การพัฒนาวิธีการวิเคราะห์ปริมาณโคเดอีนฟอสเฟตและโปรเมธาซีนฮัยโดรคลอไรด์ในยาน้ำแก้ไอด้วยวิธีไมเซลลาร์อิเลคโทรไคเนติกโครมาโตกราฟี

นางสาว พร้อมพร จำนงธนาโชติ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเคมี ภาควิชาเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2544

ISBN 974-17-0337-6

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

THE DEVELOPMENT OF MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND PROMETHAZINE HYDROCHLORIDE IN COUGH SOLUTIONS

Miss Promporn Jamnongtanachot

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy in Pharmaceutical Chemistry

Department of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University

Academic Year 2001
ISBN 974-17-0337-6

Thesis Title	THE DEVELOPMENT OF MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND PROMETHAZINE HYDROCHLORIDE IN COUGH SOLUTIONS
By	Miss Promporn Jamnongtanachot
Field of Study	Pharmaceutical Chemistry
Thesis Advisor	Walapa Tatong, Ph.D.
Thesis Co-advisor	Assistant Professor Wallee Vanichseni, M.Phil.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of Faculty of Pharmaceutical Sciences

(Associate Professor Boongyong Tantisira, Ph.D.)

THESIS COMMITTEE

……Chairman

(Assistant Professor Mitr Pathipvanich, Ph.D.)

……Thesis Advisor

(Walapa Tatong, Ph.D.)

……Thesis Co-advisor

(Assistant Professor Wallee Vanichseni, M.Phil.)

……Member

(Associate Professor Darawan Tanyavutti, M.S.)

……Member

(Yaowalak Wattanapisit, M.Sc. in Pharm)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

พร้อมพร จำนงธนาโชติ : การพัฒนาวิธีการวิเคราะห์ปริมาณโคเดอีนฟอสเฟตและโปรเมธาซีนฮัยโดรคลอไรด์ในยาน้ำแก้ไอด้วยวิธีไมเซลลาร์อิเลคโทรไคเนติกโครมาโตกราฟี (THE DEVELOPMENT OF MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND PROMETHAZINE HYDROCHLORIDE IN COUGH SOLUTIONS). อ.ที่ปรึกษา : ดร. วัลลภา ทาทอง, อาจารย์ที่ปรึกษาร่วม : ผศ. วัลลีย์ วาณิชเสนี 83 หน้า. ISBN 974-17-0337-6

การพัฒนาวิธีไมเซลลาร์อิเลคโทรไคเนติกโครมาโตกราฟีสำหรับวิเคราะห์ปริมาณโคเดอีนฟอสเฟตและโปรเมธาซีนฮัยโดรคลอไรด์ในยาน้ำแก้ไอ โดยใช้แคพิลลารีชนิดฟิวส์ซิลิกา (37 เซนติเมตร x 75 ไมโครเมตร) ที่อุณหภูมิ 20 องศาเซลเซียส แบคกราวอิเลคโตรไลท์ประกอบด้วยโซเดียมโดเดคซิลซัลเฟต 50 มิลลิโมลาร์ที่ละลายอยู่ในบอเรตบัฟเฟอร์ 25 มิลลิโมลาร์ พีเอช 10.0 และอะซีโตไนไทรล์ 10 เปอร์เซ็นต์ ใช้การตรวจวัดชนิดอัลตราไวโอเลตที่ความยาวคลื่น 214 นาโนเมตร มีกัวฟีนีซีนเป็นสารมาตรฐานอินเทอร์นอล โคเดอีนฟอสเฟตและโปรเมธาซีนฮัยโดรคลอไรด์แยกได้ดีจากสารที่ไม่ออกฤทธิ์และสารสลายตัวเนื่องจากแสง ความสัมพันธ์ระหว่างความเข้มข้นและค่าอัตราส่วนพื้นที่ของพีคเป็นเส้นตรงในช่วงความเข้มข้นของโคเดอีนฟอสเฟตระหว่าง 45 ถึง 135 ไมโครกรัมต่อมิลลิลิตร และสำหรับโปรเมธาซีนฮัยโดรคลอไรด์ระหว่าง 18 ถึง 54 ไมโครกรัมต่อมิลลิลิตร ความเที่ยงตรงของวิธีวิเคราะห์แสดงในรูปของค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ภายในวันเดียวกัน (จำนวน 6 การวิเคราะห์) และระหว่างวัน (จำนวน 3 วัน) สำหรับโคเดอีนฟอสเฟตมีค่า 1.14% และ 1.45% และสำหรับโปรเมธาซีนฮัยโดรคลอไรด์มีค่า 1.12% และ 2.15% ความถูกต้องของวิธีวิเคราะห์แสดงในรูปของค่าเฉลี่ยของการกลับคืนที่ความเข้มข้น 3 ระดับ สำหรับโคเดอีนฟอสเฟตมีค่า 99.2% และโปรเมธาซีนฮัยโดรคลอไรด์มีค่า 98.2% ปริมาณโคเดอีนฟอสเฟตและโปรเมธาซีนฮัยโดรคลอไรด์ที่วิเคราะห์ได้ด้วยวิธีที่พัฒนาขึ้นนี้ไม่มีความแตกต่างจากค่าที่วิเคราะห์ได้ด้วยวิธีไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโตกราฟีอย่างมีนัยสำคัญที่ระดับความเชื่อมั่น 95% วิธีไมเซลลาร์อิเลคโทรไคเนติกโครมาโตกราฟีที่พัฒนาขึ้นเป็นวิธีวิเคราะห์ที่รวดเร็ว มีความจำเพาะ เที่ยงตรง ถูกต้องไม่ต้องมีการเตรียมตัวอย่างที่ยุ่งยากก่อนการวิเคราะห์ และไม่ถูกรบกวนด้วยสารสลายตัวของโปรเมธาซีนฮัยโดรคลอไรด์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา……เภสัชเคมี……....ลายมือชื่อนิสิต……
สาขาวิชา……..……เภสัชเคมี……..……...ลายมือชื่ออาจารย์ที่ปรึกษา…….
ปีการศึกษา……2544……ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..…..

4176570133 : MAJOR PHARMACEUTICAL CHEMISTRY
KEY WORD: MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD/ CODEINE PHOSPHATE/ PROMETHAZINE HYDROCHLORIDE/ COUGH SOLUTIONS

PROMPORN JAMNONGTANACHOT : THE DEVELOPMENT OF MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND PROMETHAZINE HYDROCHLORIDE IN COUGH SOLUTIONS. THESIS ADVISOR : WALAPA TATONG, Ph.D. THESIS CO-ADVISOR : ASST. PROF. WALLEE VANICHASENI, M.Phil., 83 pp. ISBN 974-17-0337-6

The developed micellar electrokinetic chromatographic (MEKC) method for the simultaneous analysis of codeine phosphate and promethazine hydrochloride in cough solutions was performed on a fused silica capillary (37cm x 75 μm I.D.) at 20°C with background electrolyte consisting of 50 mM sodium dodecyl sulfate in 25 mM borate buffer pH 10.0 and 10% acetonitrile. UV detection was performed at 214 nm. Guaifenesin was used as an internal standard. Codeine phosphate and promethazine hydrochloride were well separated from inactive ingredients and photodegradation products. The relationship between concentration and peak area ratio was linear in the range of 45 – 135 μg/ml of codeine phosphate and 18 – 54 μg/ml of promethazine hydrochloride. The relative standard deviation of the intra - day precision (n=6) and inter - day precision (n=3) were 1.14 and 1.45 % for codeine phosphate and were 1.12 and 2.15 % for promethazine hydrochloride. Accuracy of the method expressed as mean recovery at three concentration levels were 99.2% for codeine phosphate and 98.2% for promethazine hydrochloride. The content of codeine phosphate and promethazine hydrochloride which were analysed by MEKC method was not significantly different from the one obtained from high performance liquid chromatographic method at 95% confidence level. The developed MEKC method was rapid, specific, precise, accurate, no sample pretreatment required and no interference from photodegradation products of promethazine hydrochloride.

Department…...Pharmaceutical Chemistry…… Student's signature……...
Field of study…Pharmaceutical Chemistry…… Advisor's signature……...
Academic year……2001…… Co-advisor's signature……..

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Walapa Tatong, of the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulanongkorn University for her excellent guidance, encouragement and suggestion through the course of this study.

I am also very grateful to Assistant Professer Wallee Vanichseni, my research co-advisor for her for her advice, guidance and helpful suggestion.

A special appreciation is also extended to Mrs Sarunyaporn Kitchaiya of the Drug Analysis Section, Chonburi Regional Medical Sciences Center, for Capillary Electrophoresis Instrument, her kindness and encouragement.

I would like to express my gratitude to the staffs of the Department of Pharmaceutical Chemistry for their help.

I also would like to thank Graduate School, Chulalongkorn University for granting partial financial support to conduct this investigation.

Finally, I wish to express my profound appreciation and graditude to my parents for their continuing encouragement and love and to all those who help me making this study a reality.

CONTENTS

Page

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

LIST OF FIGURES

Figure No. page

Figure No. page

Figure No. page

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

MW	=	molecular weight
g	=	gram
ml	=	millilitre
$^{\circ}C$	=	degree Celsius
UV	=	ultraviolet
nm	=	nanometre
GLC	=	gas-iquid chromatography
HPLC	=	high-performance liquid chromatography
IR	=	infrared
TLC	=	thin-layer chromatography
SPE	=	solid phase extraction
CE	=	capillary electrophoresis
cm	=	centimetre
µm	=	micrometre
BGE	=	background electrolyte
kv	=	kilovolt
EOF	=	electroosmotic flow
CZE	=	capillary zone electrophoresis
MEKC	=	micellar electrokinetic chromatography
CGE	=	capillary gel electrophoresis
CIEF	=	capillary isoelectric focusing
CITP	=	capillary isotachophoresis
PI	=	isoelectric point
TBA	=	tetrabutylammonium hydrogensulfate
CTAB	=	cetyltrimethylammonium bromide

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

I.D.	=	internal diametre
µl	=	microlitre
mm	=	millimetre
mg	=	milligram
p.s.i.	=	pounds per squre inch
s	=	second
M	=	molar
mM	=	millimolar
SDS	=	sodium dodecyl sulfate
v/v	=	volume by volume
µA	=	microampere
r	=	correlation coefficient
SD	=	standard deviation
RSD	=	relative standard deviation

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Codeine phosphate and promethazine hydrochloride are common ingredients employed as cough suppressants in compound preparations for the symptomatic treatment of coughs and the common colds. These preparations are usually in dosage forms of linctuses or solutions.

Codeine

Codeine (Figure 1) is an alkaloid which occurs naturally in opium, but the amount present is usually too small to be of commercial importance. Consequently, most commercial codeine is prepared from morphine by methylation of the phenolic hydroxyl group. (Delgado and Remers, 1998)

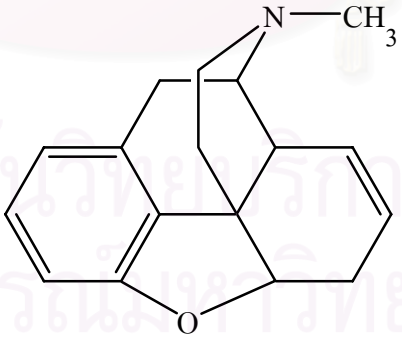


Figure 1 Structure of codeine

$C_{18}H_{21}O_3N$

MW = 299.4

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Codeine has a reputation as an antitussive, depressing the cough reflex, and is used in many cough preparations. It is considerably less addicting than morphine and in the usual doses respiratory depression is negligible.

Codeine is a mono-acidic base and readily forms salts with acids, the most important salts being the sulfate and the phosphate.

Codeine phosphate ($C_{18}H_{21}O_3N.H_3PO_4$) occurs as fine, needle-shaped, white crystals, or as a white, crystalline powder. It is odorless and has a bitter taste. It is efflorescent and is sensitive to light.

Its solubility is 1 g in 4 ml of water, 1 g in 0.5 ml of hot water, 1 g in 450 ml of alcohol and 1 g in 125 ml of boiling alcohol. The pK_a value at 20°C is 8.2. Log P (octanol/pH 7.4) at 20°C is 0.6. It exhibits a characteristic UV spectrum in water with a maximum at 284 nm (Muhtadi and Hassan, 1981). Because of its high solubility in water as compared with the sulfate, codeine phosphate is used widely, especially for liquid preparations.

Both codeine linctus and codeine phosphate oral solution preparations are official in the British Pharmacopoeia 1999. Their assay procedures are performed by high-performance liquid chromatographic method.

The mixture of codeine phosphate and acetaminophen in oral liquid preparations, including the mixture of codeine phosphate and guaifenesin in syrup are both official in the United States Pharmacopoeia 24 (2000). The analysis method for the determination of codeine in these preparations is high-performance liquid chromatography.

Sisco et al. (1986) studied thermal decomposition of codeine phosphate in the Acetaminophen with Codeine Phosphate Elixir by placing the elixir in a 60°C oven for two weeks. Codeine phosphate, as well as decomposition products: p-aminophenol, codeine N-oxide, and codeinone were analysed by high-performance liquid chromatography with a diode-array detector at 214 nm, while the C18 column was heated at 50°C.

Numerous methods for the determination of codeine in pharmaceutical preparations are reported, including colorimetric method using acid dyes (Matsui and French, 1971; Mathew et al, 1972), GLC method (Steven, 1975; Galant, Visalli and Patel, 1979) and several HPLC methods (Gupta, 1980; Halstead, 1982; Sisco, Rittenhouse and Everhart, 1985). However, these methods are not applicable to analyse the combination of codeine and promethazine in cough solutions.

Promethazine

Promethazine (Figure 2) is a phenothiazine derivative that used as histamine H1-receptor antagonist and antiemetic. It is decomposed primarily by oxidation and/or photolysis. Aqueous solutions of promethazine hydrochloride, stored at room temperature in diffused daylight for two days to six months, decomposed to promethazine sulfoxide, 9,9-dioxopromethazine, N-demethyl-promethazine and several more unidentified compounds. Phenothiazine was identified as a major degradation product when a solution of promethazine hydrochloride was exposed to sunlight (Shearer and Miller, 1976).

Promethazine hydrochloride is a white to faint yellow crystalline powder, practically odorless. It is slowly oxidized and acquired a blue color, on prolonged exposure to air. (Shearer and Miller, 1976). Its solubility is 1 g in 0.6 ml of water, 1 g

in 9 ml of alcohol, 1 g in 2 ml of chloroform, very soluble in hot dehydrated alcohol, practically insoluble in acetone, ether and ethyl acetate (Reynolds, 1989). The pK_a value is 9.1. Log P (octanol/pH 7.4) at 20°C is 2.9. It exhibits a characteristic UV spectrum in water with a maximun at 249 and 297 nm (Shearer and Miller, 1976).

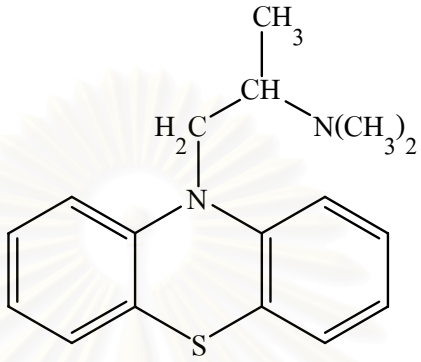


Figure 2 Structure of promethazine

$C_{17}H_{20}N_2S$

MW = 284.4

Promethazine hydrochloride is decomposed by photolytic oxidation. It can be stabilized by the addition of ascorbic acid, cysteine, sodium metabisulfite or rongolite (AHFS, 1998).

Promethazine hydrochloride oral liquid preparations that are official in the British Pharmacopoeia 1999 and the United States Pharmacopoeia 24 are assayed by liquid-liquid extraction prior to spectrophotometric method. The method found to be lacked of specificity and interfered by other UV absorbing drugs, coloring and flavoring agents and oxidative products.

Davidson (1976) determined phenothiazine drugs by difference spectrophotometric technique based upon the absorbance of sulfoxide derivative.

Sperling (1967) developed a sample preparation technique using column chromatography to separate promethazine hydrochloride in official syrups from the interfering degradation products. Promethazine hydrochloride was subsequently identified and determined by IR and UV spectrophotometry, respectively.

The oxidative degradation products of promethazine hydrochloride were investigated and analysed using GLC (Stavechansky, Wallace and Wu, 1983). Underberg (1978) used various techniques, including TLC, GLC, IR, UV and mass spectroscopy to analyze these degradation products.

Several HPLC methods were developed for determination of promethazine hydrochloride in pharmaceutical preparations (Pound and Sears, 1973; Wallace and Shinuk, 1981; Yang, Wilken and Clarke, 1985; Mathew, Gupta and Bethea, 1994). These methods were effective for analysis of promethazine hydrochloride but not suitable for simultaneous assay of the combination of codeine phosphate and promethazine hydrochloride in cough solutions.

The analysis of a mixture of codeine phosphate and promethazine hydrochloride in cough solutions presents many difficulties. Liquid-liquid extraction is normally used for sample preparation prior to instrumental analysis. Traditional liquid-liquid extractions are tedious, time consuming and costly. These methods not only require several sample handling steps but may also present problems of phase emulsions and large amount of toxic and expensive organic solvents to analysts. The assay of such preparation is also quite tedious because each drug is separately determined by spectrophotometric method. However, these active ingredients in the original formulation product, Phensedyl® cough linctus, are simultaneously determined by liquid-liquid extraction prior to gas-liquid chromatographic (GLC) analysis.

Sooksri Ungboriboonsri (1992) developed a simple, convenient and effective analytical method utilized the XAD-2 solid phase extraction (SPE) and isocratic reversed-phase high-performance liquid chromatography for the simultaneous analysis of ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride in cough syrups.

Capillary electrophoresis (CE) has received increased attention for the determination of drugs in the pharmaceutical field. CE offers several advantages, including highly efficient and fast separation, relatively inexpensive and long lasting capillary column, small sample size requirements. High-resolution separations of a wide variety of sample types can be done by CE. It can be used for analysis of polar ionic, polar nonionic, and nonpolar nonionic compounds, as well as high molecular weight biomolecules, and chiral compounds.

CE is a method in which ions are separated by differences in their rates of migration through a narrow-bore silica capillary, typically 30 – 100 cm long with 25 – 75 μm inner diameter and 375 μm outer diameter under the influence of an electric field. The capillary is filled with an electrolyte solution, called the background electrolyte (BGE) or run buffer, and each end of the capillary dips into an electrolyte reservoir. The content of the reservoirs is identical to that within the capillary. The reservoirs also contain the electrodes used to make electrical contact between the high voltage power supply (0 – 30 kV) and capillary. Sample is loaded onto the capillary by replacing one of the reservoir (usually at the anode) with a sample reservoir and applying either an electric field, electrokinetic injection, or an external pressure, hydrodynamic injection. After replacing the buffer reservoir, the electric field is applied and the separation performed. The sample ions to be determined migrate at different velocities toward the electrode of opposite charge. The sample ions are

detected spectrophotometrically as they pass through a cell near the opposite end of the capillary.

When a buffer is placed inside a capillary, the inner surface of the capillary acquires a charge, due to ionization of the capillary surface or adsorption of ions from the buffer onto the capillary. The surface silanol (Si-OH) groups are ionized to negatively charged silanoate ($Si\text{-}O^-$) groups at $pH > 4$. The silanoate anions attract cations from the buffer, which form and inner layer of cations at the capillary. When an electric field is applied, the outer layer of cations are solvated, they drag the bulk buffer solution with them, thus causing electroosmotic flow (EOF).

Electroosmotic flow are measured using a neutral marker which moves through the capillary under the influence of only EOF. The main criteria in choosing a neutral marker are that it should be uncharged at the pH of the buffer, detectable by whatever type of detector is used, pure, have no reaction with the capillary wall, and be soluble in buffer. A variety of neutral markers have been used, including benzene, pyridine, phenol, methanol, mesityl oxide, and formaldehyde.

Under the influence of an electric field, an electrically charged solute will migrate through a buffer with an electrophoretic velocity, v_{EP}, in cm/s, given by

$$v_{EP} = \mu_{EP}E$$

where μ_{EP} is the electrophoretic mobility and E is the applied electric field. Separation is achieved because solutes migrate through the capillary at different velocities. Electrophoretic mobility is given by

$$\mu_{EP} = q/6\pi\eta r$$

where q is the charge of the ionized solute, η is the buffer vicosity, and r is the solute radius. Small, highly charged molecules move through the capillary the fastest, and large molecule with a lower charge move slower.

