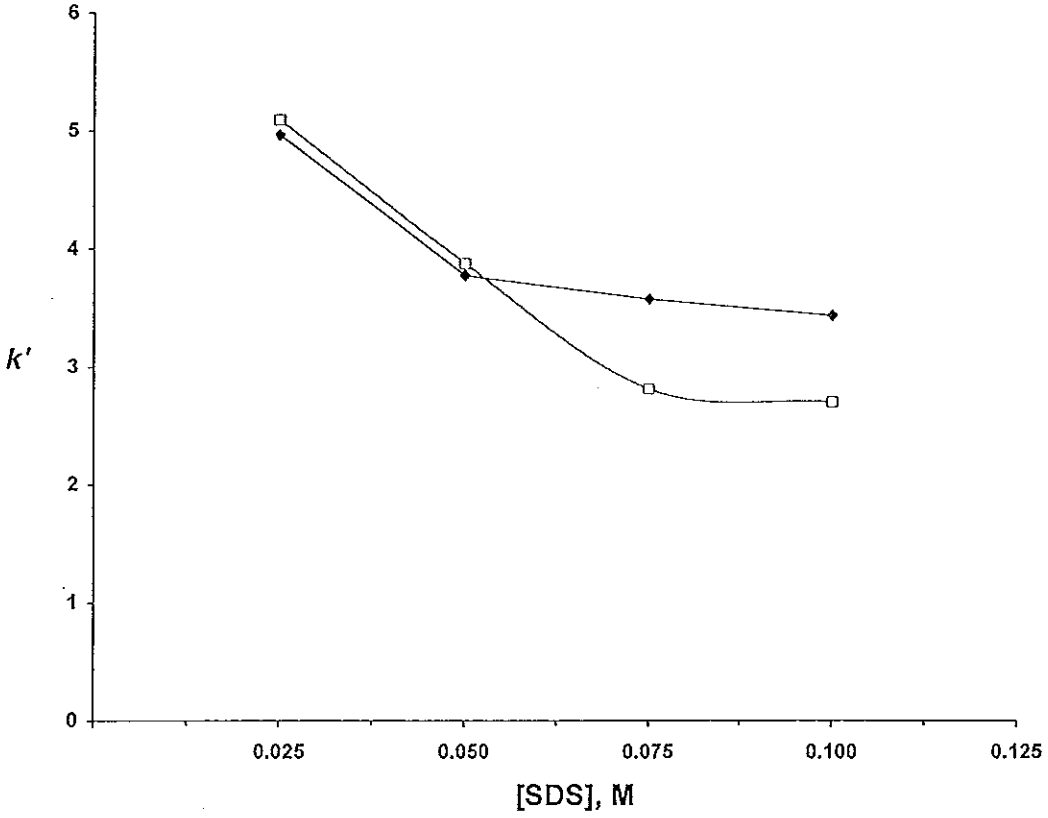


Figure 9. Influence of SDS concentrations on capacity factor : (◆) acetaminophen, (□) chlorpropamide on CN column with flow rate of 1.0 mL/min.

2. Addition of modifiers to micellar mobile phase.

A comparative study is performed to observe the effect of different alcohols added to the SDS micellar mobile phase on the retention time and the efficiency of chromatographic peak of chlropropamide and acetaminophen. For the preparation of mobile phase, a 0.05 M SDS solution was selected. The alcohols used are 1% methanol, 1% propanol and 1% butanol. Results are shown in Table 3.

The result showed that the addition of alcohol in SDS micellar mobile phase produced a decrease in capacity factor. Besides the significantly alteration of the equilibrium of the solute away from the stationary phase and the micelle toward the bulk aqueous phase, the bulky aqueous phase is less polar with the addition of an organic modifier, which lead to a reduction in the capacity factor (*Domingo, et al., 1992 : 843 - 847*).

For acetaminophen, its capacity factor was slighly affected by addition of different alcohols. With 1% butanol the lowest capacity factor was obtained and 1% methanol give the best efficiency.

When the organic modifier was added, the decreasing order of capacity factor of chlorpropamide are as following : 1% butanol < 1% propanol < 1% methanol and order of increased efficiency is as following : 1% propanol > 1% butanol > 1% methanol. In comparision of propanol, butanol has more viscoscity which lead to

high back-pressure of column and it has solvent strength higher than propanol. Thus, it cause protein precipitated in plasma. Then, propanol is selceted as modifier for determination of chlorpropamide.

With respect to the efficiency, the behaviour is variable, but frequently the best effciency corresponded to a micellar mobile phase of 3% propanol, as indicated by other workers *(Dorsey, et al. 1983 : 924-928)*

The effect of variation of the percentage of propanol in the mobile phase on capacity factor of acetaminophen and chlorpropamide are illustrated in Table 4. A decrease in capacity factor is observed by a corresponding increase in percentage of propanol concentration. From this data, the plot of k' versus %v/v propanol is illustrated in Figure 10 and Figure 11 for acetaminophen and chlorpropamide, respectively.

Table 3. Influence of the organic modifier in 0.05 M SDS on the values of capacity factor (k'), and column efficiency (N) of acetaminophen and chlorpropamide on C_{18} column.

drugs	no modifier		1% methanol		1% propanol		1% butanol	
	k'	N	k'	N	k'	N	k'	N
acetaminophen	1.26	1552	1.25	1541	1.10	1339	0.85	1229
chlorpropamide	7.86	130	4.86	373	4.18	636	3.19	480

* $t_M = 2.43$ min

Table 4. Influence of propanol on the capacity factor of acetaminophen and chlorpropamide on C_{18} column with various SDS concentrations in the mobile phase.

		capacity factor	
[SDS], M	propanol (%v/v)	acetaminopen	chlorpropamide
0.025	0	1.60	9.11
	1	1.14	5.23
	3	0.78	4.12
	5	0.66	2.44
0.05	0	1.26	7.86
	1	1.10	4.18
	3	0.70	3.21
	5	0.44	2.04
0.10	0	0.88	7.02
	1	0.47	3.35
	3	0.42	1.95
	5	0.39	1.25

* t_m = 2.43 min.

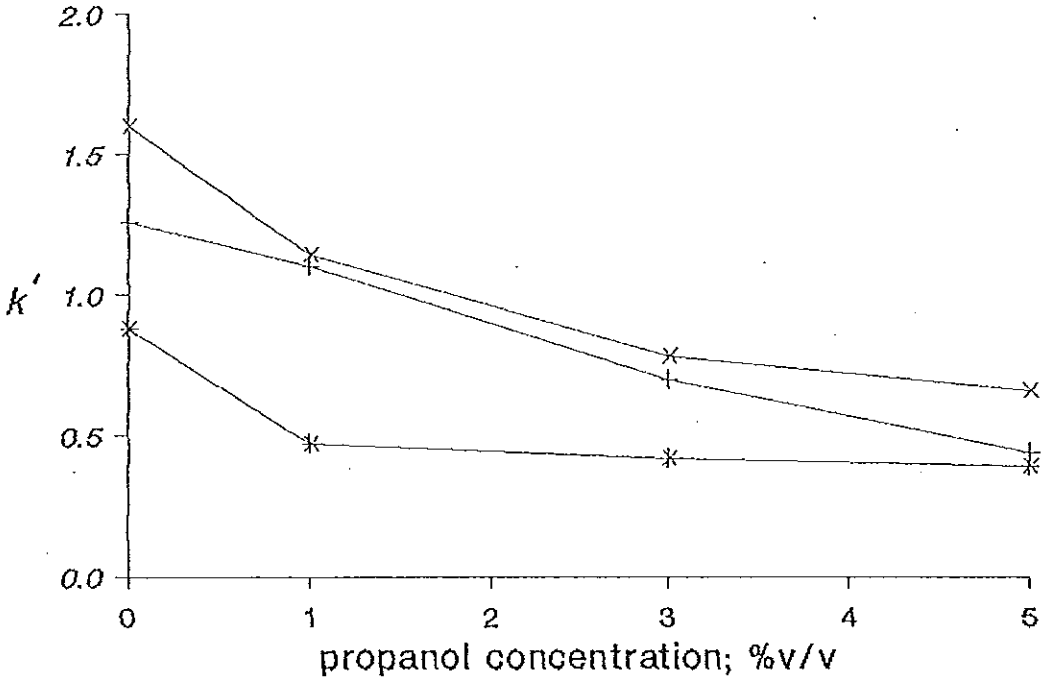


Figure 10. Influence of propanol on capacity factor of acetaminophen. : (x) 0.025, (+) 0.050, and (∗) 0.100 M SDS on C_{18} column with the flow rate of 1.0 mL/min.

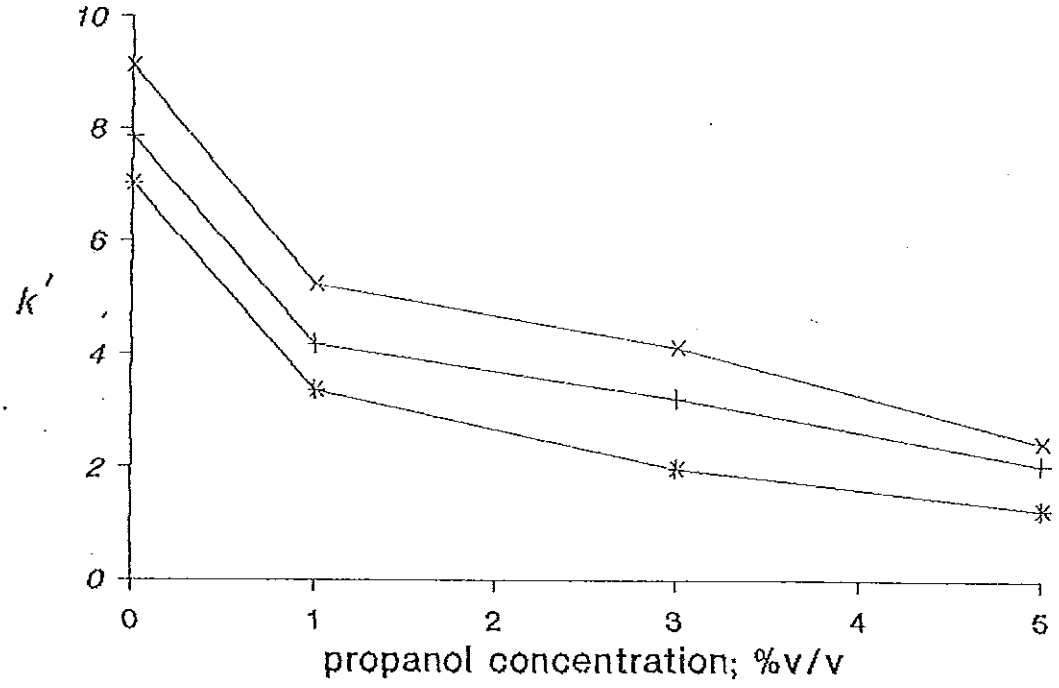


Figure 11. Influence of propanol on capacity factor of chlorpropamide: (x) 0.025, (+) 0.050, and (∗) 0.100 M SDS on C_{18} column with the flow rate of 1.0 mL/min.

3. Influence of pH.

The pH of the micellar mobile phase is an important factor to control the selectivity of ionisable drugs such as acetaminophen (pK_a = 9.5), and chlorpropamide (pK_a = 5.0) on C_{18} column.

A comparative study is performed to observe the effect of different buffers for adjusting the pH in micellar mobile phase on the retention time of both drugs and the profile of the chromatogram. The buffers tested were phosphate buffer, acetate buffer and phosphoric acid.

The results from the present study showed that retention time of both drugs has no significant difference and have no effect on separation system. So in this study, phosphoric acid is choosen for adjusting pH in micellar mobile phase, because it is convenient for preparation.

The pH dependence on retention times were examined with buffer solution containing 0.025 to 0.10 M SDS in the pH range of 3.0-7.0. The results were shown in Table 5 which indicated the effect of various pHs on capacity factor of both drugs. The values of capacity factor at various SDS mobile phase concentration were plotted with pH values, as shown in Figure 12 and 14 for acetaminophen and chlorpropamide, respectively.

For acetaminophen, as the pH increase, from 3.0 to 7.0, the capacity factor hardly altered at 0.025 - 0.100 M SDS as shown in

Figure 12. Because the range of pH under study is lower than its pK_a or isoeluting point*, therefore, the ratio of two species (acid and conjugate base) is negligibly altered. Figure 13 illustrates that capacity factor of acetaminophen is independent on the pH.

For chlorpropamide, Figure 14 reveals that the capacity factor decreases with the increase in pH. When the pH increases from 3.0 to 5.0, capacity factor slightly decrease and rapidly decrease when the pH increased from 5.0 to 6.2 while the pH from 6.2 to 7.0, capacity factor slowly decreased. From these data, it can be explained that chlorpropamide is a weak acid, so in acidic solution where the neutral form is presence, k' values decrease and remain constant in more basic solution where the anionic acid form is present, therefore, it is electrostatically repulsed by both the negative micelle and stationary phase. These behavior can be seen in Figure 15, with the noticeable that at low pHs (3.0, 4.0, and 5.0) the capacity factor of chlorpropamide is markedly decrease but at pH 6.2 and 7.0, capacity factor is slighly decrease while the SDS concentration increase.

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* In the intermediate pH value range. There is an isoeluting point where the capacity factor is completely independent of SDS concentration. This point is the pH where two species (acid and conjugate base or base and conjugate acid) in equilibrium with each other have the same k' value *(Arunyanart and Cline Love 1985 : 2457-2483).*

Table 5. Influence of the pH on the capacity factor of acetaminophen and chlorpropamide on C_{18} column with various SDS concentration in the mobile phase.

		capacity factor	
[SDS], M	pH	acetaminophen	chlorpropamide
0.025	3.0	1.54	57.13
	4.0	1.60	53.74
	5.0	1.60	45.93
	6.2	1.60	9.11
	7.0	1.54	4.82
0.05	3.0	1.51	24.13
	4.0	1.54	24.23
	5.0	1.53	22.62
	6.2	1.26	7.86
	7.0	1.53	3.21
0.10	3.0	0.84	12.78
	4.0	0.85	13.06
	5.0	0.84	12.65
	6.2	0.86	7.02
	7.0	0.84	2.46

* t_m = 2.43 min.

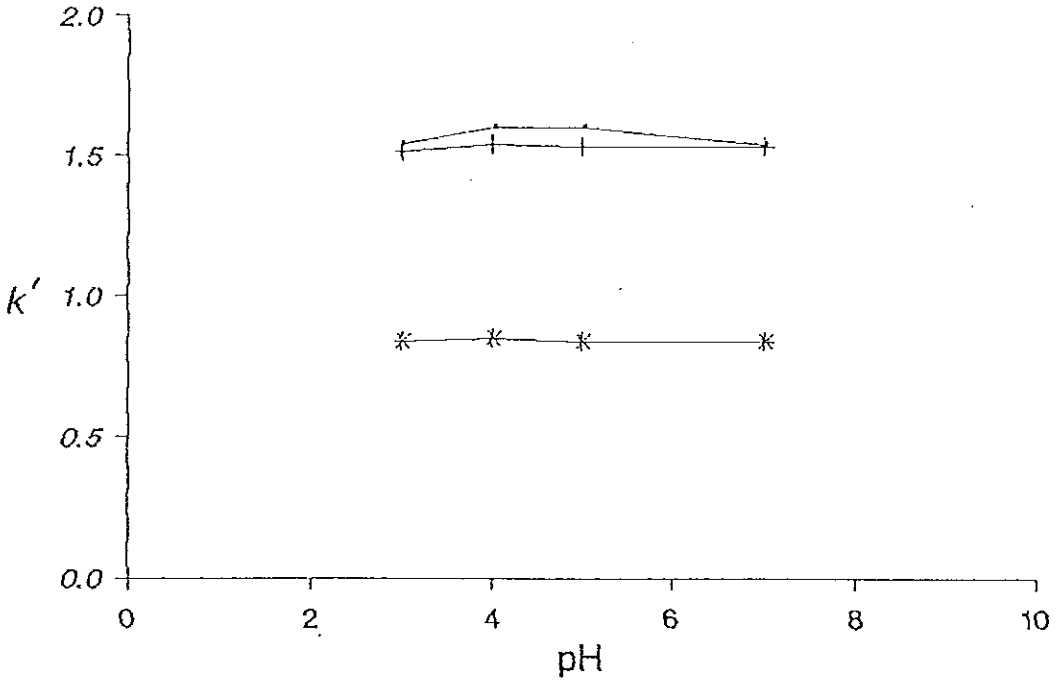


Figure 12. Plot of the capacity factor of acetaminophen versus pH at various SDS concentration : (·) 0.025, (+) 0.05, and (*) 0.10 M SDS on C_{18} column ; flow rate, 1.0 mL/min.

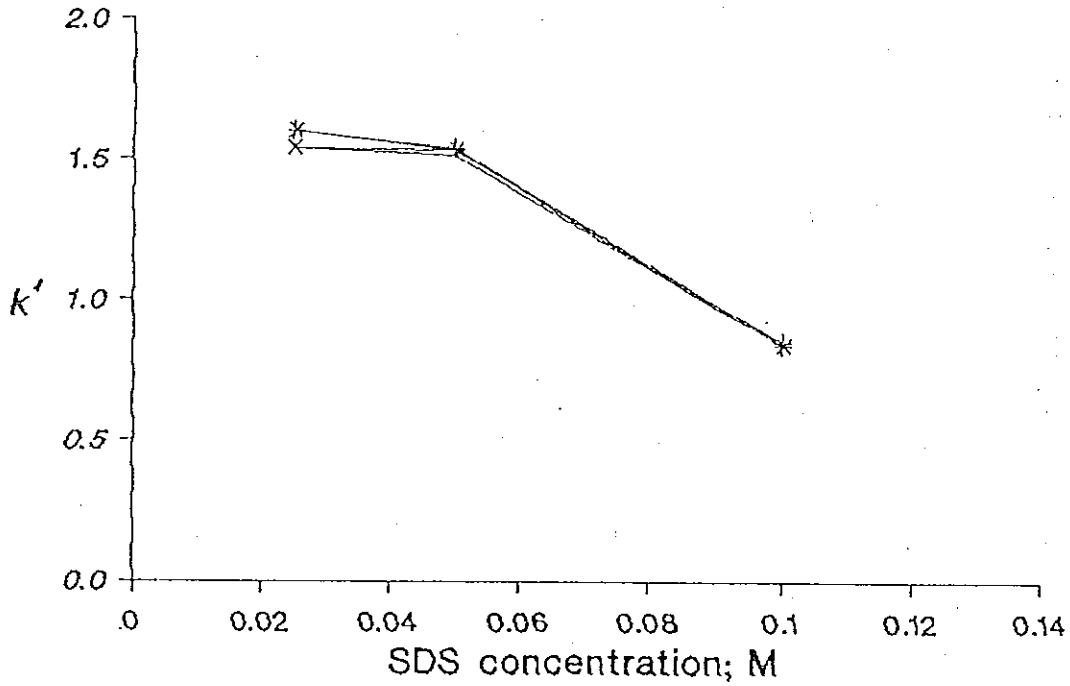


Figure 13. Plot of the capacity factor of acetaminophen versus SDS concentration at (·) pH 3.0, (+) pH 4.0, (∗) pH 5.0, and (×) pH 7.0 on C_{18} column ; flow rate, 1.0 mL/min.

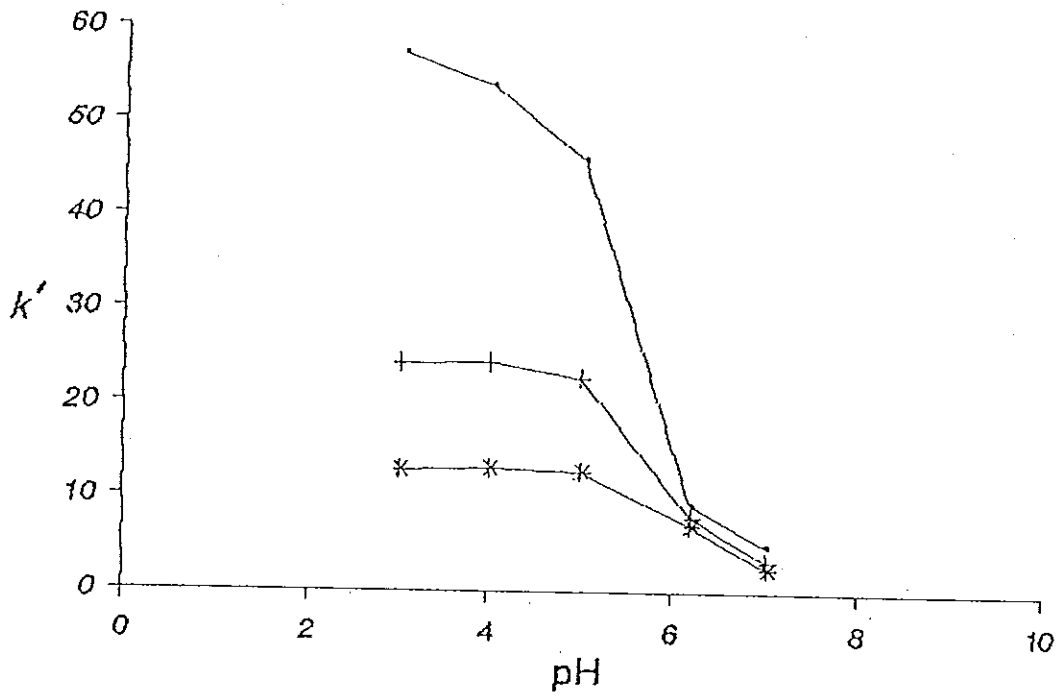


Figure 14. Plot of the capacity factor of chlorpropamide versus pH at various SDS concentration : (·) 0.025, (+) 0.05, and (*) 0.10 M SDS on C_{18} column ; flow rate, 1.0 mL/min.

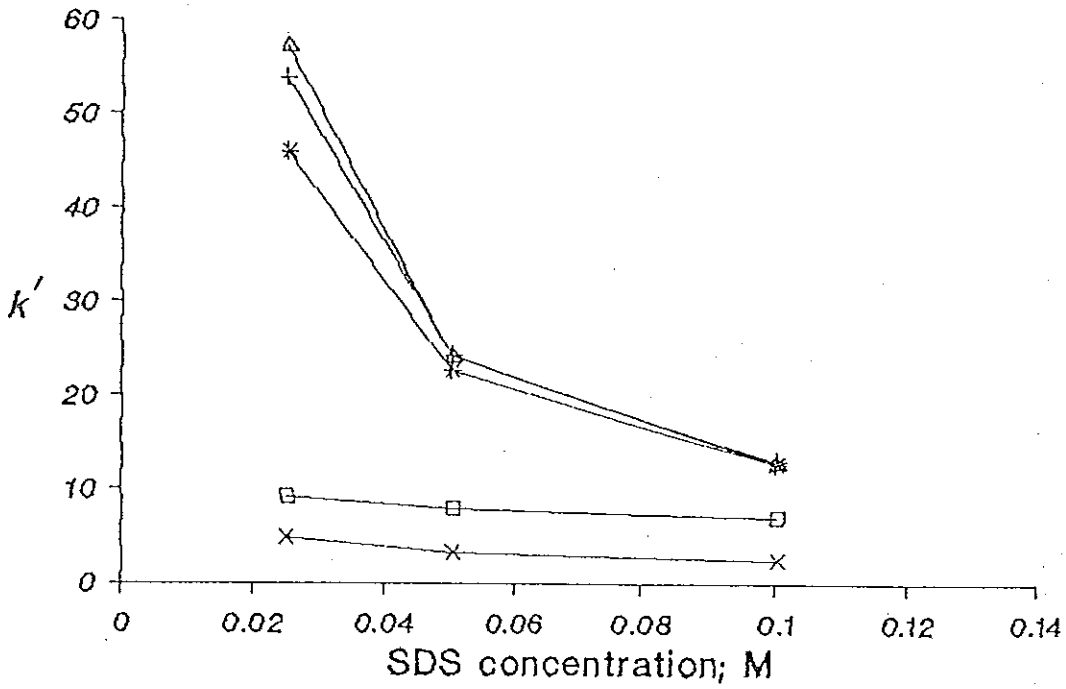


Figure 15. Plot of the capacity factor of chlorpropamide versus SDS concentration at (Δ) pH 3.0, (+) pH 4.0, (*) pH 5.0, (□) pH 6.2, and (×) pH 7.0 on C_{18} column ; flow rate, 1.0 mL/min.

4. Effect of sodium chloride on capacity factor.

The effect of sodium chloride on capacity factor of both drugs are shown in Table 6 and retention behavior of acetaminophen and chlorpropamide; plotted between capacity factor versus sodium chloride concentration; are shown in Figure 16 and 17, respectively.

In general, capacity factor of solutes will increase when sodium chloride are added to the micellar mobile phase. But in the case of acetaminophen, as shown in Figure 16, the addition of sodium chloride has no effect on its capacity factor. *Armstrong and Stine (1983 : 1317-1320)* explained that frequently the added salt resulted in an antibinding solute, changing from an antibinding to a binding type of behavior. However, not all compounds show this same simple trend, indicating that more than simple, electrostatic effect must be consider. For example, interaction of Napthol Green B with SDS micelles appears to be unaffected by the addition of salt *(Hernandez, 1992 : 871-837).*

The retention behavior of chlorpropamide showed in Figure 17, its capacity factor increases with the increase sodium chloride concentration, indicate that chlorpropamide can be less soluble in the bulk aqueous (salting-out effect). In another reason, added sodium chloride can cause a decrease in the interfacial potential at the micelle surface which will allow stronger solute-micelle interaction

which mean that at higher sodium chloride concentrations, retention of solute increased *(Arunyanart and Cline Love 1985 : 2457-2483).*

Table 6. Influence of the sodium chloride in SDS mobile phase on the capacity factor of acetaminophen and chlorpropamide on C_{18} column with SDS concentration in the mobile phase.

		capacity factor	
[SDS], M	[NaCl], %w/v	acetaminophen	chlorpropamide
0.05	0	1.26	7.86
	1	1.27	10.35
	2	1.26	14.37
0.10	0	0.88	7.02
	1	0.86	9.49
	2	0.88	11.11

* t_m = 2.43 min.

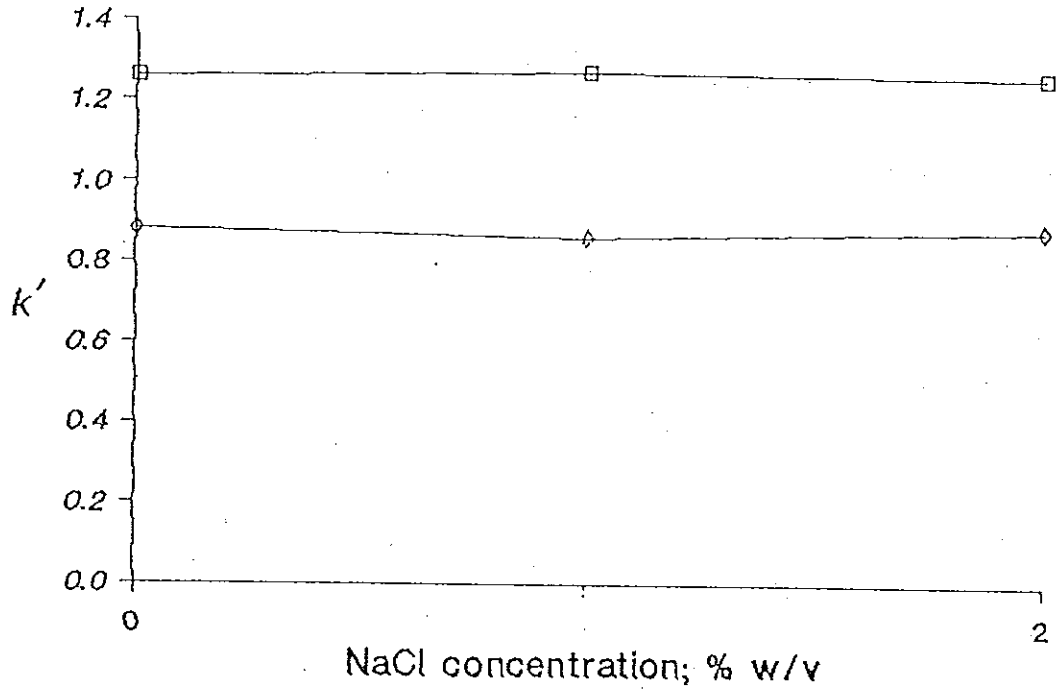


Figure 16. Plot of the capacity factor of acetaminophen versus sodium chloride concentration (%w/v) at (□) 0.05, (◊) 0.10 M SDS on C_{18} column ; flow rate, 1.0 mL/min.