In CE system, with the detector side of the capillary negatively charged (cathode) and the electroosmotic flow from the source (anode) to the detector, cations and neutral molecules migrate through the detector in the same direction to cathode. The neutral molecule moves at the same velocity as the electroosmotic flow. Anions migrate in the same direction of electroosmotic flow with lower velocity because their electrophoretic mobility are usually less than the electroosmotic mobility.

The versatility of CE is partially derived from its numerous modes of operation. The separation mechanisms of each mode are different and thus can offer orthogonal and complementary information. The basic methods encompassed by CE include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). All of these 5 modes, except MEKC can separate ionic or charged substances only.

CZE is the most widely used mode due to its simplicity of operation and its versatility. The separation principle of CZE is based on the differential electrophoretic mobility and, therefore, only charged compounds or ions can be separated by this method. CZE is not applicable to the separation of uncharged compounds, because neutral compounds have no electrophoretic mobility.

MEKC, since initiated by Terabe et al. in 1984, is the only electrophoretic technique, based on micellar solubilization and electrophoretic migration of the micelle, that can be used for the separation of neutral solutes as well as charged ones.

The separation of neutral species by MEKC is accomplished by the use of surfactants in the running buffer. At concentrations above the critical micelle concentration, aggregates of individual surfactant molecules, micelles are formed and used as pseudo-stationary phase. Micelles are generally spherical with hydrophilic groups of the surfactant molecules, being on the outside of the micelle, oriented forward the aqueous buffer, and the hydrophobic groups oriented towards the center of the micelle to avoid interaction with the hydrophilic buffer. The solutes are separated by their differential distribution between the micelle and the surrounding aqueous phase and the differential migration of the two phase.

CGE has principally been employed in the biological sciences for the size-based separation of macromolecules such as proteins and nucleic acids. The size separation is obtained by electrophoresis of the solutes through a suitable polymer which acts as a molecular seive.

CIEF is a high resolution electrophoretic technique used to separate peptides and proteins on the basis of isoelectric point (PI).

CITP is a moving boundary electrophoretic technique. In CITP, a combination of two buffer systems is used to create a state in which the separated zones all move at the same velocity. The zones remain sandwiched between so called leading and terminating electrolytes. A fundamental attribute which distinguishes CITP from CZE is the fact that all sample zones migrate with the same electrophoretic velocity if equiribrium is established. This is expressed by the name iso-tacho. In a single CITP experiment either cations or anions can be analyzed.

In summary, in theory, any compound that differ in their charge-to-size ratios can be separated by CZE. Compounds that vary in their degree of partitioning

between a micelle and a buffer may be separated by MEKC. If molecules have the same charge-to-size ratios but are of different sizes, they may be separated by CGE. And, if the compounds differ in their isoelectric points, they may be separated by CIEF.

An immense number of practical applications of CE have been developed for the analysis of pharmaceutical drug substances and drug products. Applications include antibiotics (Miyashita, Terabe and Nishi, 1990; Swartz, 1991; Ackermans, Everaerts and Beckers, 1992; Ng, Lee and Li, 1992), analgesics (Swartz, 1991; McLaughlim et al., 1992), steroids (Miyashita et al., 1990; Nishi and Matsuo, 1991; Vindevogel and Sandra, 1991; Terabe, 1991), cold-relief medications (Nishi et al., 1990; Nishi and Terabe, 1990) and chiral separations (Stalcup and Gahm, 1996; Gausepohl and Blaschke, 1998; Vela, Yanes and Stalcup, 2001).

There have been several CE methods for the individual determination of codeine and promethazine. A separation of codeine and other illicit drugs was reported using MEKC, with a phosphate-borate buffer containing sodium dodecyl sulfate as a surfactant and acetonitrile, and CZE (Weiberger and Lurie, 1991; Krogh et al., 1994; Fagliaro et al., 1996; Lurie, 1998).

Korman, Vindevogel and Sandra (1993) studied the separation of codeine and its by-products using CZE and MEKC.

Persson-Stubberud and Åström (1998) reported the development and optimization of the MEKC method for the separation of codeine phosphate, ibuprofen, their degradation products and impurities. Additionally, they validated the proposed method, according to the International Conference on Harmonisation's guidance on the

validation of analytical methods. The method was intended for the determination of codeine phosphate hemihydrate and ibuprofen in commercial tablet formulations.

Ong et al. (1991) investigated the effect of β-cyclodextrin and tetrabutylammonium hydrogensulfate (TBA) on the separation of nine antihistamines using MEKC. In addition, quantitative analysis of promethazine hydrochloride in commercial drug samples was performed.

The determination of promethazine hydrochloride and other structurally related phenothiazines in pharmaceutical preparations was reported using CZE and MEKC with cetyltrimethylammonium bromide (CTAB) as micelle forming agent (Muijselaar, Claessens and Cramers, 1996).

None of the CZE and MEKC methods in these studies have been able to determine simultaneously codeine phosphate and promethazine hydrochloride in compound pharmaceutical preparations.

Disadvantages of the simultaneous determination of codeine phosphate and promethazine hydrochloride in cough solutions using reversed-phase HPLC were the resulting chromatogram with poor peak shape espectially the peak of promethazine hydrochloride and long analysis time. The optimisation of HPLC conditions was tedious and time comsuming due to long column equilibrating time when changing from one mobile phase to another. Furthermore, the sample pretreatment was needed for the cough solution prior to HPLC analysis.

CE provides advantages over liquid chromatography in terms of high efficiency, relatively inexpensive and long lasting capillary, small sample size requirements and low reagent consumption. Separation by CE are fast and it is

relatively easy to adjust experimental conditions to obtain an adequate separation of analytes. Many CE applications do not require sample pretreatment other than a possible dilution. Therefore, the method can be employed as an alternative for analysis of the compound of interest.

The purpose of this study is to develop a convenient and reproducible method for analysis of codeine phosphate and promethazine hydrochloride in cough solutions using MEKC, one of the most widely used mode of CE for pharmaceutical analysis.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

EXPERIMENTATION

Material and Method

Material

1. Apparatus and Instruments

- P/ACE™ 5010 CE System (Beckman Coulter, CA, USA)
  - eCAP™ Capillary cartridge (Beckman Coulter, CA, USA)
  - eCAP™ Capillary tubing 37 cm x 50 µm I.D., 375 µm O.D. (Beckman Coulter, CA, USA)
- High Performance Liquid Chromatograph, consists of
  - LC-10AD Liquid Chromatograph (Shimadzu)
  - SPD-10A UV-Vis detector (Shimadzu)
  - Chromatopac C-R6A (Shimadzu)
  - Injection system, Rheodyne 7167 equipped with a 20 µl loop
  - Column, Spherisorb S10, ODS2, 4.6 x 250 mm I.D. (Waters, MA,USA)
- Analytical balance, Sartorius AC211S (Sartorius AG, Goettingen, Germany)
- pH-meter, PerpHect® Meter Models 350 (Orion, MA, USA)
- Ultrasonicator, Sornicor® SC-52 (Sornicor, New York, USA)

2. Chemicals and Reagents

- Acetic acid (Merck, Germany)
- Acetonitrile HPLC grade (Lab-Scan, Bangkok, Thailand)
- Ammonium acetate (Merck, Germany)
- Boric acid powder (May & Baker, Dagenham, England)
- Codeine phosphate, hemihydrate, working standard
- Dichloromethane (J.T. Baker, NJ, USA)
- Disodium hydrogen phosphate (Merck, Germany)
- Guaifenesin, working standard
- Methanol HPLC grade (Lab-Scan, Bangkok, Thailand)
- Nylon filter membrane, 0.45 μm, 13mm (Alltech, IL, USA)
- Promethazine hydrochloride, working standard
- Sodium dodecyl sulfate (Sigma, MO, USA)
- Sodium hydroxide, pellets 'Baker Analyzed'® A.C.S. reagent (J.T. Baker, NJ, USA)
- Sodium tetraborate (Merck, Germany)
- Sudan III (Fluka, Switzerland)

3. Test samples

Three brands of cough solutions were selected for this study. Product A was Phensedyl® cough linctus. Product B and C were local made products. All products were purchased from drug stores and all have labelled amount of 9.0 mg codeine phosphate and 3.6 mg promethazine hydrochloride in 5 millilitres.

Method

Analytical procedure

The CE system was a P/ACE™ 5010 series equipped with a UV-detector set at 214 nm. Analysis was carried out in an uncoated fused-silica capillary of 75 μm I.D. and 30 cm effective length with the detector window at 7 cm from the outlet. Sample introduction was accomplished by hydrodynamic injection at the anodic end of the capillary with applying pressure of 0.5 p.s.i. for 5 s. A thermostating liquid was used to maintain the capillary at the stated temperature. Before use, the solution was filtered through a 0.45 μm pore size filter membrane. Prior to each experiment, the capillary was flushed with 0.1 M sodium hydroxide and water for 5 minutes, each and equilibrated with the background electrolyte (BGE) for 10 minutes. Electropherograms were recorded and processed with the capillary electrophoresis software for the P/ACE™ System 5000 series. This analysis method was used in the subsequent experiment.

1. Determination of MEKC conditions

1.1 Determination of buffer composition

Determination of buffer composition includes choice of buffer ions, concentration and pH.

In this study, disodium hydrogen phosphate, sodium tetraborate and boric acid were employed as buffer ions.

Buffer solutions

Various buffer solutions were prepared from disodium hydrogen phosphate, sodium tetraborate and boric acid. The concentration of each buffer type was varied from 10 to 100 mM with a pH range of 8.5 –10.5, adjusting to the desired pH with 1 M sodium hydroxide.

Background electrolyte

The BGEs were prepared by adding 50 mM sodium dodecyl sulfate (SDS) to the prepared buffer solutions of each buffer type. Filtered each buffer solution through a 0.45 μm membrane filter.

Standard mixture solution

The standard mixture solution of 90 μg/ml codeine phosphate and 36 μg/ml promethazine hydrochloride in deionized water was prepared and filtered through a 0.45 μm membrane filter.

1.1.1 Determination of buffer ion

The prepared standard mixture solution was analyzed. The BGEs prepared from each buffer type at concentration of 25 mM, pH 10.0 containing 50 mM SDS were used as running electrolytes. The instrument was operated at 20°C and 15 kV.

Ohm's law plots on the buffer systems used were run by injecting the BGE of each buffer type at concentration of 25 mM and pH 10.0. The current profiles at several voltages were monitored.

1.1.2 Determination of buffer concentration

The standard mixture solution was analyzed, using the BGEs of borate buffer preparing from boric acid at concentrations ranging from 10 to 75 mM and adjusting to pH 10.0 with 1 M sodium hydroxide. The analysis was performed at 20°C, 15 kV.

1.1.3 Determination of buffer pH

The standard mixture solution was analyzed and operating at 20°C, 15 kV, using the BGEs of borate buffer preparing from boric acid at concentration of 25 mM and adjusting to pH from 8.5 to 10.5.

1.2 Determination of SDS concentration

The standard mixture solution of codeine phosphate and promethazine hydrochloride was analyzed, using the BGEs consisting of 25 mM borate buffer, pH 10.0 with different concentrations ranging from 10 – 80 mM of SDS , and operating at 20°C, 15 kV.

1.3 Determination of organic modifier

The standard mixture solution of codeine phosphate and promethazine hydrochloride was analyzed, using the BGEs consisting of 25 mM borate buffer, pH 10.0, 40 mM SDS with different concentrations ranging from 5 – 20 %v/v of acetonitrile or methanol, and operating at 20°C, 15 kV.

1.4 Optimisation of MEKC conditions

1.4.1 Borate buffer

Borate buffer solutions of 25 mM of boric acid were prepared and adjusted to pH 9.5, 10.0 or 10.5 with 1 M sodium hydroxide.

1.4.2 Background electrolyte

Various BGEs were prepared. The BGE consisted of borate buffer at pH 9.5, 10.0 or 10.5, SDS of 30, 40 or 50 mM and acetonitrile of 8, 10 or 12 %v/v. The composition of the BGEs used in optimisation of MEKC conditions was summarized in Table 1. The nominal BGE was a buffer pH 10.0 containing 40 mM SDS and 10 %v/v acetonitrile. These solutions were filtered through a 0.45 μm membrane filter.

1.4.3 Standard mixture solution

The aqueous standard solution containing 90 μg/ml of codeine phosphate, 36 μg/ml of promethazine hydrochloride, 10 μg/ml sudan III and 5% methanol was prepared. The solution was prepared in 10-fold diluted nominal BGE.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.4.4 Procedure

The Plackett-Burman design was used to study the influence on the separation of codeine phosphate and promethazine hydrochloride. Five factors were selected. The buffer pH was 9.5, 10.0 or 10.5. The SDS concentration was 30, 40 or 50 mM. Organic modifier (8, 10 or 12 %v/v) used was acetonitrile. During CE

separation, the temperature of the capillary was maintained at 15, 20 or 25°C and the applied voltage was 10, 15 or 20 kV.

Table 1 Composition of the background electrolytes for optimisation of MEKC conditions.

BGE number	SDS concentration (mM)	Acetonitrile Concentration (%v/v)	pH of 25 mM borate buffer
1	40	10	10.0
2	30	10	10.0
3	30	8	10.0
4	40	8	9.5
5	30	10	9.5
6	40	8	10.0
7	40	10	9.5
8	30	8	9.5
9	50	12	10.5
10	40	12	10.5
11	40	10	10.5
12	50	10	10.0
13	40	12	10.0
14	50	10	10.5
15	50	12	10.0

The nominal values of the operating conditions were a nomimal BGE and operated at 20°C and 15 kV.

The Plackett-Burman design was presented in Table 2 as a seven-variable array for five selected factors (F1 - F5) along with two dummy factors (D1 and D2). Two complementary designs were constructed around the nominal values of the operating conditions which were used as (+1) level in design I and (-1) level in design II. Table 2 also shows the values of the factors to be implemented for design I and II, respectively. As shown in Table 1, BGE number 1 – 8 were used in design I and BGE number 1, 9 – 15 were used in design II. The experiments were carried out in the same order as indicated in Table 2, using the standard mixture solution as the test solution.

The effect of changing a factor from a low to a high level value was examined on the resolution (R_s), the analysis time (t_m), considered as the migration time of the last eluting peak, and also the asymmetry factor (A_s) of the last peak. The last peak was found to be the peak of promethazine.

2. Establishment of MEKC condition

Background electrolyte

The borate buffer consisting of 25 mM boric acid was prepared, adjusted to pH 10.0 with 1 M sodium hydroxide. Then 50 mM SDS and 10% v/v acetonitrile were added to the borate buffer. Filtered through a 0.45 μm membrane filter.

Analytical Procedure

Sample introduction was hydrodynamic injection with injection time of 5 sec and operated at 20°C, 15 kV. To stabilized the MEKC system, two injections of BGE were made before starting the analysis. Vials of BGE were replaced for approximately every twentieth injection to maintain the level of BGE in the vial and to

avoid evaporation of the BGE during the whole run. Separately hydrodynamically injected six replicate injections of standard mixture solution into the capillary.

Table 2 Eight - experimental Plackett- Burman design for seven factors and its two implementation.

Exp.	Factors						
	F1	F2	F3	F4	F5	D1	D2
1	+1	+1	+1	-1	+1	-1	-1
2	-1	+1	+1	+1	-1	+1	-1
3	-1	-1	+1	+1	+1	-1	+1
4	+1	-1	-1	+1	+1	+1	-1
5	-1	+1	-1	-1	+1	+1	+1
6	+1	-1	+1	-1	-1	+1	+1
7	+1	+1	-1	+1	-1	-1	+1
8	-1	-1	-1	-1	-1	-1	-1

Factors	Design I Level		Design II Level	
	(-1)	(+1)	(-1)	(+1)
F1 SDS concentration (mM)	30	40	40	50
F2 Acetonitrile concentration (%v/v)	8	10	10	12
F3 pH of BGE	9.5	10.0	10.0	10.5
F4 Capillary temperature ($^{\circ}$C)	15	20	20	25
F5 Applied voltage (kV)	10	15	15	20
D1	-	-	-	-
D2	-	-	-	-

2.1 Selection of the internal standard

To compensate for various analytical errors, six compounds, including phenylephrine hydrochloride, guaifenesin, phenylpropanolamine hydrochloride, chlorpheniramine maleate, brompheniramine maleate and diphenhydramine hydrochloride, were randomly screened as the internal standard. Each compound was dissolved in deionized water to have concentration of 80 µg/ml and was filtered through a 0.45 µm membrane filter prior to injection into the capillary using the optimized condition.

The criteria for selecting the internal standard were: the compound must not be present in the samples of cough solution studied, the compound must be stable and nonreactive and could be completely resolved from other compounds in the samples as symmetrical peak under the experimental conditions.

3. Analytical method validation

The validation was performed according to the International Conference on Harmonisation 's guidance on the validation of analytical procedures (ICH, 1996).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Preparation of standard mixture solution

Stock solution of codeine phosphate and promethazine hydrochloride were separately prepared in deionized water with the concentrations of 1.8 and 0.7 mg/ml, respectively. Transferred 5.0 ml of each stock solution and 5.0 ml of the internal standard solution to a 100-ml low actinic volumetric flask, diluted to volume with deionized water mixed and filtered through a 0.45 µm membrane filter. The final

concentrations of codeine phosphate and promethazine hydrochloride were 90 and 36 μg/ml, respectively.

Preparation of sample solution

Determined accurately weight per ml of each cough solutions. Transferred an accurately weighed portion of the sample, equivalent to 5.0 ml of sample solution, to a 100-ml low actinic volumetric flask. Added 5.0 ml of the internal standard solution, diluted to volume with deionized water, mixed and filtered through a 0.45 μm membrane filter.

Preparation of the solution of photolytic degradation products of promethazine hydrochloride

(a) Standard solution of promethazine hydrochloride

The standard solution of 0.7 mg/ml of promethazine hydrochloride was prepared in deionized water, and placed on a table for exposure to light at room temperature for one week.

Following the exposure time, 5.0 ml of the resulting solution was diluted and adjusted to 100 ml with deionized water. Filtered through a 0.45 μm membrane filter.

(b) Sample solution

Three cough solutions, including Phensedyl® cough linctus and two locally made preparations representing different manufactures, were placed on a table for

exposure to light at room temperature for one week.

Following the exposure time, transferred an accurately weighed portion of the sample, equivalent to 5.0 ml of sample solution, diluted and adjusted to 100 ml with deionized water. Filtered through a 0.45 μm membrane filter.

3.1 System suitability

System repeatability was evaluated by six replicate injections of the standard mixture solution. Measured the peak area of codeine, promethazine and internal standard from electropherograms and calculated the percentage of relative standard deviation of peak area ratios.

Calculated resolution (R_s), theoretical plate (N) and asymmetry factor (A_s) from electropherograms of the standard mixture solution.

3.2 Selectivity

The solution of standard, sample and photolytic degradation products of promethazine hydrochloride were analysed according to the proposed method.

3.3 Linearity and range

Linearity was assessed by preparing five standard solutions with concentration range of 45 – 135 μg/ml for codeine phosphate and 18 - 54 μg/ml for promethazine hydrochloride, to which 100 μg/ml of the internal standard was added. The solutions were triplicate injected into the capillary using the condition described in section 2. Measured the peak area of codeine, promethazine and internal standard

from electropherogram and calculated the peak area ratios. Ploted the peak area ratios of each compound and internal standard versus the concentration.

3.4 Accuracy

The recoveries, at three concentration levels, of codeine phosphate and promethazine hydrochloride were determined using standard addition method. Transferred accurately weighed portions of Phensedyl® cough linctus equivalent to 5.0 ml into three 100-ml low actinic volumetric flasks. Added 7.2, 9.0, and 10.8 mg of codeine phosphate separately to each flask. Then separately added 2.8, 3.6, and 4.3 mg of promethazine hydrochloride to each of those flasks. Finally 5.0 ml of the internal standard was added, diluted to volume with deionized water, mixed and filtered through a 0.45 μm membrane filter. The solutions were triplicate injected into the capillary using the MEKC condition described in section 2.

The percentage of recoveries of codeine phosphate and promethazine hydrochloride were determined from the ratio of the amount found and the amount added.

3.5 Precision

Intra-day precision was evaluated by six replicate analyses (n=6) of Phensedyl® cough linctus, using the described MEKC method in section 2.

Inter-day precision was similarly evaluated for three non-consecutive days (n=3). Six replicates of the sample were used.

The content of each compound and the percentage of relative standard deviation were determined.