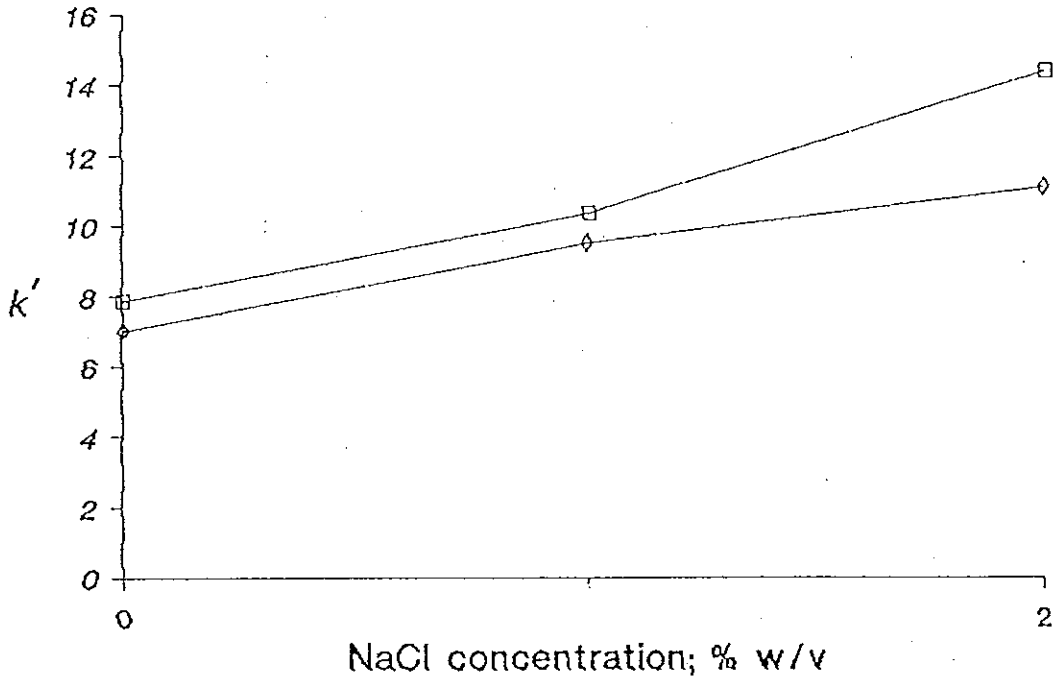


Figure 17. Plot of the capacity factor of chlorpropamide versus sodium chloride concentration (%w/v) at (□) 0.05, (◊) 0.10 M SDS on C_{18} column ; flow rate, 1.0 mL/min.

Section B. The quantitative determination of acetaminophen and chlorpropamide in human plasma.

1. Absorption spectra.

The wavelength of maximum absorbance for chlorpropamide and acetaminophen in aqueous solution are 230 and 245 nm, respectively, which are obtained from UV-VIS spectrophotometer by scanning wavelength from 200 to 300 nm. The maximum absorption of chlorpropamide and acetaminophen are shown in Figure 18 and 19, respectively.

The chromatograms of chlorpropamide in plasma at various wavelength are shown in Figure 20. It is noticable that at the shorter wavelength, the more plasma and impurities in plasma can be absorbed. Thus, the optimum wavelength of chlorpropamide at 230 nm is not suitable and should shift to longer wavelength which is at 240 nm. For the determination of acetaminophen in plasma, the maximum wavelength of 245 nm can be used because chromatographic peak of acetaminophen is not interfered by protein and impurities in plasma (Figure 27).

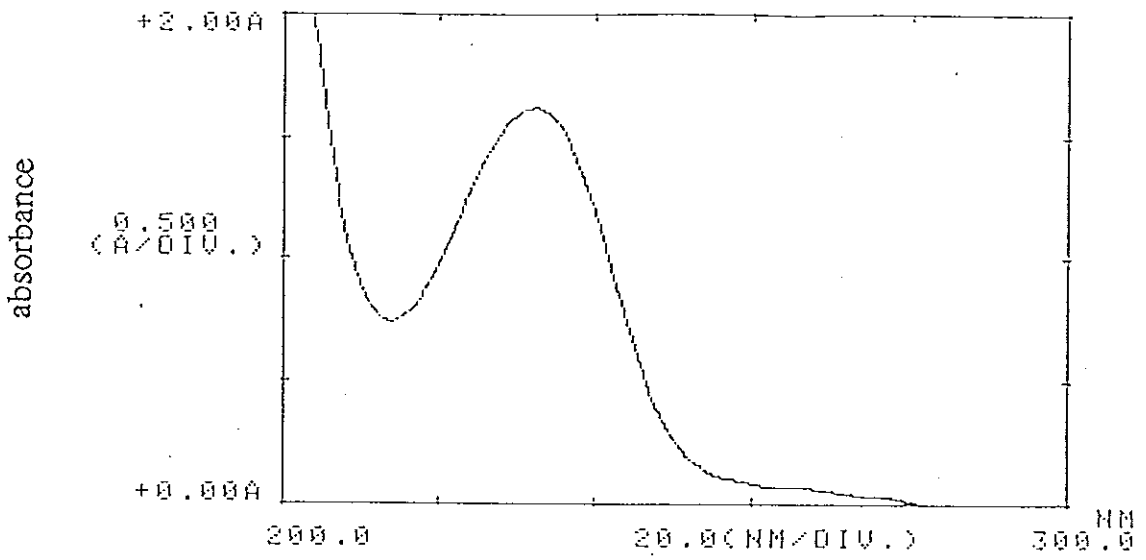
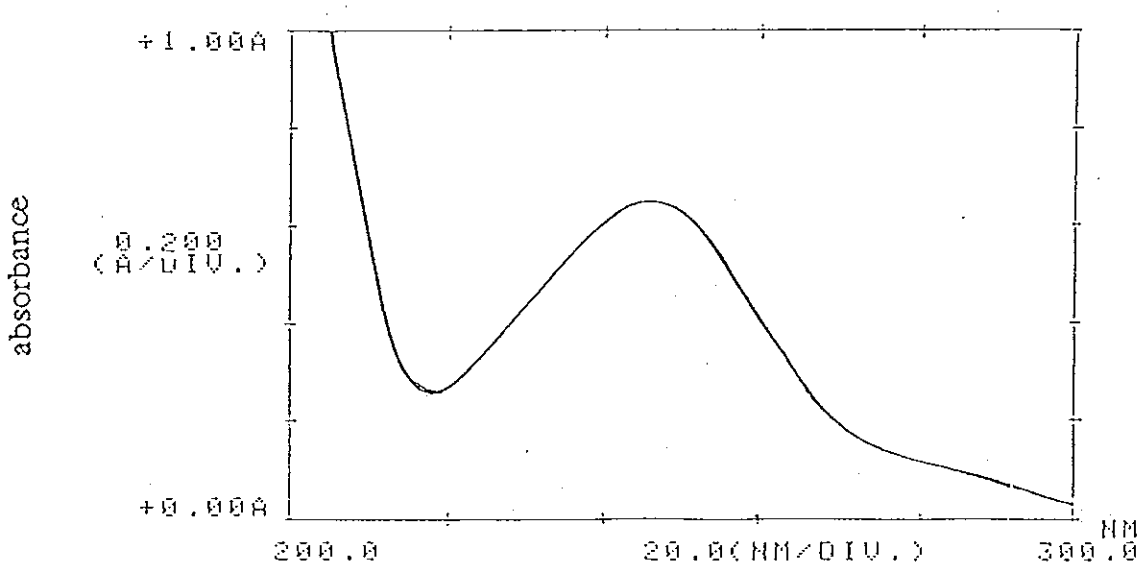
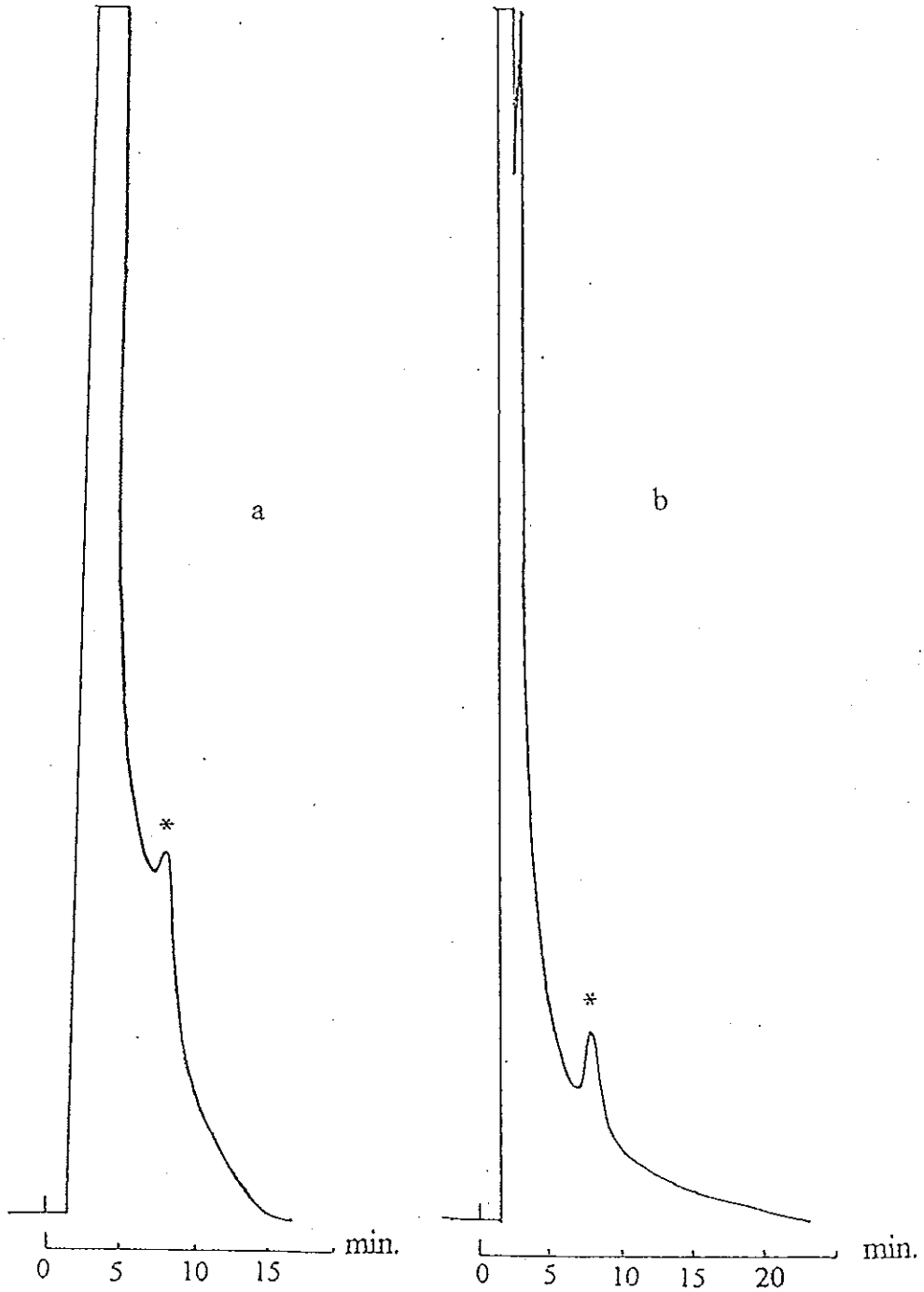


Figure 18. UV spectrophotometric absorption spectra of chlorpropamide in aqueous solution.



wavelength (nm)

Figure 19. UV spectrophotometric absorption spectra of acetaminophen in aqueous solution.


a
b
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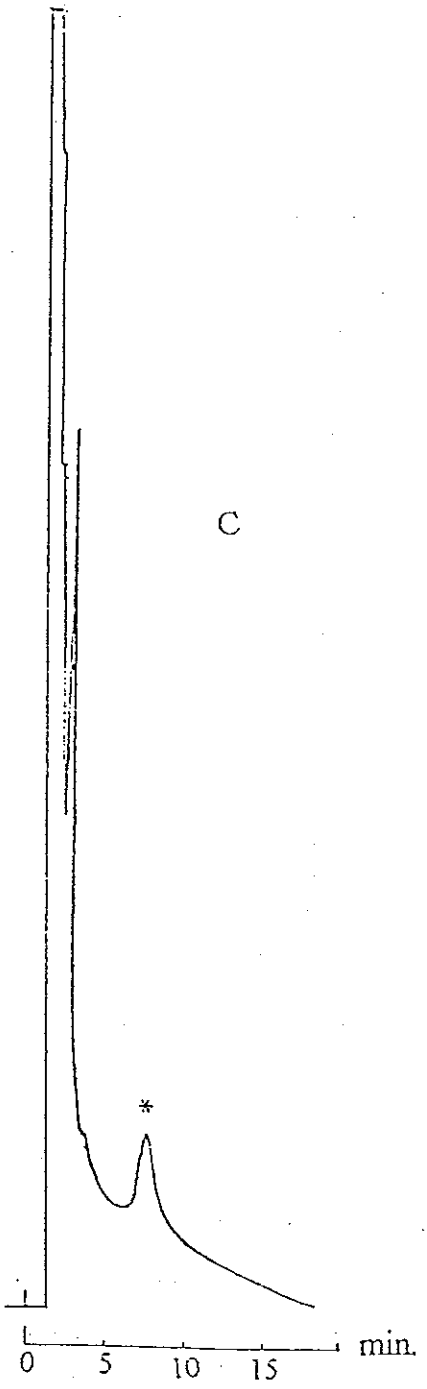


Figure 20. Chromatograms of chlorpropamide in plasma at (a) 230, (b) 235, and (c) 240 nm in 0.10 M SDS + 2% propanol + 2% NaCl on C_{18} column; flow rate is 1.0 mL/min, (*) = chlopropamide.