4. Quantitative analysis of cough solutions by optimised MEKC method

The optimum MEKC condition in section 2 was used for analysis of three cough solutions, including Phensedyl® cough linctus and two locally made preparations, representing the different manufactures. All of these solutions contained the same concentration of codeine phosphate and promethazine hydrochloride.

Each cough solution was aliquoted twice, and each was duplicate injected with optimum condition. The amount of codeine phosphate and promethazine hydrochloride was calculated using peak area ratios obtained from assay preparation compared with those obtained from standard solution.

5. Quantitative analysis of cough solutions by HPLC method

5.1 Chromatographic condition

Acetate buffer (40 mM) was prepared by dissolving ammonium acetate in deionized water 1,000 ml and adjusting the pH to 7.0 with glacial acetic acid. The mobile phase was prepared by mixing 150 ml of methanol and 850 ml of acetate buffer and the mixture was filtered through a 0.45 µm membrane filter and degassed by sonication. A 4.6 x 250 mm I.D. stainless-steel column packed with Spherisorb S10 ODS2 was used. The UV detector was set at a wavelength of 254 nm. The mobile phase was used at a flow rate of 2.0 ml per min.

5.2 Internal standard solution

Diphenhydramine hydrochloride was dissolve in distilled water at concentration of 1.0 mg per ml.

5.3 Standard preparation

Stock solutions of codeine phosphate and promethazine hydrochloride were prepared separately in deionized water with the concentration of 1.8 and 0.7 mg/ml, respectively.

Transferred 5.0 ml of each stock solution, 5.0 ml of internal standard solution and 5.0 ml of 1 M sodium hydroxide to a 250-ml separatory funnel, and mixed. Extracted with 5x5.0 ml of dichloromethane. The extracts were collected in a 50-ml erlenmeyer flask and evaporated to dryness by a stream of nitrogen. The residue was reconstituted in 25.0 ml of mobile phase.

5.4 Assay preparation

Determined accurately weight per ml of each cough solutions. Transferred an accurately weight portion of the sample, equivalent to 5.0 ml of sample solution, to a 250-ml separatory funnel. Added 5.0 ml of internal standard solution and 5.0 ml of 1 M sodium hydroxide, and mixed. Extracted with 5x5.0 ml of dichloromethane. The extracts were collected in a 50-ml erlenmeyer flask and evaporated to dryness by a stream of nitrogen. The residue was reconstituted in 25.0 ml of mobile phase. The assay preparation of each cough solution was duplicately prepared.

5.5 Assay procedure

Duplicate injected equal volume (20 µl) of each of the standard preparation and assay preparations into the chromatograph, recorded the chromatograms and measured the responses of codeine and promethazine. Calculated the quantity of codeine phosphate and promethazine hydrochloride using peak area ratio obtained from the assay preparation and the standard preparation.

6. Comparison of quantitative analysis of cough solutions by MEKC and HPLC method

The test results obtained from MEKC method were compared with those obtained from HPLC method by using statistical t – test.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

RESULTS AND DISCUSSION

1. Development of MEKC condition

1.1 Determination of buffer composition

1.1.1 Determination of buffer ion

The running buffer selection is extremely important to the success of any CE separation. Most buffer systems have significantly buffer capacity only in a limited pH range. Due to the logarithmic definition of pH, buffer capacity decreases by a factor of 10 for every pH unit away from the pK. A board variety of buffer systems can be used in CE. For this study, the three buffer systems, including disodium hydrogen phosphate, sodium tetraborate and boric acid were investigated.

Criterion for selecting the buffer for use in this study was that it should possess the following properties: good buffering capacity in the pH range of 8.5 – 10.5, low absorbance at 214 nm and low mobility to minimize current generation.

The pH range of 8.5 – 10.5 was chosen to elute the micelle and maintain a suitable elution time window for codeine and promethazine. First, the drug analysis was performed using three different buffer system: disodium hydrogen phosphate, sodium tetraborate and boric acid, at nearly constant pH (pH 10.0) and SDS concentration 50 mM.

As shown in Figure 3a, poor peak shape of codeine and promethazine as well as unstable baseline were obtained using disodium hydrogen phosphate as a buffer system. The results obtained, a form of band broadening known as electrodispersion, were due to differences in sample zone and BGE conductivities which caused by mismatching buffer ion mobility to solute mobility. In this case, the sample zone anion had a lower mobility than the running buffer anion, thus a lower conductive solute zone with higher resistance and higher electric field than in the BGE. As a result, peak shape distortions occurred.

Comparing Figure 3b with Figure 3a, using sodium tetraborate as a buffer system, the peak shape of the codeine was improved but not for promethazine peak. The results implied that the problem of electrodispersion still existed. But symmetrical peaks of codeine and promethazine were observed with a 25 mM boric acid run buffer at pH 10.0 as shown in Figure 3c, a consequence of matching the buffer and sample conductivities.

The mobility of buffer ions not only had an effect on electrophoretic dispersion, but also on the resulting current at a given field strength. This is demonstrated in Figure 4 by an Ohm's law plot of voltage to current of different buffer systems used in this study. The plot should be linear with a zero intercept and a slope of 1/R, where R is the resistance. Joule heating is a consequence of the resistance of the buffer system to the flow of current. The heat produced is directly proportional to the applied voltage. When excessive heat is produced, the resistance goes down, causing an increase in current which is indicated by a deviation from linearity and an increase in the slop of the plot. The point at which there is a positive deviation (about 5%) from linearity, is the optimal voltage for the separation.

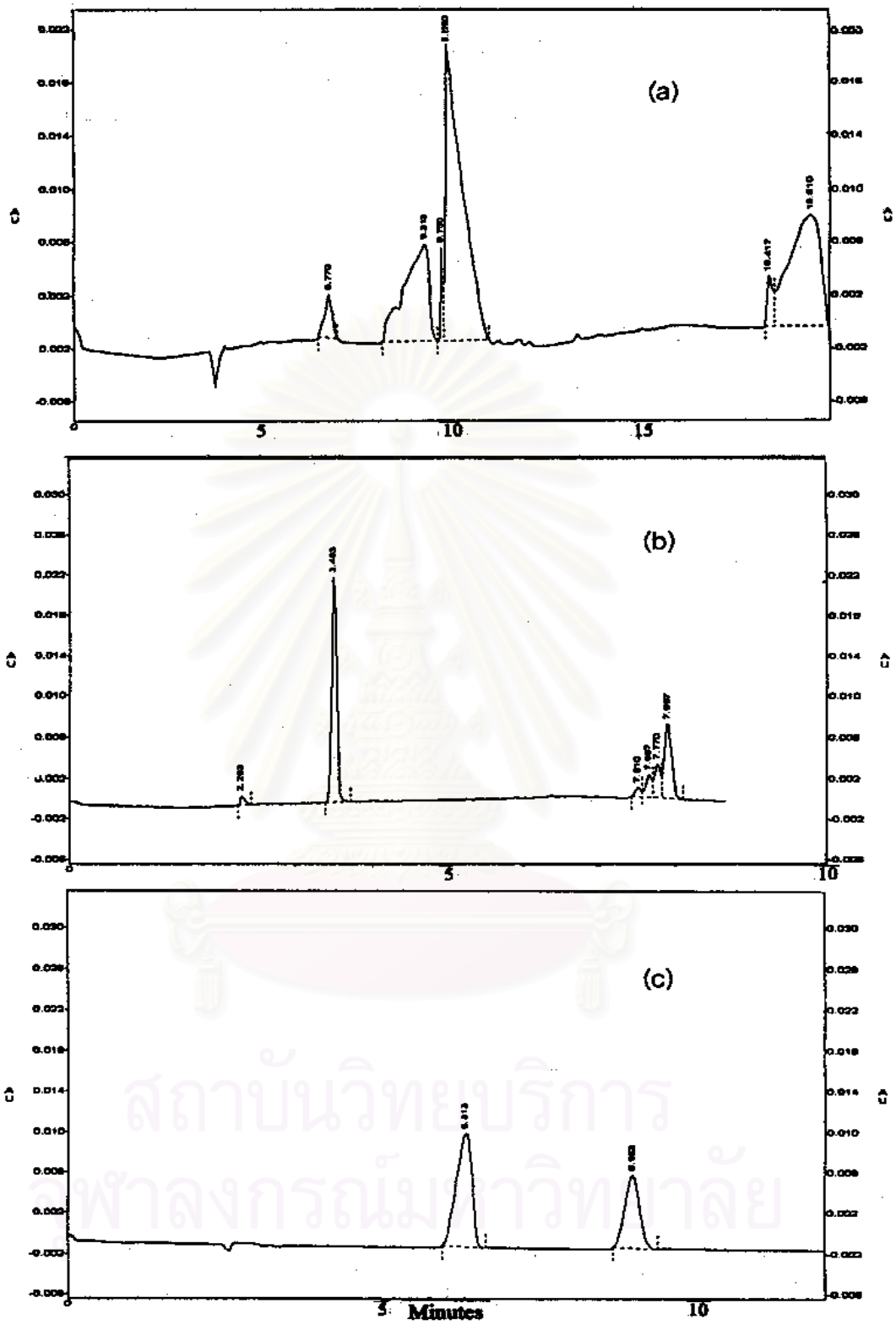


Figure 3 Electropherograms of the standard mixture seprrated with BGE consisting of 50 mM SDS, 25 mM of (a) disodium hydrogen phosphate, (b) sodium tetraborate and (c) boric acid; pH 10.0.

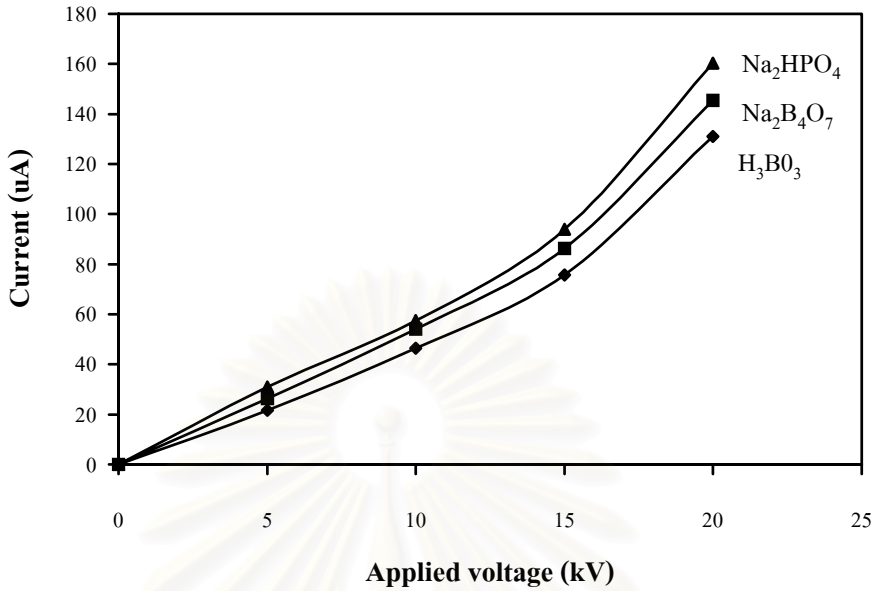


Figure 4 Ohm's law plots of applied voltage (kV) versus the resulting current (μA), for 25 mM of three buffer systems ; disodium hydrogen phosphate (Na_2HPO_4), sodium tetraborate ($Na_2B_4O_7$), and boric acid (H_3BO_3) at the same pH (10.0).

Because the equivalent concentration of all buffer systems in this study was 25 mM with sodium as the common cation, differences in the slopes were effected solely by the different ionic equivalent conductance of the anions. The influence of heat generation on the curve shape was more pronounced for disodium hydrogen phosphate, which had a high conductance, than for sodium tetraborate and sodium borate. Sodium borate seemed to have lowest conductivity and mobility and thus producing lowest current with a linear relationship of Ohm's law plot up to the applied voltage of 15 kV where Joule heating became significant.

Form Figure 3c and 4, a boric acid buffer system, subsequently called borate buffer, was therefore selected as a component of the BGE with the optimal applied voltage of 15 kV.

1.1.2 Determination of buffer concentration

Various buffer concentrations were also studied. Generally, the concentration should be high enough to maintain adequate buffering capacity but not too high so as to increase Joule heating significantly. The ionic strength is an important tool that can be used to improve efficiency, resolution and sensitivity of the separation system. Variation of the ionic strength induces several effects such as temperature increase and viscosity changes, which in turn influence the mobility.

Using a borate buffer at pH 10.0, the concentration was varied from 10 to 100 mM. Concentrations of SDS in the BGEs was maintained at 50 mM.

Figure 5 shows the separation of codeine and promethazine at several concentrations. The migration time of the analytes increased noticeably with increasing buffer concentration due to the decrease EOF as a result of the buffer ions shielding the capillary wall charge. This method can be used to improve resolution by decreasing EOF and retaining the solutes in the capillary for a longer period of time. In addition, higher resolution was obtained with increasing buffer concentration which might be caused by a better suppression of electrophoretic dispersion.

At 10 mM borate, as shown in Figure 5a, peak of both compounds were broad due to less buffer capacity and less suppress ion of electrophoretic dispersion. Increasing in the concentration of borate buffer improved peak shape. But when buffer concentration was higher than 50 mM, higher current was produced which resulted in

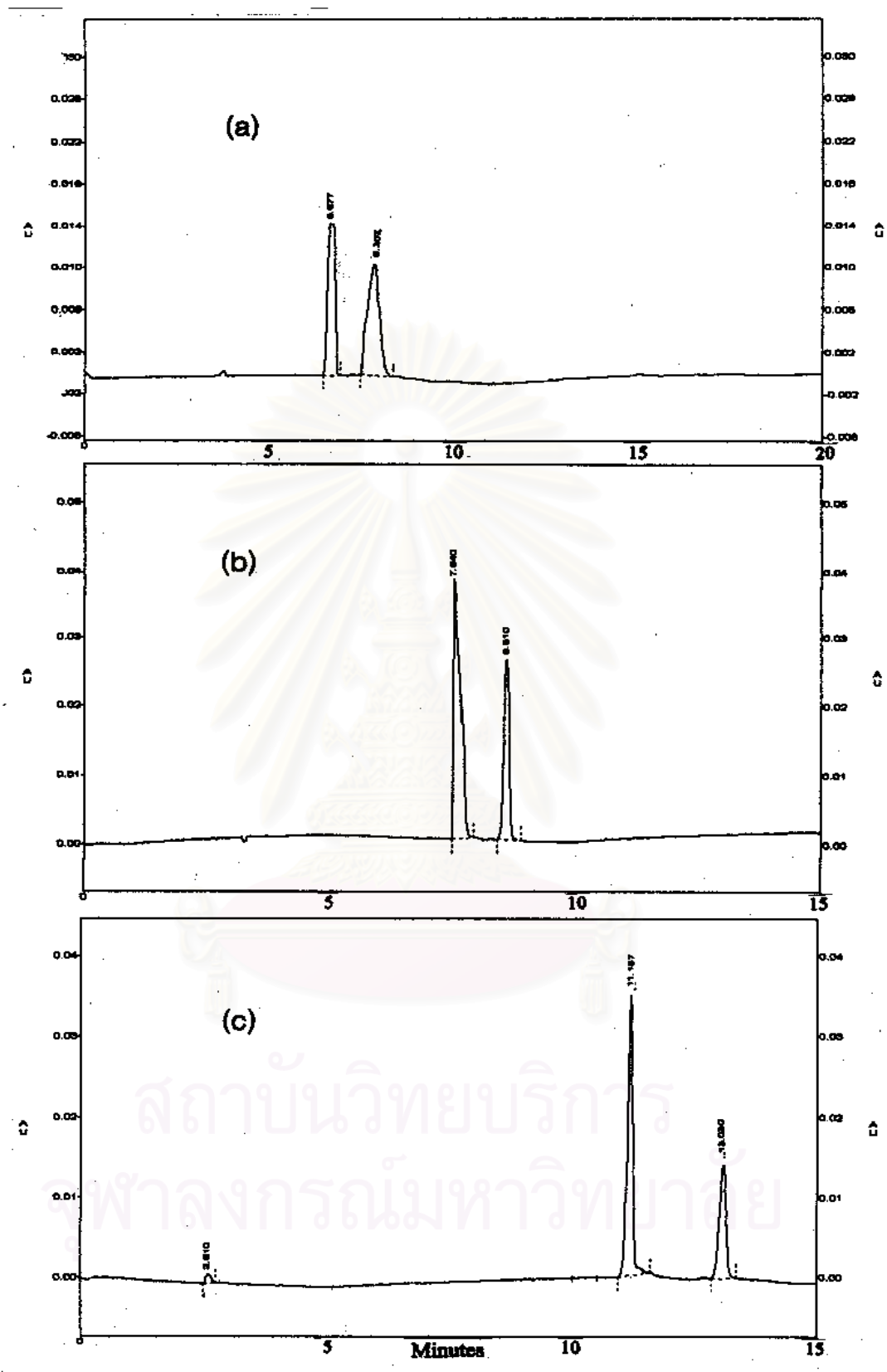


Figure 5 Electropherograms of the standard mixture separated with BGE consisting of 50 mM SDS in varied concentration of borate buffer (a) 10 mM, (b) 25 mM and (c) 50 mM; pH 10.0.

unstable baseine. At 75 mM of borate buffer, the Joule heating became significant and the analysis at this concentration was omitted. Due to generated current, the chosen concentration should be the lowest that can separate codeine and promethazine with better peak shape.

Therefore, the borate buffer concentration of 25 mM was chosen to separate codeine and promethazine in this study.

1.1.3 Determination of buffer pH

Buffer pH has a significant effect on electroosmotic flow because it changes the zeta potential. As pH increases, electroosmotic flow increases, primarily because at high pH, there is more dissociation of Si-OH to $Si\text{-}O^-$ on the inner capillary wall. The zeta potential is proportional to surface charge on the capillary wall. At higher pH, there are more charge $Si\text{-}O^-$ groups and, consequently, a greater zeta potential, and an increase in electroosmotic velocity. At lower pH, there is less surface ionization and, consequently, a lower zeta potential, and a decrease in electroosmotic velocity.

Various pH (8.5 - 10.5) of 25 mM borate buffer was studied. At this pH range the ionization of both codeine (pKa 8.2) and promethazine (pKa 9.1) was quite low and consequently, the separation of the two compounds based on differential electrophoretic mobility was primarily depended on their lipophilicity.