2. Optimum organic modifier and sodium chloride in mobile phase.

Organic modifer and sodium chloride were added to micellar mobile phase for controlling retention and selectivity of both drugs. Another important reason is to improve the resolution of adjacent chromatographic bands.

The optimum micellar mobile phase for the determination of acetaminophen in plasma is 0.05 M SDS without any modifier, because acetaminophen 's peak is not interfered by impurities or protein in plasma (Figure 21) and can be determined in a short analysis time. In the determination of chlorpropamide, organic modifier and sodium chloride must be added in mobile phase, because in mobile phase without modifier (0.10 M SDS), chlorpropamide 's peak overlap with peak of impurities or protein in plasma (Figure 22).

In this study, addition of propanol and sodium chloride was limited by the precipitation of protein in plasma. If propanol $\geq$ 5% (v/v) is added, protein in plasma will precipitate. From the determination of chlorpropamide in plasma, a separated peak of chlorpropamide from peak of impurity or protein can not be obtained by addition of propanol (Figure 23 and Figure 24) or sodium chloride (Figure 25 and Figure 26) in 0.10 M SDS.

Thus, mixing both propanol and sodium chloride in mobile phase were tested and the results are shown in Figure 27, 28, 29, and Figure 30.

According to the results of separation mentioned above, optimum modifier in mobile phase of chlopropamide is 2% (v/v) propanol and 2% (w/v) sodium chloride containing 0.1 M SDS which is selected for quantitative determination of chlorpropamide (Figure 30).

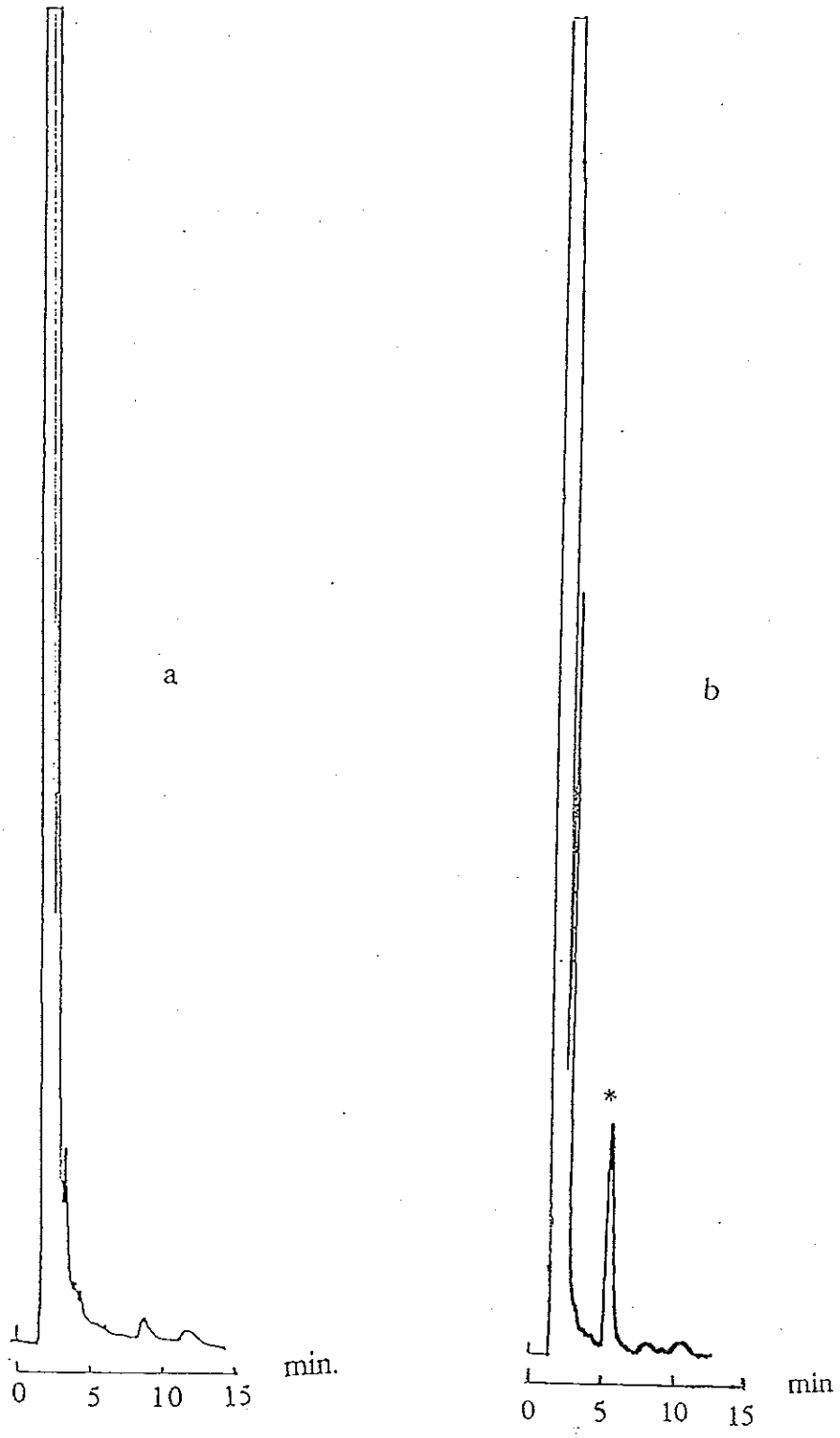


Figure 21. Chromatograms showing the separation of acetaminophen with mobile phase : 0.05 M SDS, (a) blank plasma. (b) acetaminophen in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = acetaminophen.

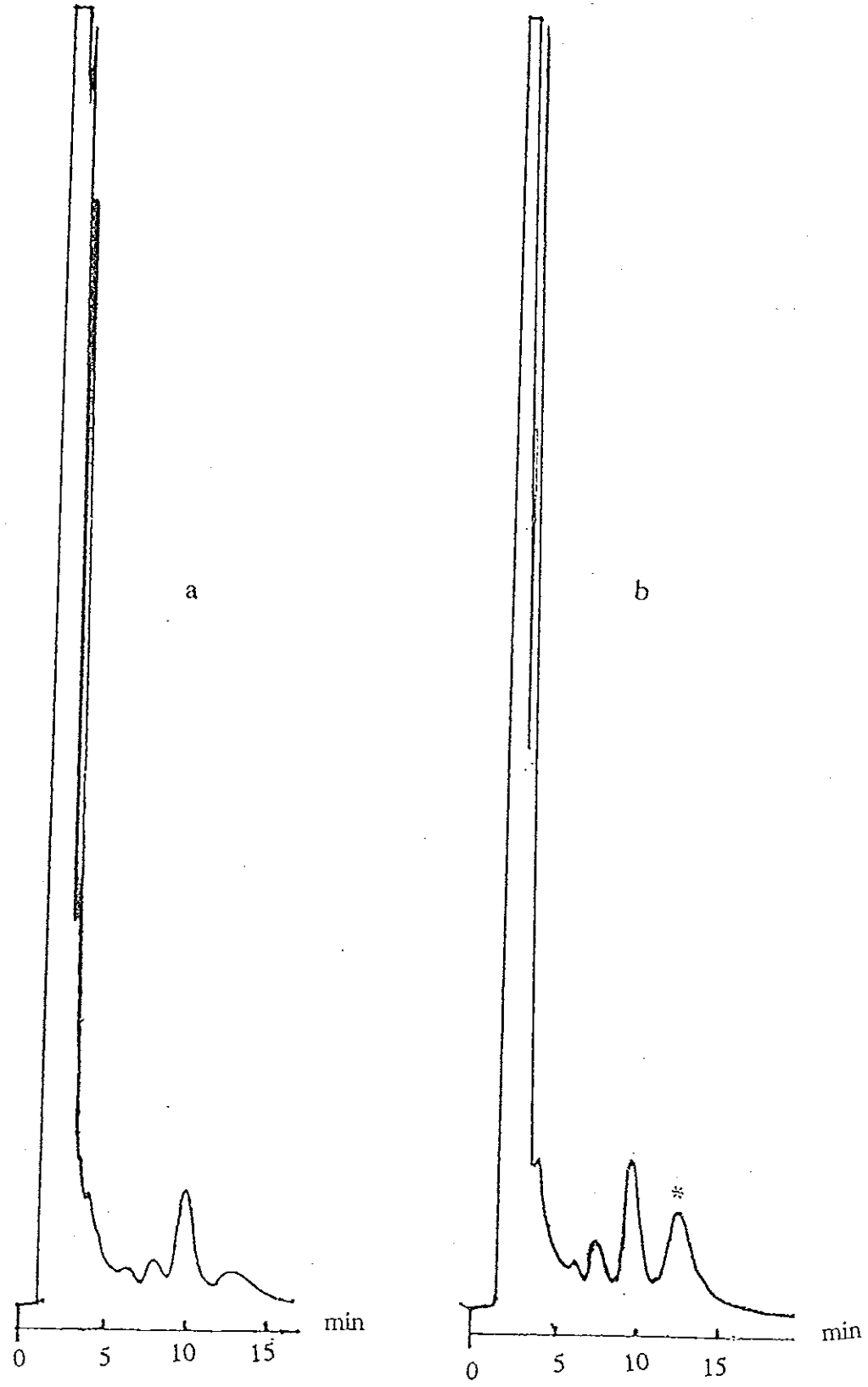


Figure 22. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS (a) blank plasma. (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide

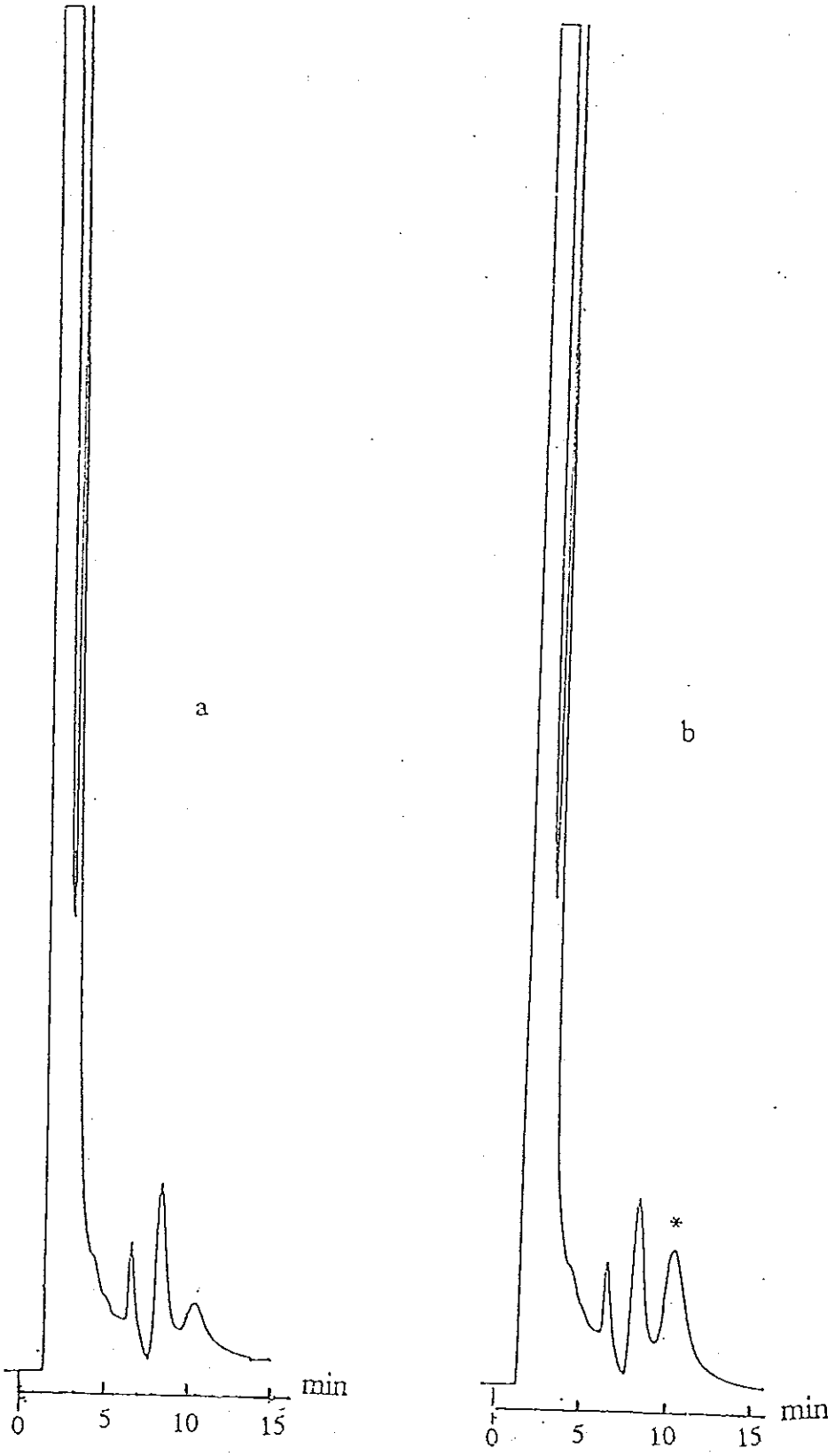


Figure 23. Chromatograms showing the separation of chlorpropamide with mobile phase :0.1 M SDS + 1% propanol (v/v), (a) blank plasma. (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

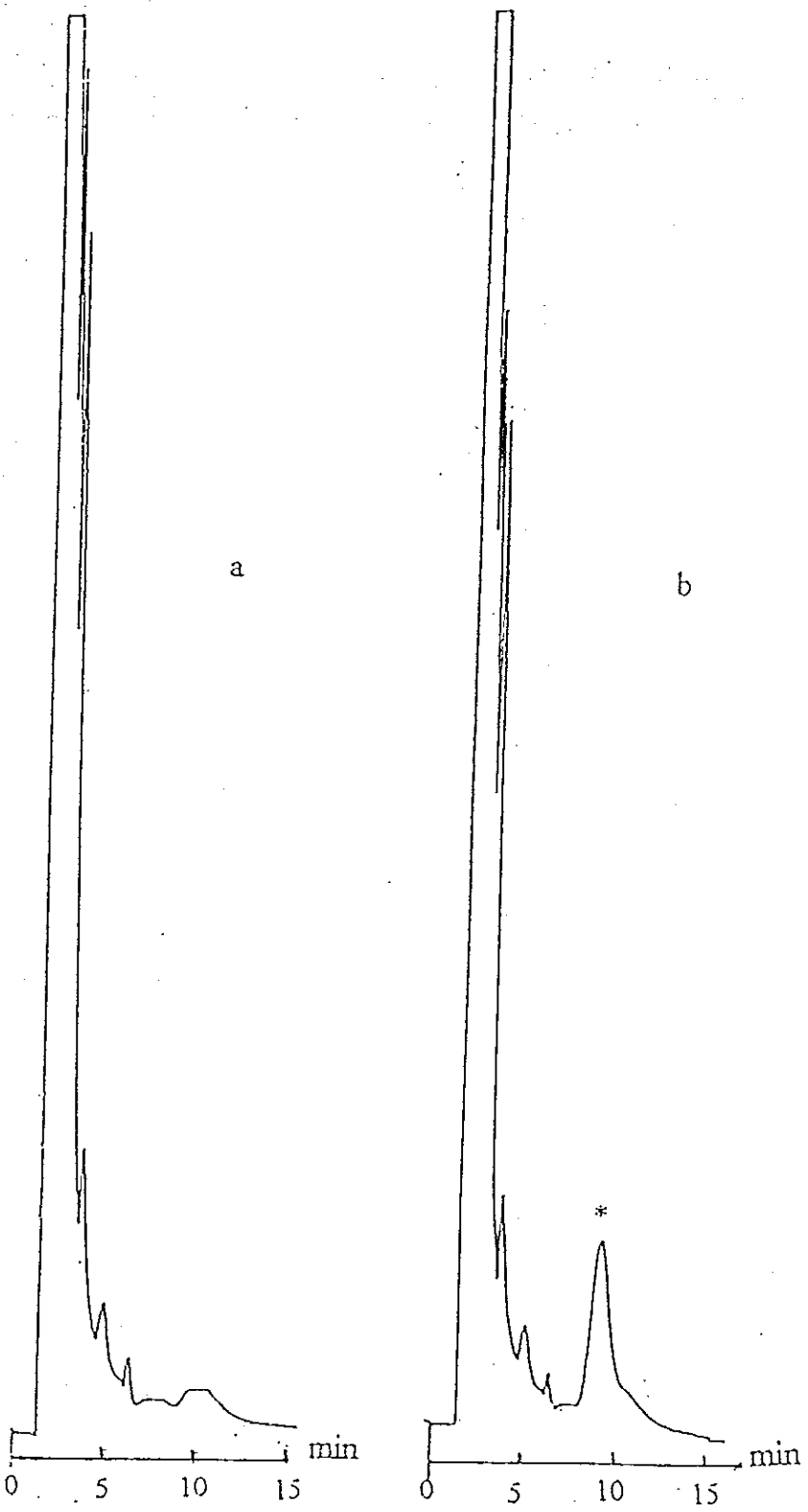


Figure 24. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS + 2% propanol (v/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

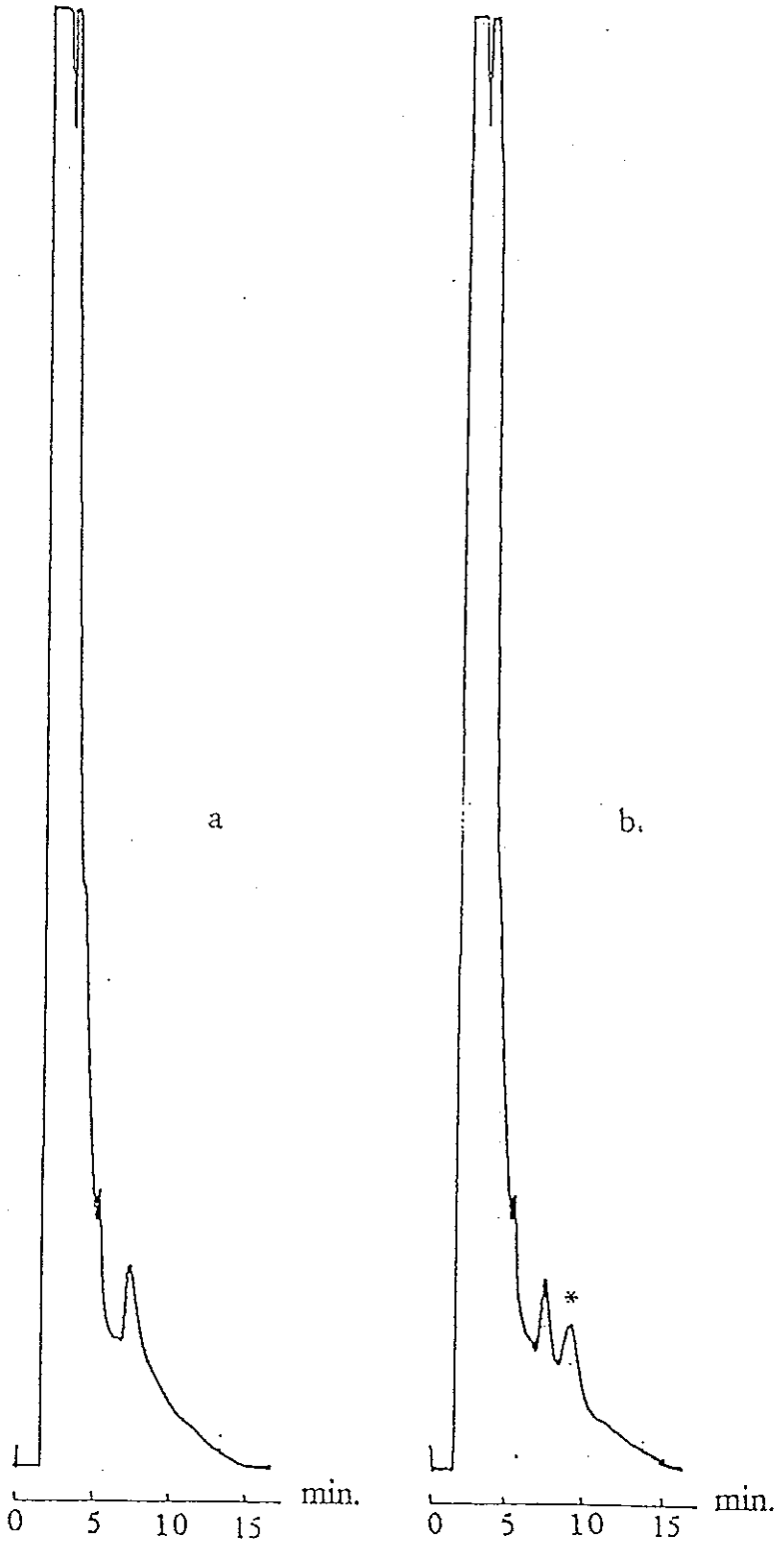


Figure 25. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS + 1% NaCl (w/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

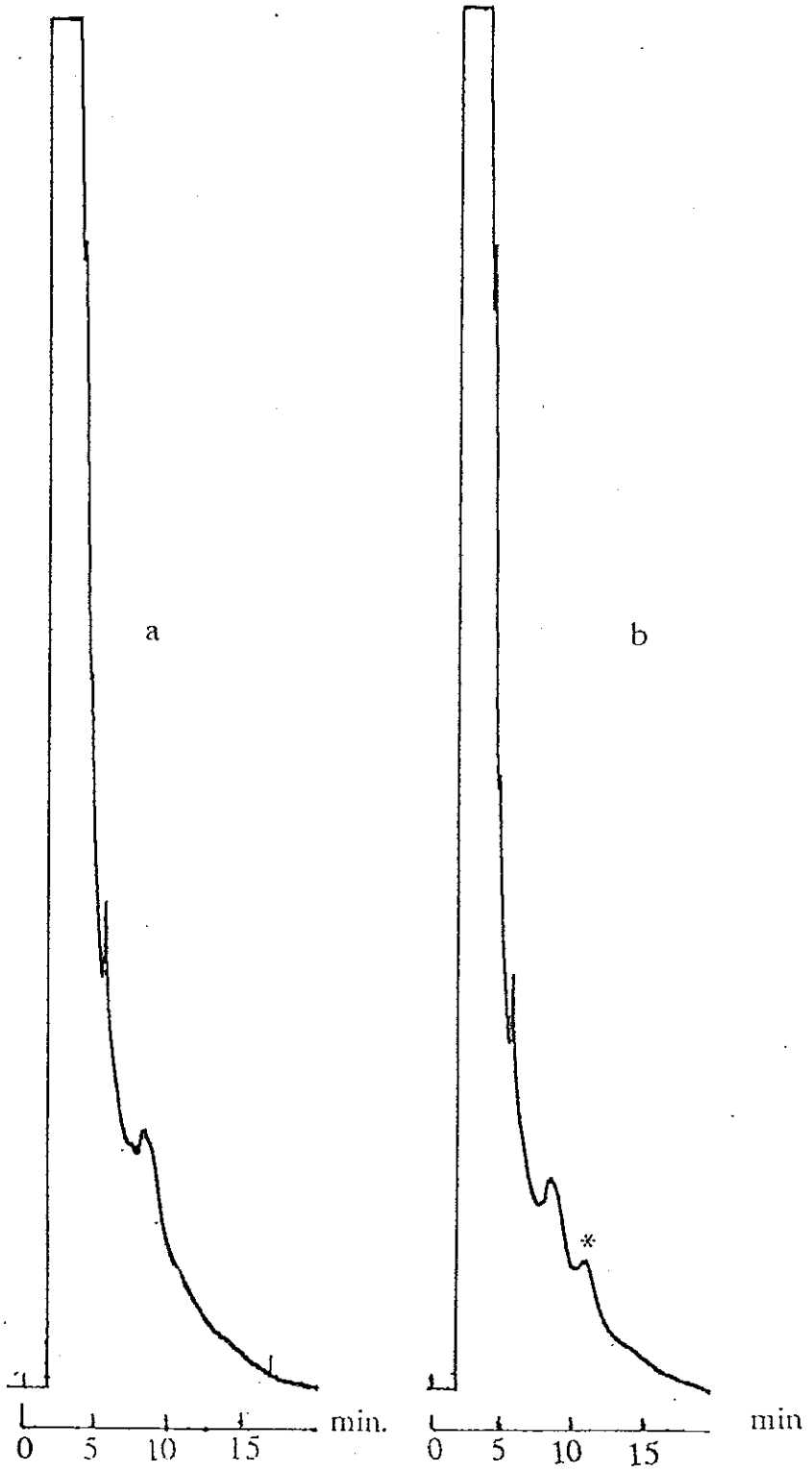


Figure 26. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS + 2% NaCl (w/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