Figure 6 shows the electrophoretic separation of codeine and promethazine at different pH values ranging from pH 8.5 to 10.5. At pH 8.5. the peak of codeine and promethazine could not be resolved. Promethazine was partially ionized form, thus mechanism affected migration were its mobility interactions with the micelles and

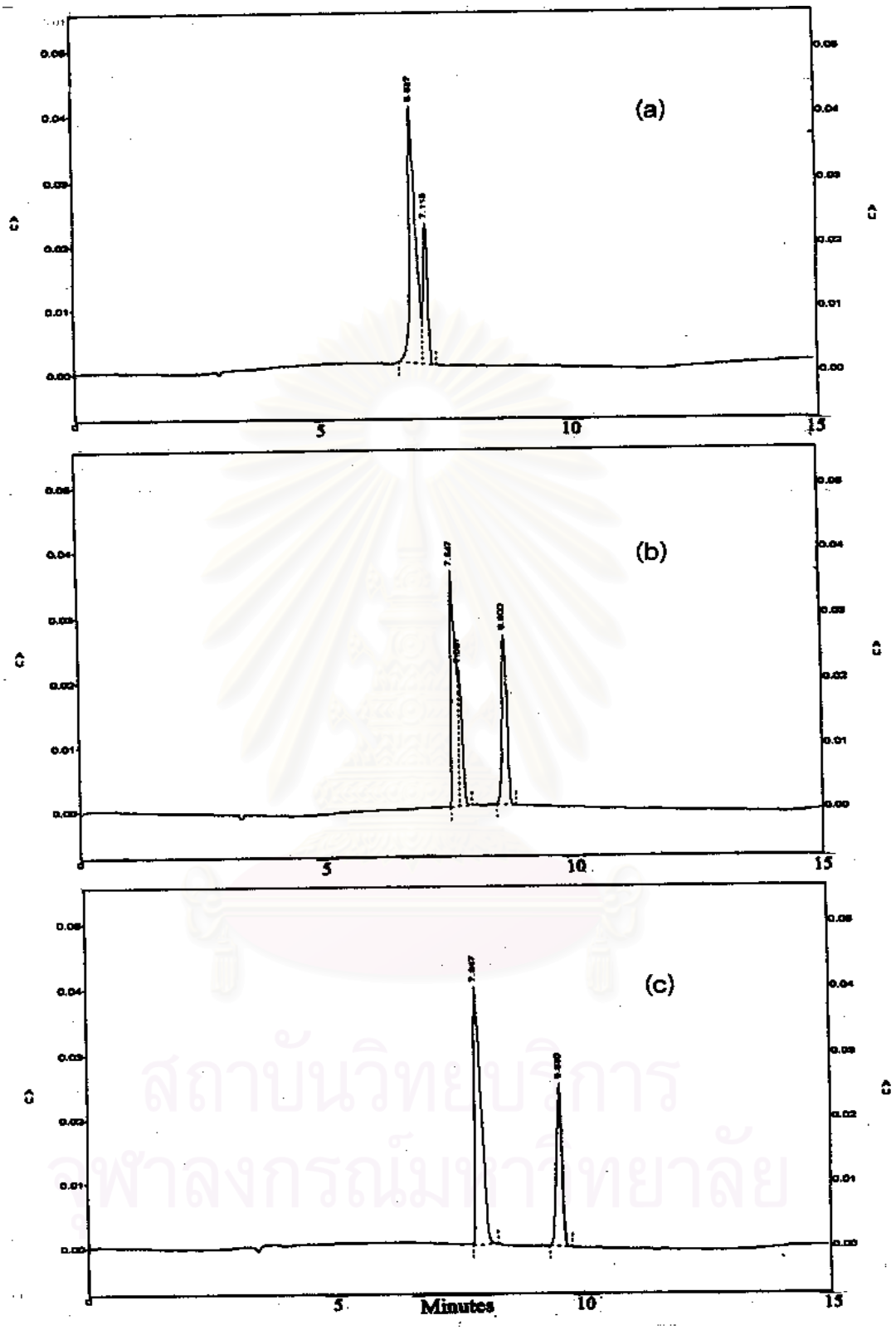


Figure 6 Electropherograms of the standard mixture separated with BGE consisting of 50 mM SDS, 25 mM borate buffer (a) pH 8.5, (b) pH 9.5 and (c) pH 10.5

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

partitioning into the micellar phase. As one can readily see, even small changes of the pH have a dramatic influence on the resolution of the two drugs. The pH 10.0 of borate buffer was selected based on the suitability of both resolution, analysis time and extending the lifetime of capillary.

1.2 Determination of SDS concentration

SDS, the most widely used anionic surfactant, was used in this study. SDS is an attractive choice because it gives excellent selectivity for a wide range of compounds, and is available in high purity. Although, the minimum surfactant concentration is defined by the critical micellar concentration (CMC, 8 mM for SDS in water at 25°C), the optimum concentration is the best determined experimentally because the actual CMC is affected by pH and ionic strength of the buffer. In this study, the SDS concentration was varied from 10 to 80 mM using a 25 mM borate buffer of pH 10.0.

The effect of SDS concentration on the separation of codeine and promethazine was shown in Figure 7. Without the addition of SDS to the BGE, the analytes were not resolved, as shown in Figure 7a. Increasing the SDS concentration affected partitioning of codeine and promethazine resulting in prolong their migration times, as shown in Figure 7b – f. At pH 10.0, both drugs existed largely in the unionized form, especially codeine. The more hydrophobic promethazine interacted more strongly with the micelle and was retained longer than codeine. Increasing the concentration above 20 mM greatly improved peak shape. Increasing the SDS concentration may have other effects on the analysis. The most important is the increase in associated current because the surfactant is charged. In this study, the current level increased from 34.0 μA at 10 mM SDS to 85.7 μA at 80 mM SDS. For this study, the addition of organic modifier was intended for improving the resolution

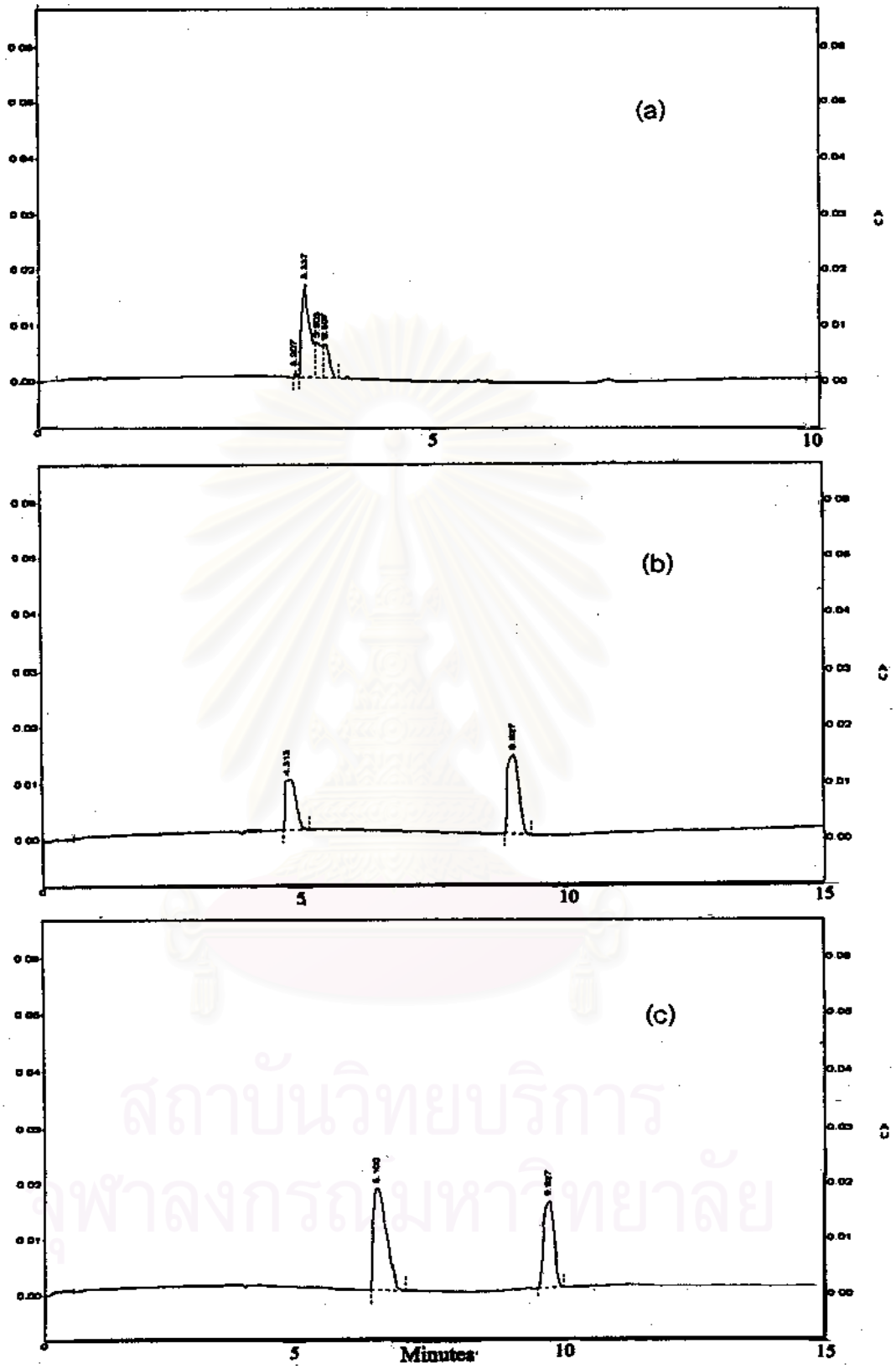


Figure 7 Electropherograms of the standard mixture separated with BGE consisting of 25 mM borate buffer pH 10.0 with containing (a) 0 mM SDS, (b) 5 mM SDS, (c) 10 mM SDS.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

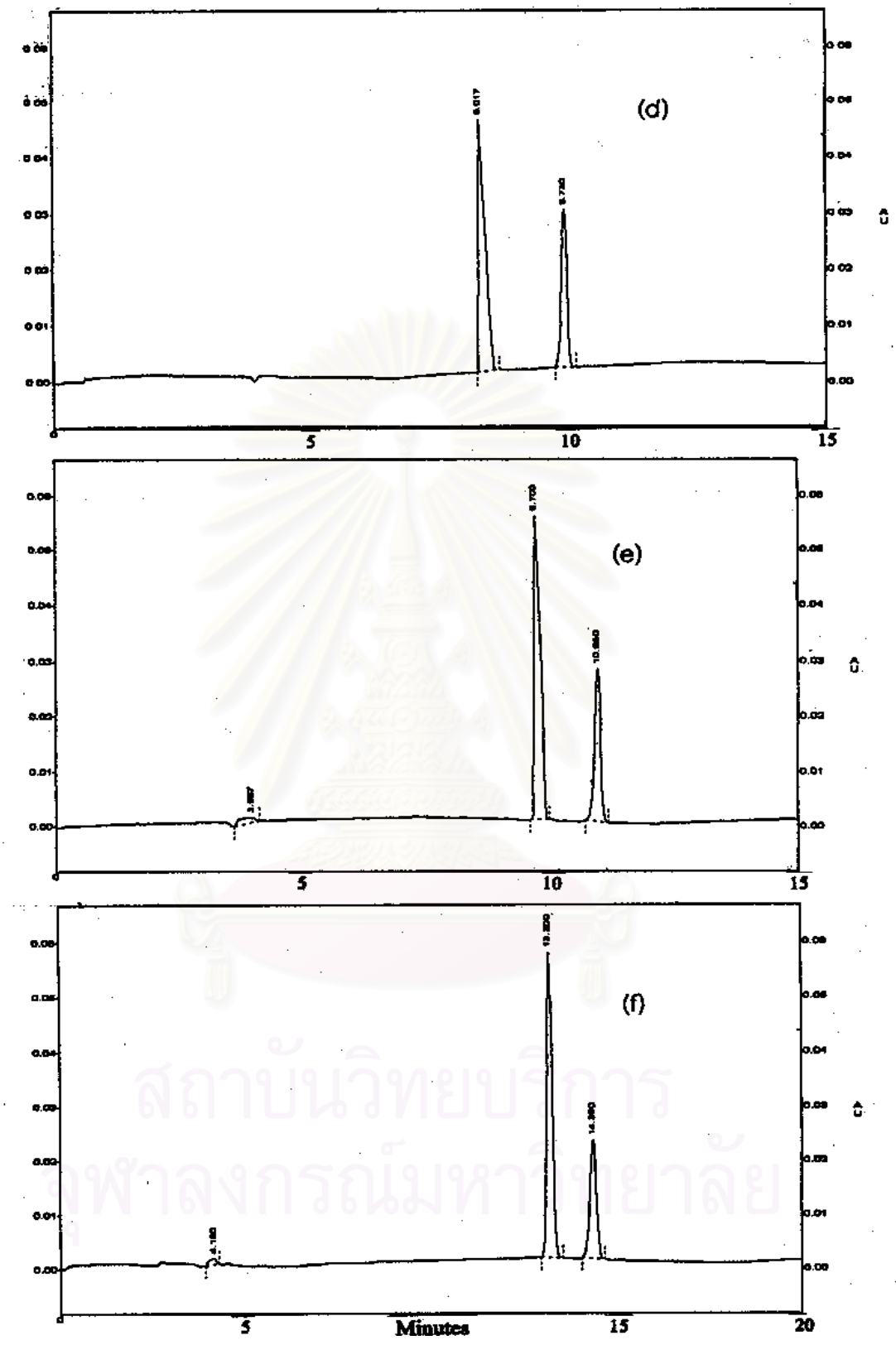


Figure 7 (Continued) Electropherograms of the standard mixture separated with BGE consisting of 25 mM borate buffer pH 10.0 with containing (d) 20 mM SDS, (e) 40 mM SDS and (f) 80 mM SDS.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

but at the same time this would destabilize the micellar system. The SDS concentration of 40 mM was therefore selected with respected to the generated current and the number of available micelles for further optimisation study.

1.3 Determination of organic modifier

Organic modifiers, such as methanol and acetonitrile added to the running buffer to alter retention mechanism by changing the viscosity and the polarity of aqueous phase. The effect of adding an organic solvent to the buffer depends on which and how much solvent is added. Adding methanol to water increases the viscosity of the solution until the percent of methanol exceeds about 50%v/v, then viscosity decreases up to 100% methanol. In contrast, adding acetonitrile to water decreases the viscosity of the mixture from 0 –100% acetonitrile (Baker, 1995). Hence, organic modifiers affect the time window by changing the viscosity of the buffer system.

In this study, methanol and acetonitrile as organic modifiers were investigated.

As shown in Figure 8a - c, increasing the concentration of acetonitrile from 5 – 20% v/v improved resolution between codeine and promethazine and migration time window due predominantly to an increase in the micellar electrophoretic mobility and, consequently, the micelle migration time. At 20 %v/v acetonitrile, the migration time of promethazine was more than 40 min, and was not shown in Figure 8. Acetonitrile of 10 %v/v seemed to give a reasonable analysis time and resolution.

In this study, when methanol (5 – 20 %v/v) was used as an organic modifier, the unstable baseline was obtained. Therefore, acetonitrile of 10 %v/v was chosen as the organic modifier for preparing BGE for optimisation study.

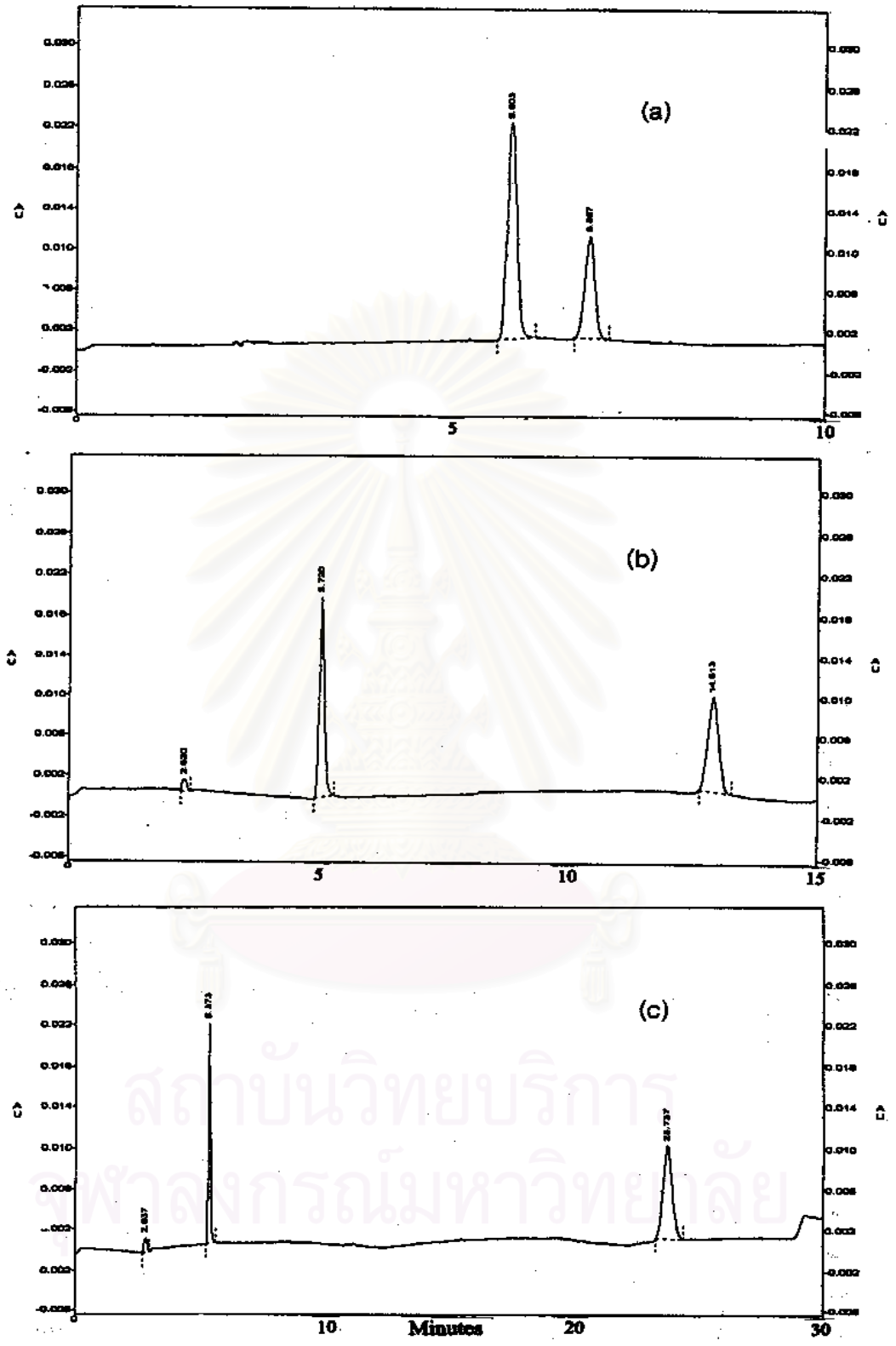


Figure 8 Electropherograms of the standard mixture separated with BGE consisting of 40 mM SDS, 25 mM borate buffer pH 10.0 with varied concentration of acetonitrile (a) 5 %v/v, (b) 10 %v/v and (c) 15 %v/v.

1.4 Optimisation of MEKC conditions

Five factors at 3 levels were selected for study the optimisation of the MEKC conditions for the separation of codeine phosphate and promethazine hydrochloride. These factors were the concentrations of SDS and acetonitrile, pH of the BGE, temperature of the capillary and the applied voltage. These factors did not independently affect electrophoretic separation of analytes. Therefore, it was inappropriate to do the traditional one-factor-at-a-time experimentation, which ordinarily requires tremendous experimental effort to obtain less-than-perfect knowledge of the conditions. In this case the experimental design can be applied to determine in an efficient way the set of conditions that are required to obtain a process with desirable, often optimal characteristic. For this study using factorial design, when each of the 5 factors were investigated at two levels only, all possible combinations would still required 2^5 experiments. Further reduction of the number of experiments can be obtained by focusing on the main effects, ignoring the effects that are specifically due to interaction between factors. The Plackett-Burman design (Massart et al., 1997) is an example of this type of statistical design and used in this study. With five selected factors at three levels, two complementary designs of the Plackett-Burman design was selected for this study.

As shown in Table 2, the concentrations of SDS and acetonitrile, pH of the BGE, capillary temperature and the applied voltage were factor F1, F2, F3, F4 and F5, respectively for both design I and II. Dummy factors (D1 and D2) are imaginary variables for which the difference between the high level (+1) and the low level (-1) is zero. The effect does not represent a physical difference, so it can be used to estimate the variability of the system and the significance of the effects found for the true physical parameters.

In this study, methanol (5 %v/v) and sudan III were added to the standard mixture solution to serve as neutral markers for measuring the migration time of electroosmotic flow (t_0) and the migration time of micelles (t_{mc}), respectively. Both compounds were uncharged at the pH (9.5 – 10.5) of the buffer, detectable by UV-detector at 214 nm, pure, have no interaction with the capillary walls, and were soluble in the buffer. Methanol was considered an insoluble solute in the SDS micelles that spent most of its time in the aqueous buffer and eluted at time t_0. Sudan III was very lipophilic and totally solubilized by SDS micelles and eluted at time t_{mc}.

The nominal levels of the operating conditions were a borate buffer of pH 10.0, containing 40 mM SDS, 10 %v/v acetonitrile and operated at 20°C and 15 kV. These values were compared with the lower levels and higher levels in design I and II, respectively.

The effect of changing a factor from a low to a high level value was examined on some selected quality responses such as the resolution (R_s) of codeine and promethazine, the analysis time (t_m), considered as the migration time of the last eluting peak, promethazine, and also the tailing factor (A_s) of the promethazine peak.

Resolution and tailing factor were calculated according to the following equations;

$$R_s = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2'}{1+k_2'}\right)\left(\frac{1-t_0/t_{mc}}{1+(t_0/t_{mc})k_1'}\right) \quad \text{.....(1)}$$

While capacity factor has been shown to be related to migration times.

$$k' = \frac{t_m - t_0}{t_0(1-t_m/t_{mc})} \quad \text{.....(2)}$$

and

$$\alpha = \frac{k_2'}{k_1'} \qquad \text{.....(3)}$$

$$A_s = \frac{W_{0.1}}{2f} \qquad \text{.....(4)}$$

where N is the average theoretical plate (Balchunas and Sepaniak, 1988) of codeine and promethazine; α is the selectivity, k_1' and k_2' are the capacity factor of codeine and promethazine, respectively; t_0 and t_{mc} are the migration times of electroosmotic flow and micelle. $W_{0.1}$ is the peak width at 10% of the peak, f is the distance measured from the leading edge of the peak to the perpendicular of the peak maximum at 10% of the peak height.

A composite response, Q* was introduced which reflected the desirability to obtain sufficient resolution within a short analysis time. Therefore the experimental data of migration time and resolution, as shown in Table 3 and 4 for design I and II, respectively, were scaled between 0 and 1. For the migration time, the shortest one of the eight experiments of a design was given the value 1 and the longest, zero, whereas for the resolution the largest was assigned the value 1 and the smallest, the value zero. The transformed data t_m* and R_s* were obtained by linear interpolation. The new response, Q* was defined as:

$$Q^* = \frac{t_m^* + 2R_s^*}{3} \qquad \text{.....(5)}$$

so that $0 \leq Q^* \leq 1$. The higher the Q* value, the better the compromise between resolution and migration time. In this definition the resolution was attributed

arbitrarity two times more weight than the analysis time since the former response was considered more important from the analytical point of view.

Within each design the effect (E_x) of a particular factor X was calculated from the difference between the average result at the (+1) level ($\sum Y_{x(+1)}$)/4 and the average result at the (-1) level ($\sum Y_{x(-1)}$)/4;

$$E_x = \frac{\sum Y_{x(+1)}}{4} - \frac{\sum Y_{x(-1)}}{4} \qquad \text{.....(6)}$$

To facilitate comparison of the effects E_x of the five factors on different response a normalized effect ($\%E_x$) was calculated as follows;

$$\%E_x = \frac{E_x}{Y} \cdot 100 \qquad \text{.....(7)}$$

where Y is the average of all results for a particular response

$$Y = \frac{\sum Y_x}{8} \qquad \text{.....(8)}$$

The effects of the dummy factors (D1 and D2) were used to estimate the variability of the experiments. Therefore, the standard error (S.E.) was calculated as;

$$S.E. = \sqrt{\frac{\sum E_{(Di)}^2}{n_i}} \qquad \text{.....(9)}$$

where $E_{(Di)}$ is the effect of a dummy factor and n_i is the number of dummies involved. The effect of a factor X was considered significant if the absolute value of $\%E_x$ is greater than 2.%S.E. (Massart et al., 1997).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

The results of design I and II are shown in Table 3 and 4, respectively, and visualized by effect-plots in Figure 9 - 13.

1.4.1 Influence of SDS concentration

From the results of design I and II and the plot shown in Figure 9, it was obvious that a high concentration of SDS provided a significant increase in migration time of the last eluting peak, promethazine, than the lower one. As concentration of SDS was increased more micelles were formed and, consequently, more solubilization of the lipophilic phenothiazine in the micellar phase. As a result, the longer migration time of promethazine.

The effect of SDS concentration on the improvement in resolution was statistically significant only in the high concentration range (40 - 50 mM), whereas in the low concentration range (30 - 40 mM) an opposite but not statistically significant effect was observed. Although, the effect on resolution was significant in the high concentration range, the associated significantly longer migration time resulted in the lack of significant effects on the composite response, Q*, when changing from 40 to 50 mM. The effect on migration time was also visible in the negative effect on Q* which was statistically significant in the low concentration range (30 - 40 mM).

The asymmetric factor was not significantly affected by variation of the SDS concentration.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 3 Results of experimental design I

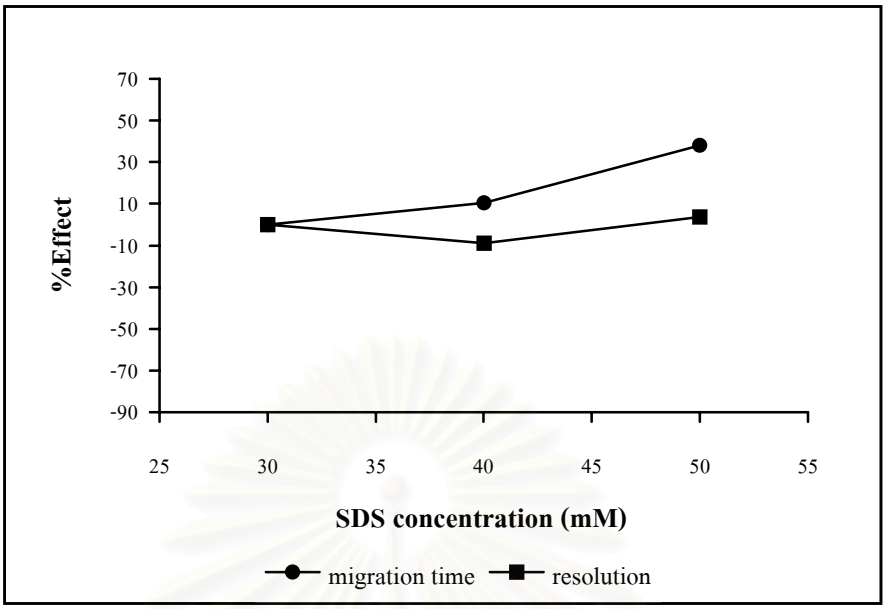
Exp	Response (Y)			Transformed data		Q*
	t_m (min)	R_s	A_s	t_m*	R_s*	
Response						
1	16.277	16.35	1.052	0.50	0.29	0.36
2	22.063	22.54	1.132	0.00	1.00	0.67
3	10.813	14.72	1.027	0.98	0.11	0.40
4	10.582	13.78	0.975	1.00	0.00	0.33
5	12.157	17.08	1.090	0.86	0.38	0.54
6	21.490	15.96	1.033	0.05	0.25	0.18
7	19.537	17.09	1.098	0.22	0.38	0.33
8	16.187	14.68	1.009	0.51	0.10	0.24
Average Y	16.138	16.52	1.052			0.38
Effects						
E(SDS)	1.67	-1.46	-0.025	-	-	-0.16
E(ACN)	2.74	3.48	0.082	-	-	0.19
E(pH)	3.05	1.74	0.018	-	-	0.04
E(T)	-0.78	1.02	0.012	-	-	0.10
E(V)	-7.36	-2.08	-0.032	-	-	0.05
E(D1)	0.87	1.63	0.011	-	-	0.10
E(D2)	-0.28	-0.63	0.020	-	-	-0.04
2.SE	1.29	2.47	0.032	-	-	0.15
Normalized effects						
%E(SDS)	**10.33**	-8.83	-2.38	-	-	**-41.9**
%E(ACN)	**16.98**	**21.08**	**7.79**	-	-	**48.7**
%E(pH)	**18.87**	10.50	1.71	-	-	11.5
%E(T)	-4.83	6.15	1.14	-	-	26.5
%E(V)	**-45.62**	-12.60	-3.04	-	-	14.4
%E(D1)	5.39	9.86	1.05	-	-	26.0
%E(D2)	-1.72	-3.79	1.90	-	-	-10.3
2.%SE	8.00	14.93	3.07	-	-	39.6

Significant effects are bold letter.

Table 4 Results of experimental design II

Exp	Response (Y)			Transformed data		Q*
	t_m (min)	R_s	A_s	t_m*	R_s*	
Response						
1	22.780	20.16	0.967	0.12	0.86	0.61
2	24.420	21.10	1.010	0.01	0.97	0.65
3	11.400	15.02	0.997	0.86	0.25	0.45
4	13.263	15.02	1.087	0.74	0.25	0.41
5	9.310	12.96	1.021	1.00	0.00	0.33
6	24.547	21.33	1.072	0.00	1.00	0.67
7	18.367	17.67	1.050	0.41	0.56	0.51
8	14.643	16.40	1.052	0.65	0.41	0.49
Average Y	17.341	17.46	1.032			0.52
Effects						
E(SDS)	4.80	2.18	0.024	-	-	0.07
E(ACN)	2.76	1.03	-0.040	-	-	0.02
E(pH)	6.89	3.89	-0.041	-	-	0.16
E(T)	-0.96	-0.51	0.008	-	-	-0.02
E(V)	-6.31	-3.34	-0.028	-	-	-0.13
E(D1)	1.09	0.29	0.031	-	-	0.00
E(D2)	-2.87	-1.43	0.006	-	-	-0.05
2.SE	4.34	2.06	0.045	-	-	0.07
Normalized effects						
%E(SDS)	**27.66**	**12.46**	2.33	-	-	13.4
%E(ACN)	15.89	5.90	-3.88	-	-	4.4
%E(pH)	**39.74**	**22.28**	-3.97	-	-	**30.8**
%E(T)	-5.52	-2.92	0.78	-	-	-3.8
%E(V)	**-36.36**	**-19.10**	-2.71	-	-	**-24.8**
%E(D1)	6.27	1.66	3.00	-	-	-0.2
%E(D2)	-16.55	-8.16	0.58	-	-	-9.9
2.%SE	25.03	11.78	4.33	-	-	14.0

Significant effects are bold letter.



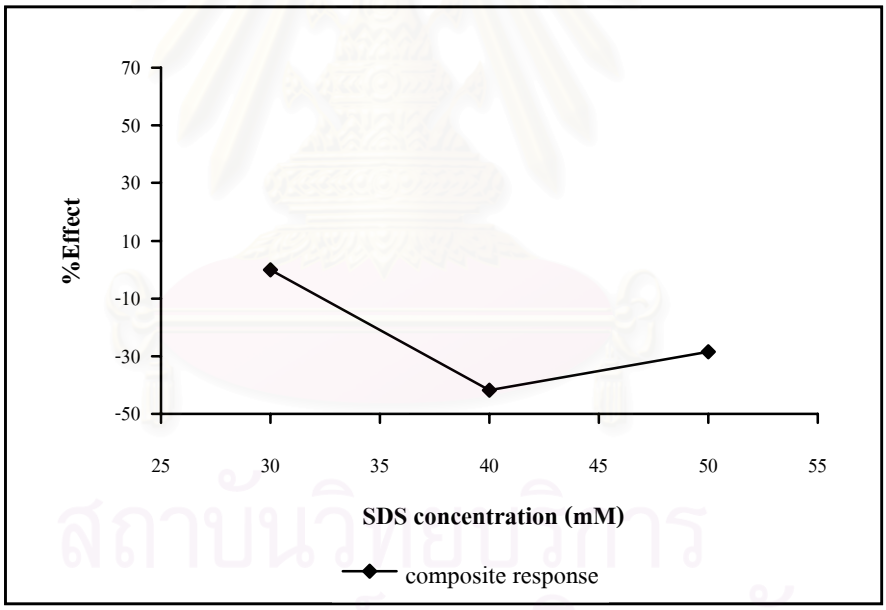


Figure 9 Effect – plots of the concentration of SDS on (a) migration time and resolution and (b) composite response, Q*

1.4.2 Influence of acetonitrile

Addition of acetonitrile to the BGE showed a significant effect on the resolution, migration time and asymmetric factor only in the low concentration range (8 - 10 %v/v). Acetonitrile increases the elution time window by increasing electrophoretic mobility of the micelle. Consequently, the increase in migration time of the micelle and resolution.

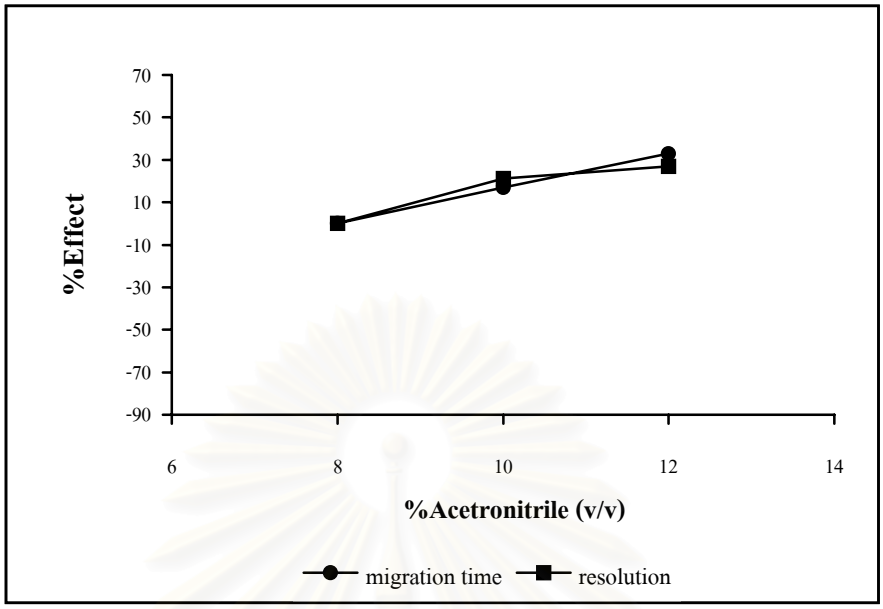
The favorable effects of acetonitrile on both resolution and migration time in the low concentration range (8 - 10 %v/v) were clearly seen in the significant effect on the composite response, Q*, whereas in the high concentration range (10 - 12 %v/v) a similar but not statistically significant effect was observed as shown in Figure 10.

1.4.3 Influence of pH

From the results of Table 3 and 4 and Figure 11, it was obvious that the BGE at higher pH provided a significantly longer migration time. At higher pH, more promethazine existed in uncharged form and so retained longer in the micellar phase.

The favorable effects of the pH on both resolution and migration time at high pH range (10.0 -10.5) were clearly seen in the significant effects on the composite response, Q*.

Variation of the pH of BGE did not significantly affect the asymmetric factor.



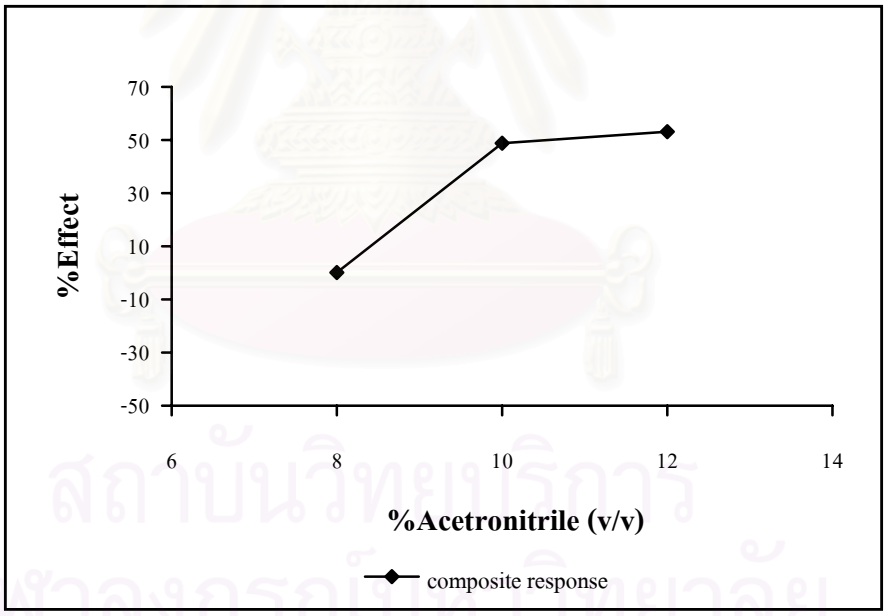
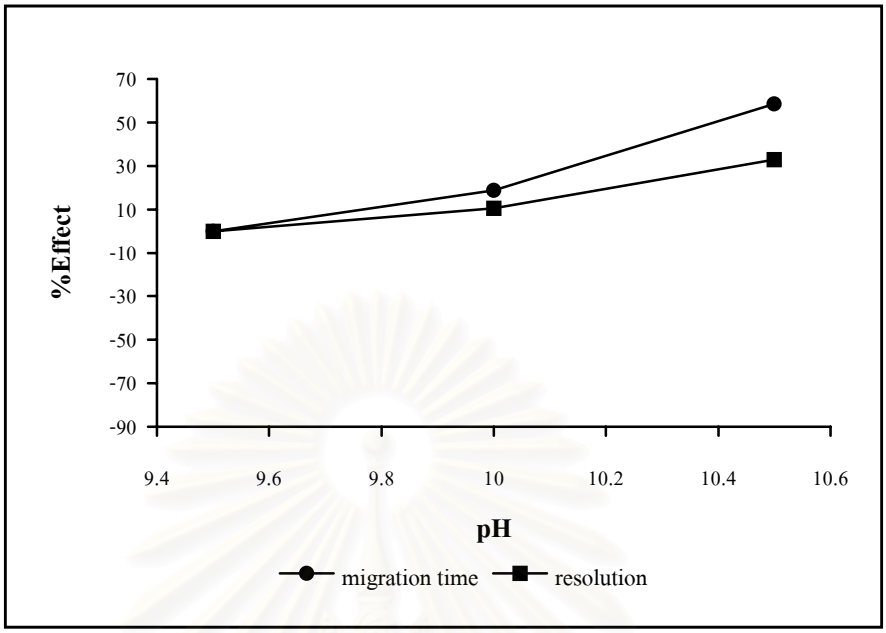


Figure 10 Effect – plots of the concentration of acetonitrile on (a) migration time and resolution and (b) composite response, Q*



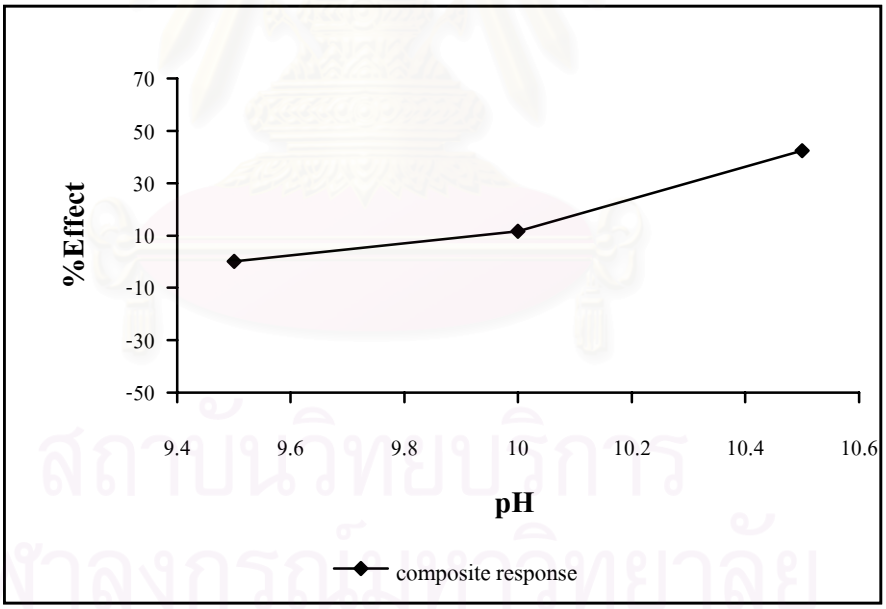


Figure 11 Effect – plots of pH on (a) migration time and resolution and (b) composite response, Q*

1.4.4 Influence of temperature

The temperature of the capillary was the only factor that did not produce any significant effects, as shown in Table 2 and 3 and Figure 12. This might be due to the rapid heat dissipation of the system or low power generation under the experimental conditions. Furthermore, the selected temperature ranges (15 - 20°C) and (20 - 25°C) might be too narrow to cause any effect on the separation.

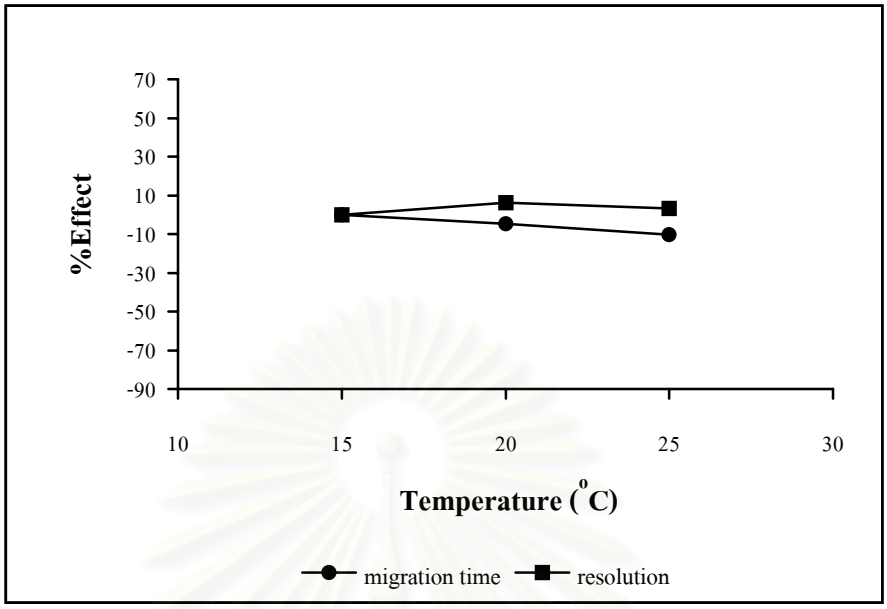
1.4.5 Influence of applied voltage

In the range of 10 - 20 kV, the migration time was reduced at higher voltage values. This observation is obvious since the velocity of the micelles or any charged compound is proportional to the electric field according to equation (10):

$$v = \mu E \qquad \text{.....(10)}$$

where, v is the ion velocity, μ is the ion electrophoretic mobility and E is the applied electric field in volts/cm.