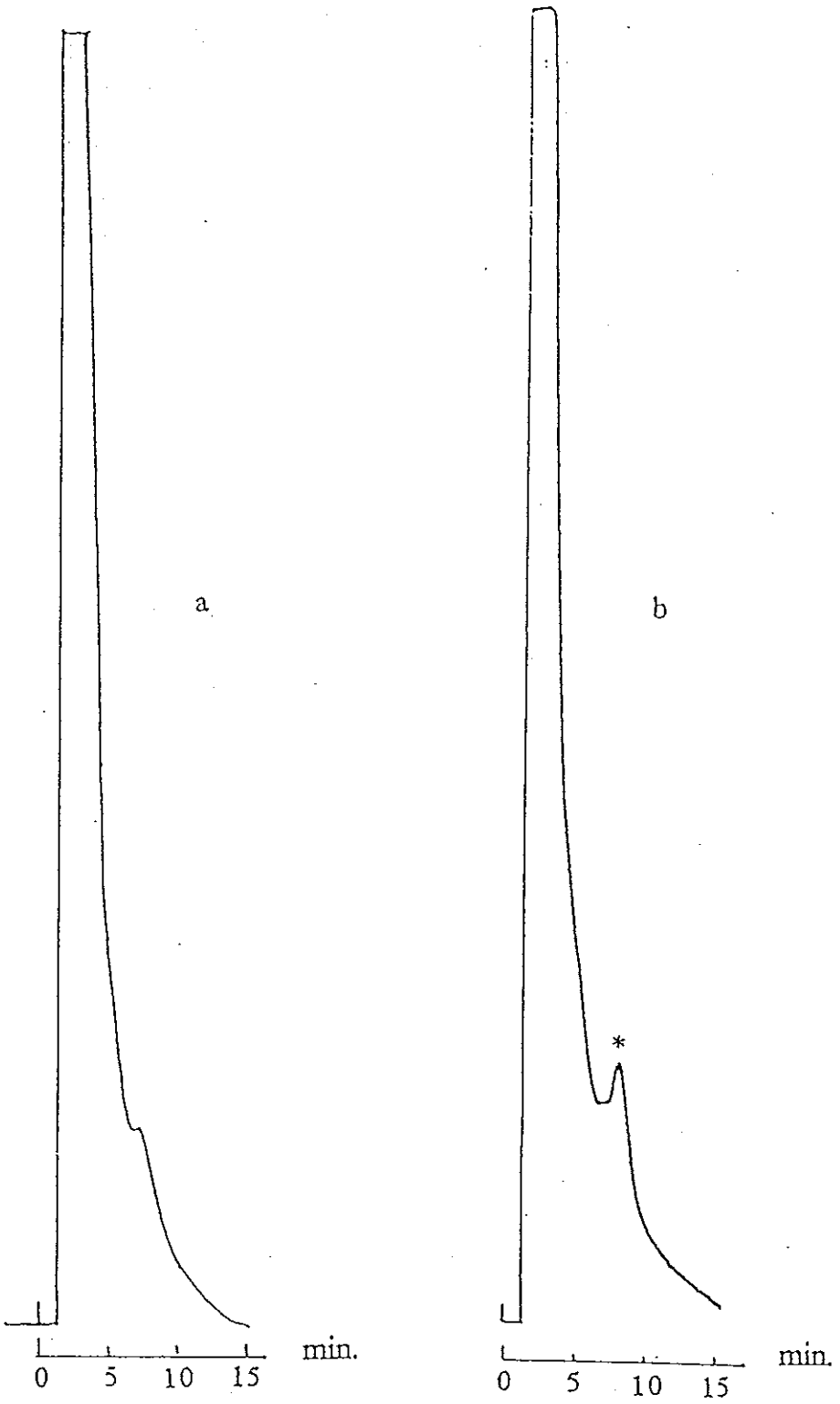


Figure 27. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS + 1% NaCl (w/v) + 1% propanol (v/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

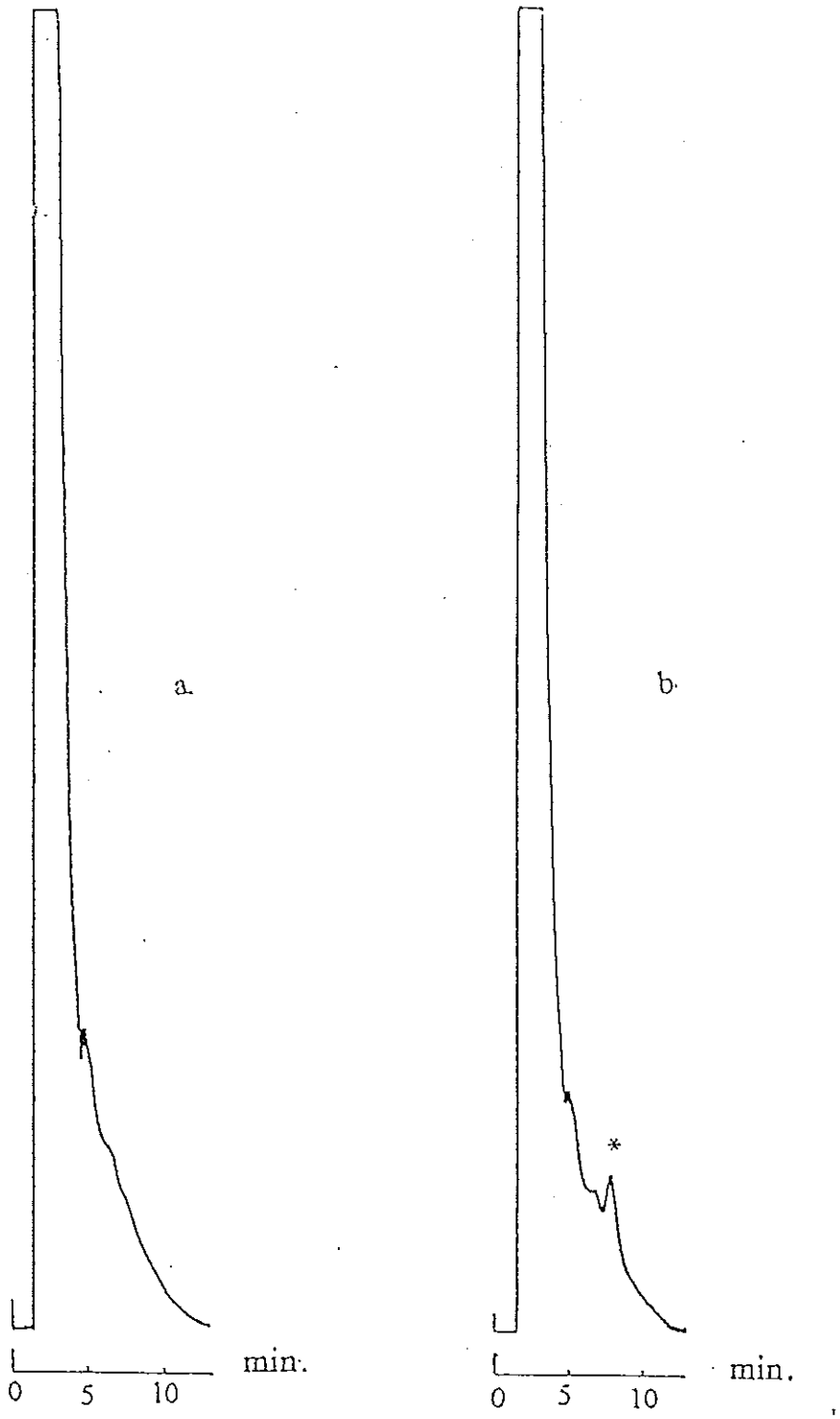


Figure 28. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.1 M SDS + 1% NaCl (w/v) + 2% propanol (v/v), (a) blank plasma. (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

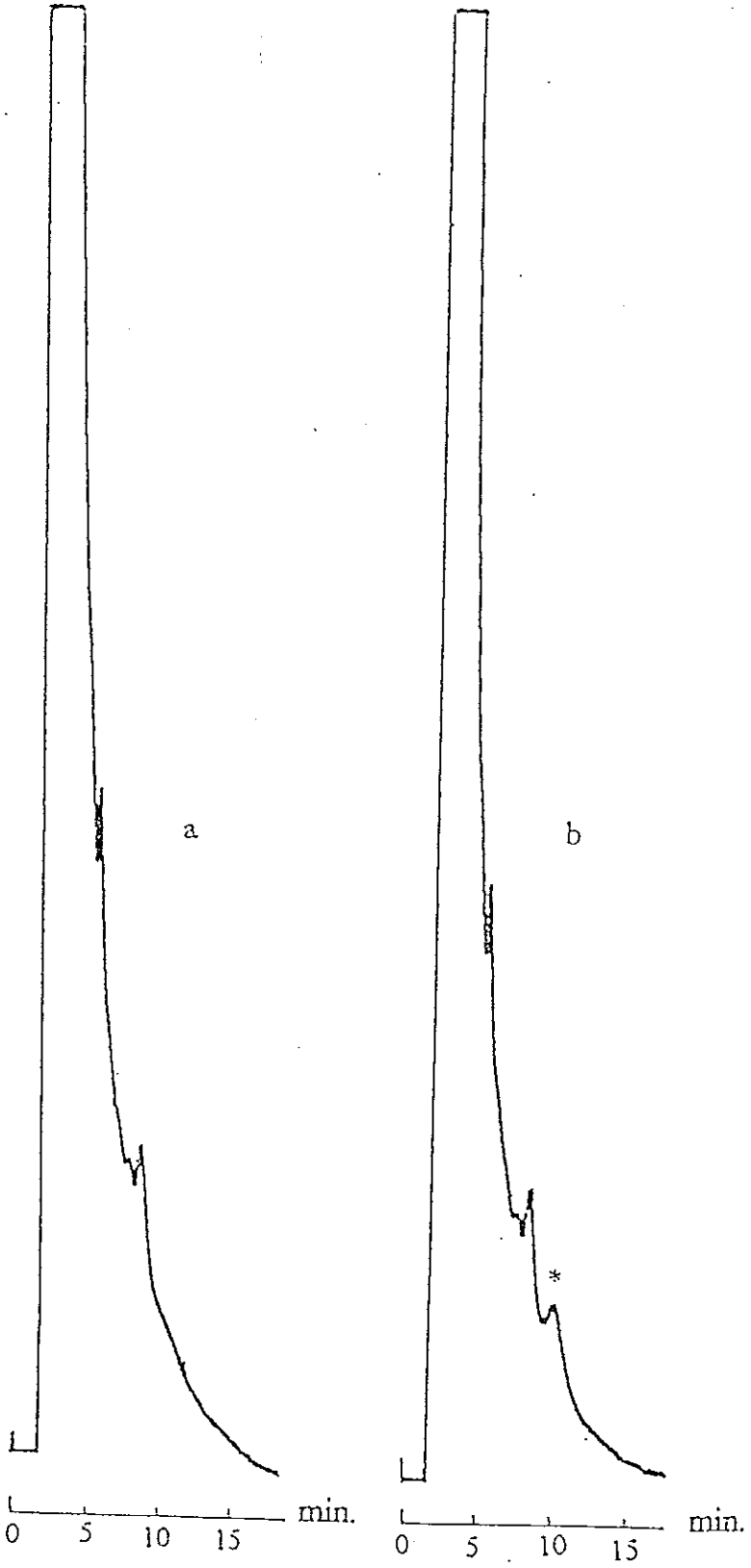


Figure 29. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.10 M SDS + 2% NaCl (w/v) + 1% propanol (v/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

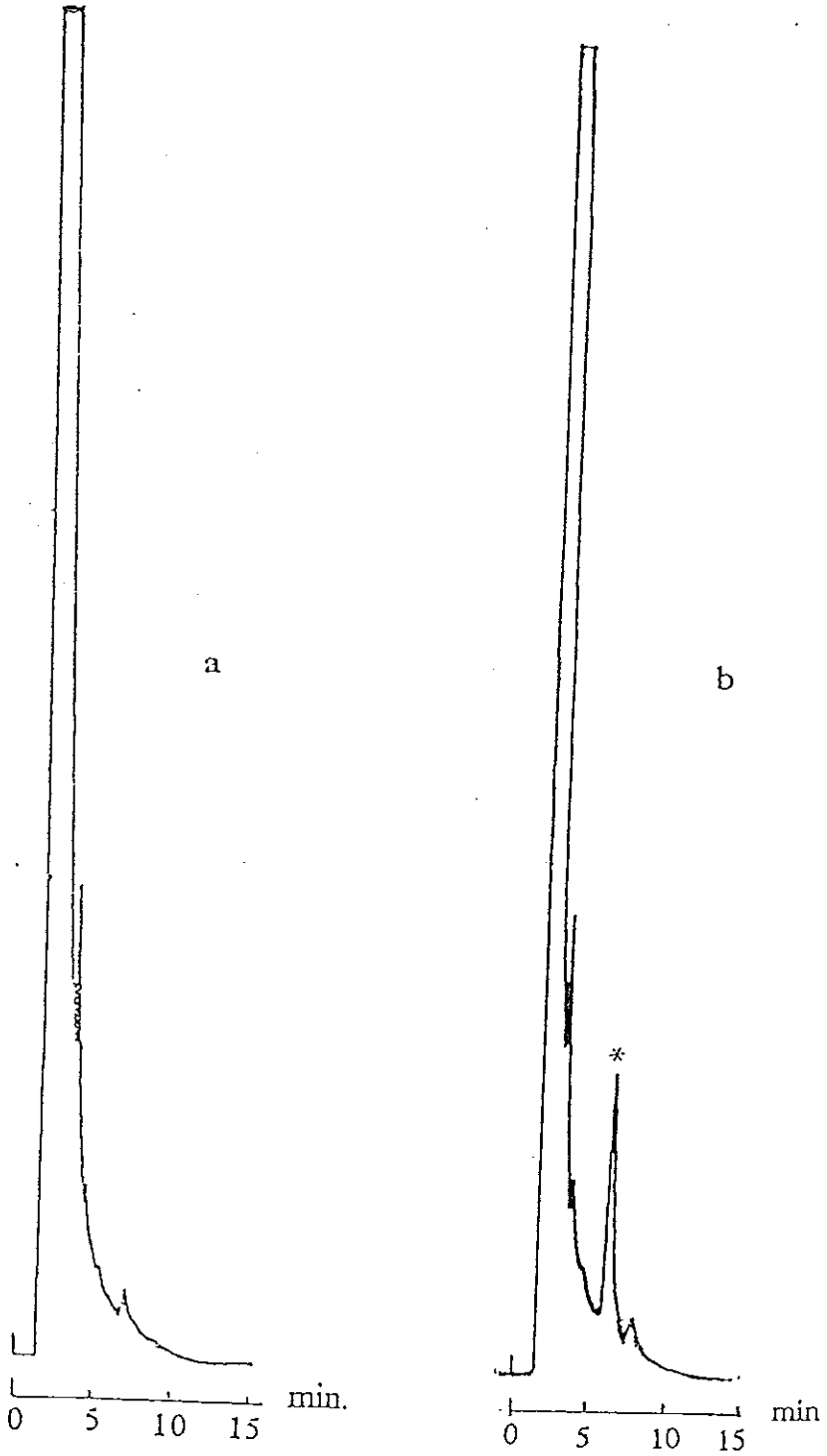
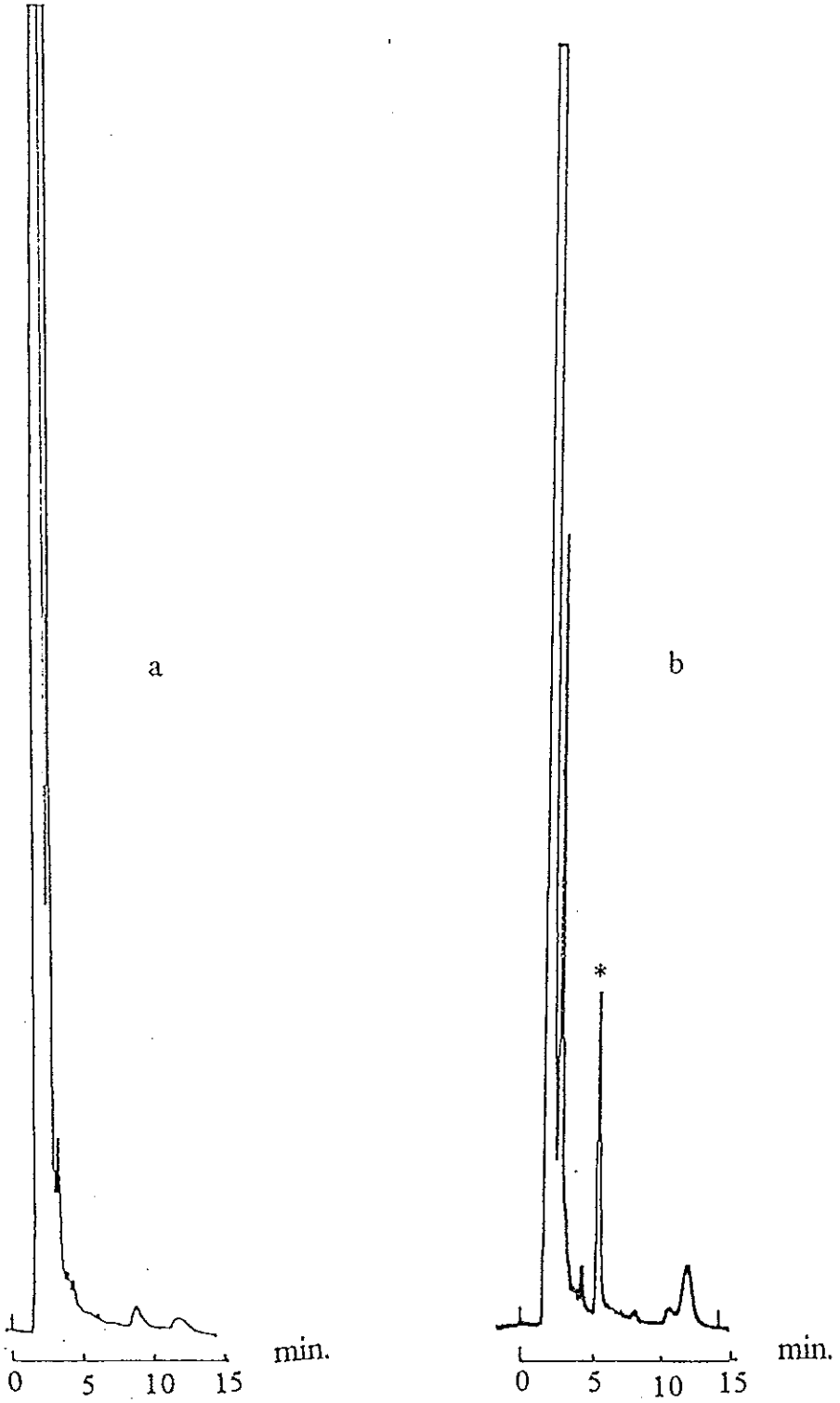


Figure 30. Chromatograms showing the separation of chlorpropamide with mobile phase : 0.10 M SDS + 2% NaCl (w/v) + 2% propanol (v/v), (a) blank plasma (b) chlorpropamide in plasma on C_{18} column; flow rate of 1.0 mL/min, (*) = chlorpropamide.

3. The quantitative determination of acetaminophen in human plasma.

Acetaminophen in human plasma is successfully separated by 0.05 M SDS micellar mobile phase on C_{18} column. It is eluted at 5.28 min. The results are shown in Figure 31. Acetaminophen concentrations in plasma sample are determined by using the calibration curve (Figure 32), which is constructed by plotting peak-height versus standard concentration of acetaminophen in plasma (Table 7) and the results are shown in Table 8.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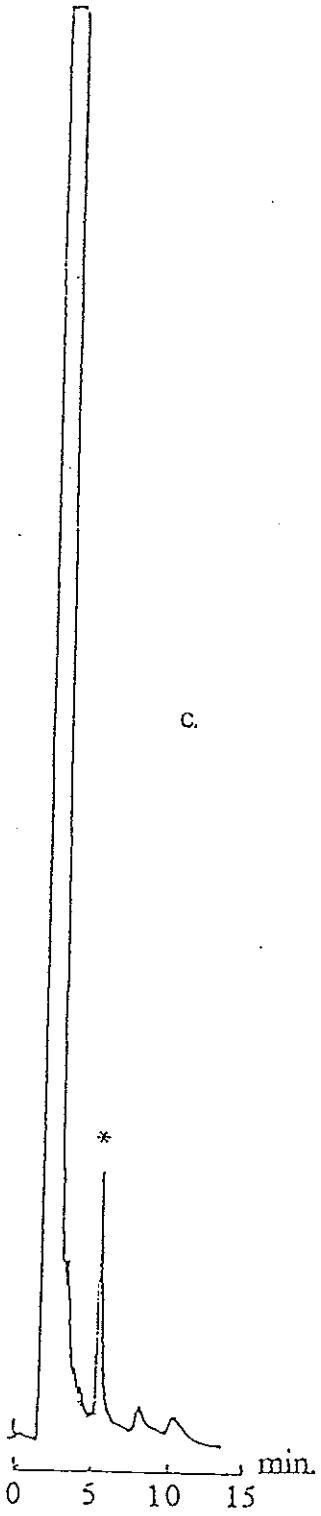


Figure 31. Chromatogram of (a) blank plasma and (b) standard acetaminophen in plasma (10 μg/mL) and (c) sample of acetaminophen in plasma (7.40 μg/mL); mobile phase 0.05 M SDS; detector sensitivity 0.16 absorbance unit full scale (AUFS); wavelength 245 nm; flow rate 1.0 mL/min; paper speed 2.0 mm/min, (*) = acetaminophen.

Table 7. Standard concentrations of acetaminophen in blank human plasma.

[APAP][a] ; μg/mL	peak height[b] (cm)
1.0	0.53 ± 0.28
5.0	2.33 ± 0.12
10.0	4.10 ± 0.36
15.0	6.27 ± 0.21

[a] [APAP] is referred to acetaminophen concentration.

[b] n = 3

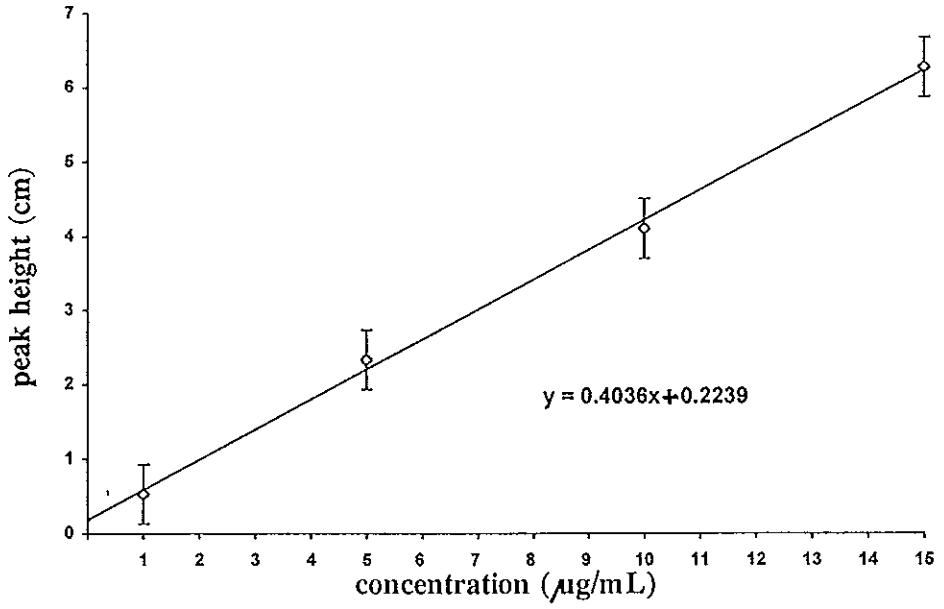


Figure 32. Plot showing the calibration curve for acetaminophen .

Table 8. Concentration of acetaminophen in human plasma samples.

sample	peak height[c] (cm)	concentration ; μg/mL
A[a]	3.27 ± 0.15	7.55
B[b]	1.20 ± 0.00	2.42
C[b]	1.93 ± 0.15	4.23
D[b]	1.43 ± 0.06	2.99

[a] sample was obtained from volunteer.

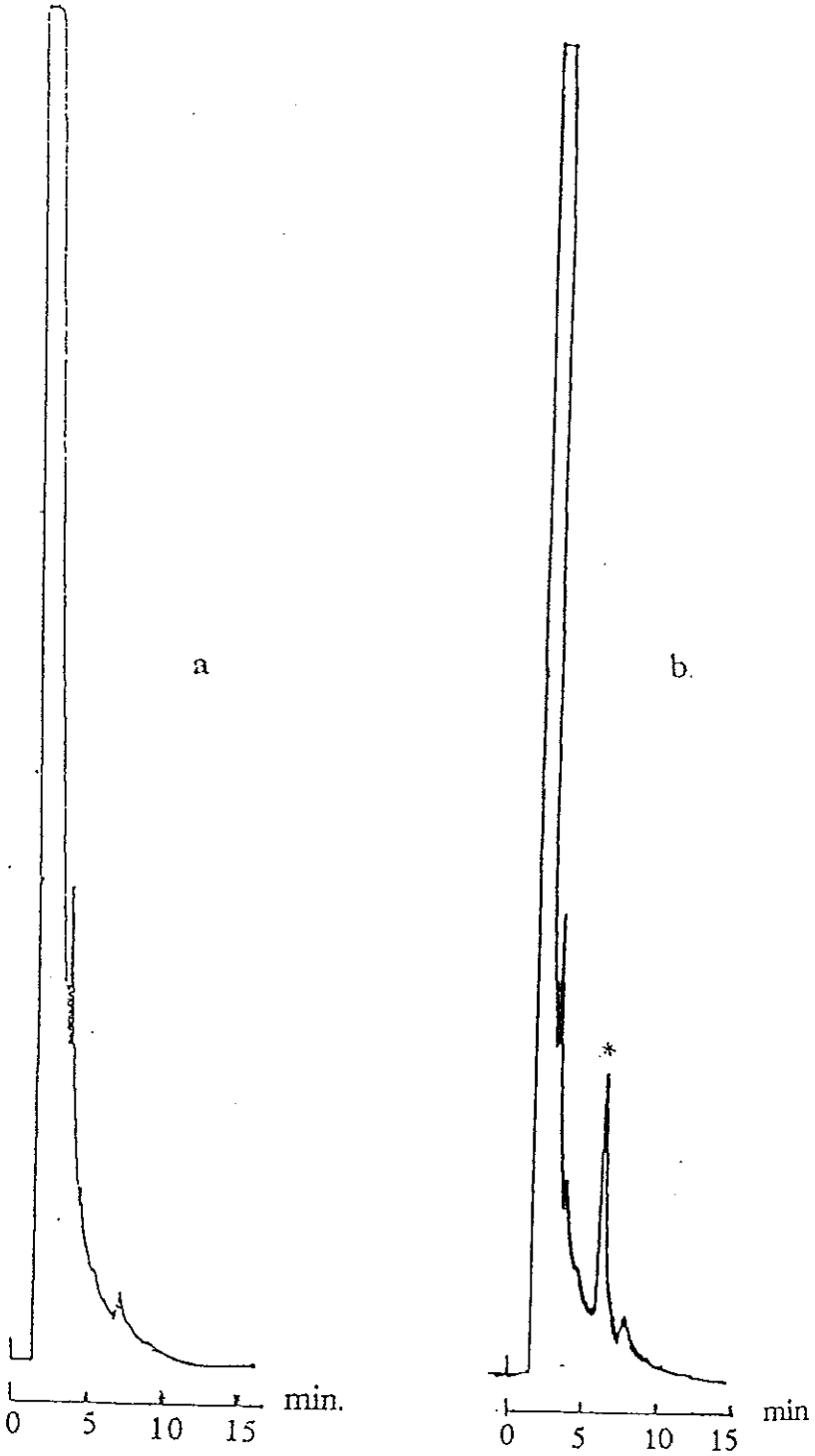
[b] sample was obtained from patients of Yala hospital, which administered orally with drug for 6 hours prior to the blood collection.

[c] n = 3

4. The quantitative determination of chlorpropamide in human plasma.

Chlorpropamide in human plasma is successfully separated by 0.10 M SDS + 2% (w/v) sodium chloride + 2% (v/v) propanol micellar mobile phase on C_{18} column. It is eluted at 6.47 min. The results are shown in Figure 33.

Chlorpropamide concentration in plasma sample is determined by using the standard addition method, which is constructed by plotting peak-height versus standard concentrations of chlorpropamide in plasma sample (Figure 34). Concentration of chlorpropamide in plasma sample is 7.87 μg/mL, which is calculated from multiplication of measured concentration with dilution factor (2.0).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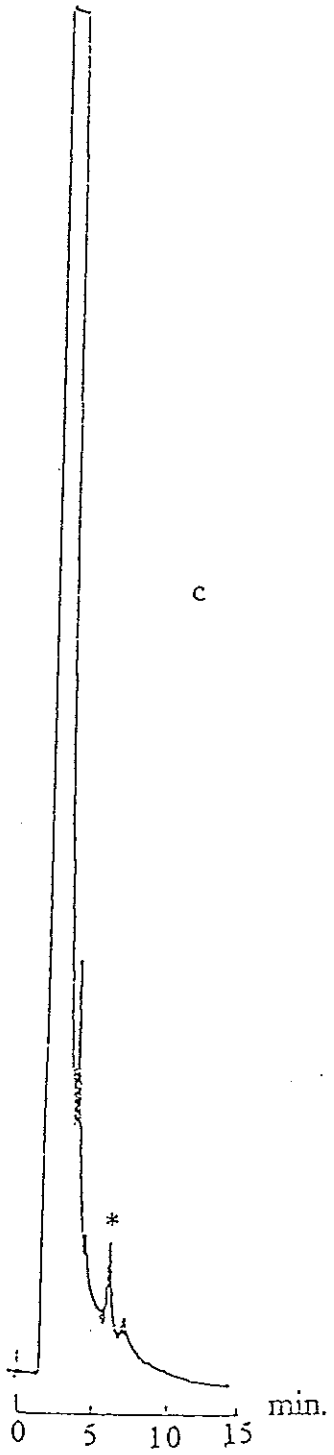

Figure 33. Chromatogram of (a) blank plasma (b) standard chlorpropamide in plasma (10 μg/mL) and (c) sample of chlorpropamide in plasma (3.93 μg/mL); mobile phase 0.1 M SDS + 2% NaCl (w/v) + 2% propanol (v/v); detector sensitivity 0.08 absorbance unit full scale (AUFS); wavelength 240 nm; flow rate 1.0 mL/min; paper speed 2.0 mm/min, (*) = chlorpropamide.

Table 9. Standard addition for chlorpropamide in human plasma.

[CPA][a] added (μg/mL)	peak height[b] (cm)
0	1.2 ± 0.05
1	1.47 ± 0.03
5	2.77 ± 0.15
10	3.90 ± 0.17
15	5.80 ± 0.17

[a] [CPA] is refered to chlorpropamide concentration.

[b] n = 3

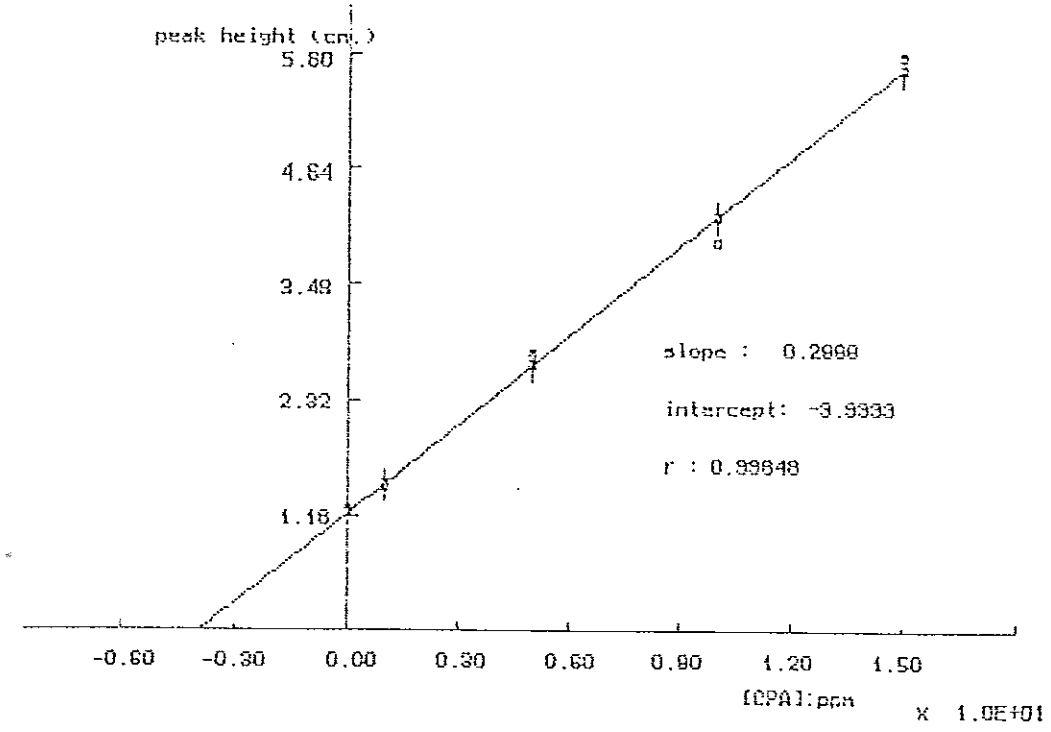

Figure 34. Plot showing the standard addition for chlorpropamide.

5. Recovery.

The recoveries of acetaminophen and chlorpropamide were studied by adding the drug into blank plasma to yield a concentration range of 1.0 - 15.0 μg/mL. The peak-height of acetaminophen and chlorpropamide in aqueous solution and blank plasma were compared and calculated for the average recoveries, as illustrated in Table 10 and Table 11, respectively.

Table 10. Recoveries of acetaminophen.

concentration (μg/mL)	mean peak height* (cm)		recovery (%)
	aqueous	plasma	
1.0	0.63	0.53	84
5.0	2.65	2.33	88
10.0	5.38	4.10	76
15.0	8.20	6.27	76

* n = 3

Table 11. Recoveries of chlorpropamide.

concentration (μg/mL)	mean peak height* (cm)		recovery (%)
	aqueous	plasma	
1.0	0.43	0.37	86
5.0	1.70	1.55	91
10.0	3.27	3.20	98
15.0	4.78	4.33	91

* n = 3

6. Linearity.

The linearity over the concentration range of interest is ascertained by adding known amounts of drugs to blank plasma. A linear relationship for the peak-height existed over tested concentration range.

The equation of acetaminophen is $y = 0.4036x + 0.2239$ with regression coefficient ; $r = 0.9990$ (Figure 35).

The equation of chlorpropamide is $y = 0.2874x + 0.1525$ with regression coefficient ; $r = 0.9972$ (Figure 36).

where y is the peak-height (cm),

x is the concentration (μg/mL).

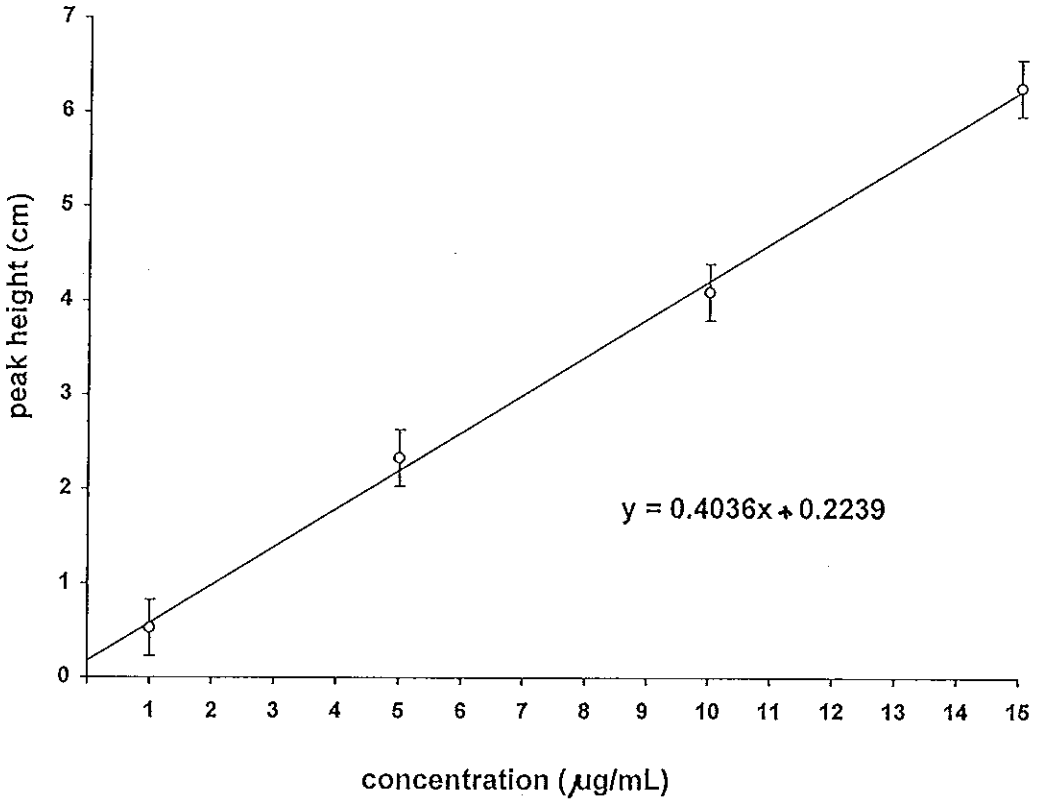

Figure 35. Plot showing the linear range of acetaminophen in plasma.

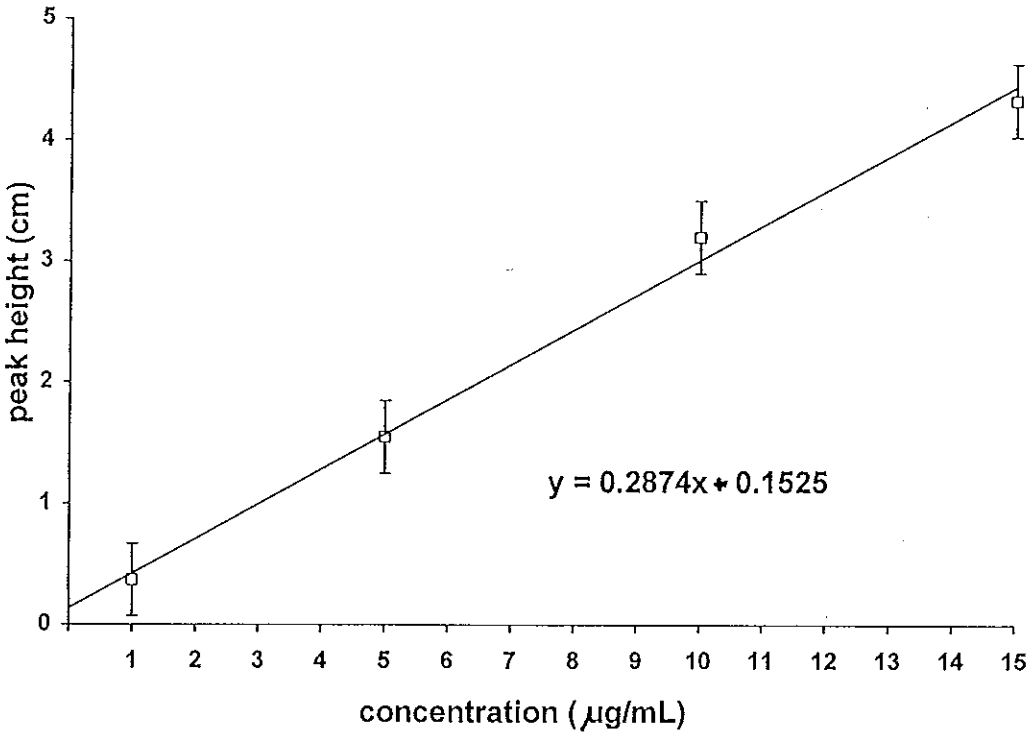

Figure 36. Plot showing the linear range of chlorpropamide in plasma.

7. Precision.

The within-day precision was calculated by analysing 10 blank plasma samples containing acetaminophen or chlorpropamide at the concentration of 10 μg/mL (Table 12). The results indicate that a precision of acetaminophen is about 2.74 % (mean = 9.50 μg/mL ; S = 0.26 μg/mL) and chlorpropamide is about 2.65 % (mean = 10.18 μg/mL , S = 0.27 μg/mL).

Table 12 Within-day precision[a].

N[b]	peak height (cm)		concentration (μg/mL)	
	acetaminophen	chlorpropamide	acetaminophen	chlorpropamide
1	3.83	3.10	8.93	10.26
2	4.13	3.00	9.68	9.91
3	3.95	3.20	9.23	10.60
4	4.05	3.20	9.43	10.60
5	4.10	3.10	9.60	10.26
6	4.10	3.07	9.60	10.15
7	4.15	3.10	9.73	10.26
8	4.15	3.00	9.73	9.91
9	4.00	3.00	9.36	9.91
10	4.13	3.00	9.68	9.91

[a] Expressed as percent CV

[b] N = Number of injection.

CHAPTER 4

CONCLUSION

Studies on retention behaviors of acetaminophen and chlorpropamide can be concluded that :

1. Capacity factor of both drugs on CN and C_{18} column decrease with the increase in SDS concentration.

2. The addition of modifier such as 1% methanol, 1% propanol and 1% butanol in SDS mobile phase reduce capacity factor and improve efficiency of both acetaminophen and chlorpropamide.

3. Salt such as sodium chloride has effect on capacity factor of chlorpropamide, in such away that capacity factor increase when sodium chloride concentration in SDS mobile phase increase.

4. The capacity factor of acetaminophen on various SDS concentration was not affected by the change in pH between 3 - 7 but the capacity factor of chlorpropamide decreases with the increasing pH.

The results of quantitative analysis from this study indicate that micellar liquid chromatography, using SDS as the anionic mobile phase, was an appropriate technique for the direct determination of acetaminophen and chlorpropamide in plasma by HPLC with UV

detector because each drug can be separated and good chromatographic performance çan be obtained :

For acetaminophen, the retention time is 5.28 min and total analysis time is 15 min with valuable selectivity and the precision, 2.74 %; detection limit is 0.52 μg/mL.

For chlorpropamide, the retention time is 6.47 min and total analysis time is 15 min with valuable selectivity and the precision, 2.65 %; detection limit is 0.67 μg/mL.

Moreover, the advantages of this technique are using a small sample volume, good precision, low mininum detectable concentrations, and selective.

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