The significantly decreased in resolution was clearly observed in the high range of 15 - 20 kV which might be due to Joule heating. Although, it was shown from Figure 13 that a voltage of 15 kV produced a significantly shorter migration time, the associated small loss in resolution resulted in the lack of significant effects on the composite response, Q* when changing from 10 to 15 kV. However, the effects on both migration time and resolution in the high applied voltage range (15 - 20 kV) was clearly visible in the significant negative effects on the composite response, Q*.



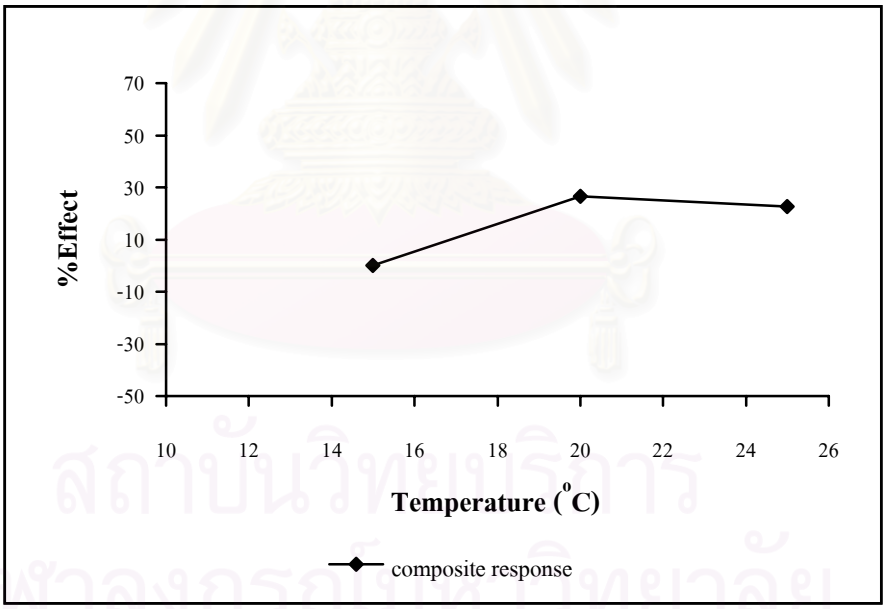


Figure 12 Effect – plots of temperature on (a) migration time and resolution and (b) composite response, Q*

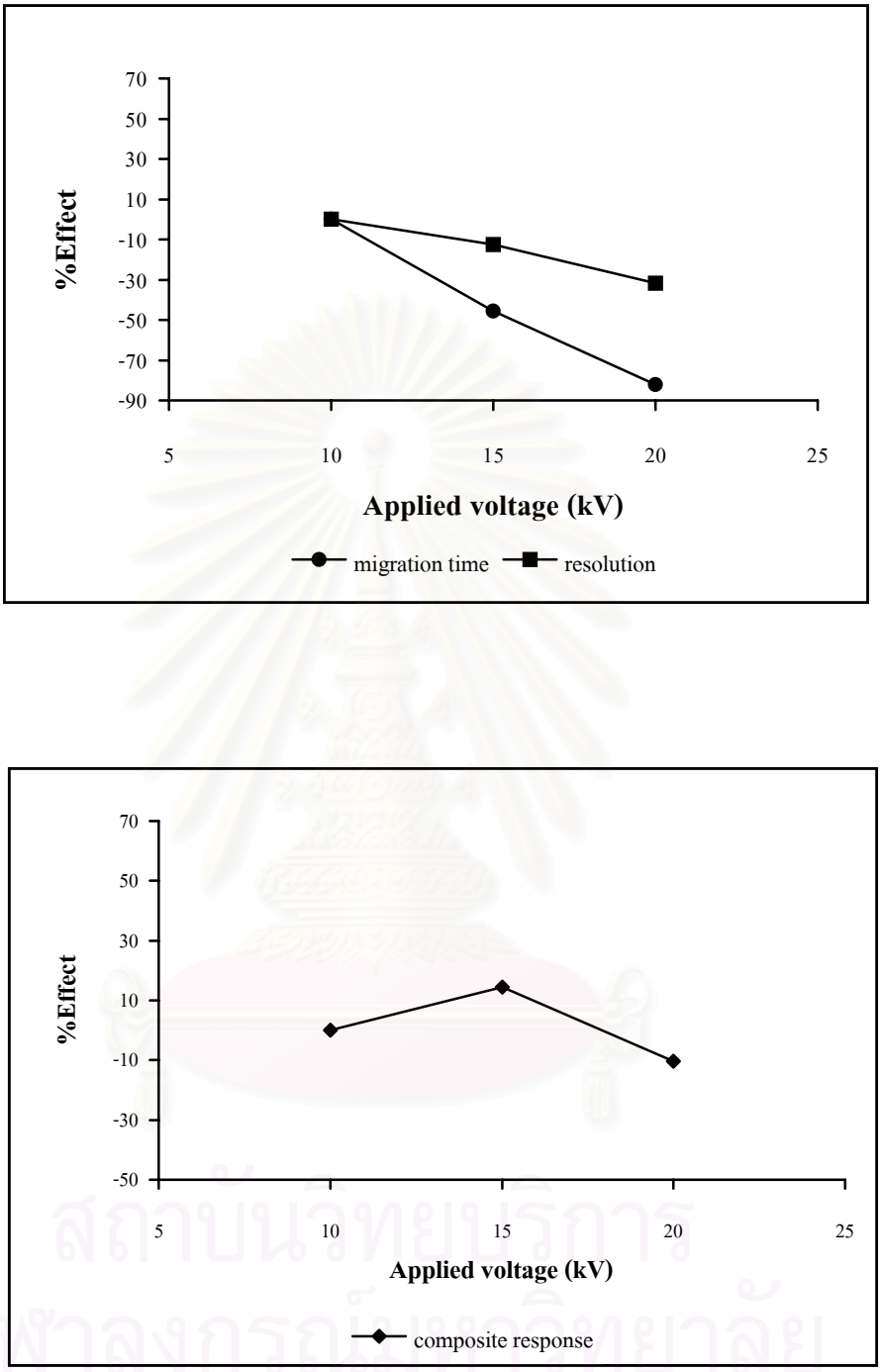


Figure 13 Effect – plots of applied voltage on (a) migration time and resolution and (b) composite response, Q*

From the results of the experimental design, the following conditions for the separation of codeine phosphate and promethazine hydrochloride were selected: high SDS concentration (50 mM) and the nominal acetonitrile concentration (10 %v/v). The capillary temperature variations remained indifferent, therefore, the nominal temperature of 20°C was remained. Although, the effect of the BGE pH of 10.5 on the separation was significant, the nominal pH 10.0 was selected to extend the lifetime of the capillary. The applied voltage was remained at the nominal value of 15 kV.

2 Selection of the internal standard

Six compounds were screened as the internal standard. The results were shown in Table 5 which included the name of compound, the migration time and associated migration time of electroosmotic flow, and the relative migration time. In addition, preservatives (methyl paraben, propylparaben) and antioxidants (ascorbic acid, benzoic acid) commonly used in liquid preparations were also tested for probability of overlapping with analyte peaks. The relative migration time of phenylephrine and guaifenesin were not too small to close to the electroosmotic flow and not too large to overlap with the peak of promethazine, codeine, or other analytes in the cough preparations. The peak of guaifenesin was sharp and symmetry but that of phenyleprine was asymmetry. Therefore, guaifenesin was finally selected as the internal standard.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

The electropherogram of a standard mixture solution of codeine phosphate, promethazine hydrochloride and the internal standard, guaifenesin, was shown in Figure 14.

Table 5 Selection of Internal Standard

Compound	Migration time t_m (min)	Migration time of EOF, t_0 (min)	Relative migration t_m/t_0 (min)
Phenylephrine HCl	3.630	2.590	1.402
Guaifenesin	3.673	2.593	1.417
Phenylpropanolamine HCl	4.583	2.587	1.772
Propylparaben	4.603	2.590	1.777
Methylparaben	4.680	2.590	1.807
Ascorbic acid	4.880	2.610	1.870
Benzoic acid	5.720	2.600	2.200
Codeine phosphate	6.473	2.603	2.487
Chlorpheniramine maleate	12.537	2.593	4.835
Brompheniramine maleate	13.023	2.597	5.015
Diphenhydramine HCl	13.230	2.600	5.088
Promethazine HCl	14.543	2.603	5.587

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

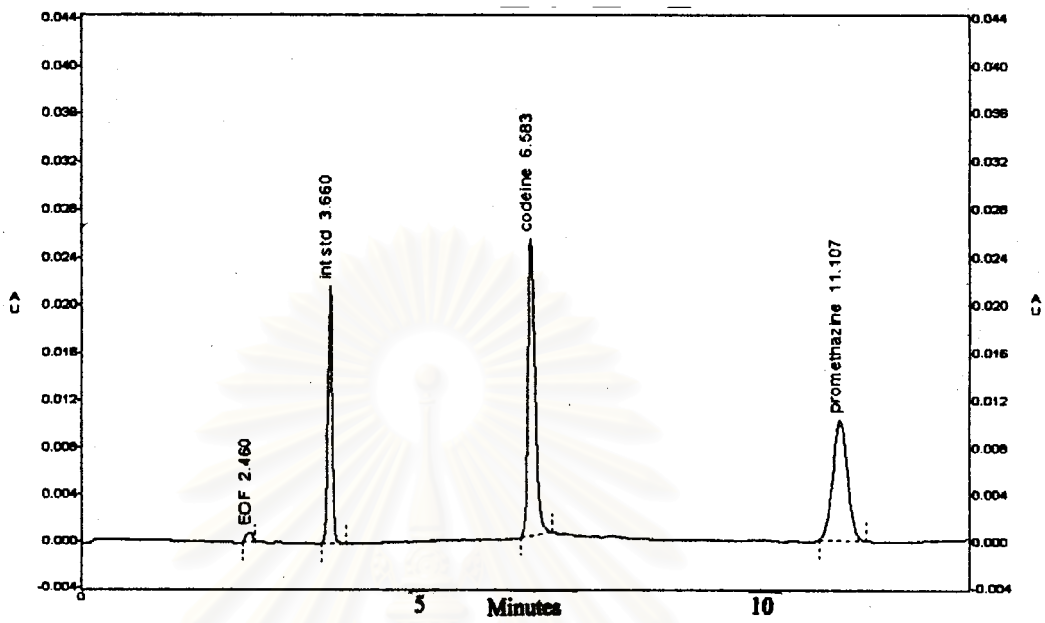


Figure 14 Electropherogram of a standard mixture of codeine, promethazine and the internal standard, guaifenesin.

3. Analytical method validation

3.1 System suitability

From electropherogram of the standard mixture solution in Figure 14, the theoretical plates, N of codeine and promethazine were 15488 and 14084, respectively. Asymmetry factor, A_s, of codeine and promethazine were 1.0. The resolution between peaks of codeine and promethazine was 8.14.

The percentage of relative standard deviation of peak area ratio from six replicate injection of standard mixture solution were 1.21 and 1.61 for codeine and promethazine, respectively.

3.2 Selectivity

The representative electropherograms in Figure 14 - 17 shown the identity of each separated peak and the separation between main peaks, the internal standard and other inactive ingredients. The relative migration time of internal standard, codeine and promethazine were 1.5, 2.7 and 4.5, respectively, with respect to t_0.

The standard solution of promethazine hydrochloride, placing on the table for exposure to light at room temperature for one week, turned from clear colorless solution to pale pink solution. The electropherograms as shown in Figure 18, presented (a) peak of standard mixture solution with internal standard and (b) peak of promethazine stock solution with its photodegradation products that did not interfere peak of internal standard, codeine and promethazine.

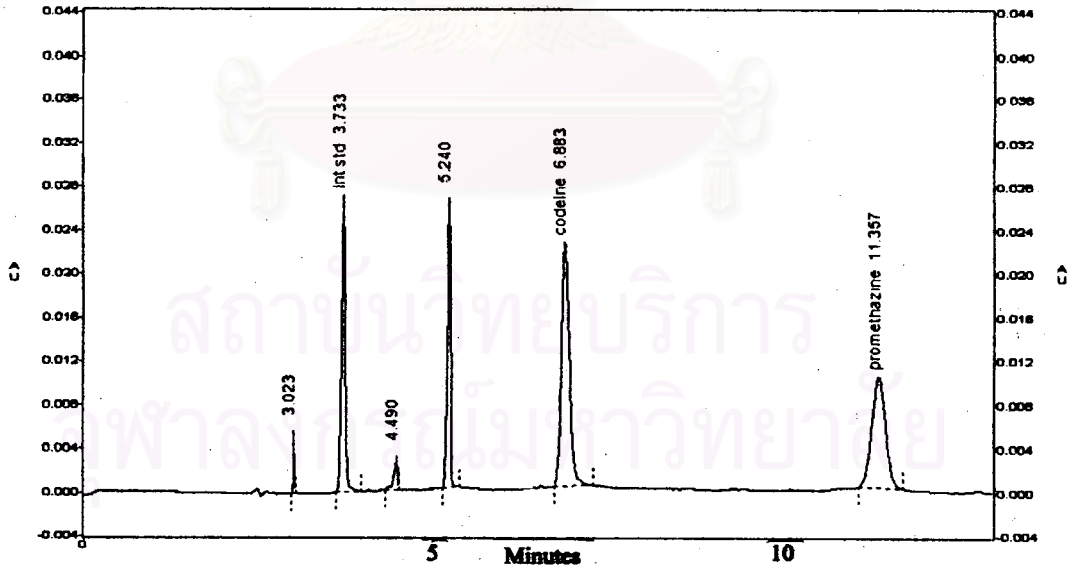


Figure 15 Electropherogram of sample No.1

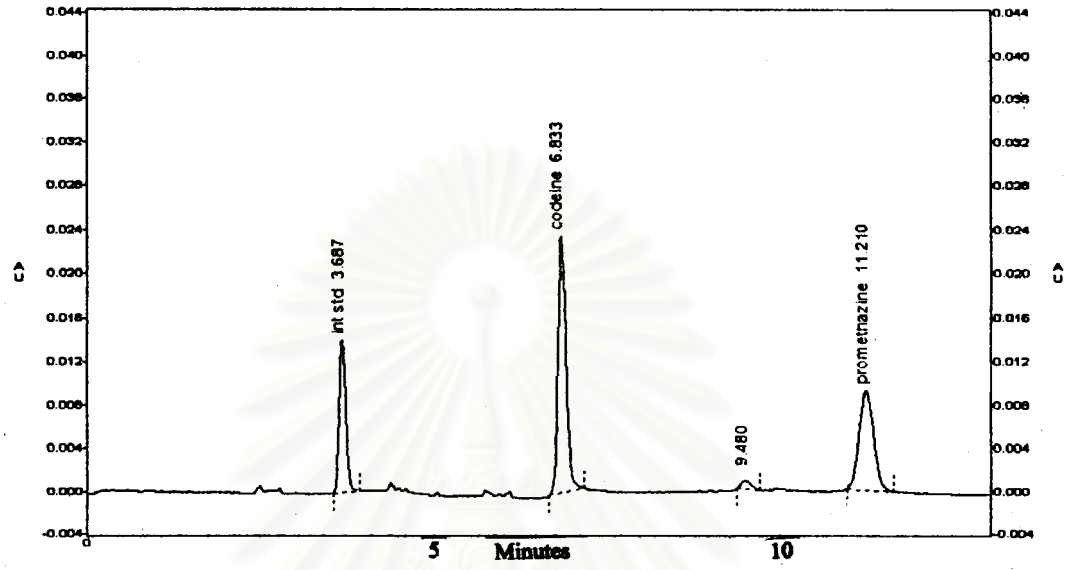


Figure 16 Electropherogram of sample No.2

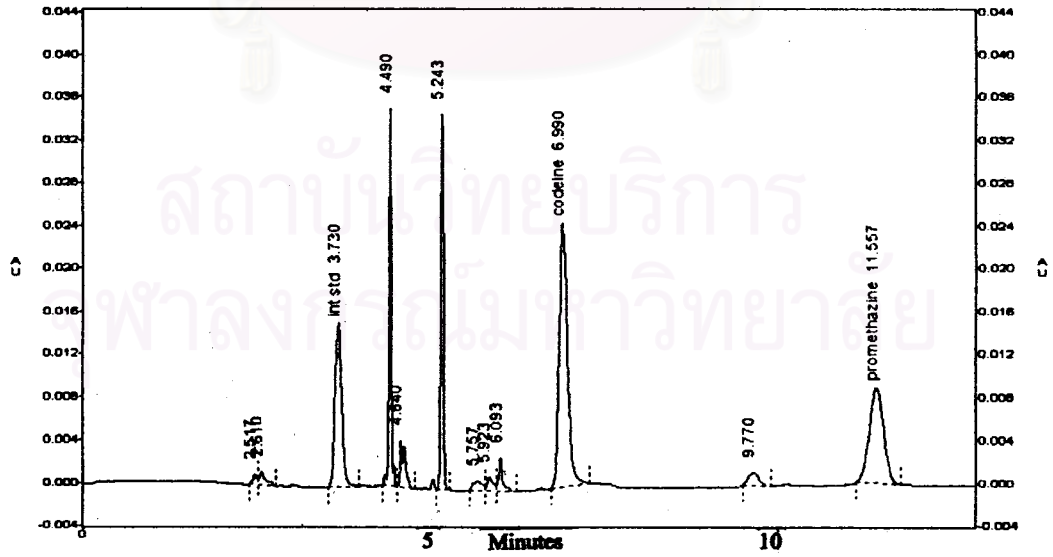


Figure 17 Electropherogram of sample No.3.

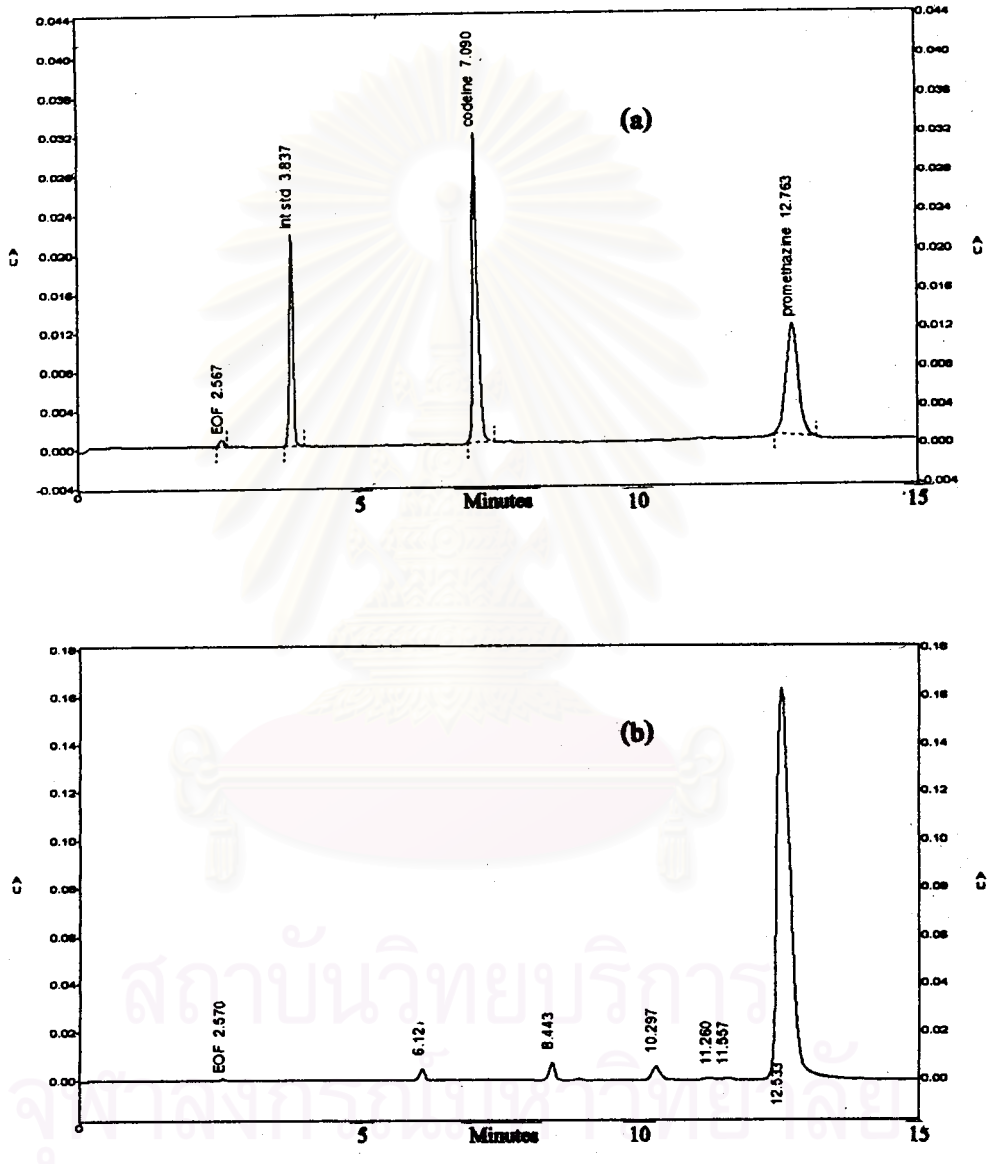


Figure 18 Electropherogram of (a) a standard mixture solution with internal standard and (b) promethazine with its photodegradation products

Electropherograms of each sample that was protected from light and was exposed to light were shown in Figure 19 – 21. Comparing the electropherogram of the sample exposed to light with that of the sample protected from light, peaks of photodegradation compounds of promethazine were clearly seen along with the reduction of peak height of promethazine. Peaks of degradation compounds were separated from codeine, promethazine and the internal standard. Therefore, the developed MEKC method has acceptable selectivity and can be used as stability indicating method.

3.2 Linearity and range

Five standard solutions were diluted to cover 50 - 150% of the expected assay concentration of codeine phosphate and promethazine hydrochloride with 100 μg/ml of the internal standard. Ploted of the ratio of peak area of each standard to that of the internal standard versus the standard concentration. The calibration curve was found to be linear over the concentration range of 45 - 135 μg/ml for codeine phosphate and 18 - 54 μg/ml for promethazine hydrochloride.

Linear regression equations of codeine phosphate:

Peak area ratio = 0.0172(concentration) + 0.0687

and that of promethazine hydrochloride;

Peak area ratio = 0.0564(concentration) – 0.3313

with the correlation coefficients of 0.9994 and 0.9975, for codeine phosphate and promethazine hydrochloride, repectively as shown in Table 6, Figure 22 and 23.

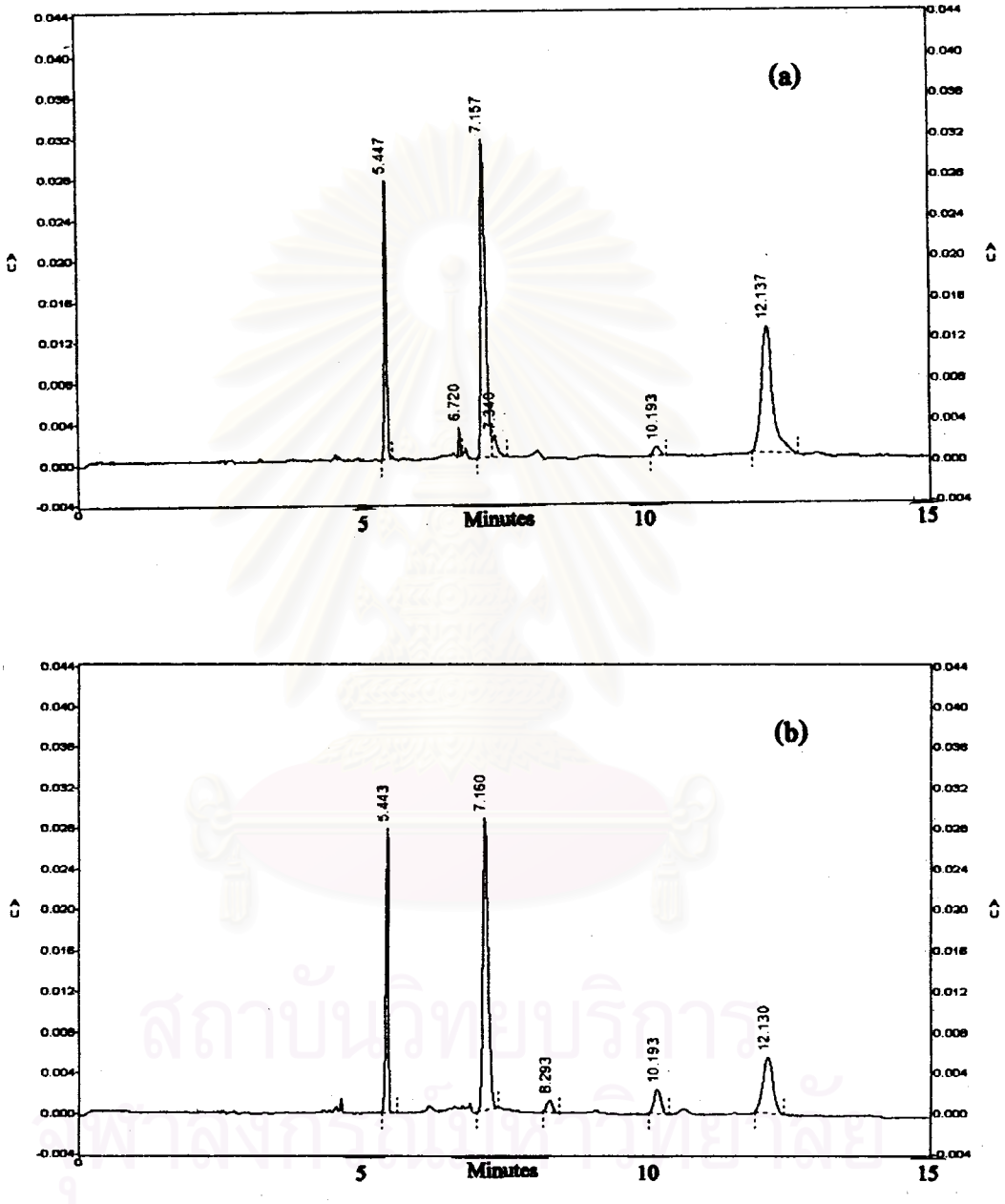


Figure 19 Electropherogram of sample No.1 (a) protected from light and (b) placed to expose the light

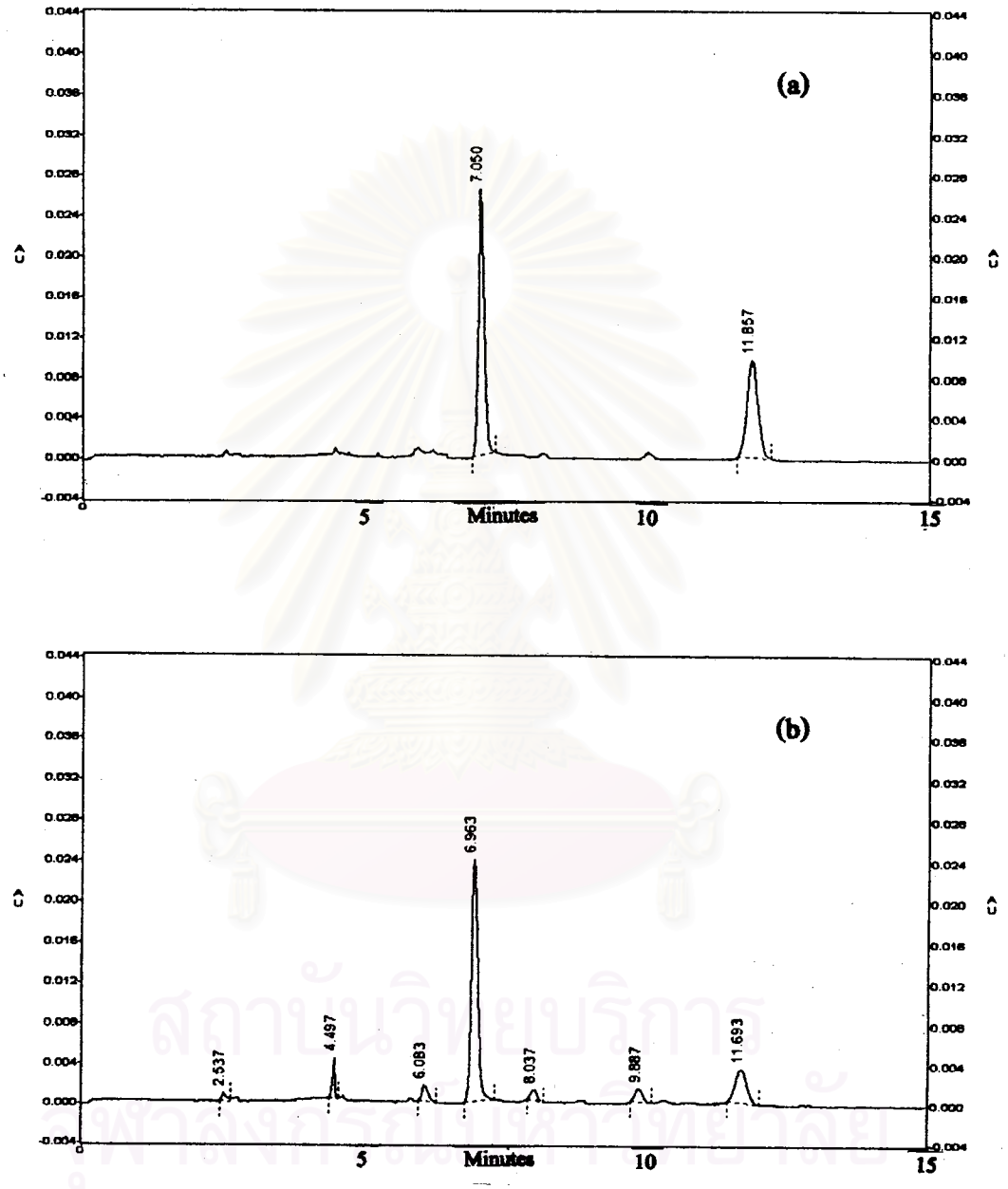


Figure 20 Electropherogram of sample No.2 (a) protected from light and (b) placed to expose the light

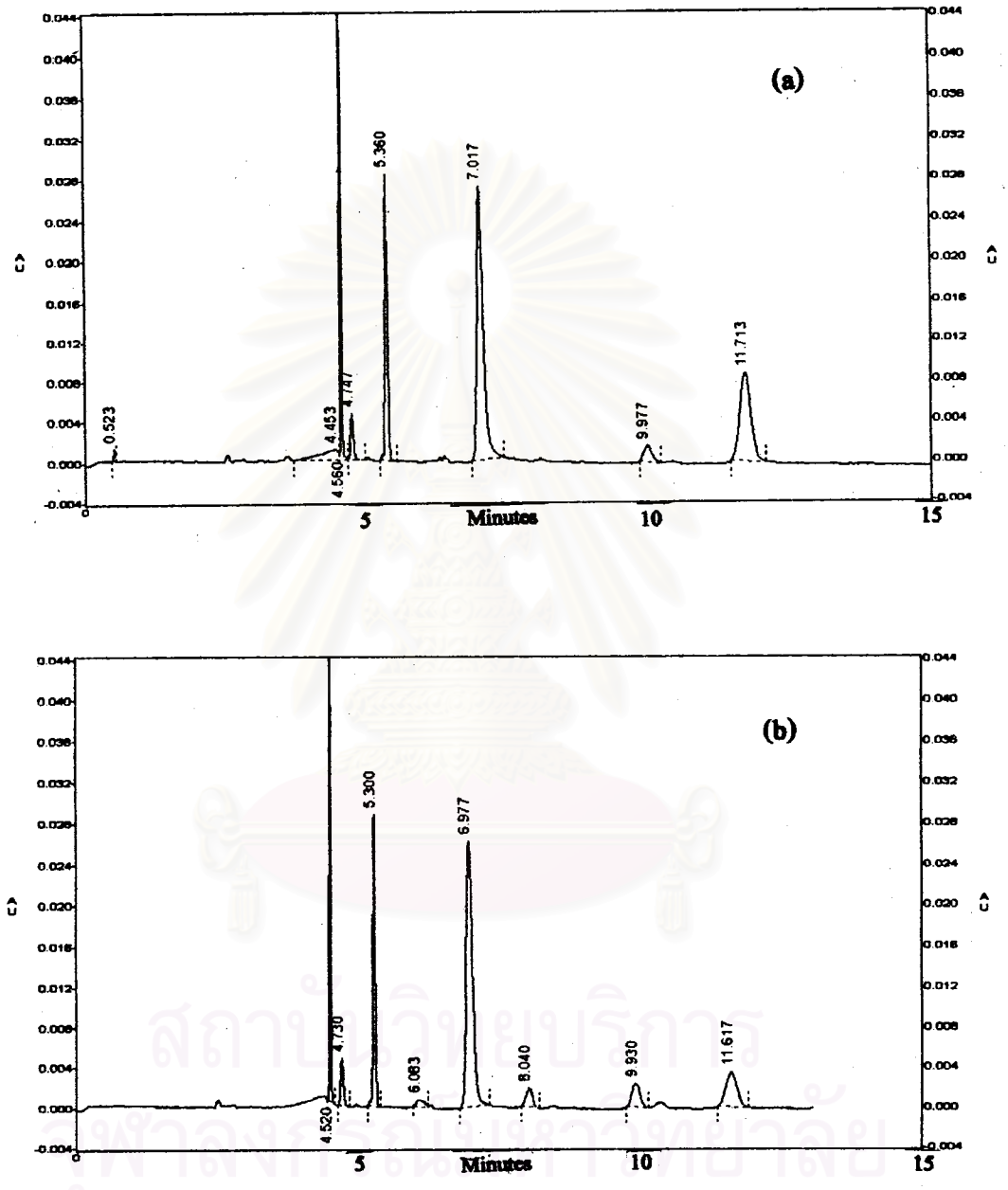


Figure 21 Electropherogram of sample No.3 (a) protected from light and (b) placed to expose the light

The linear relationships confirmed that the test results were directly proportional to the concentration.

Table 6 Relationship between concentration and peak area ratio

Codeine phosphate		Promethazine hydrochloride	
Concentration (μg / ml)	Peak area ratio[a]	Concentration (μg / ml)	Peak area ratio[a]
45.21	0.8266 ± 0.0134	18.70	0.7472 ± 0.0025
67.82	1.2354 ± 0.0007	28.05	1.1746 ± 0.0314
90.43	1.6406 ± 0.0090	37.39	1.8101 ± 0.0126
113.03	2.0291 ± 0.0561	46.74	2.3811 ± 0.1364
135.64	2.3691 ± 0.0260	56.09	2.7813 ± 0.0143
Slope	0.0171	Slope	0.0561
Y – intercept	0.0687	Y – intercept	- 0.331
r	0.999	r	0.997

[a] mean ± standard deviation (n=3)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

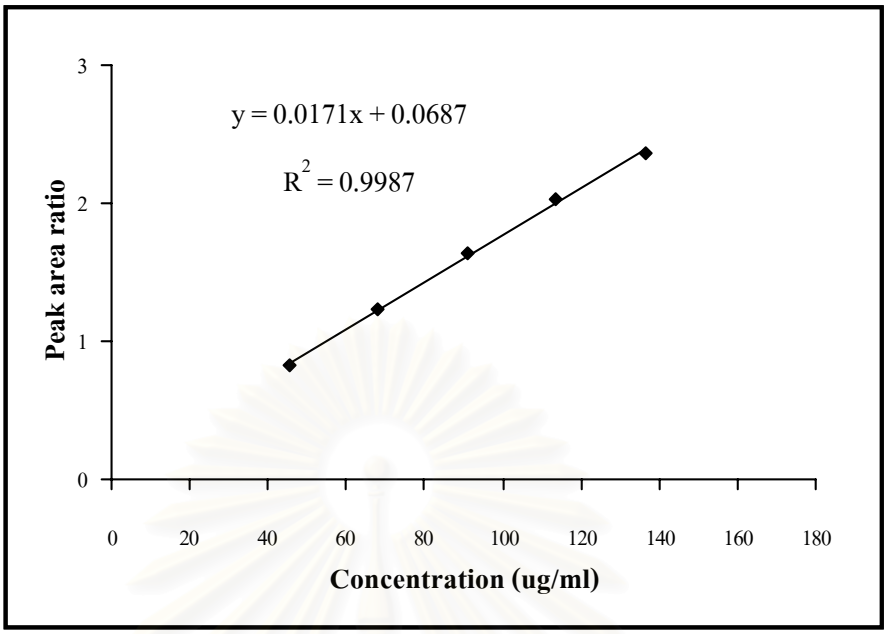


Figure 22 Relationship between concentration and peak area ratio of codeine phosphate

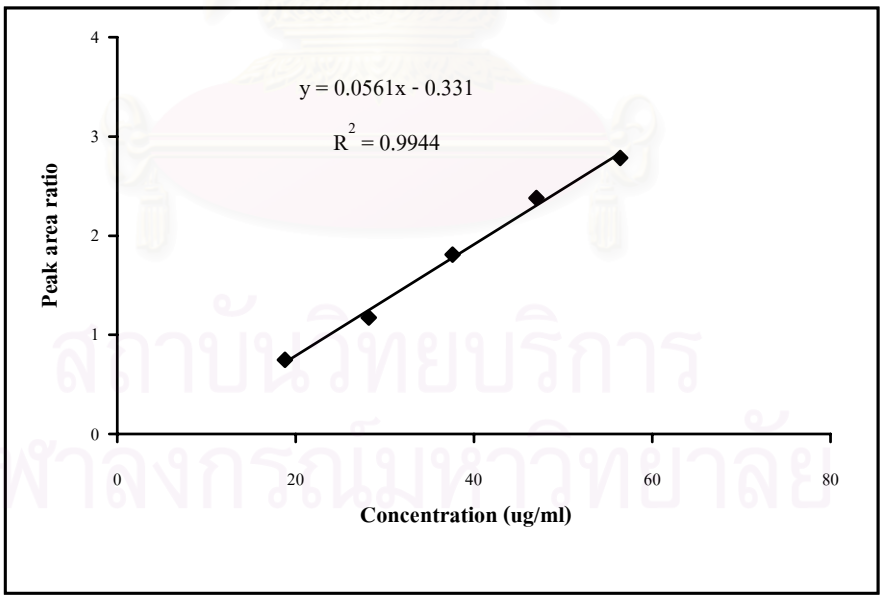


Figure 23 Relationship between concentration and peak area ratio of promethazine hydrochloride

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

3.4 Accuracy

Three different amounts of codeine phosphate and promethazine hydrochloride were added to a sample. The three concentrations covered 80 - 120% interval of the expected assay concentration. The recoveries, as shown in Table 7, ranged from 98.6% to 99.8% for codeine phosphate and from 97.8% to 98.5% for promethazine hydrochloride. The results implied that the method gave sufficient accuracy.

Table 7 Recoveries of standards from sample A, Phensedyl® cough linctus spiked with corresponding standards

Compound	Expected Concentration (%)	Spiked amount (mg)	Amount Found (mg)	Recovery (%)
Codeine phosphate	80	7.39	7.34	99.3
	100	9.24	9.22	99.8
	120	11.08	10.92	98.6
Promethazine HCl	80	3.01	2.94	97.8
	100	3.76	3.70	98.5
	120	4.51	4.43	98.3

3.5 Precision

The results of the intra-day and inter-day precision for the analysis of codeine phosphate and promethazine hydrochloride were presented in Table 8 and 9, respectively.

For the intra-day precision, six replicate analyses of Phensedyl® cough linctus were performed with the relative standard deviation (n = 6) of 1.14 and 1.12 % for codeine phosphate and promethazine hydrochloride, respectively.

As shown in Table 9, The inter-day precision was determined by analyzing six replicates of Phensedyl® cough linctus on three non-consecutive days. The relative standard deviation (n = 3) were 1.45 and 2.16 % for codeine phosphate and promethazine hydrochloride, respectively.

The precision of the proposed method was acceptable.

Table 8 Intra – day precision data of codeine phosphate and promethazine hydrochloride

Analysis No.	Percent Labeled Amount	
	Codeine Phosphate	Promethazine HCl
1	100.6	99.5
2	101.3	101.1
3	99.8	99.7
4	102.6	98.9
5	102.8	100.7
6	101.7	98.1
Mean	101.5	99.7
SD	1.16	1.11
%RSD	1.14	1.12

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 9 Inter – day precision of 3 non – consecutive days

Day	Percent Labeled Amount	
	Codeine Phosphate	Promethazine HCl
1	101.5 ± 1.16	99.7 ± 1.11
2	99.7 ± 1.75	101.8 ± 1.45
3	102.6 ± 1.41	97.5 ± 2.01
Mean	101.5	99.7
SD	1.46	2.15
%RSD	1.45	2.16

4 Quantitative analysis of cough solutions by MEKC method

With the optimum MEKC condition, three commercial brands of codeine phosphate and promethazine hydrochloride in cough solutions were analysed in duplicate with duplicate injections. The electropherograms were shown in Figure 15 – 17.

The results of analysis of three commercial brands were shown in Table 10. The mean contents of codeine phosphate were 102.7, 98.8 and 106.8 % labeled amount.

The mean contents of promethazine hydrochloride were 99.2, 92.1 and 100.2 % labeled amount.

The manufacturer's specification limits of codeine phosphate and promethazine hydrochloride were 90.0 - 110.0 % labeled amount. The mean contents

of both codeine phosphate and promethazine hydrochloride from 3 commercial samples were conformed to the specifications.

Table 10 Percent labeled amounts of 3 commercial products according to the MEKC method.

Sample No.	% labeled amount[a]	
	Codeine phosphate	Promethazine HCl
1	102.7 ± 1.13	99.2 ± 1.48
2	98.8 ± 1.27	92.1 ± 1.13
3	106.8 ± 1.34	100.2± 0.42

[a]mean ± SD of duplicate analyses

5 Quantitative analysis of cough solutions by HPLC method

With the same lot number as those that were analysed by MEKC method, the three commercial brands of codeine phosphate and promethazine hydrochloride in cough solutions were also analysed by HPLC and the results were shown in Figure 24 – 27 and Table 11. The retention times of codeine, promethazine and the internal standard, diphenhydramine, were 4.367, 15.858 and 6.225 min, respectively. The mean content of codeine phosphate were 100.3, 97.6 and 101.7 % labeled amount. The mean content of promethazine hydrochloride were 97.6, 93.1 and 99.5 % labeled amount.

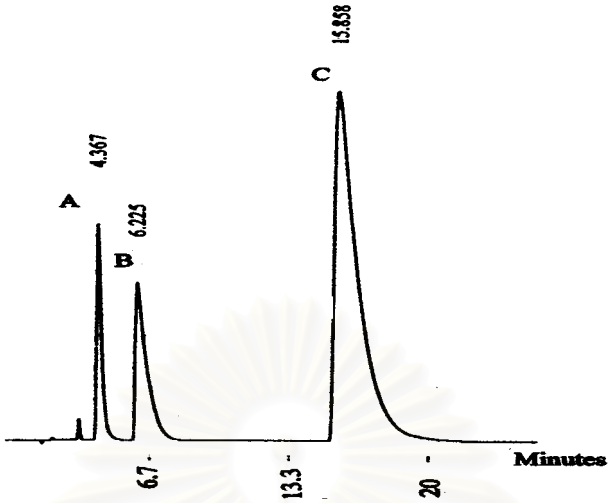


Figure 24 Chromatogram of a standard mixture with internal standard, diphenhydramine hydrochloride: A = codeine phosphate, B = internal standard and C = promethazine hydrochloride

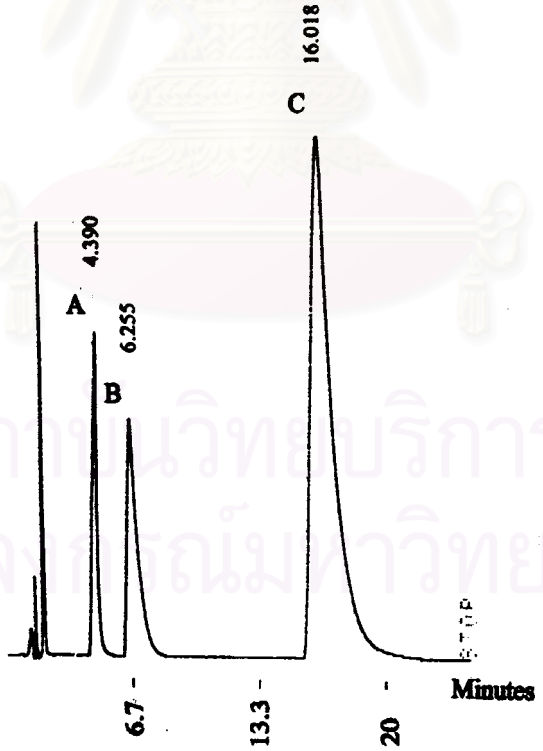


Figure 25 Chromatogram of sample No. 1

A, B, and C are the same as listed in Figure 24

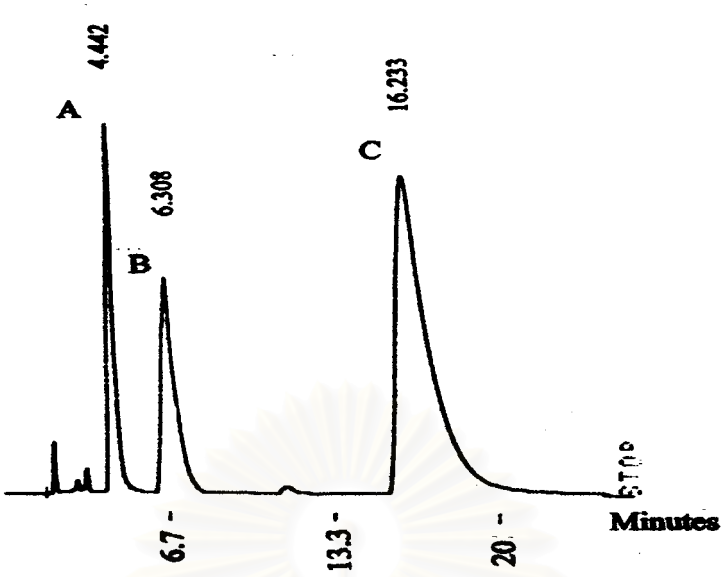


Figure 26 Chromatogram of sample No. 2

A, B, and C are the same as listed in Figure 24

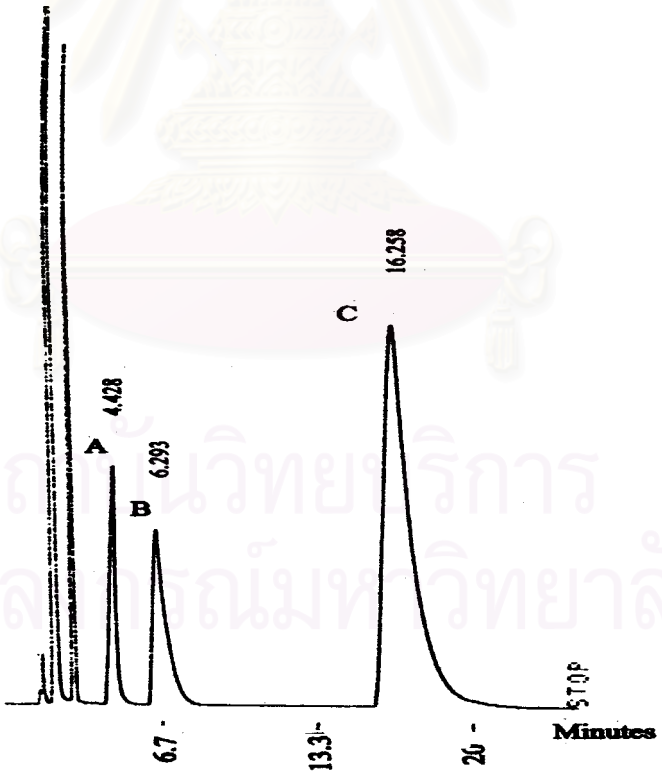


Figure 27 Chromatogram of sample No. 3

A, B, and C are the same as listed in Figure 24

Table 11 Percent labeled amounts of 3 commercial products according to the HPLC method.

Sample No.	% labeled amount[a]	
	Codeine phosphate	Promethazine HCl
1	100.3 ± 0.99	97.6 ± 1.70
2	97.6 ± 1.13	93.1 ± 1.84
3	101.7 ± 1.41	99.5 ± 1.56

[a]mean ± SD of duplicate analyses

6 Comparison of quantitative analysis of cough solutions by MEKC and HPLC method

For comparison purpose, MEKC method was performed in parallel with HPLC method on the same sample. The results obtained indicated good correlation between MEKC and HPLC method. There was no significant differences in the contents of codeine phosphate and promethazine hydrochloride in cough solutions analysing by the developed MEKC and HPLC methods, at 95% confidence level using the statistical t – test, as shown in Table 12.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 12 Statistical test of MEKC and HPLC assay

(t-test at 95% confidence)

Sample No.	Calculated t-value	
	Codeine phosphate	Promethazine HCl
1	2.2592	1.0039
2	0.9983	-0.6549
3	2.6219	0.6128

t-value (critical) = 4.303

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

CONCLUSIONS

In this study, MEKC method was developed for the analysis of codeine phosphate and promethazine hydrochloride in cough solutions. A good separation between codeine phosphate, promethazine hydrochloride and its photodegradation products, inactive ingredients and internal standard was obtained with very simple sample preparation.

An uncoated fused-silica capillary was selected because of its simplicity in preparing and conditioned prior to analysis. It was transparent to ultraviolet and visible light, so the capillary itself can be used as the detector cell when a UV-detector was used. The cell window can be made by simply burning or scraping of a small section of the polyimide outer coating. In order to control Joule heating, a capillary temperature was maintained at 20 ^{0}C using a thermostating liquid. The optimum BGE system consisted of 50 mM SDS, 25 mM borate buffer pH 10.0 and 10% acetonitrile. Because of the narrow pathlength of a fused-silica capillary, the wavelength of the ultraviolet detection was set at 214 nm with high absorptivity of codeine and promethazine. The voltage was applied at 15 kV, and the generated current was found to be about 75 μA.

The assay content from the developed MEKC method was not significantly different from that obtained from the HPLC method at 95% confidence level, but lower relative standard deviation was observed for the MEKC method.

The developed method had good precision and accuracy, the relationship between the standard concentration and peak area ratio of the peak area of the standard to that of the internal standard was found to be linear in the range of 45 - 135 μg/ml for codeine phosphate and 18 - 54 μg/ml for promethazine hydrochloride.

Micellar electrokinetic chromatographic method is precise, accurate and required short analysis time. It uses small amount of sample and organic solvent, resulting in the low cost of analysis and environmental friendly. Therefore, the MEKC method may be used as an alternative for the quantitative analysis of codeine phosphate and promethazine hydrochloride in cough solutions.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

REFERENCES

Ackermans, M.T., Everaerts, F.M. and Beckers, J.L. 1992. Determination of aminoglycoside antibiotics in pharmaceuticals by capillary zone electrophoresis with indirect UV detection coupled with micellar electrokinetic chromatography. J. Chromatogr. 606: 229-235.

Baker, D.R. 1995. Capillary electrophoresis. Canada: John Wiley & Sons.

Balchunas, A.T. and Sepaniak, M.J. 1988. Gradient elution of micellar electrokinetic capillary chromatography. Anal. Chem. 60: 617-621.

British Pharmacopoeia. 1999. London: The United Kingdom for Her Majesty's stationery Office.

Davidson, A. G. 1976. The determination of phenothiazine drugs in pharmaceutical preparation by a difference spectrophotometric method. J. Pharm. Pharmaco. 28: 795-800.

Delgado, J.N. and Remers, W.A. 1998. Textbook of organic medicinal and pharmaceutical chemistry. 10nd ed. New York: Lippincott-Raven.

Fagliaro, F., Smith, F.P., Turrina, S., Equisetto, V. and Marigo, M. 1996. Complementary use of capillary zone electrophoresis of results in forensic drug analysis. J.Chromatogr A. 735: 227-235.

Galente, R.N., Visalli, A.J., and Patel, D.M. 1979. Solid state acetylation of codeine phosphate by aspirin. J. Pharm. Sci. 68: 1494-1498.

Gausepohl, C. and Blaschke, G. 1998. Stereoselective determination of clenbuterol in human urine by capillary electrophoresis. J. Chromatogr B. 713: 443-446.

Gupta, V.D. 1980. Simultaneous quantitation of acetaminophen, aspirin, caffeine, codeine phosphate, phenacetin and salicylamide by high performance liquid chromatography. J. Pharm. Sci. 69: 110-112.

Halstead, G.W. 1982. Determination of amine ingredients in cough-cold liquids by reversed-phase ion-pair high performance liquid chromatography. J. Pharm.Sci. 71: 1108-1112.

International Conference on Harmonisation of Technical requirements of pharmaceuticals for human use at Step 4. 1996. Validation of analytical procedures: Methodology.

Korman, M., Vindevogel, J. and Sandra, P. 1993. Seperation of codeine and its by products by capillary zone electrophoresis as a quality control tool in pharmaceutical industry. J. Chromatogr A. 645: 366-370.

Krogh, M., Brekke, S., T∅nnesen, F. and Rasmussen, K.E. 1994. Analysis of drug seizures of heroin and amphetamine by capillary electrophoresis. J. Chromatogr A. 674: 235-240.

Lurie, I.S. 1998. Capillary electrophoresis of illicit drug seizures. Forensic Sci. Int. 92: 125-136.

Massart, D.L. et al. 1997. Handbook of Chemometrics and Qualimetrics: Part A . Amsterdam: Elsevier.

Matsui, F. and French, W.N. 1971. Analysis of binary mixtures of pharmaceutical amines by the acid dye technique. J. Pharm. Sci. 60: 287-290.

Mathew et al. 1972. Rapid method for the quantitative measurement of numerous pharmaceutically important amines. J. AOAC. 55: 789-793.

Mathew, M.; Gupta, V.D. and Bethea, C. 1994. Quanttation of promethazine hydrochloride in pharmaceutical dosage forms using high performance liquid chromatography. Drug Dev. Ind. Pharm. 20: 1693-1698.

McLaughlin, G.M. et al. Pharmaceutical drug separation by HPCE: Practical guidlides. J. Liq. Chromatogr. 15: 961-1021.

Miyashita, Y.; Terabe, S. and Nishi, H. 1990. Separation of some antibiotics and corticosteroids by micellar electrokinetic capillary chromatography using P/ACE System 2000, Beckman Instruments, Appl. Brief DS-766.

Muijselaar, P.G.H.M., Claessens, H.A. and Cramers, C.A. 1996. Determination of structurally related phenothiazines by capillary zone electrophoresis and micellar electrokinetic chromatography. J.Chromatogr A. 735: 395-402.

Muhadi, F.J. and Hassan, M.M.A. 1981. Codein phosphate in K. Florey (ed.). Analytical profile of drug substances. Vol.10. New York Academic Press.

Ng, C.L., Lee, H.K. and Li, S.F.Y. 1992. Systemic optimization of capillary electrophoretic separation of sulfonamides. J. Chromatogr. 598: 133-138.

Nishi, H., Fukuyama, T., Matsuo, M. and Terabe, S. 1990. Separation and determination of the ingredients of a cold medicine by micellar electrokineticchromatography with bile salts. J. Chromatogr. 498: 313-323.

Nishi, H. and Matsuo, M. 1991. Separation of corticosteroids and aromatic hydrocarbons by cyclodextrin-modified micelllar electrokinetic chromatography. J. Liq. Chromatogr. 14: 973-986.

Nishi, H. and Terabe, S. 1990. Application of micellar electrokinetic chromatography to pharmaceutical analysis. Electrophoresis. 69: 1-670.

Ong, C.P., Ng, C.L., Lee, H.K. and Li, S.F.Y. 1991. Determination of antihistamines in phamaceuticals by capillary electrophoresis. J. Chromatogr. 588: 335-339.

Persson-Stubberud, K. and Astrom, O. 1998. Seperation of ibuprofen, codeine phosphate, their degradation products and impurities by capillary electrophoresis: I Method development and optimization with fractional factorial design. J. Chromatogr A. 798: 307-314.

Persson-Stubberud, K. and Astrom, O. 1998. Seperation of ibuprofen, codeine phosphate, their degradation products and impurities by capillary electrophoresis: II Validation. J. Chromatogr A. 826: 95-102.

Pound, N.J. and Sears, R.W. 1973. Analysis of promethazine hydrochloride formulation by high speed liquid chromatography. Canadian J. Pharm. Sci. 8: 84-88.

Shearer, C.M. and Miller, S.M. 1976. Promethazine hydrochloride in K. Florey (ed.). Analytical profile of drug substances. vol. 5. New York: New York Academic Press.

Sisco, W.R., Rittenhouse, C.T. and Everhart, L.A. 1985. Simultaneous high performance liquid chromatographic stability-indicating assay of acetaminophen and codeine phosphate in tablets and capsules. J. Chromatogr. 348: 253-263.

Sisco, W.R., Rittenhouse, C.T., Everhart, L.A. and Mclaughlin, A.M. 1986. Simultaneous high performance liquid chromatographic stability-indicating assay of acetaminophen, codeine phosphate and sodium benzoate in elixer. J. Chromatogr. 354: 335-356.

Sooksri Ungboriboonsri. 1992. The development of solid phase extraction and HPLC method for the simultaneous analysis of basic drugs in syryp formulation. Master's Thesis, Department of Pharmaceutical Chemistry, Graduate School, Chulalongkorn University.

Sperling, A.R. 1967. Analysis of promethazine hydrochloride in syrups. J. Pharm. Sci. 56: 98-100.

Stalcup, A.M. and Gahm, K.H. 1996. Application of sulfated cyclodextrins to chiral separations by capillary zone electrophoresis. Anal. Chem. 68: 1360-1368.

Stavchansky, S., Wallace, J.E. and Wu, P. 1983. Thermal and photoretic degradation studies of promethazine hydrochloride: A stability indicating. J. Pharm. Sci. 72: 546-548.

Stevens, M.R. 1975. GLC analysis of caffeine and codein phosphate in pharmaceutical preparations. J. Pharm. Sci. 64: 1686-1687.

Swartz, M.E. 1991. Method development and selectivity control for small molecule pharmaceutical separation by capillary electrophoresis. J. Liq. Chromatogr. 14: 923-938.

Terabe, S., Ishihama, Y., Nishi, H., Fukuyama, F. and Otsuka, K. 1991. Effect of urea addition in micellar electrokinetic chromatography. J. Chromatogr. 545: 359-368.

Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando, T. 1984. Electrokinetic separations with micellar solution and open tubular capillary. Anal. Chem. 56 : 111-113.

The United States Pharmacopiea 24 and The National Formulary 19. 2000 Philadephia: Mack.

Underburg, W.J.M. 1978. Oxidative degradation of pharmaceutically important phenothiazine. J. Pharm. Sci. 67: 1128-1138.

Vela, J., Yanes, E.G. and Stalcup, A.M. 2001. Quantitative determination of clenbuterol, salbutamol and tulobuterol enantiomers by capillary electrophoresis. Fresenius J. Anal. Chem. 369: 212-219.

Vindevogal, J. and Sandra, P. 1991. Resolution optimization in micellar electrokinetic chromatography: use of Plackett-Burman statistical design for the analysis of testosterone esters. Anal. Chem. 63: 1530-1536.

Wallace, J.E., Shinuk, E.L.Jr., Stavchansky, S. and Harris, S.C. 1981. Determination of promethazine and phenothiazine compounds by liquid chromatography with electrochemical detection. Anal. Chem. 53: 960-960.

Weinberger, R. and Lurie, I.S. 1991. Micellar electrokinetic capillary chromotography of illicit drug substances. Anal. Chem. 63: 823-827.

Yang, S.L.; Wilken, L.O. and Clark, C.R. 1985. A high performance liquid chromatographic method for simulataneous assay of aspirin, caffeine, dihydrocodeine bitartrate and promethazine hydrochloride in capsule formulation. Drug Dev. Ind. Pharm. 11: 799-814.

VITAE

Miss Promporn Jamnongtanachot was born on the 17th April, 1973 in Bangkok. She graduated with a B.Sc. in Pharmacy from the faculty of Pharmaceutical Sciences, Prince of Songkla University in 1996. From 1996 to 1998. She was employed by Drug Analysis Division, Department of Medical Sciences, Ministry of Public Health.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย