# การหาปริมาณของสเตอริโอไอโซเมอร์ของทรามาดอลในรูปแบบยาเตรียม ด้วยแคปพิลลารีโซนอิเลคโทรโฟรีซิส

นางสาว ศศิดา สุขสว่าง

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จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

# DETERMINATION OF STEREOISOMERS OF TRAMADOL IN PHARMACEUTICAL DOSAGE FORMS BY CAPILLARY ZONE ELECTROPHORESIS

Miss Sasida Suksawang

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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การแยกอิแนนทิโอเมอร์ของซีส-และทรานส์-ทรามาดอลด้วยแคปพิลลารีโซนอิเลคโทรโฟรีซีส ได้จากการศึกษาโดยใช้ไซโคลเด็กซ์ทรินที่เป็นกลาง (เบต้า-ไซโคลเด็กซ์ทรินและไฮดรอกซีโพลพิล-เบต้า-ไซโคลเด็กซ์ทริน)และชนิดประจุลบ (คาร์บอกซีเมทิล-เบต้า-ไซโคลเด็กซ์ทริน) เป็นสารแยกไครัล การแยกสารเหล่านี้จนถึงเส้นฐานสามารถทำได้โดยใช้แคปพิลลารีชนิดฟิวส์ซิลิกา (50 เซนติเมตร x เส้นผ่าศูนย์กลางภายในขนาด 50 ไมโครเมตร) ฟอสเฟตบัฟเฟอร์ความเข้มข้น 65 มิลลิโมลาร์ พีเอช 2.5 ซึ่งมีคาร์บอกซีเมทิล-เบต้า-ไซโคลเด็กซ์ทริน 8.5 มิลลิโมลาร์ผสมอยู่ ศักย์ไฟฟ้าที่ให้ขนาด 18 กิโลโวลต์และอุณหภูมิของแคปพิลลารีเป็น 30 องศาเซลเซียส

การตรวจสอบความถูกต้องของสภาวะการทดลองที่เหมาะสมที่สุด ได้จากการศึกษาโดยใช้ตัวอย่างยาทรามาดอลที่เป็นยาฉีดและยาแคปซูลที่มีวางจำหน่าย โดยประเมินจากหัวข้อความจำเพาะเจาะจง ภาวะเชิงเส้นและพิสัย ความแม่นยำ ความถูกต้อง ลิมิตการตรวจวัด ลิมิตการวิเคราะห์เชิงปริมาณ ความคงตัวของสารละลายและความเหมาะสมของระบบ พบว่า ภาวะเชิงเส้นสำหรับซีส-และทรานส์-ทรามาดอลมีค่า 70-130 และ 2.8-7.5 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ความแม่นยำของการวิเคราะห์ภายในวันเดียวและการวิเคราะห์ต่างวันของแต่ละอิแนนทิโอเมอร์ที่คำนวณได้จากค่าสัมพัทธ์การเบี่ยงเบนมาตรฐาน มีค่าน้อยกว่า 1.5% ลิมิตการตรวจวัดและลิมิตการวิเคราะห์เชิงปริมาณสำหรับทรานส์-ทรามาดอล มีค่าเป็น 0.48 และ 1.46 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ วิธีวิเคราะห์ที่นำเสนอนี้สามารถนำไปวิเคราะห์ปริมาณซีส-และทรานส์-ทรามาดอลในตำรับยาฉีดและยาแคปซูลได้โดยตรง ไม่ต้องผ่านขั้นตอนการทำความสะอาดตัวอย่าง

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| ภาควิชา เภสัชเคมี | ลายมือชื่อนิสิต ..... |
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The separation of the enantiomers of cis-and trans-tramadol was investigated by capillary zone electrophoresis employing neutral (β-cyclodextrin and hydroxypropyl-β-cyclodextrin) and negatively charged (carboxymethyl-β-cyclodextrin) cyclodextrins as chiral selectors. The baseline separation of these analytes was achieved with 8.5 mM carboxymethyl-β-cyclodextrin in 65 mM phosphate buffer at pH 2.5, using a fused silica capillary (50 cm x 50 μm, i.d.), applied voltage of 18 kV and capillary temperature of 30°C.

Validation of the optimum condition was performed on commercially available tramadol injection and capsule formulations. Validation of the method was assessed from the selectivity, linearity and range, precision, accuracy, limit of detection and quantification, stability of solution and system suitability. The linear ranges of 70-130 and 2.8-7.5 μg/ml, respectively, were found for cis-and trans-tramadol. The intra-day and inter-day precision of each enantiomer calculated from the relative standard deviation was less than 1.5%. The limit of detection and limit of quantification of trans-tramadol were 0.48 and 1.46 μg/ml, respectively. The proposed method was successfully applied to the direct determination of cis- and trans-tramadol in injection and capsule formulations without any sample clean-up procedures.

สถาบันวิทยบริการ
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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| $^{O}C$ | = | degree Celsius |
| σ | = | standard deviation of y-intercept |
| μg/ml | = | microgram per millilitre |
| μl | = | microlitre |
| μm | = | micrometre |
| β-CD | = | β-cyclodextrin |
| BGE | = | background electrolyte |
| CDs | = | cyclodextrins |
| CE | = | capillary electrophoresis |
| CGE | = | capillary gel electrophoresis |
| CIEF | = | capillary isoelectric focusing |
| CITF | = | capillary isotachophoresis |
| cm | = | centimetre |
| CM-β-CD | = | carboxymethyl-β-cyclodextrin |
| CZE | = | capillary zone electrophoresis |
| EOF | = | electroosmotic flow |
| g | = | gram |
| GC | = | gas chromatography |
| HP-β-CD | = | hydroxypropyl-β-cyclodextrin |
| HPLC | = | high-performance liquid chromatography |
| hr | = | hour |
| i.d. | = | internal diametre |
| kV | = | kilovolt |
| LOD | = | limit of detection |
| LOQ | = | limit of quantification |

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| | | |
|---|---|---|
| M | = | molar |
| mg/ml | = | milligram per millilitre |
| min | = | minute |
| ml | = | millilitre |
| mm | = | millimetre |
| mM | = | millimolar |
| MW | = | molecular weight |
| MEKC | = | micellar electrokinetic chromatography |
| nm | = | nanometer |
| NPA | = | normalized peak area |
| PI | = | isoelectric point |
| p.s.i. | = | pounds per square inch |
| $r^2$ | = | correlation coefficient |
| RSD | = | relative standard deviation |
| s | = | second |
| S | = | slope of regression line |
| SD | = | standard deviation |
| SFC | = | supercritical fluid chromatography |
| TLC | = | thin-layer chromatography |
| UV | = | ultraviolet |
| v/v | = | volume by volume |
| w/v | = | weight by volume |

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# CHAPTER I

# INTRODUCTION

Enantiomers refer to the pair of opposite (mirror image) forms of a substance possessing a chiral carbon atom. A 1:1 mixture of the two enantiomers is called a racemic mixture. Enantiomers have identical physical and chemical properties, in an isotropic environment. Its chirality is only observed when the molecule is subjected to a chiral influence. A well known example is the optical rotation of polarized light. Polarized light is rotated when passing through solutions containing chiral molecules (but not when passing through racemic mixtures). Based on this property the two enantiomers are also called 'optical isomers'. Optical isomers rotate the light in an equal degree but in opposite direction. If the enantiomer rotates the light to the right, it will be indicated as dextrorotatory, "d" or "(+)". Optical isomers that rotate light to the left, on the other hand will be indicated as levorotatory, "l" or "(-)". Howerver, optical rotation is a physical property: the degree of rotation depends on the solvent used, sample concentration, temperature and wavelength of light used to measure rotation. Even the direction of rotation may change under different conditions, e.g., by changing the pH of solution.

Enantiomers are often readily distinguished by biological systems, which is predominantly a chiral environment, and may have different pharmacokinetic properties (absorption, distribution, biotransformation and excretion) and quantitatively or qualitatively different pharmacologic or toxicologic effects. It is well known that the (-)-enantiomer of the β–blocker propanolol is about 100 times more active than the (+)-form. Another example is given by the thalidomide tragedy in the early 1960s. Thalidomide was administered as a racemate. However, only the (R)-

(+)-enantiomer possessed the sleep inducing action. The (S)-(-)-enantiomer possessed teratogenic action, responsible for serious malformation in newborn babies of women who took the drug during pregnancy.

**Separation of optical isomers**

These examples underline the need for chiral separation methods. Since enantiomers have identical physical properties, they cannot be separated by traditional method. Nonetheless, they differ in their molecular shape, optical polarity and their interactions with other chiral substance. Thus they can be separated chiroselective chromatographic technique either by the direct or the indirect separation method. The indirect separation method is based on the formation of a covalent bond between the optical antipodes on the one hand and a pure chiral compound, called the chiral selector, on the other hand. This chemical reaction will result in a product consisting of two isomeric compounds which are not mirror images anymore. They are known as diastereoisomers and they can, in principle, be separated by any analytical method using achiral separation mechanism. This method is time consuming since sample pretreatment involving a chemical reaction is necessary. Furthermore, the chiral selector has to be very pure, since optical impurity will result in two more diastereomeric products.

In the direct separation mode, the separation of the optical isomers is based on complex formation between the enantiomers and a chiral selector, resulting in the formation of labile diastereoisomers. Separation can be accomplished if the complexes possess different stability constants. The afore mentioned disadvantages do not apply for the direct separation mode. The chiral purity of the selector only influences the resolution.

Analytical methods used so far for the enantiomeric separation include high performance liquid chromatography (HPLC) (Han,1997), thin-layer chromatography (TLC) (Aboul-Enein et al., 1999), gas chromatrography (GC) (Schurig, 2001), supercritical fluid chromatography (SFC) (Liu et al., 2002), and capillary electrophoresis (CE) (Blaschke and Chankvetadze, 2000). TLC, HPLC and CE can separate enantiomers by using chiral selector for stationary phase and also for mobile phase additive. While GC and SFC can separate enantiomers by using chiral selector only for stationary phase. GC and SFC have certain disadvantages relative to HPLC. The most critical of which is temperature selection. They often require high temperature, which can result in racemization or decomposition of the chiral analyte. The application of GC is mainly restricted to more volatile compound. HPLC and CE offer higher column efficiency and greater reproducibility as compared to TLC. The main drawback of CE compared to HPLC is that until now, CE has not shown to be useful as a preparative separation tool. Another advantage of HPLC over CE is the low detection limit, due to the much longer path length of the detection cell and the much higher injection volume. In HPLC technique the disadvantages of chiral column are more expensive, poorer column–to–column reproducibility, lower chemical selectivity and efficiency than conventional HPLC column (Han, 1997). In addition it may be difficult to find HPLC separation conditions where the trace enantiomer elutes before the main component – a condition that more readily allows maximum sensitivity for a component that has significant tailing (Rickard and Bopp, 1994). However the very high efficiencies usually obtained in CE, and the ease of method development, make it a very good alternative for analytical separation of enantiomers. Other advantages of CE over HPLC are the low consumption of both analyte and chiral selector and the short analysis times. Moreover, CE has no need for expensive chiral stationary phases, since the chiral selector is simply added to the buffer.

**Capillary electrophoresis (CE)**

CE as a separation technique utilizes the differences in migration rates of charged species in an electric field in a narrow-bore silica capillary, typically 30 – 100 cm long with 25 – 75 μm inner diameter and 375 μm outer diameter. 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 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 > 2. The silanoate anions attract cationic species from the BGE and the static layer created is called the Stern layer. Another layer with more mobile cationic ions, the so called diffuse layer is formed next to the Stern layer. When the voltage is applied the cations in the diffuse layer will migrate towards the cathode and drag the bulk liquid along, thus causing electroosmotic flow (EOF) as shown in Figure 1.

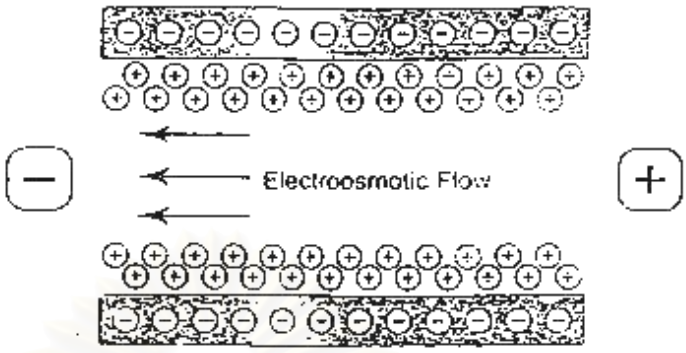



Figure 1 Development of electroosmotic flow

The velocity and direction of the EOF can be affected by changing pH and ionic strength of the BGE as well as by the addition of organic solvents, micelles, cyclodextrins or neutral polymers (Heiger, 1997).

Electroosmotic flow is 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 $\mu_{EP}$ is the electrophoretic mobility of the ion 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, $\eta$ is the buffer viscosity, 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 the presence of an EOF the measured mobility, called the apparent mobility, $\mu_{app}$, is given by

$$\mu_{app} = \mu_{ep} + \mu_{EOF}$$

$$\text{or} \quad \mu_{app} = \nu_{app}/E = (L_d * L_t)/V * t_m$$

where $\mu_{EOF}$ is the electroosmotic mobility, $\nu_{app}$ is the apparent velocity, $L_d$ is the capillary length to the detector, $L_t$ is the total capillary length, V is the applied voltage and $t_m$ is the migration time. The unit of $\mu_{app}$ is ($cm^2$/Vs).

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.

MEKC 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 phases.

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 separate zones all move 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 equilibrium is established. This is expressed by the name iso-tacho. In a single CITP experiment either cations or anions can be analyzed.

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 (charge-to-size ratio). The order of elution is cations, neutrals, then anions. Cation elute in order of their charge-to-size ratios, with small, highly charged cations eluting first. Neutral molecules, which move through the capillary under the influence of only the electroosmotic flow and are not separated from each other, elute after the cations. Anions elute last. Anions elute in reverse order to their charge-to-size ratios, with small, highly charged anions eluting last.

CZE is also the most frequently applied CE techniques for chiral separations. Numerous attempts have been made to develop the CZE methods for stereoisomer separations, including the separation of cis/trans isomers of retinoic acid (Bempong, Honigberg and Meltzer, 1993), pilocarpine (Baeyens et al., 1993) and clomiphene (Bempong and Honigberg, 1996), positional isomers of aminopyridines and diaminopyridine (Sabbah and Scriba, 2001) as well as enantiomers of clenbuterol, salbutamol and tulobuterol (Vela, Yanes and Stalcup, 2001), ephedrine (Flurer et al., 1995), levodopa and carbidopa (Sarac, Chankvetadze and Blaschke, 2000), amino acid (Perrin et al., 2000), peptides (Loukas, Sabbah and Scriba, 2001) and neurotransmitters (Maruszak et al., 2001).

In contrast to the use of chiral stationary phases as in HPLC, chiral analysis by CZE usually involves the addition of a chiral selector to the BGE in order to selectively modify the mobility of the two enantiomer forming labile diastereoisomeric complexes. These selectors can be cyclodextrins (CDs), crown ethers, macrocyclic antibiotics and heparin. This method has proven to be an effective method for a wide range of chiral compounds. The resolution of enantiomers has been obtained in CZE through inclusion - complexation mechanism. The separation of the two enantiomers can take place only if the two diastereomeric complexes, formed during the electrophoretic process, possess different stability constants. The enantiomer separation is obtained due to the formation of secondary bonds between the substituent groups on the chiral center of the analytes and those of the chiral selectors positioned outside the cavity. Selectivity can be tuned by adjusting the type and concentration of the chiral additive, and also by the addition of modifiers such as alcohol, surfactants, urea and metal ions.

**Cyclodextrins (CDs)**

CDs represent the most frequently used chiral selectors. Native CDs are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD) or eight (γ-CD) glucopyranose units with a truncated cone providing a hydrophobic cavity. The shape and chemical structure of β-CD are shown in Figure 1(a) and (b), respectively. Due to the presence of hydroxyl groups the outside of the CD is hydrophilic. The chiral recognition mechanism is based on inclusion of a bulky hydrophobic part of the molecules, preferably aromatic moieties, in the hydrophobic cavity of the CD. An additional requirement is that secondary interactions have to take effect; these include dipole-dipole interactions or hydrogen bonds between the hydroxyl groups at position 2 or 3 at the mouth of the CD and polar substituents close to the chiral center of the analyte. Physical properties of the three native CDs are quite different, i.e. width of

the cavity, solubility, molecular mass, however they possess the same depth as shown in Table 1 (Fanali, 1997, 2000).

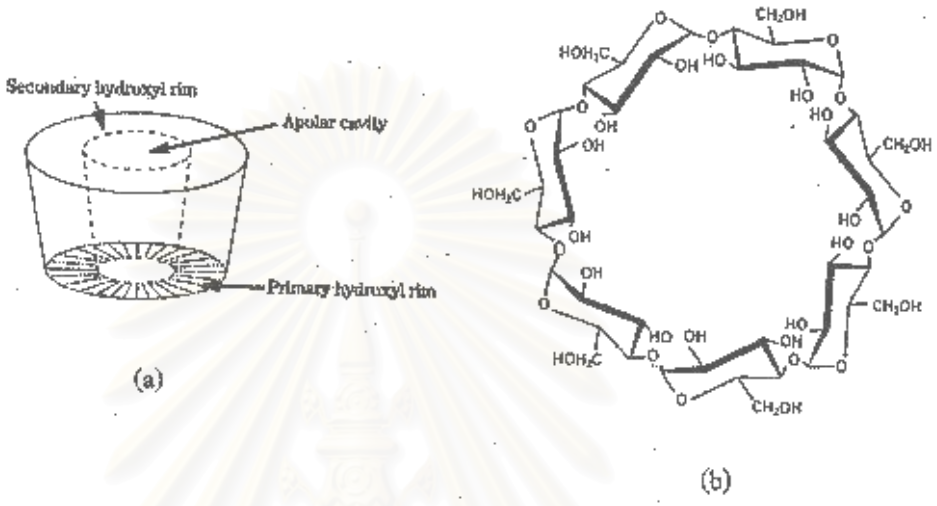



Figure 2 β-cyclodextrin, (a) shape of β-cyclodextrin, (b) chemical structure

Table 1 Physical properties of native cyclodextrins.

| Cyclodextrin type | Number of glucopyranose | Cavity (mm) | | Molecular weight | Solubility (g/100 ml in water, 25° C) |
|---|---|---|---|---|---|
| | | Diameter | Depth | | |
| α | 6 | 0.47-0.6 | 0.78 | 972 | 14.5 |
| β | 7 | 0.8 | 0.78 | 1135 | 1.85 |
| γ | 8 | 1.0 | 0.78 | 1297 | 23.2 |

The hydroxyl groups in positions 2,3 and 6 are available for derivatization, giving CD derivatives with increasing depth of the cavity and solubility. Among derivatized CDs that have been used are : neutral CDs (heptakis-O-methyl

cyclodextrin, M-CD; heptakis (2, 6-di-O-methyl) cylcodextrin, DM-CD; heptakis (2, 3, 6-tri-O-methyl) cyclodextrin, TM-CD; hydroxyethyl cyclodextrin, HE-CD; and hydroxypropyl cyclodextrin, HP-CD), negatively charged CDs (carboxymethyl-β-cyclodextrin, CM-β-CD; carboxyethyl-β-cyclodextrin, CE-β-CD; succinyl-β-cyclodextrin, Succ-β-CD; sulfobutyl-β-cyclodextrin, SBE-β-CD; and sulfated-β-cyclodextrin, S-β-CD), positively charged CDs (heptamethylamino-β-CD; mono (6-amino-6-deoxy)-β-CD) and amphoteric CDs (mono-(6-glutamylamino-6-deoxy)-β-CD, Glu-β-CD; amphoteric-β-CD, AM-β-CD). Detailed information and applications of derivatized CDs can be found in recent reviews (Gübitz and Schmid, 1997, 2000).

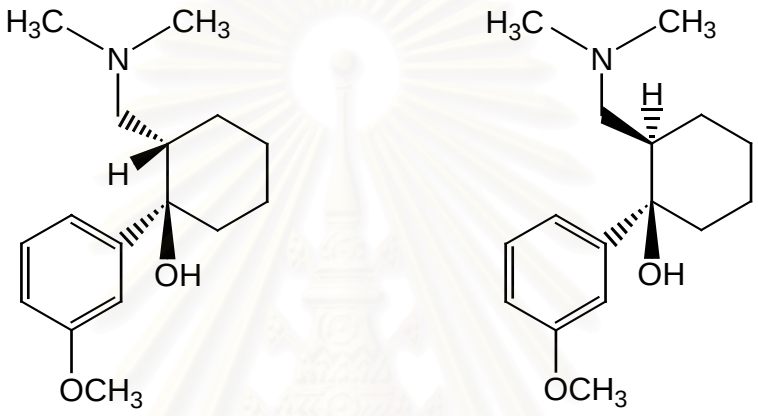
CDs as chiral selectors have many advantages when used in CE. First CD and derivatives are commercially available. They are easy to handle and can be used as normal buffer additive. They does not absorb in the UV range commonly used for capillary electrophoretic detection. And also they are stable over a wide pH range, resistive to light and non-toxic. The solubility is sufficient and can be increased by substitution and by addition of urea (Schmitt and Engelhardt, 1993).

The most commonly used CD for chiral separations in CE is β-CD. The reason for this is the relatively low cost of β-CD compared to the other CDs and the cavity of β-CD is appropriate to host a wide number of chemical compounds especially of pharmaceutical interest. Furthermore, functionalization of the various native CDs has expanded the utility of these chiral selectors, allowing for the CE enantioseparation of neutral as well as charged compounds.

The ability to resolve enantiomers of chiral analytes can vary considerably when comparing β-CDs, and functionalized β-CDs. Moreover, compounds that were not resolved by native β-CD are often successfully resolved by functionalized β-CD. There are many reports on the separation of enantiomers using CZE and various CDs

as chiral selectors such as β-CD (Aturki and Fanali, 1994), sulfated β-CD (Zhou et al., 2000), carboxymethyl β-CD (Manuszak et al, 2001), phosphate-β-CD (Yanes et al., 2001) and hydroxypropyl β-CD (Palmarsdottir and Edholm, 1994; Rickard and Bopp, 1994).

**Tramadol**




trans-tramadol cis-tramadol

$C_{16}H_{25}NO_2$ MW = 264

Figure 3 Chemical structure of tramadol

Tramadol [2-(dimethylamino)methyl]-1-[3-methoxyphenyl)cyclohexanol] is a centrally acting opioid analgesic drug that possesses an analgesic action with a potency ranging between weak opioid and morphine. Unlike the typical opioid analgesic, tramadol does not have clinically significant side effects such as respiratory depression, constipation (even after long term administration) or depression. Its structural formula is shown in Figure 3.

Tramadol hydrochloride is a white, bitter, crystalline and odorless powder with a melting point of about 180-181 °C. It is readily soluble in water and ethanol and has a pKa of 9.41. The water/n-octanol partition coefficient is 1.35 at pH 7 (Sifton, 2001).

Tramadol has two chiral C atoms- at each of these a R or S configuration is possible. Thus, 4 stereoisomers occur, (1R, 2R), (1R, 2S), (1S, 2R) and (1S, 2S), existing as two pairs of enantiomers. It also has a pair of geometric (cis/trans) isomers. Tramadol was introduced in Germany in the late 1970s and the biological active isomer was [(1R, 2R), (1S, 2S)] by Grunenthal, (Flick and Frankus, 1972).

Tramadol, the racemic hydrochloride salt of cis-isomer [(1R, 2R) and (1S, 2S)] was approved for use in the United States in 1995 and is currently marketed as Ultram® by Ortho-McNeil Pharmaceuticals, Inc. (Sifton, 2000). The trans-isomer [(1R, 2S) and (1S, 2R)] was a related substance. It is known that the (+)-enantiomer of the cis-isomer [(+)-(1R, 2R)] exhibits analgesic activity 10-fold higher than that of the (-)-enantiomer [(-)-(1S, 2S)] (Goeringer, Logar and Christian, 1997). The (+)-cis-tramadol has higher affinity for the μ–receptor and preferentially inhibits serotonin uptake and enhances serotonin release while the (-)-cis-tramadol preferentially inhibits norepinephrine uptake, stimulating $\alpha_2$–adrenegic receptors. The action of these two enantiomers is both complementary and synergistic (Dayer et al., 1994; Ground et al., 1997; Lewis and Han, 1997; and Raffa et al., 1993).

Tramadol is not official in the United States Pharmacopoeia (USP) but official as a raw material in the British Pharmacopoeia 2002 (BP). Tramadol in the BP 2002 is [(1R, 2R), (1S, 2S)] racemate compound while [(1R, 2S) and (1S, 2R)] racemate compound is an impurity and must be present at not more than 0.2%, determining by the HPLC method.

Elasing and Blashke (1993) developed HPLC method to determine the enantiomers of tramadol and its main metabolites (N-demethyltramadol and O-demethyltramadol) in urine by using chiral column in the normal-phase mode. The separations of enantiomers of tramadol and N-demethyltramadol were achieved on the

amylose tris-3,5-dimethylphenyl carbamate chiral stationary phase (ChiralPak AD® column) with different mobile phase compositions. The mobile phases for enantiomeric separation of tramadol and N-demethyl tramadol were n-hexane-2-propanol-diethylamine (97.5 : 2.5 : 0.01, v/v) and n-hexane-ethanol-diethylamine (94.0 : 6.0 : 0.01, v/v), repectively. The enantiomers of O-demethyltramadol was resolved on a cellulose tris-3,5-dimethylphenyl carbamate column (Chiralcel® column) with a mobile phase of n-hexane-ethanol-water (96.0 : 4.0 : 0.1, v/v).

Ceccato et al. (1997) developed a sensitive and automated method for the separation and individual determination of tramadol enantiomers in plasma by using solid-phase extraction in combination with chiral liquid chromatography. The enantiomeric separation of tramadol was achieved using a Chiracel OD® column containing cellulose tris-(3,5-dimethyphenyl carbamate) as a chiral stationary phase and the mobile phase was a mixture of 0.2 M sodium perchlorate in phosphate buffer, pH 6.0 and acetonitrile (75 : 25).

Guo et al. (1998) studied the separation of cis- and trans-isomers of tramadol hydrochloride by CZE without using any chiral selector. Because of their configuration difference, cis- and trans-isomers would possess different hydrodynamic radii, although the charges carried by such isomers are the same (at suitable pH). Therefore, their electrophoretic mobilities would be different allowing an electrophoretic separation to be possible. The separation was achieved by using a fused silica capillary (94 cm x 75 μm i.d.) with 40 mM borate buffer at pH 9.14, the temperature of 25$^{o}$C and applied voltage of 15 kV. The method was finally applied to the analysis of samples from industrial waste.

Chan and Ho (1998) reported the enantiomeric separation of tramadol hydrochloride and its major metabolites, O-demethyltramadol and N-

demethyltramadol using cyclodextrins as chiral selectors in CZE. They also studied the influences of type and concentration of CD, capillary temperature, length of capillaries, buffer pH and the addition of polymer modifier by varying the factor individually. They found that tramadol and the metabolites could be baseline-separated simultaneously by using 50 mM phosphate buffer (pH 2.5) containing 75 mM methyl-β-CD, 220 mM urea and 0.05% ( w/v) hydroxypropylmethyl cellulose.

Rudaz et al. (1998) developed and optimized the CZE method for the enantiomeric resolution of racemic tramadol. Both uncoated and polyacrylamide-coated capillaries were tested using either negatively charged (carboxymethyl-β-CD and sulfobutylether-β-CD), neutral (hydroxypropyl-β-CD and heptakis (2,6-di-O-methyl)-β-CD) and native (α, β and γ-CD) cyclodextrins added to the BGE. Among the CDs tested, carboxymethyl-β-CD allowed the baseline separation of tramadol enantiomers. The optimized method was validated in a coated capillary for enantiomeric analysis of tramadol enantiomers in pharmaceutical formulation. They reported that the precision of the validated method was less than 2%, with an accuracy higher than 99%. The method was used to determine the enantomeric ratio of tramadol in capsules as well as to quantify tramadol enantiomers content.

Rudaz et al. (1999) studied the simultaneous enantiomeric resolution of tramadol and its phase I metabolites (O-demethyl tramadol, N-demethyltramadol and O-demethyl-N-demethyltramadol) in urine by CZE using carboxymethylated-β-cyclodextrin as a chiral selector. Baseline resolution of tramadol and its metabolites was obtained in less than 30 min using a 50 mM phosphate buffer (pH 2.5) containing 5 mM carboxymethyl β-CD in a fused silica capillary polyacrylamide coated 40.5 cm x 50 μm i.d. Chiral determination of tramadol and its main three metabilites were performed in urine after a simple double liquid-liquid extractions of 200 μl of biological material.

Kurth and Blaschke (1999) developed a chiral separation for the determination of tramadol and its main metabolite O-demethyltramadol in urine sample by CE with UV detection. The simultaneous chiral separation of tramadol and O-demethyltramadol was achieved using 25 mM borate buffer, pH 10.1 containing 30 mg/ml carboxymethyl β–CD. The method was validated and its applicability was shown by the determination of tramadol and O-demethyltramadol in urine sample.

Despite the many studies already published on the subject of enantiomeric separation of tramadol using CZE, hardly any data is available on simultaneous enantiomeric separation of cis-and trans-tramdol.

The objectives of this study were to develop the CZE procedure for separation of the four tramadol stereoisomers and to apply the developed method to the simultaneous enantiomeric separation and determination of tramadol in pharmaceutical dosage forms.

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# CHAPTER II

# EXPERIMENTATION

## Material and method

### Material

#### 1. Apparatus and Instrument

- Bio-Rad Biofocus 3000 automatic capillary electrophoresis system (Bio-Rad Laboratories, Inc., USA.)
- BioCap®, uncoated fused-silica capillary tubing, 50 cm × 50 μm i.d. (Bio-Rad Laboratories, Inc., USA.)
- pH meter, Titroprocesser (Metrohm, Switzerland)
- Analytical balance, Mettler AT 200 (Mettler-Toledo, Switzerland)
- Analytical balance Mettler M3 (Mettler-Toledo, Switzerland)

#### 2. Chemicals and Reagents

- Acetic acid, glacial 100% (Merck, Germany)
- Boric acid, powder (May & Baker, Dagenham, England)
- Carboxymethyl-β-cyclodextrin (Fluka, Switzerland)
- Cis-tramadol (DMSc, Thailand)
- β-cyclodextrin (Fluka, Switzerland)
- Dopamine working standard (DMSc, Thailand)
- Hydrochloric acid, 37% (Merck, Germany)
- Hydroxypropyl-β-cyclodextrin (Fluka, Switzerland)

- Phosphoric acid, 85% ( Carlo Erba, Italy)
- Sodium dihydrogen phosphate (Carlo Erba, Italy)
- Sodium hydroxide, pellets (Mallinckrodt, Mexico)
- Trans-tramadol, working standard (Mikromol GmbH, Germany)

**3. Test samples**

Capsule and injection dosage forms of tramadol were selected for this study. Three brands of each dosage form were purchased from hospitals in Bangkok. The labeled amount of tramadol were 50 mg for each capsule and 50 mg per millilitre for injection.

## Method

### 1. Analytical procedure

CZE was performed on a Biofocus 3000 automatic electrophoresis apparatus equipped with a multiwavelength UV-visible detector set at 196 nm. Separations were carried out in an uncoated fused-silica capillary with a total length of 50 cm, effective length of 45.5 cm, inner diameter of 50 μm and an outer diameter of 375 μm. A thermostating liquid was used to maintain the capillary at the stated temperature. For preliminary experiment the instrument was operated at 25°C and 15 kV of the applied voltage. Injections were performed by hydrodynamic injection at the anodic capillary inlet with applying pressure of 5 p.s.i. for 5s. At the beginning of the work day, the capillary was washed with water for 5 min and with buffer without chiral selector for 2 min and then equilibrated with running buffer containing the chiral selector for 15 min. Between runs the capillary was treated successively with buffer without chiral selector for 1 min and running buffer containing the chiral selector for 2 min. Vials containing anode and cathode buffer were changed for approximately

every tenth injection. The separation conditions were optimized by varying the electrolyte pHs, concentrations, chiral selectors, capillary temperatures and the applied voltages as described in section 3 and 4.

## 2. Standard preparation

### 2.1 Stock standard solutions

Stock standard solutions of cis-tramadol and trans-tramadol were prepared at concentration of 1 mg/ml in deionized water and kept in a refrigerator at 4°C.

### 2.2 Standard mixture solution

2.2.1 Standard mixture solution containing 50 µg/ml each of cis and trans-tramadol was prepared daily in deionized water from appropriate dilution of standard stock solutions. Before use, all solutions were filtered through a 0.45 µm pore size filter. This standard mixture solution was used for selection of background electrolyte pH and chiral selector.

2.2.2 Standard mixture solution containing 150 µg/ml of cis-tramadol and 7.5 µg/ml of trans-tramadol, concentrations found in the real sample, was prepared in deionized water from appropriate dilution of standard stock solutions. This standard mixture solution was used for determination of the influences of concentrations of chiral selector and buffer and optimization of secondary experimental parameters.

## 3. Optimization of primary experimental parameters

The effects of buffer (pH and concentration) and chiral selector (type and concentration) on the enantiomer separation of the interested compounds were investigated. The migration time $t_m$ and resolution ($R_s$) from each condition were compared.

### 3.1 Effect of background electrolyte pH

Various buffer systems in the acidic and basic regions at pH 2.5 – 10.2 were studied. These buffers were prepared with constant ionic strength of 50 mM. The composition of the buffers is listed in Table 2. A standard mixture solution of trans- and cis-tramadol of 50 μg/ml each was analyzed.

Table 2. Buffer solutions

| pH | cation | anion |
|---|---|---|
| 2.5 | 50 mM, $H^+$ | Phosphate |
| 4.0 | 50 mM, $H^+$ | Acetate |
| 7.0 | 50 mM, $Na^+$ | Phosphate |
| 9.0 | 50 mM, $Na^+$ | Borate |
| 10.2 | 50 mM, $Na^+$ | Borate |

### 3.2 Effect of chiral selector type and concentration

Three types of CDs namely β-CD, carboxymethyl-β-CD (CM-β-CD) and hydroxypropyl-β-CD (HP-β-CD) were selected in order to find the most appropriate chiral selector for the enantiomeric separation of tramadol. Experiments

were performed with the BGE composed of 2 mM chiral selector at the pH 2.5, 9.0 and 10.2. A standard mixture solution of trans- and cis-tramadol of 50 μg/ml each was analyzed.

For the evaluation of the influence of concentration of CM-β-CD on the stereoselective interaction with tramadol, BGEs composed of 50 mM $H_3PO_4$ and CM-β-CD concentration in the range of 7-13 mM at pH 2.5 were tested. A standard mixture solution of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) was analyzed.

### 3.3 Effect of background electrolyte concentration.

The electrolyte concentration generally has a marked effect on migration time, since it directly affects the EOF. The phosphate buffer concentration in the range of 50-65 mM at pH 2.5 were investigated. A standard mixture solution of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) was analyzed.

## 4. Optimization of secondary experimental parameters

After evaluation of the primary experimental parameters (i.e., pH, CD type and concentration), secondary experimental parameters, can be adjusted to complete the optimization of the separation. These parameters are capillary temperature and applied voltage.

The Plackett-Burman design, a two-level fractional factorial design for studying 7 factors in 8 runs, was used to study the optimization of secondary parameters. Seven factors studied were five experimental parameters and two dummies. Five experimental parameters were capillary temperature (20, 25 or 30° C),

applied voltage (12, 15 or 18 kV), buffer pH (2.2, 2.5 or 2.8), buffer concentration (55, 65 or 75 mM) and CM-β-CD concentration (7.5, 8.0 or 8.5 mM). The first two parameters were secondary parameters. The last three parameters were included in the design for fine-tuning the stereoisomers separation. The composition of BGEs used in Plackett-Burman design was summarized in Table 3.

Table 3 Composition of the background electrolytes for optimization of CZE condition

| BGE number | Concentration of carboxymethyl-β-cyclodextrin | pH of phosphate buffer | Concentration of phosphate buffer |
|---|---|---|---|
| 1 | 8.0 | 2.5 | 65 |
| 2 | 7.5 | 2.5 | 65 |
| 3 | 7.5 | 2.2 | 65 |
| 4 | 8.0 | 2.2 | 55 |
| 5 | 7.5 | 2.5 | 55 |
| 6 | 8.0 | 2.2 | 65 |
| 7 | 8.0 | 2.5 | 55 |
| 8 | 7.5 | 2.2 | 55 |
| 9 | 8.5 | 2.8 | 75 |
| 10 | 8.0 | 2.8 | 75 |
| 11 | 8.0 | 2.5 | 75 |
| 12 | 8.5 | 2.5 | 65 |
| 13 | 8.0 | 2.8 | 65 |
| 14 | 8.5 | 2.5 | 75 |
| 15 | 8.5 | 2.8 | 65 |

The nominal values of the operating conditions were the BGE composing of 8 mM CM-β-CD and 65 mM phosphate buffer at pH 2.5, the capillary temperature of 25 °C and the applied voltage at 15 kV.

The Plackett-Burman design was presented in Table 4 as a seven-variable array for five selected factors ($F_1$-$F_5$) along with two dummy factors ($D_1$ and $D_2$). 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 as shown in Table 5. As shown in Table 3, BGE number 1-8 were used in design I and BEG number 9-15 were used in design II. The experiments were carried out in the same order as indicated in Table 4, using the standard mixture solution containing 150 μg/ml of cis-tramadol and 7.5 μg/ml of trans-tramadol preparing in 10% diluted BGE (chiral selector free), as the test solution.

The effect of changing a factor from a low to a high level value was examined on the resolution ($R_s$) and the analysis time ($t_a$), considered as the migration time of the last eluting peak. The last two peaks were found to be the peak of enantiomers of cis-tramadol.

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Table 4 Eight-experimental Plackett-Burman design for seven factors

| 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 |

Table 5 Factor values for the two complementary designs

| Factors | Design I Level | | Design II Level | |
|---|---|---|---|---|
| | (-1) | (+1) | (-1) | (+1) |
| F1, Carboxymethyl-β-CD concentration(mM) | 7.5 | 8.0 | 8.0 | 8.5 |
| F2, pH of phosphate buffer | 2.2 | 2.5 | 2.5 | 2.8 |
| F3, Concentration of phosphate buffer | 55 | 65 | 65 | 75 |
| F4, Applied voltage (kV) | 12 | 15 | 15 | 18 |
| F5, Capillary temperature (°C) | 20 | 25 | 25 | 30 |
| D1 | - | - | - | - |
| D2 | - | - | - | - |

**5. Selection of the internal standard**

To compensate for various analytical errors, seven compounds including phenformin, procaine, pyrimethamine, d-norpseudoephedrine, dopamine, levodopa and metformin were randomly screened in order to find the most appropriate internal standard. The molecular weight of each compound is closed to or less than tramadol. Each compound was dissolved in deionized water to have concentration of 0.1 mg/ml. The solution was filtered through a 0.45 μm membrane filter prior to inject into the capillary by using the optimized condition.

The criteria for selecting the internal standard were as follows. The compound should ionized in phosphate buffer at pH 2.5 to have positive charge, must not be present in the sample of tramadol preparations studied, must be stable and nonreactive, could be completely resolved from other compounds in the samples and must have shorter migration time than the interested peaks under the experimental condition.

**6. Analytical Method Validation**

The validation was performed according to a procedure recommended by the International Conference on Harmonization (ICH, 1996). The following measures of method performance were assessed: selectivity, linearity and range, precision, accuracy, determination of limit of detection (LOD), and quantification (LOQ), stability of standard and sample solutions and system suitability.

### 6.1 Selectivity

A standard mixture solution of 100 µg/ml cis-tramadol and 5 µg/ml trans-tramadol (nominal concentration), including 30 µg/ml of the internal standard was prepared in 10% diluted BGE (chiral selector free). Sample solutions of Tramal ® Capsules and Injection containing a norminal concentration of cis-tramadol and 30 µg/ml of the internal standard were also prepared.

The specificity was determined by analyzing a standard mixture and sample solutions according to the proposed method. Electropherograms of standard and samples were then compared.

### 6.2 Linearity and range

Linearity was obtained with calibration curves using five standard mixture solutions prepared with cis-tramadol and trans-tramadol over the range of 70-130 µg/ml (70-130%) and 2.8-7.5 µg/ml (56-150%), respectively. The standard mixture solutions contained 30 µg/ml of dopamine as an internal standard. Each solution was injected in triplicate. Least square linear regression analysis was performed by plotting peak area ratios, normalized peak area ratio and peak height ratios of cis-tramadol and trans-tramadol/internal standard versus concentrations. The coefficients of determination ($r^2$) were calculated and should be 0.990 or greater.

### 6.3 Precision

Precision was accessed from the % relative standard deviation (%RSD), which was determined from the following formula:

$$\%RSD = \frac{SD \times 100}{\bar{X}}$$

Where SD is the standard deviation of the observed data. The %RSD should be less than 2% for cis-tramadol enantiomers and 5% for trans-tramadol enantiomers.

The precision was assessed by the following methods:

(1) System precision

This was determined by triplicate injections of three standard mixture concentrations of trans-tramadol (2.8, 5.0 and 7.5 μg/ml) and cis-tramadol (130, 100 and 70 μg/ml).

(2) Intra-day and inter-day precision

Six sample preparations of Tramal ® Capsules and Injection at the nominal concentration of cis-tramadol (100 μg/ml), spiked with 5.0 μg/ml of trans-tramadol, were analyzed for determination of the intra-day precision. These studies of the same batches of samples were also repeated on three non-consecutive days to determine the inter-day precision.

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**6.4 Accuracy**

Accuracy was calculated from percent recovery using standard addition method. Three different amounts of cis-tramadol and trans-tramadol were added to solutions of Tramal® Capsules and Injection. The three concentrations covered an 80-120% interval of the expected assay concentrations. The solutions were prepared in triplicate at each concentration. Percent recovery was calculated from the ratio of the

amount found and the amount added. The mean recoveries should be within 98-102% and 95-105% for cis-tramadol and trans-tramadol, respectively.

### 6.5 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of trans-tramadol were obtained with a calibration curve constructed by analysing standard solutions of known concentration of cis-tramadol and trans-tramadol, including the internal standard. Seven-point calibration was performed over the range 1.85 to 5.0 μg/ml of trans-tramadol. Each standard solution also contained 100 and 30 μg/ml of cis-tramadol and the internal standard, respectively. The solutions were injected in triplicate. Normalized peak area ratio of trans-tramadol/internal standard was plotted against concentration. Least square linear regression analysis was performed to determine slope, intercept and the standard deviation of the y-intercept.

The LOD and LOQ of trans-tramadol were calculated by following equations :

$$LOD = 3.3\,\sigma/S$$

$$LOQ = 10\,\sigma/S$$

Where $\sigma$ is the standard deviation of the y-intercept and S is the slope of the regression line. At the LOQ, the %RSD of the intra-day precision should be less than 10%.

### 6.6 Stability of the standard mixture and sample solutions

A standard mixture solution of cis-tramadol and trans-tramadol at nominal concentration, including the internal standard was prepared and divided into two portions. A portion of the solution was stored in laboratory glass at room temperature

and analyzed at hourly interval up to 4 hours. Another portion of the solution was stored in vials, put in the autosampler and analyzed at 6 hrs. interval up to 12 hrs. Freshly prepared standard mixture solution was analyzed and compared as reference.

The same procedure was repeated for Tramal® Capsules and freshly prepared sample solution of Tramal® Capsules was analyzed and compared as reference.

### 6.7 System suitability

System suitability testing was evaluated by six replicate injections of a standard mixture solution at nominal concentration of cis-tramadol and trans-tramadol, including the internal standard. The %RSD of normalized peak area ratio of cis-tramadol and trans-tramadol/internal standard and resolutions were determined. The %RSD should be less than 3% and $R_s$ between the last two peaks of enantiomers of cis-tramadol should be at least 1.49.

## 7. Determination of cis-tramadol and trans-tramadol in pharmaceutical preparations

### Preparation of sample

For capsules, an accurately weighed quantity equivalent to about 50 mg of cis-tramdol, was transferred to a 25-ml volumetric flask, dissolved in about 20 ml of deionized water, shaked well and sonicated for 5 min. Diluted with deionized water to volume and mixed to obtain a stock solution of capsule sample.

For injection, an accurately measured volume equivalent to about 50 mg of cis-tramadol, was transferred to a 25-ml volumetric flask, diluted with deionized water to volume and mixed to obtain a stock solution of injection sample.

For the assay of cis-tramadol, 5.0 ml of the stock solution of capsule or injection sample was diluted with deionized water to 10.0 ml. One ml of the diluted solution was transferred to a 10-ml volumetric flask, containing 0.3 mg of internal standard, diluted with 10% diluted BGE (chiral selector free) to volume, mixed and filtered through a 0.45 μm pore size filter.

There are two specification limits for the content of trams-tramadol. For the assay of trans-tramadol with the specification limit of 0.5%, 5 ml of the stock solution of capsule or injection sample was pipetted into a 10 ml volumetric flask containing 0.3 mg internal standard, diluted with 10% diluted BGE (chiral selector free) to volume, mixed and filtered through a 0.45 μm pore size filter. The assay of trans-tramadol with the specification limit of 0.25%, 6 ml of the sample stock solution was pipetted and followed the above procedure.

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# CHAPTER III

# RESULTS AND DISCUSSION

A capillary zone electrophoresis (CZE) method was demonstrated for separation of the four stereoisomers of tramadol. Optimization of CZE conditions was investigated by varying experimental parameters, which included concentration and pH of the BGE, type and concentration of CD, capillary temperature and applied voltage. The optimized method was validated and applied to pharmaceutical formulations.

**A: UV spectrum of tramadol**

The UV spectra of cis-tramadol (5 μg/ml) and trans-tramadol (5 μg/ml), in deionized water were shown in Figure 4a and 4b, respectively.

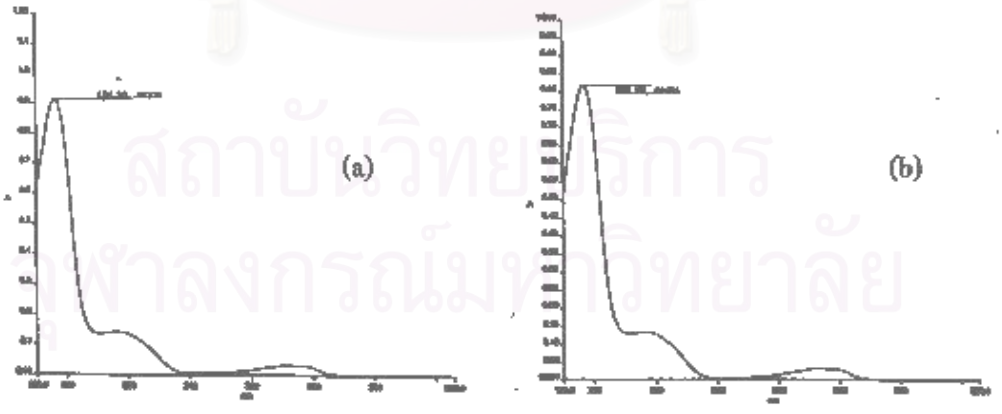


Figure 4 The UV spectra of (a) cis-tramadol and (b) trans-tramadol

Both cis-tramadol and trans-tramadol exhibited maximum absorbance at 196 nm. Therefore, the detection wavelength at 196 nm was selected in order to obtain the highest sensitivity.

**B: Optimization of primary experimental parameters**

The optimization of CZE condition was performed using standard mixture of cis-tramadol and trans-tramadol. The detection wavelength was set at 196 nm. The following parameters were consecutively optimized: pH of the background electrolyte, type and concentration of chiral selector and background electrolyte concentration. Migration time ($t_m$) and resolution ($R_s$) for each condition were compared.

**1. Effect of background electrolyte pH**

The buffer pH is one of the most important parameters for improving resolution in CE. Buffer pH has a significant effect on electroosmotic flow (EOF) because it changes zeta potential which is proportional to surface charge on the inner capillary wall. The inner surface of a fused silica capillary is covered with silanol groups (SiOH), which are ionized to $SiO^-$ at pH > 2. The electroosmotic mobility in a fused silica capillary is known to have a sigmoid relationship with pH. At the low pH, the ionization of the surface silanol groups is suppressed and the EOF approaches zero. Under alkaline conditions, the silanol groups are fully charged and the EOF reaches a plateau value (Lambert and Middleton, 1990). For a 50 mM phosphate buffer solution, a sigmoid curve shape is as follows: the inflection point of the curve is at approximately pH 5.2, small changes of the buffer pH have an immense effect on the electroosmotic mobility in the pH range of 3-7, and the EOF does not change significantly for pH values higher than pH 7 and lower than pH 3 (Kuhn and Hoffstetler-Khun, 1993).

In addition to affecting the EOF, changing the buffer pH will also cause a concomitant change in the charge of analytes which is dependent on the degree of ionization of the analytes and, hence, their electrophoretic mobilities.

Figure 5 shows the electrophoretic separation of cis-and trans-tramadol using buffers at different pH values ranging from pH 2.5 to 10.2 in the absence of chiral additive. Tramadol which is a weak base with pKa value of 9.4 would be totally ionized throughout the pH range of 2.5 - 7.0. The degree of ionization of tramadol drops to 71 and 14% at pH 9.0 and 10.2, respectively.

At pH 2.5 (Figure 5a), a partial resolution of trans-and cis-tramadol was achieved with migration times of 9.38 and 9.48 min, respectively (Table 6). The EOF approached zero at pH 2.5 and therefore the partial resolution of these analytes was solely based on differences in their electrophoretic mobilities or size-to-charge ratios. Trans-and cis-tramadol have the same charge, so the partial separation was on the basis of differences in their sizes. But this difference was not large enough to allow for baseline resolution.

At pH 4.0, the EOF became significant and reached a maximum at pH 7.0. Both trans-tramadol and cis-tramadol were fully ionized throughout this pH range and had different electrophoretic mobilities. The EOF of the buffer was much greater than the electrophoretic mobilities of analytes. Because of strong EOF of buffers, trans-tramadol and cis-tramadol were carried along the capillary at the same rate with migration times of 5.51 min at pH 4.0 and 3.26 min at pH 7.0 as shown in Figure 5b and 5c, respectively. Not surprisingly the migration time of the analytes at pH 7.0 was shorter than that at pH 4.0.

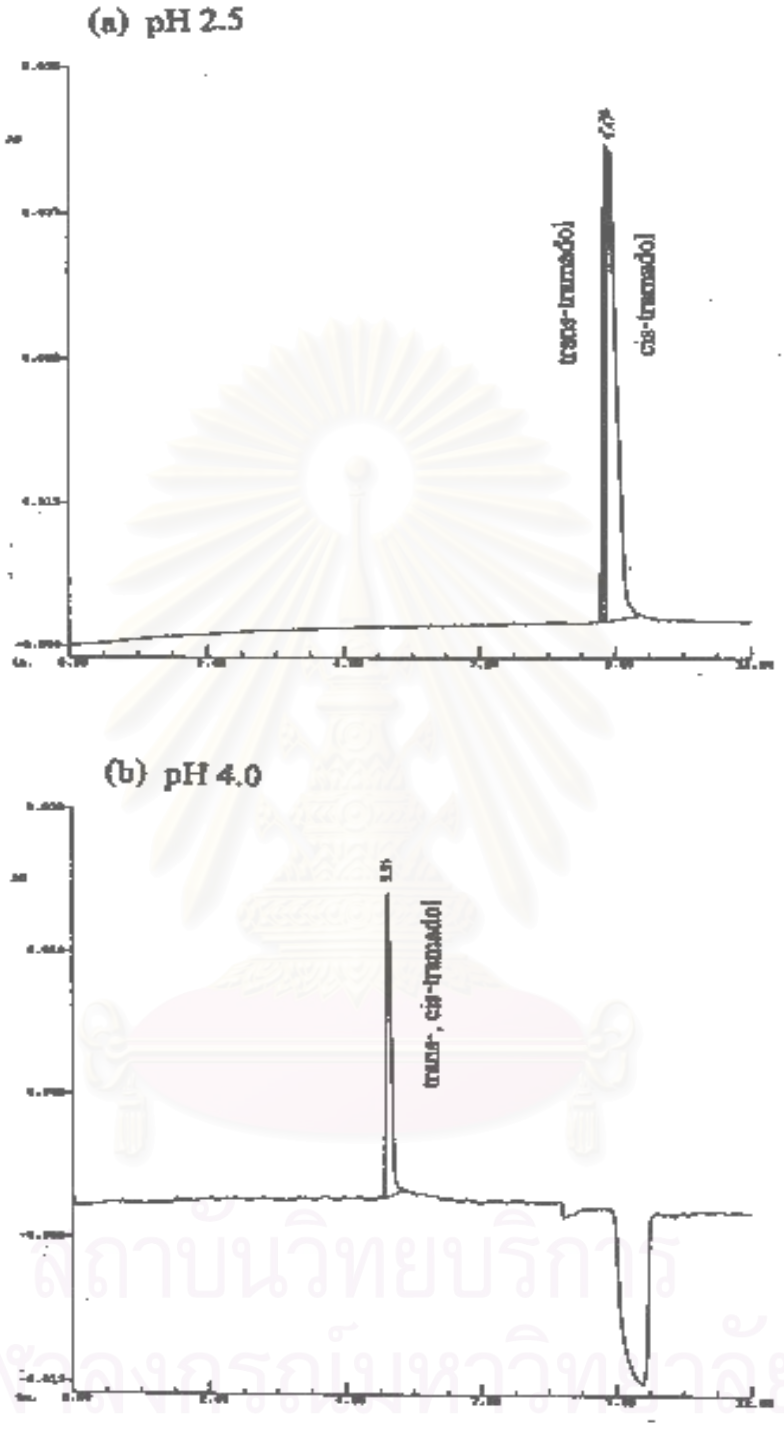


Figure 5 Effect of buffer pH on the separation of tramadol enantiomers in absence of chiral selector using 50 mM running buffer, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

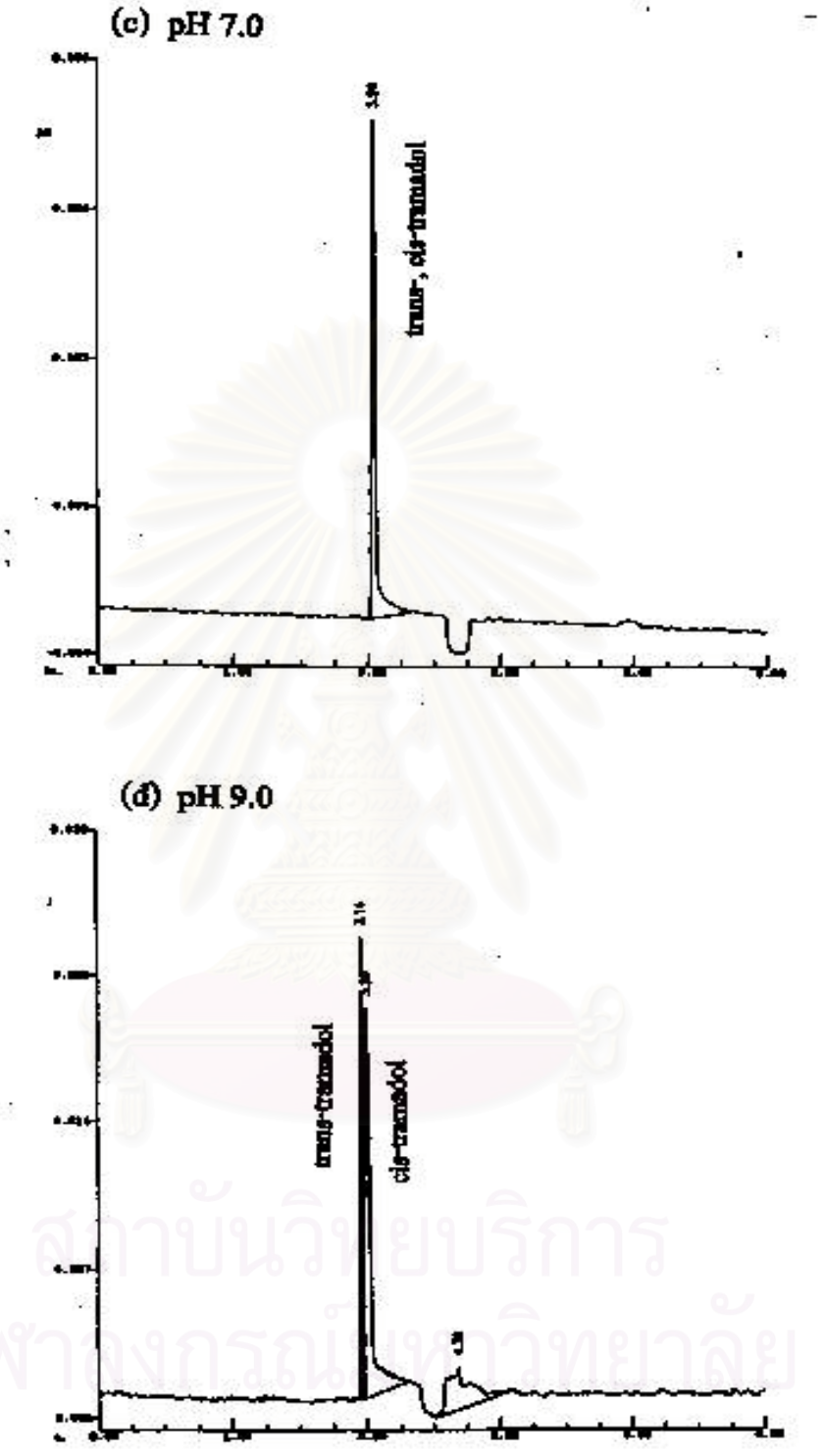


Figure 5 (Continued) Effect of buffer pH on the separation of tramadol enantiomers in absence of chiral selector using 50 mM running buffer, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

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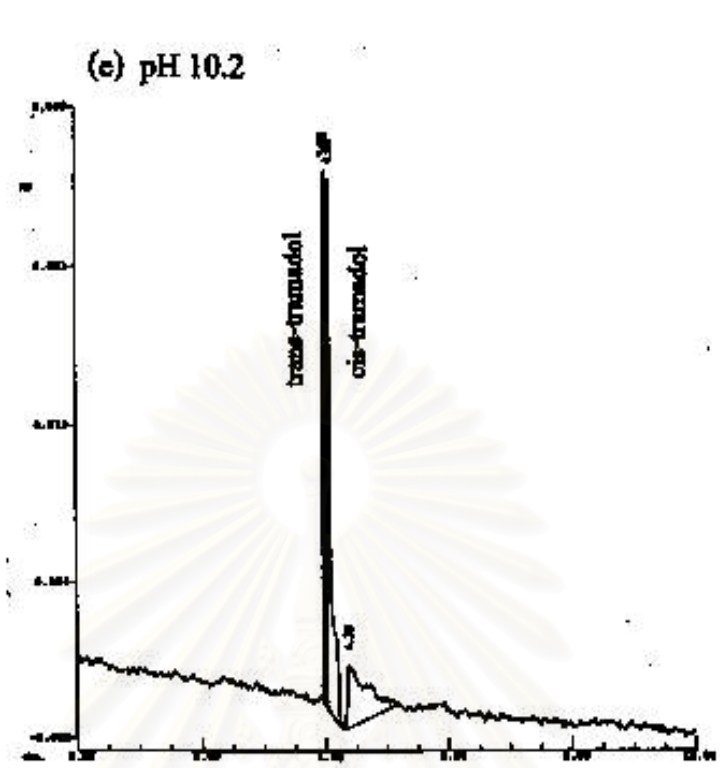


Figure 5 (Continued) Effect of buffer pH on the separation of tramadol enantiomers in absence of chiral selector using 50 mM running buffer, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

Table 6: Migration time and resolution of tramadol at different buffer pHs

| buffer pH | % ionization | $t_m$ (min) | | $R_s$ |
|---|---|---|---|---|
| | | trans-tramadol | cis-tramadol | |
| 2.5 | ~ 100 | 9.38 | 9.48 | partial resolution |
| 4.0 | 99.999 | 5.51 | 5.51 | No resolution |
| 7.0 | 99.6 | 3.26 | 3.26 | No resolution |
| 9.0 | 71.4 | 3.14 | 3.20 | Partial resolution |
| 10.2 | 13.7 | 3.98 | 4.06 | Partial resolution |

Changing the pH of the buffer from 7.0 to 9.0 or higher resulted in a drastic reduction in the degree of ionization of trans-tramadol and cis-tramadol and, hence their electrophoretic mobilities, but insignificant effect on the EOF of the buffer. The electrophoretic mobility, $\mu_{ep}$ ($cm^2/Vs$), of an ion is dependent on its degree of ionization according to equations (1) and (2).

$$\nu_{ep} = \alpha_i \nu_{ep}(0) \quad \text{.....①}$$

$$\mu_{ep} = \nu_{ep}/E = \nu_{ep} \cdot L/V \quad \text{.....②}$$

Where, $\nu_{ep}$ — electrophoretic velocity of the ion (cm/s)

$\alpha_i$ — the fraction of the solute that is ionized

$\nu_{ep}(0)$ — electrophoretic velocity of the solute that is totally ionized (cm/s)

E — applied electric field (V/cm)

L — total capillary length (cm)

V — applied voltage (V)

As the degree of ionization of trans-tramadol and cis-tramadol is decreased, the difference in their electrophoretic mobilities became obvious. Consequently, a partial resolution of trans-tramadol and cis-tramadol was observed with migration times of 3.14 and 3.20 min, respectively (Figure 5d). Further reduction in a degree of ionization of trans-tramadol and cis-tramadol, the electrophoretic mobility difference between these analytes was increased, leading to a better resolution (although not a baseline resolution) of these analytes with migration times of 3.98 and 4.06 min, respectively (Figure 5e).

From Figure 5a-5e, it was obvious that the baseline resolutions of trans-tramadol and cis-tramadol as well as their enantiomers were impossible using the

background electrolyte at pH range of 2.5 – 10.2, without the addition of chiral selector. Partial resolution of trans-tramadol and cis-tramadol, but not their enantiomers was achieved at the buffer pHs 2.5, 9.0 and 10.2. Therefore further separation optimizations would be studied at these three pH values.

**2. Effect of chiral selector type and concentration**

In this study, both uncharged and charged β-cyclodextrins were investigated in order to find the most appropriate chiral selector for enantiomeric separation of trans-and cis-tramadol. The uncharged β-cyclodextrins selected were β-cyclodextrins (β-CD) and hydroxypropyl-β-cyclodextrins (HP-β-CD) while the charged β-cyclodextrins was carboxymethyl-β-cyclodextrins (CM-β-CD). The background electrolytes studied were buffers at pH 2.5, 9.0 and 10.2, containing 2 mM of each β-CD, HP-β-CD and CM-β-CD.

Figures 6-8 show electropherograms of the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of each β-CD, HP-β-CD and CM-β-CD as chiral selector in the running buffer at pH 2.5, 9.0 and 10.2, respectively. No stereoisomeric separation of tramadol was observed at pH 9.0 and 10.2 (Figure 7 and 8). At pH 2.5, chiral recognition was only observed with CM-β-CD, but not with the other two CDs (Figure 6c). With CM-β-CD in 50 mM phosphate buffer at pH 2.5, baseline chiral separation of trans-tramadol was achieved but only partially chiral separation of cis-tramadol was obtained. Although baseline resolution was not achieved, the racemic cis-tramadol was clearly enantioseparated with CM-β-CD under selected conditions.

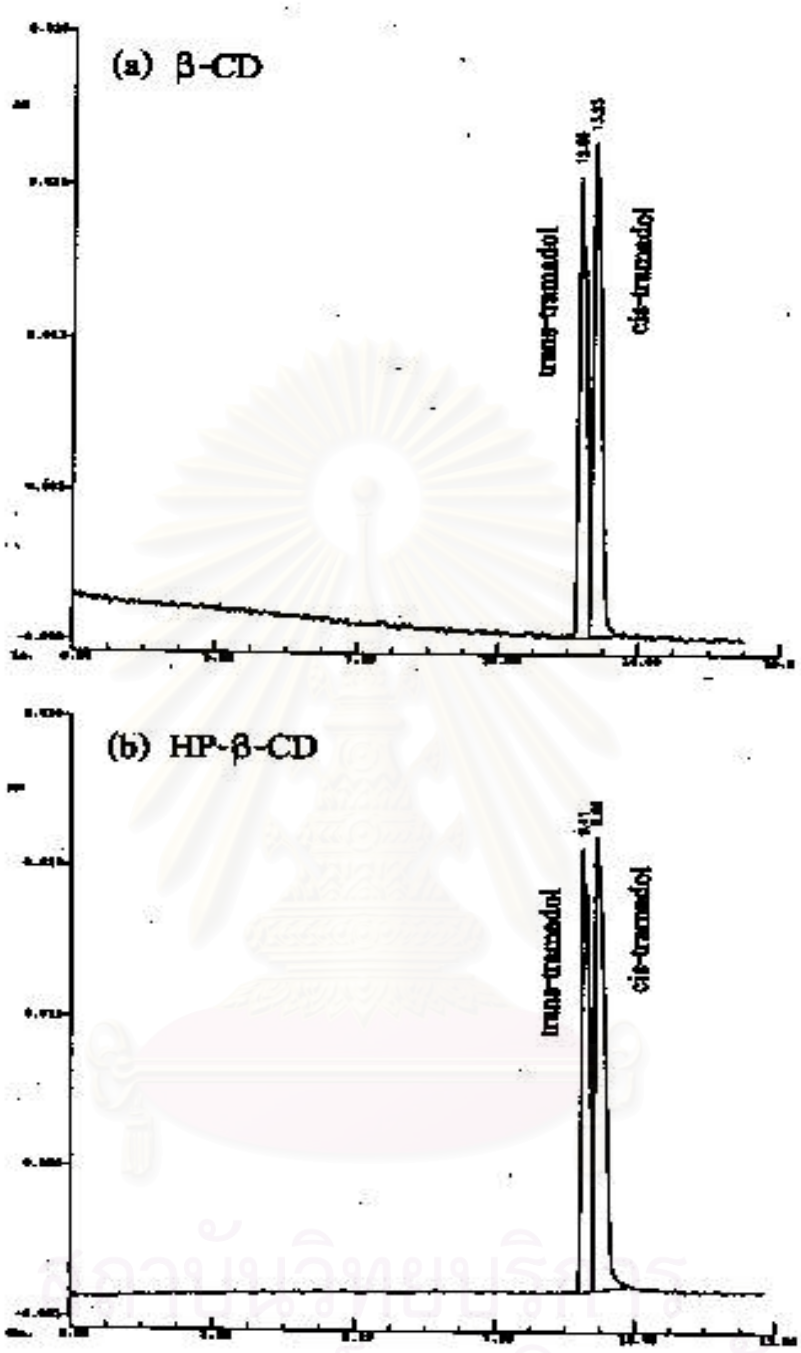


Figure 6 Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM phosphate buffer pH 2.5, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

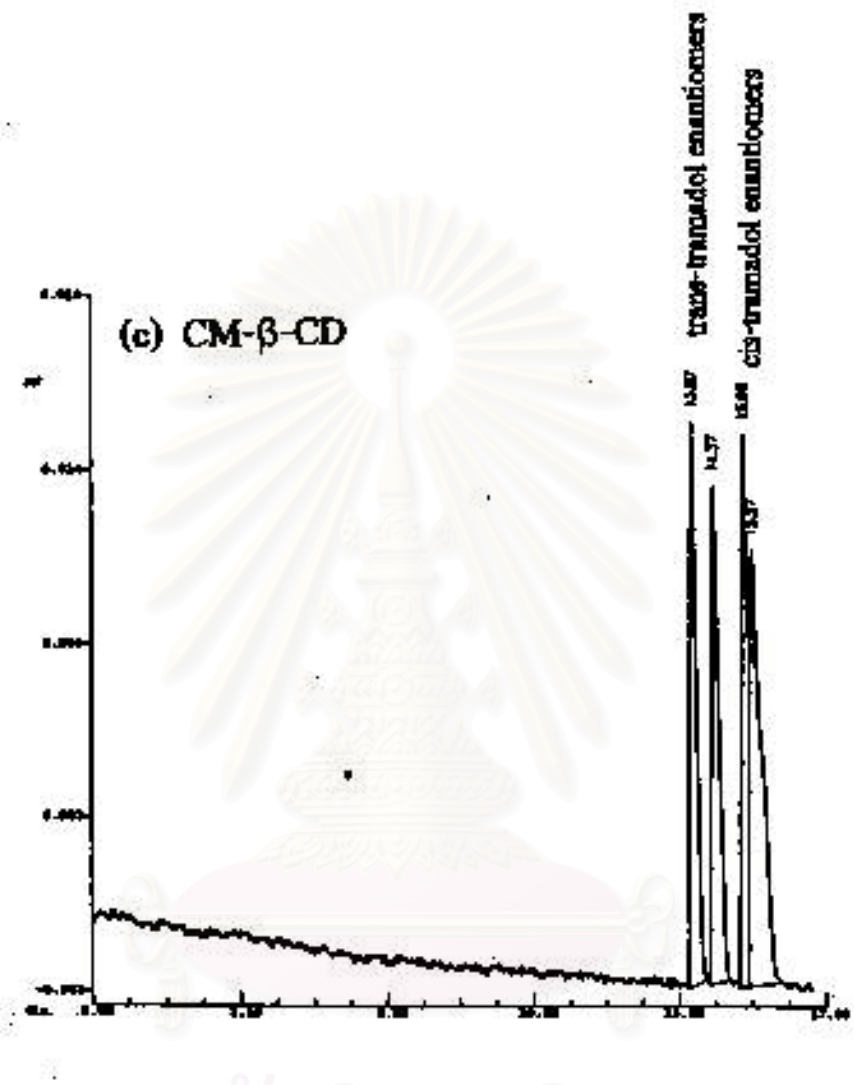


Figure 6 (Continued) Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM phosphate buffer pH 2.5, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

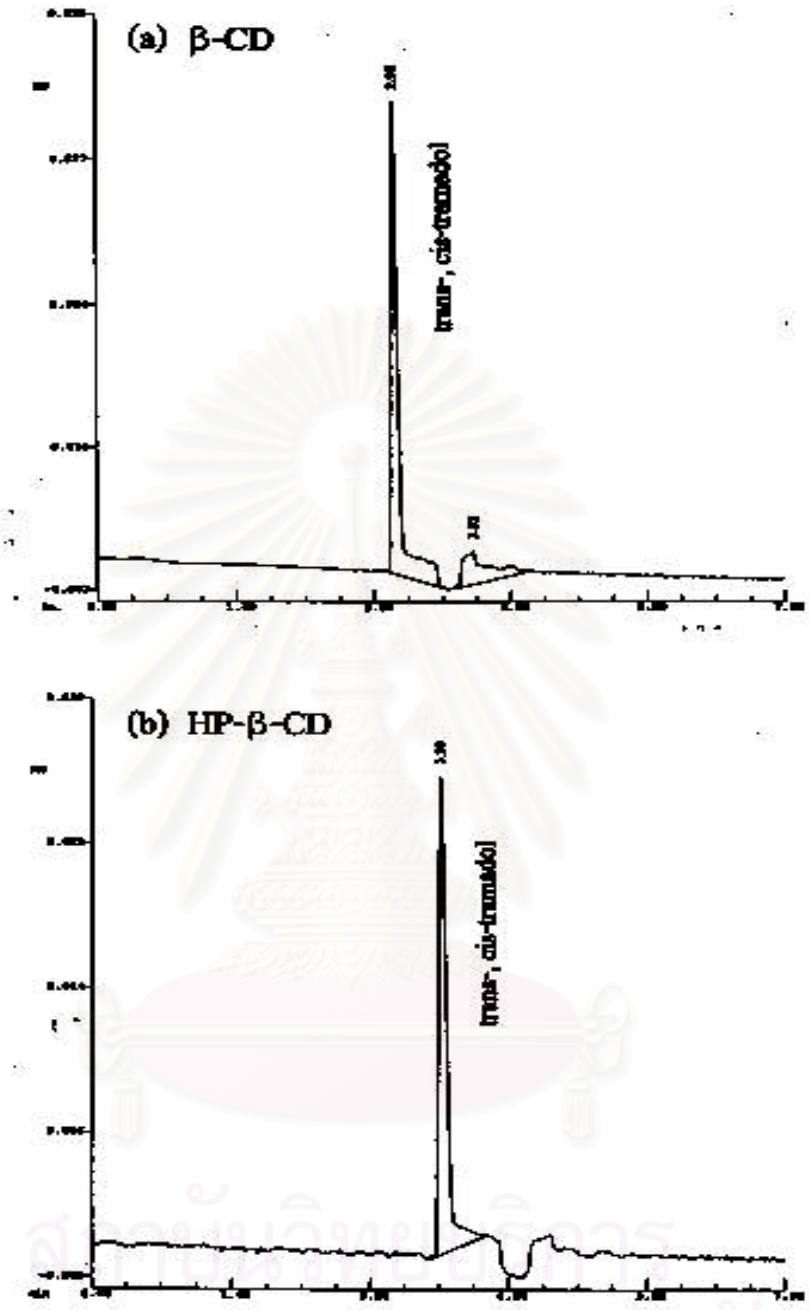


Figure 7 Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM borate buffer pH 9.0, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

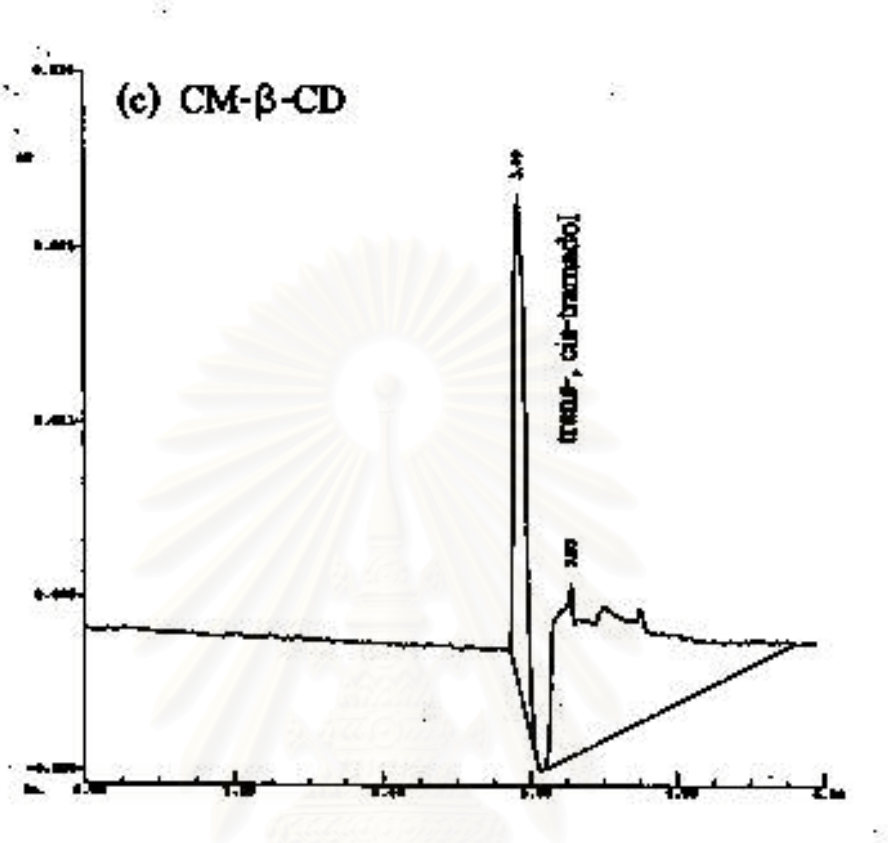


Figure 7 (Continued) Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM borate buffer pH 9.0, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

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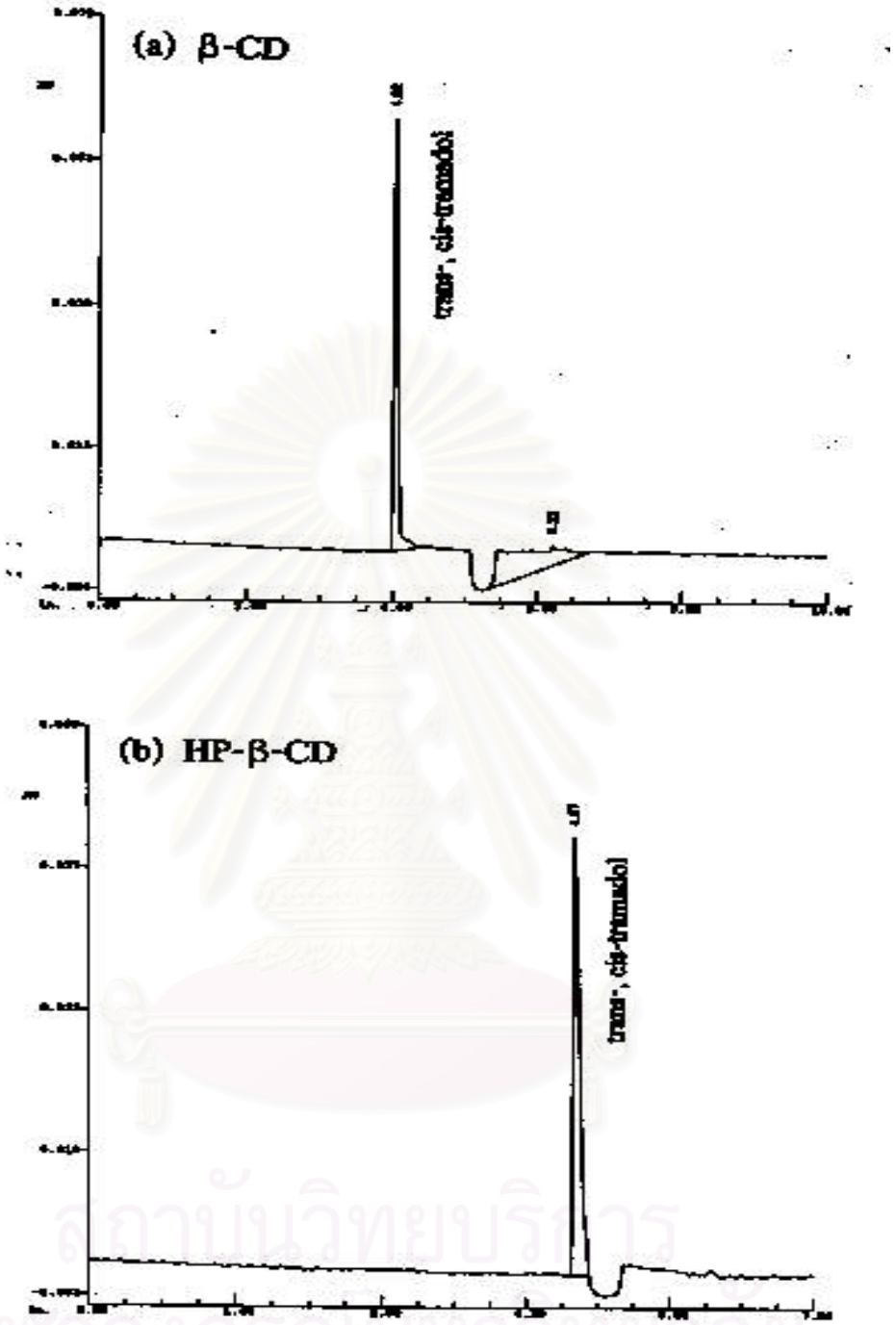


Figure 8 Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM borate buffer pH 10.2, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

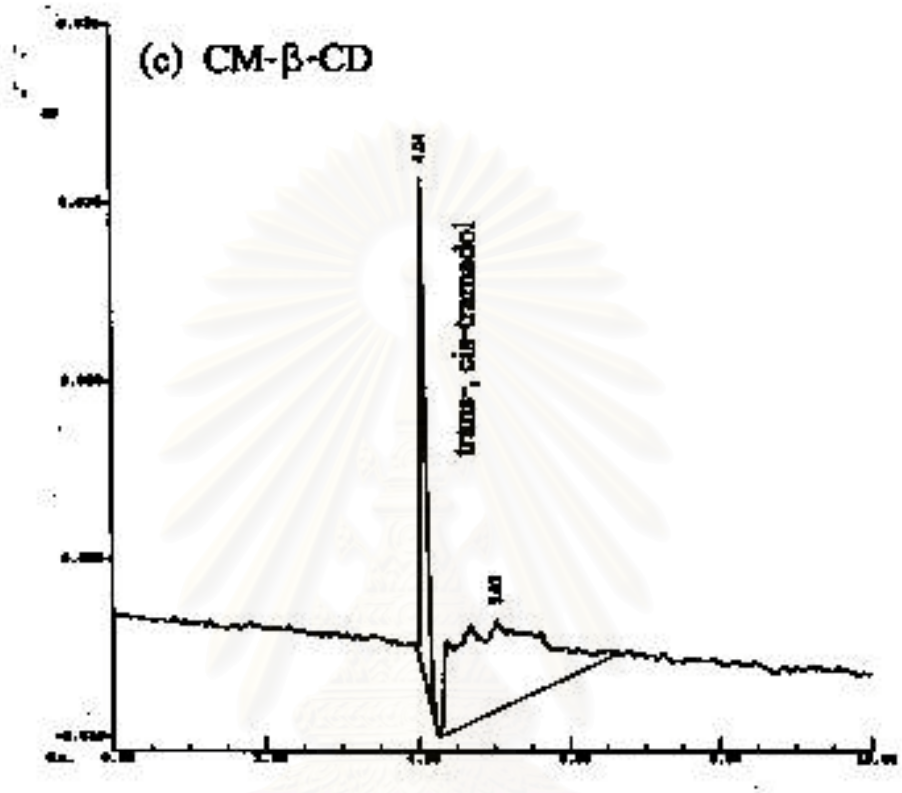


Figure 8 (Continued) Effect of cyclodextrin type on the enantiomeric separation of a racemic mixture of trans-tramadol (50 μg/ml) and cis-tramadol (50 μg/ml) using 2 mM of CD in 50 mM borate buffer pH 10.2, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary 50 cm x 50 μm i.d.

CM-β-CD can be used both in an ionized or a neutral mode depending on the buffer pH used. At pH > 5, the carboxylic functions are deprotonated and the appearing charges lead to a CD mobility enabling the separation of neutral species At pH <4, CM-β-CD is protonated and can be used for ionic species (Maruszak et al., 2001). In contrast to CM-β-CD, β-CD and HP-β-CD are neutral in the whole pH range available in CZE.

Under the experimental operating conditions of pH 2.5, the chiral selector, CM-β-CD was protonated. Enantioseparation of tramadol involved interactions that based on the inclusion of a bulky hydrophobic part, the aromatic ring, in the hydrophobic cavity of CM-β-CD. This inclusion alone was not enough for chiral recognition. An additional requirement was the secondary interactions had to occur, especially the hydrogen bonding between the carboxy functional groups of CM-β-CD and the hydrogen atom attached to the N (ammonium ion moiety) of tramadol. This hydrogen bonding seemed to be fundamental for producing the effective stereoselective interaction for tramadol enantiomeric resolving by stabilizing the complex formed between CM-β-CD and enantiomers of trans- and cis-tramadol. The net velocity of the complex differed from the velocity of the free analyte. Therefore, complex formation would result in an average velocity of the analyte, which is different from the velocity of the free analyte. As a consequence, a sufficient difference in complex stability between the two enantiomers of trans- and cis-tranmadol, would result in a difference in the average velocity of these compounds. Moreover, the EOF was relatively low compared to the average velocity of the complexes formed. Hence, the simultaneous enantioseparation of trans- and cis-tramadol was achieved.

As the pH of the running buffer was increased to 9.0 and 10.2, the degree of ionization of tramadol was decreased while that of CM-β-CD was increased. The amount of positively charged tramadol was relative low compared to that of the

negatively charge CM-β-CD. As a consequence, the formation of the inclusion complex was less favored by a drastic decrease in the H-bonding, even though there was somewhat the electrostatic attraction between negatively charged CM-β-CD and positively charged cis- and trans-tramadol enantiomers. At these two pHs the EOF was very high, that the tramadol enantiomers and the chiral selector were swept through the capillary too fast, there might not be sufficient time for both formation of inclusion complex between CM-β-CD and tramadol enantiomers and separation of the analyte zones. Therefore, no enantiomeric separation of tramadol was osberved at pH 9.0 and 10.2 of the running buffer.

At pH 2.5, chiral separation of cis- and trans-tramadol was achieved with CM-β-CD but not with β-CD and HP-β-CD. Therefore the presence of the carboxy groups on the CM-β-CD rims seemed to be fundamental for producing effective stereoselective interaction for trans- and cis-tramadol enantiomeric resolution. At low pH, these interactions were probably mainly due to the hydrogen bonding capabilities of the carboxy functional groups of the CM-β-CD and also to the different steric discrimination properties towards the trans- and cis-tramadol enantiomers when located in the CD cavity.

From the experimental results, the CM-β-CD in 50 mM phosphate buffer at pH 2.5 was found to be the best chiral selector for the stereoselective determination of trans- and cis-tramadol enantiomers.

Optimization of CD concentration is extremely important. There is often an optimum CD concentration, above and below which resolution may be decreased. At optimum CD concentration, the difference in the apparent mobility between the two enantiomers reaches a maximum.

To study the influence of the CM-β-CD concentration, the analyses were performed using a BGE at pH 2.5 supported with different amounts of CM-β-CD in the range of 7-13 mM.

Figure 9 shows the influence of the CM-β-CD concentration on the enantioseparation of trans- and cis-tramadol, using the running buffer at pH 2.5. At any concentration of CM-β-CD, trans-tramadol exhibited a shorter migration time and a higher enantiomeric separation than cis-tramadol. The results implied that the cis-tramadol exhibited a stronger CD complex than trans-tramadol.

As the CM-β-CD concentration was increased, the migration time increased in an almost linear fashion (Figure 10a). The higher cyclodextrin concentration naturally favors complex formation so the analyte spends more time in complexed form. The charge-to-size ratio is reduced and likewise its mobility. In order to maximize enantioselectivity, one should obviously maximize the difference in mobility between the two enantiomers. The difference in the apparent mobility between the two enantiomers of cis-tramadol seemed to reach a maximum at 8 mM of CM-β-CD as shown in Figure 10b.

Increasing the concentration of CM-β-CD to 13 mM increased the migration time of both trans- and cis-tramadol enantiomers with resulted in unacceptable long analysis time. Hence, CM-β-CD at the concentration of 13 mM was excluded from the study. Consequently, a 8 mM CM-β-CD was selected as it provided acceptable resolution within a reasonable analysis time.

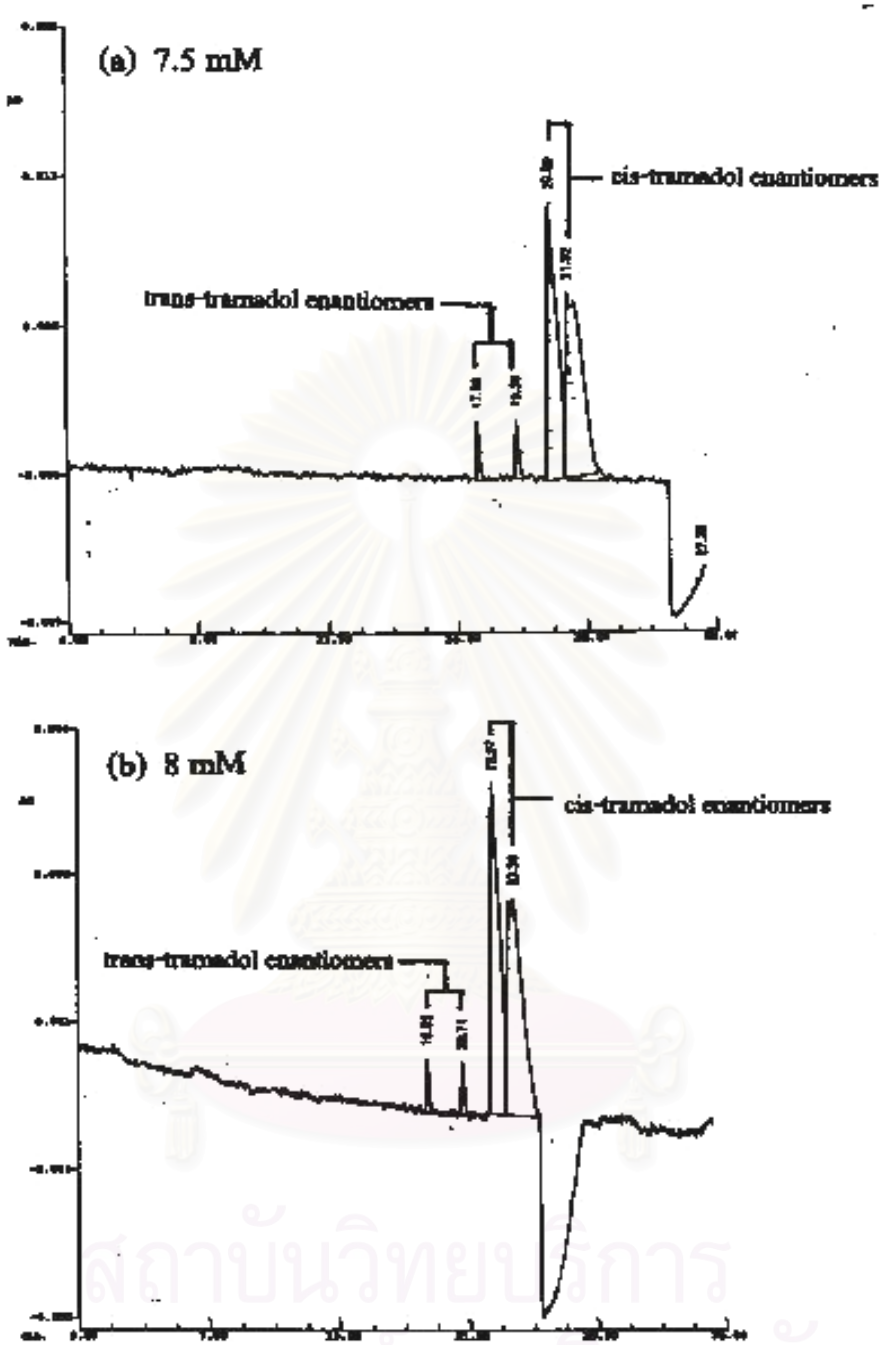


Figure 9 Effect of CM-β-CD concentration on the enantioseparation of a racemic mixture of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) using 50 mM phosphate buffer pH 2.5, temperature 25 °C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 μm i.d.

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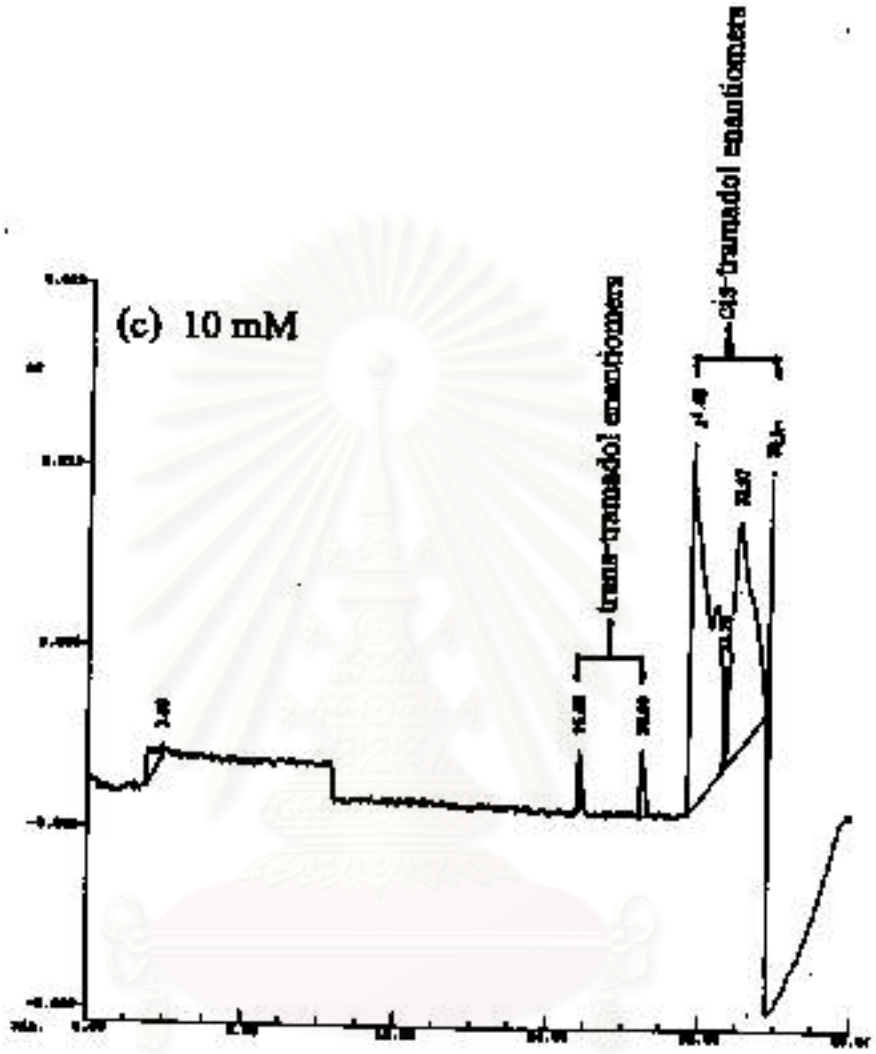
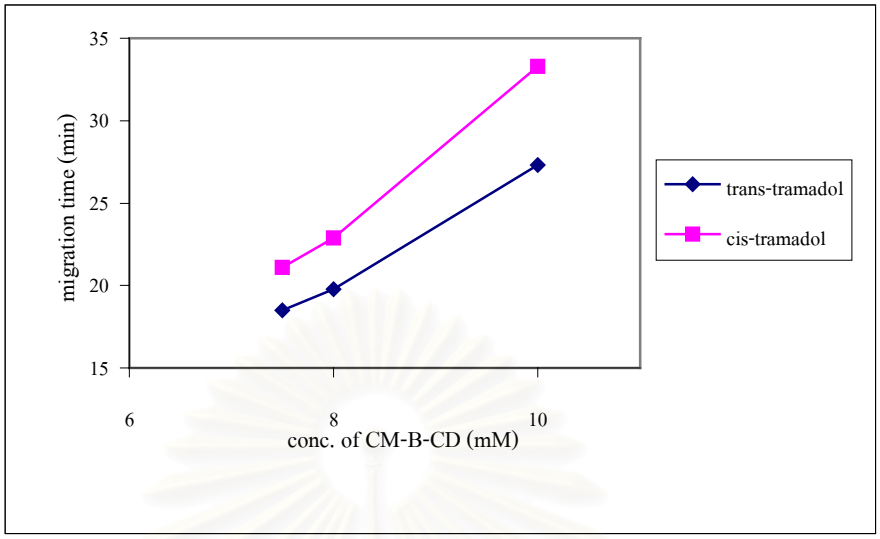
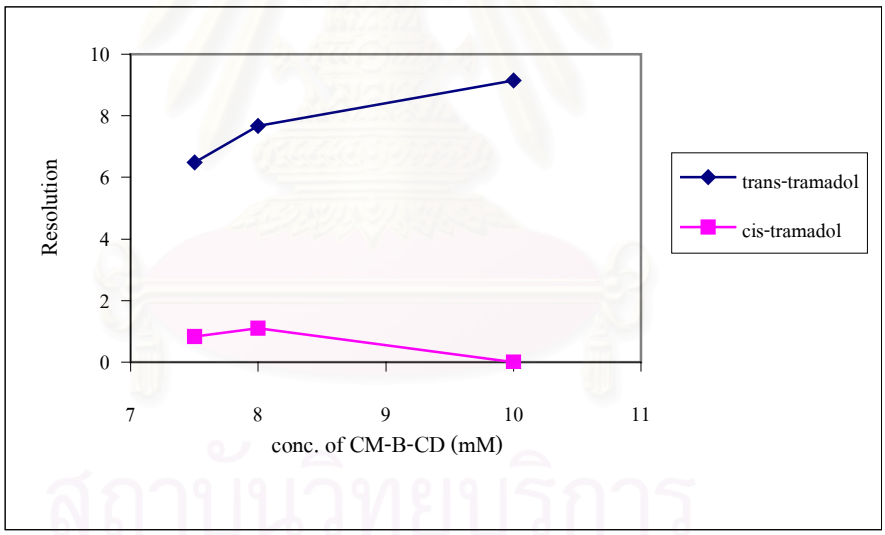



Figure 9 (Continued) Effect of CM-β-CD concentration on the enantioseparation of a racemic mixture of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) using 50 mM phosphate buffer pH 2.5, temperature 25 $^{o}$C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 μm i.d.




(a)




(b)

Figure 10 Effect of CM-β-CD concentration on (a) migration time and (b) resolution of a racemic mixture of trans-tramadol (7.5 µg/ml) and cis-tramadol (150 µg/ml)

### 3. Effect of buffer concentration

Figure 11 shows the impact of buffer ionic strength (50-65 mM) on the enantioseparation of trans- and cis-tramadol. As expected, increasing the ionic strength decreased the mobility by shielding the effective charge and therefore an increase in migration time. At the same time, resolution increases with increasing phosphate concentration in the BGE (Figure 12).

The phosphate buffer concentration of 65 mM was selected for providing better resolution of enantiomers, especially cis-tramadol enantiomers.

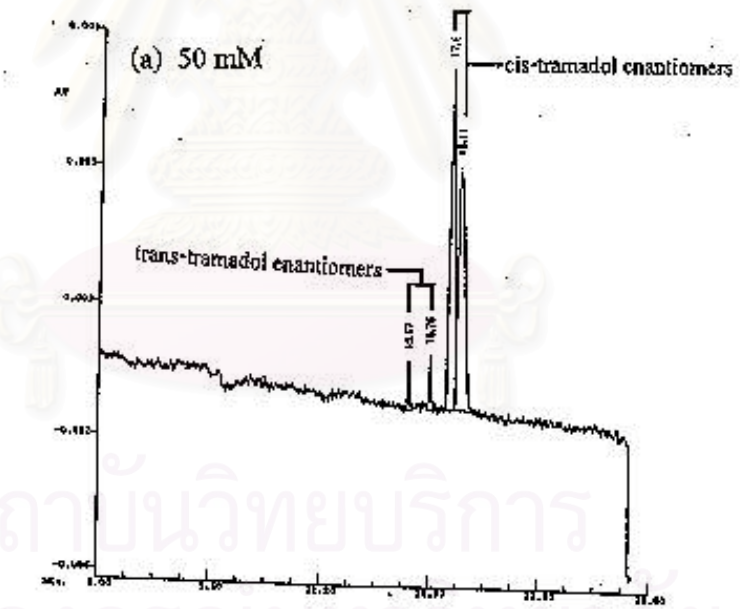



Figure 11 Effect of buffer concentration on the enantioseparation of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) using phosphate buffer at pH 2.5 containing 8 mM of CM-β-CD, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 μm i.d.

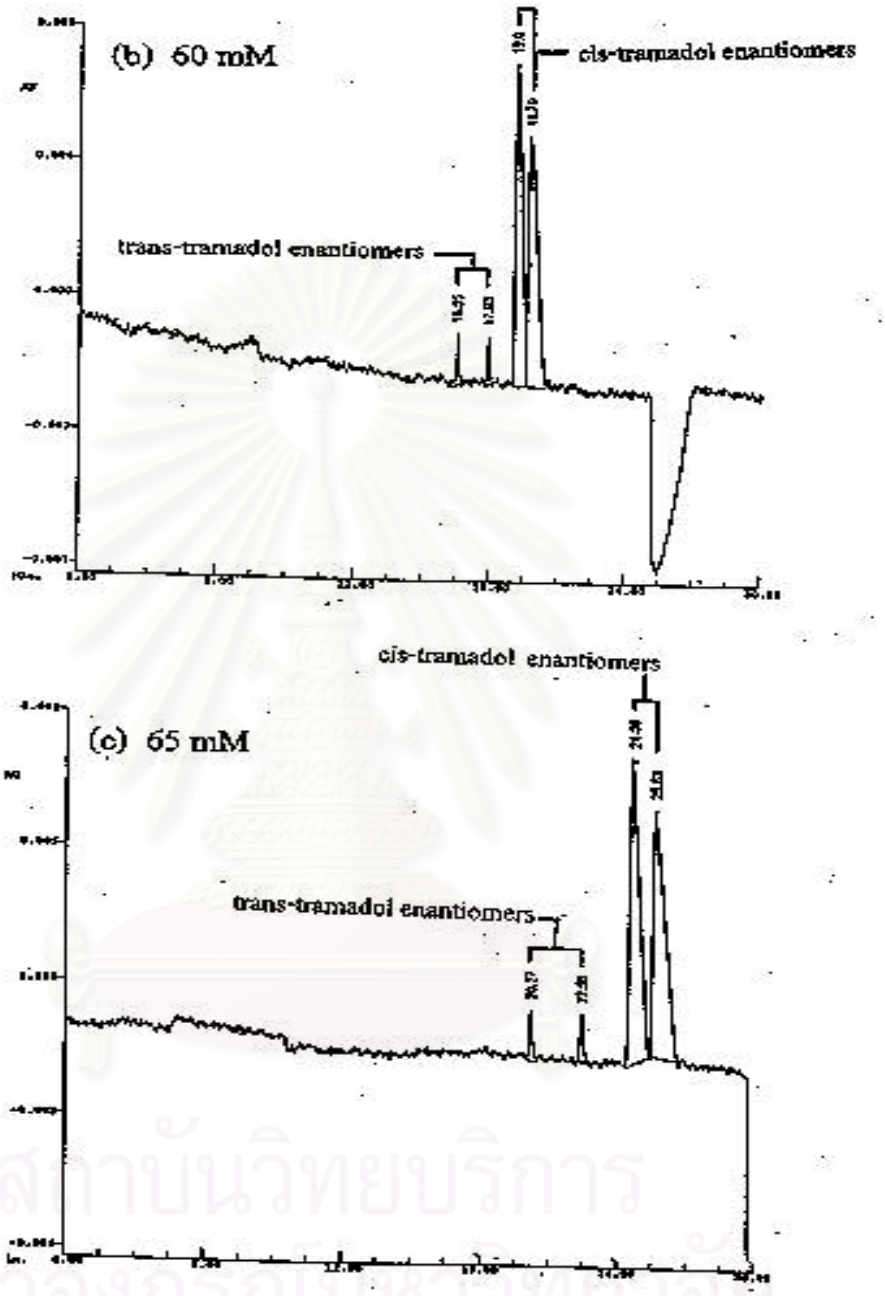



Figure 11 (Continued) Effect of buffer concentration on the enantioseparation of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml) using phosphate buffer at pH 2.5 containing 8 mM of CM-β-CD, temperature 25°C, applied voltage 15 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 μm i.d.

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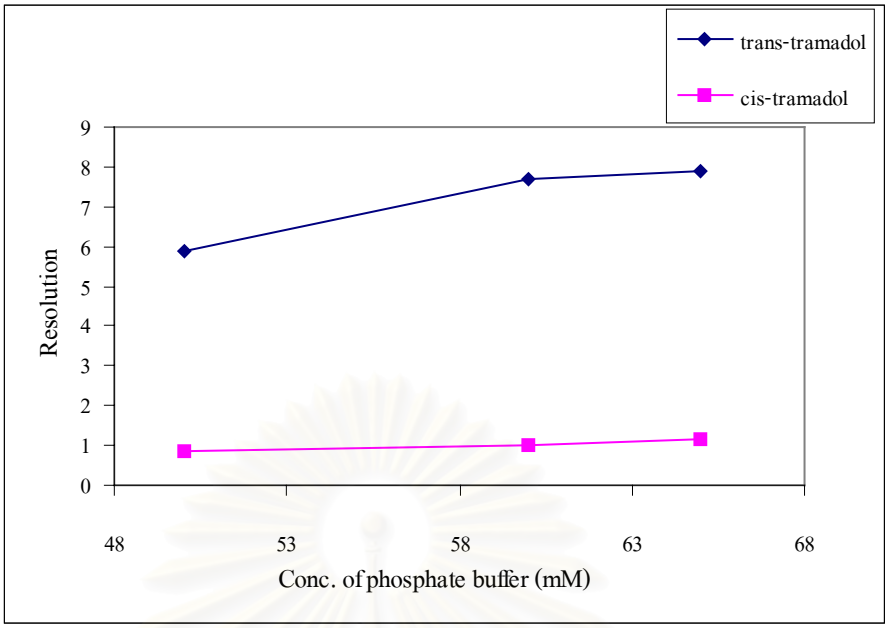



Figure 12 Effect of buffer concentration on the resolution of a racemic mixture of trans-tramadol (7.5 μg/ml) and cis-tramadol (150 μg/ml)

### C. Optimization of secondary experimental parameters

Five factors at 3 levels were selected for study the optimization of the CZE conditions for the separation of enantiomers of tramadol hydrochloride. These factors were the concentrations of CM-β-CD and phosphate buffer, 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 4, the concentrations of CM-β-CD, pH of BGE, the concentration of phosphate buffer, the applied voltage and capillary temperature 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.

The nominal levels of the operating conditions were a 65 mM phosphate buffer at pH 2.5, containing 8 mM CM-β-CD and operated at $25^0$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 the enantiomers of cis-tramadol and the analysis time considered as the migration time of the last eluting peak ($t_m$)

Resolution was calculated as :

$$R_s = \frac{1.18\ (t_2 - t_1)}{W_{0.5}(1) + W_{0.5}(2)} \qquad \text{..... (3)}$$

Where $t_1$ and $t_2$ are the migration times of the first and second eluting enantiomer, $W_{0.5}(1)$ and $W_{0.5}(2)$ are peak widths measured at half 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 7 and 10 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:

$$Q^* = \frac{T_m^* + 2R_s^*}{3} \qquad \text{..... (4)}$$

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 arbitrarily 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;

$$E_x = \frac{\sum Y_{x(+1)}}{4} - \frac{\sum Y_{x(-1)}}{4} \qquad \text{.....(5)}$$

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

$$\%E_x = \frac{E_x}{\bar{Y}} \cdot 100 \qquad \text{.....(6)}$$

where $\bar{Y}$ is the average of all results for a particular response

$$\bar{Y} = \frac{\sum Y_x}{8} \qquad \text{.....(7)}$$

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;

$$\text{S.E.} = \sqrt{\frac{\sum E_{(Di)}^{2}}{n_i}} \qquad \text{.....(8)}$$

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 $2 \cdot \%$S.E. (Massart et al., 1997).

The results of design I and II are shown in Table 7-9 and 10-12, respectively, and visualized by effect-plots in Figure 13-17.

Table 7 Results of experimental design I

| Experiment | Response (Y) | | Transformed data | | Q* |
|---|---|---|---|---|---|
| | $T_m$(min) | $R_s$ | $T_m$* | $R_s$* | |
| 1 | 24.68 | 0.94 | 1 | 0.20 | 0.47 |
| 2 | 38.55 | 1.32 | 0.36 | 0.80 | 0.66 |
| 3 | 26.08 | 0.94 | 0.94 | 0.19 | 0.44 |
| 4 | 26.95 | 1.01 | 0.90 | 0.31 | 0.51 |
| 5 | 38.67 | 1.22 | 0.36 | 0.66 | 0.56 |
| 6 | 37.20 | 0.82 | 0.42 | 0 | 0.14 |
| 7 | 46.42 | 1.44 | 0 | 1 | 0.67 |
| 8 | 42.54 | 0.87 | 0.18 | 0.09 | 0.12 |
| Average Y | 35.14 | 1.07 | - | - | 0.44 |

Table 8 Effects of factor calculated from results of experimental design I

| Effects | $T_m$ | $R_s$ | Q* |
|---|---|---|---|
| F1 (CD) | -2.65 | -0.04 | 0.00 |
| F2 (pH) | 3.89 | 0.32 | 0.28 |
| F3 (PO4) | -7.02 | -0.13 | -0.04 |
| F4 (Volt) | -1.27 | 0.21 | 0.24 |
| F5 (Temp) | -12.08 | -0.08 | 0.10 |
| F6 (D1) | 0.41 | 0.04 | 0.04 |
| F7 (D2) | 3.91 | 0.07 | 0.01 |
| 2・SE | 5.56 | 0.11 | 0.06 |

Table 9 Normalized effects of factor calculated from results of experimental design I

| Normalized effects | $T_m$ | $R_s$ | Q* |
|---|---|---|---|
| %F1 (CD) | -7.53 | -3.29 | 0.60 |
| % F2 (pH) | 11.06 | ***29.98*** | ***64.12*** |
| % F3 (PO4) | ***-19.97*** | ***-12.45*** | -8.02 |
| %F4 (Volt) | -3.62 | ***19.65*** | ***55.20*** |
| %F5 (Temp) | ***-34.39*** | -7.69 | ***21.76*** |
| %F6 (D1) | 1.17 | 4.14 | 9.27 |
| %F7 (D2) | 11.14 | 6.28 | 2.77 |
| 2・%SE | 15.83 | 10.64 | 13.68 |

Significant effects are bold and italic letters.

Table 10 Results of experimental design II

| Experiment | Response (Y) | | Transformed data | | Q* |
|---|---|---|---|---|---|
| | $T_m$(min) | $R_s$ | $T_m$* | $R_s$* | |
| 1 | 60 | 0 | 0 | 0 | 0 |
| 2 | 60 | 0 | 0 | 0 | 0 |
| 3 | 17.03 | 0.93 | 1 | 0.73 | 0.82 |
| 4 | 23.3 | 1.27 | 0.85 | 1 | 0.95 |
| 5 | 60 | 0 | 0 | 0 | 0 |
| 6 | 24 | 1.05 | 0.84 | 0.82 | 0.83 |
| 7 | 60 | 0 | 0 | 0 | 0 |
| 8 | 18.08 | 0.90 | 0.98 | 0.71 | 0.80 |
| Average | 40.30 | 0.52 | - | - | 0.42 |

Table 11 Effects of factor calculated from results of experimental design II

| Effects | $T_m$ | $R_s$ | Q* |
|---|---|---|---|
| E(CD) | 3.05 | 0.12 | 0.04 |
| E(pH) | 39.40 | -1.04 | -0.85 |
| E(PO4) | -0.09 | -0.05 | -0.02 |
| E(Volt) | -0.44 | 0.06 | 0.04 |
| E(Temp) | -0.44 | 0.06 | 0.04 |
| E(D1) | 3.05 | 0.12 | 0.04 |
| E(D2) | -0.09 | -0.05 | -0.02 |
| 2・SE | 4.31 | 0.18 | 0.07 |

Table 12 Normalized effects of factor calculated from results of experimental design II

| Normalized effects | $T_m$ | $R_s$ | Q* |
|---|---|---|---|
| %E(CD) | 7.56 | 23.34 | 9.40 |
| % E(pH) | ***97.76*** | ***-200*** | ***-200*** |
| %E(PO4) | -0.22 | -9.48 | -5.92 |
| %E(Volt) | -1.08 | 11.89 | 8.41 |
| %E(Temp) | -1.08 | 11.89 | 8.41 |
| %E(D1) | 7.56 | 23.34 | 9.40 |
| %E(D2) | -0.21 | -9.48 | -5.91 |
| 2・%SE | 10.70 | 35.63 | 15.70 |

Significant effects are bold and italic letters.

**1. Influence of CM-β-CD concentration.**

The CM-β-CD concentration was the only factor that did not produce any significant effects on the migration time and resolution of tramadol enantiomers as shown in Table 9 and 12 and Figure 13. The favorable effects of CM-β-CD on both resolution and migration time in the high concentration range (8.0 – 8.5 mM) were observed but not statistically significant effect. This might be due to the selected CM-β-CD concentration ranges (7.5 – 8.0 mM) and (8.0 – 8.5 mM) were too narrow to cause any effect on the viscosity of the BGE as well as the EOF.

A 8.5 mM of CM-β-CD was selected as a compromise between resolution and migration time.

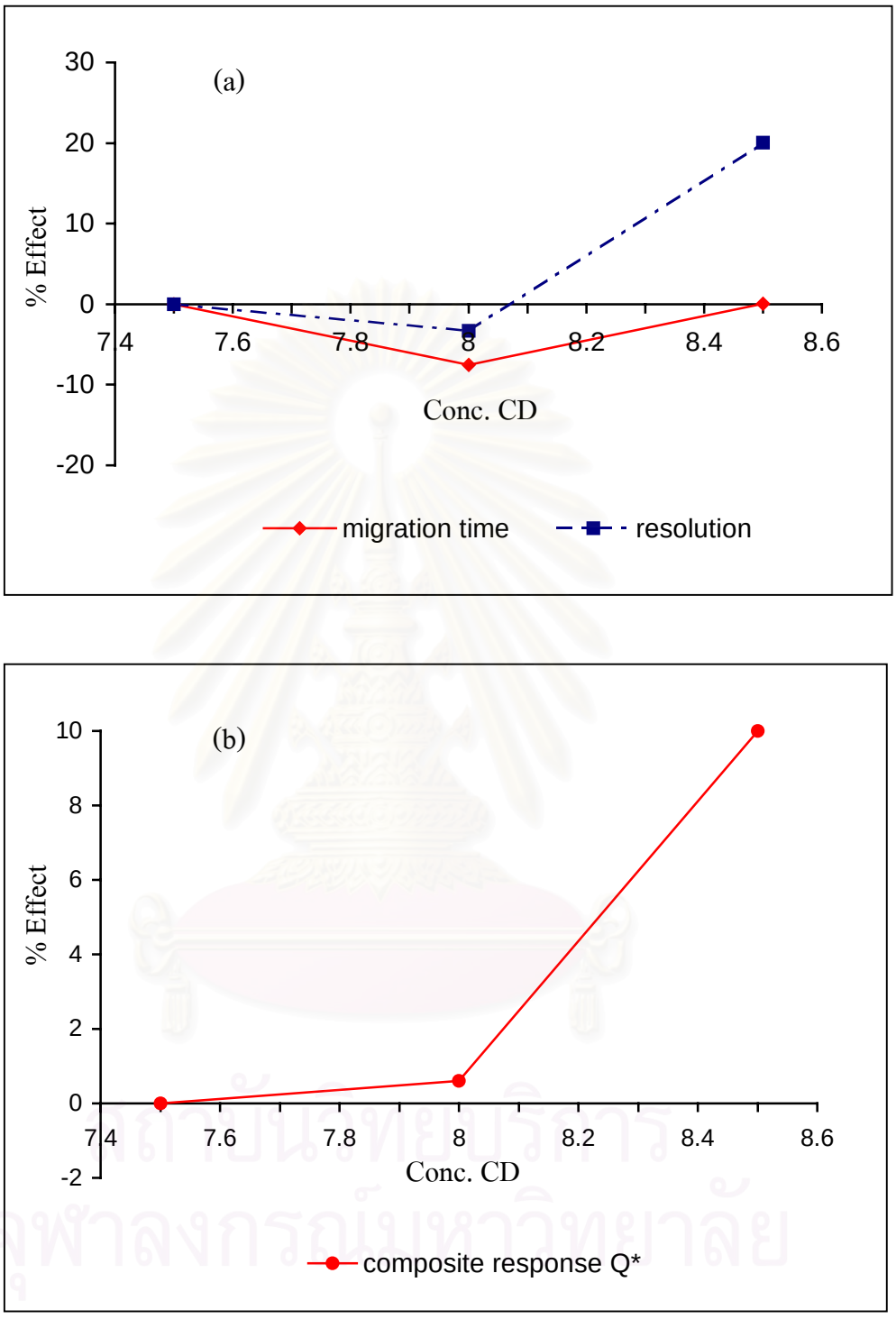



Figure 13 Effect – plots of the concentration of CM-β-CD on (a) migration time and resolution and (b) composite response, Q*

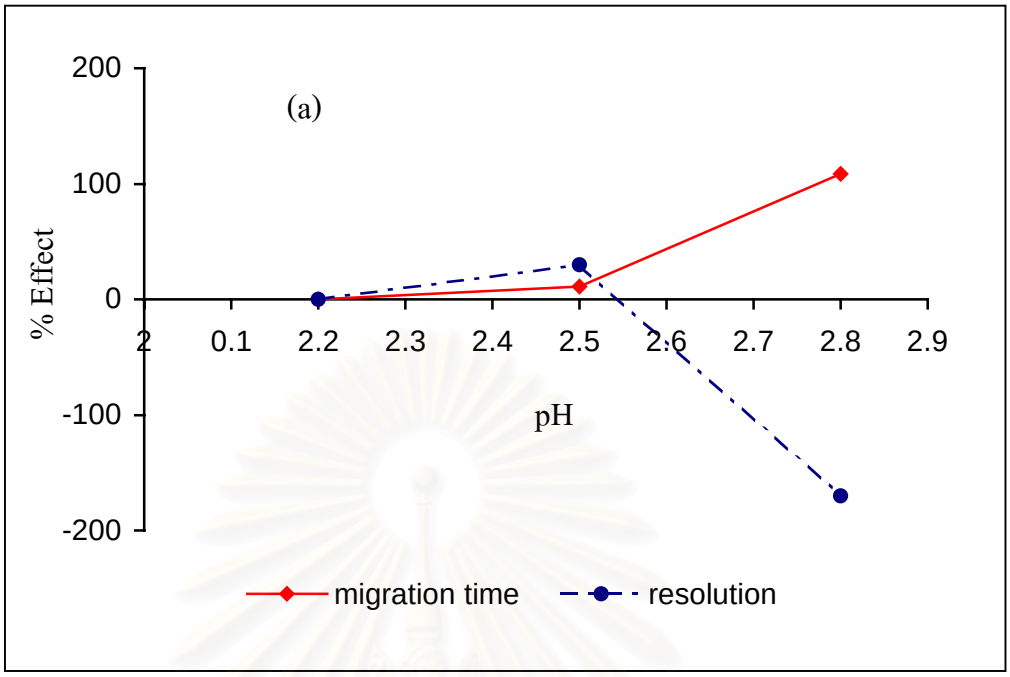
**2. Influence of pH**

From the results of Table 9 and 12 and Figure 14 , it was obvious that the BGE at higher pH (2.5 – 2.8) provided a significantly longer migration time. A similar but not statistically significant effect was also observed in the lower pH range (2.2 – 2.5). Tramadol hydrochloride which has a pKa of 9.4 would be essentially protonated throughout the pH range used in this study. As the pH is increased, carboxylic functional groups of CM-β-CD are probably partially deprotonated, leading to an enhancement of the stability of the labile diastereomeric complexes formed between tramadol enantiomers and the CD. Consequently, a decrease in the electrophoretic mobility and an increase in the migration time.

The effect of pH on the improvement in resolution was statistically significantly only in the low pH range which was clearly seen in the significant effect on the composite response, Q*.

In the high pH range, the significant negative effect on the composite response, Q*, was evident that the increasing pH produced a significant increase in migration time and decrease in resolution. The decreased resolution was very obvious that the higher pH could have a negative influence on the enantioselectivity of CM-β-CD towards tramadol.

The best pH value found from the study was 2.5.



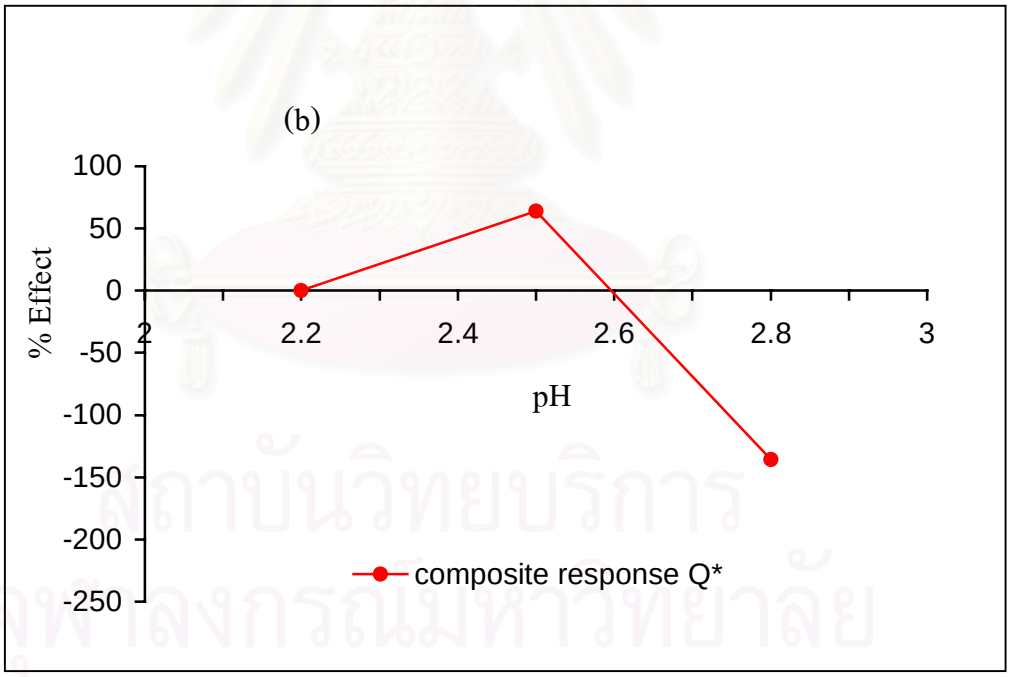




Figure 14 Effect – plots of pH on (a) migration time and resolution and (b) composite response, Q*

**3. Influence of phosphate buffer concentration.**

As shown in Table 9 and 12 and Figure 15, concentration of phosphate buffer showed a significant effect on the shorter migration time and a loss of resolution only in the low phosphate buffer concentration range (55 – 65 mM) but lack of significant negative effects on the composite response, Q*. In the high concentration range (65 – 75 mM) a similar, but not statistically significant effect was observed.

Increasing the buffer ionic strength, the migration time and resolution decreased, presumably as a result of increased current generation and Joule heating.

The optimized ionic strength of phosphate buffer selected was 65 mM.

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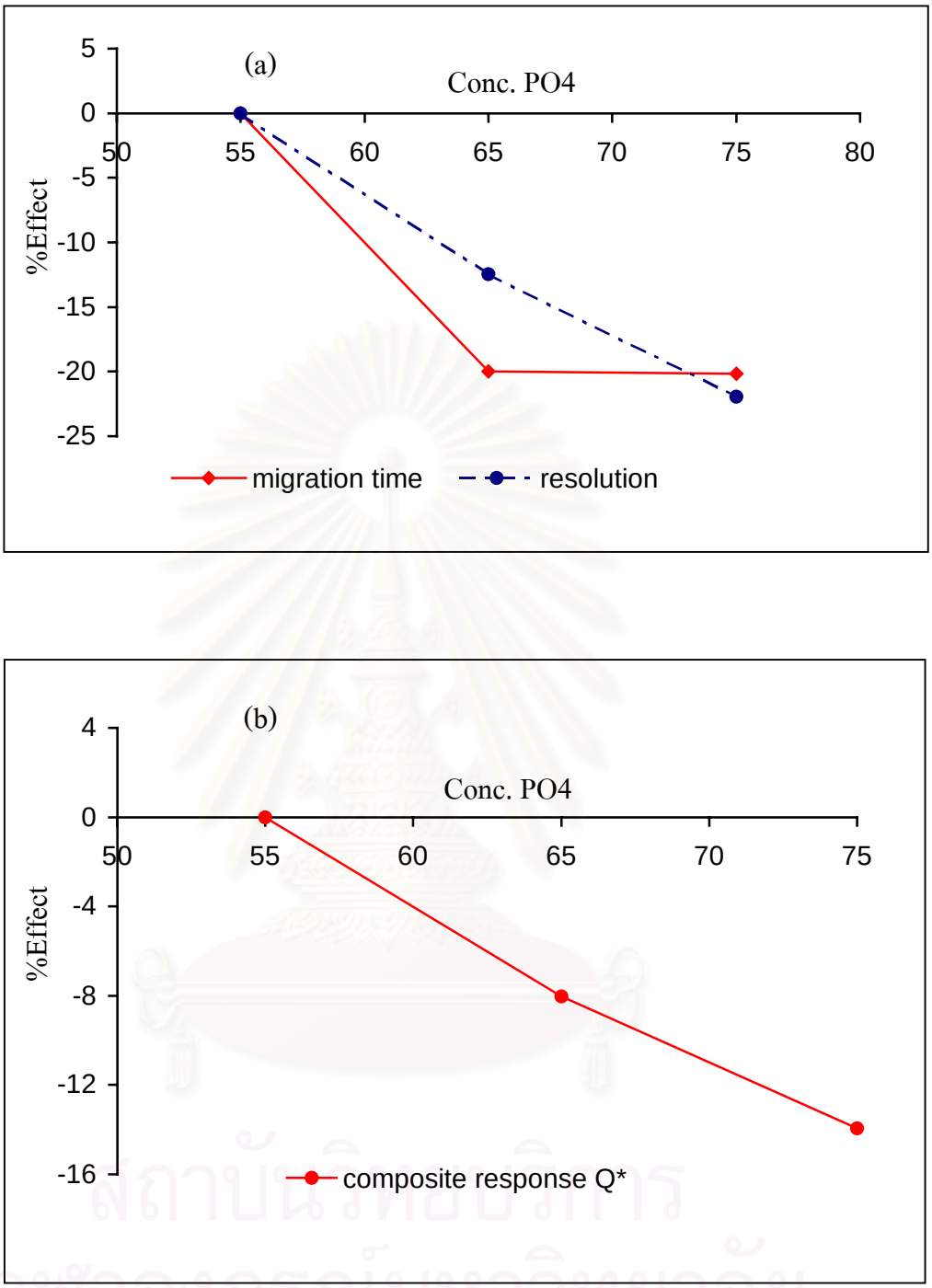


Figure 15 Effect – plots of concentration of phosphate buffer on (a) migration time and resolution and (b) composite response, Q*

**4. Influence of applied voltage**

From the results of Table 9 and 12 and Figure 16, the significant increase in resolution was obvious in the low applied voltage range (12 – 15 kV). A similar result but not statistically significant was observed in the high applied voltage range (15 – 18 kV).

The driving force behind the migration of ions in CE is the field strength (E) applied across the capillary, which is related to the applied voltage by dividing by the total capillary length. Since both the electrophoretic velocity and the EOF are directly proportional to the electric field, higher field strengths will bring about shorter analysis times and higher efficiencies (number of theoretical plates). However, higher applied voltages led to higher currents and increased Joule heating, resulting in a decreased resolution.

The applied voltage of 18 kV was selected for providing shorter migration time with reasonable resolution.

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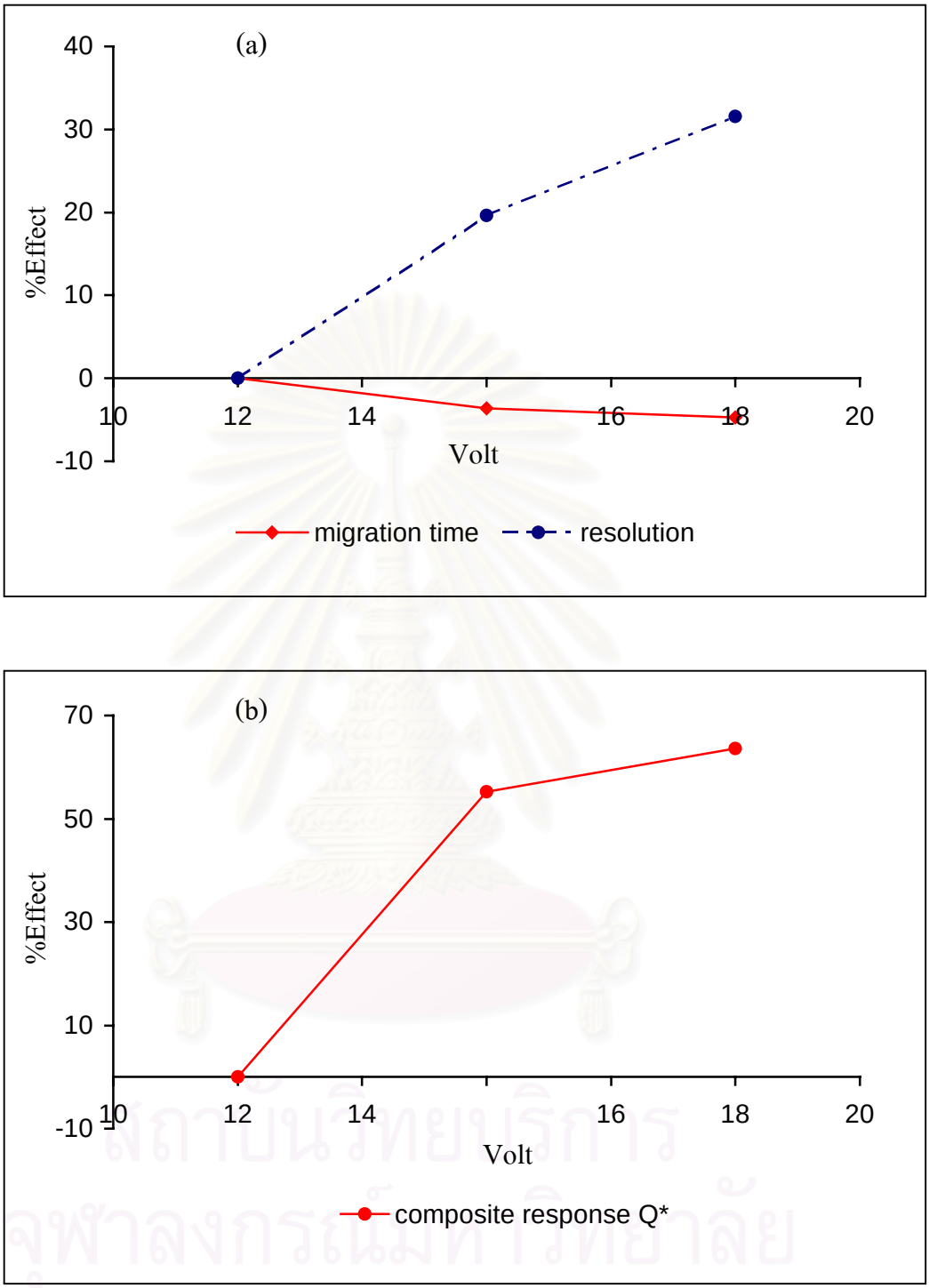


Figure 16 Effect – plots of applied voltage on (a) migration time and resolution and (b) composite response, Q*

**5. Influence of temperature**

As expected, the migration time decreased by increasing the temperature of the capillary due to a decrease in the viscosity of the running buffer, resulting in an increase in electrophoretic mobility of analytes and the EOF. In this study, (Table 9 and 12 and Figure 17) the significant decrease in migration time was only observed in the low temperature range (20 – 25$^{o}$C) which led to a significant effect on the composite response, Q*.

In the low temperature range, the resolution was decreased but not statistically significant. The reduction in resolution with increasing temperature was due to a decrease in the formation constant of the complexes formed between tramadol enantiomers and CM-β-CD. Surprisingly, in this study the resolution obtained from the high temperature range (25–30$^{o}$C) was increased, although not statistically significant.

The temperature at 30$^{o}$C was chosen since it provided shorter migration time and better resolution

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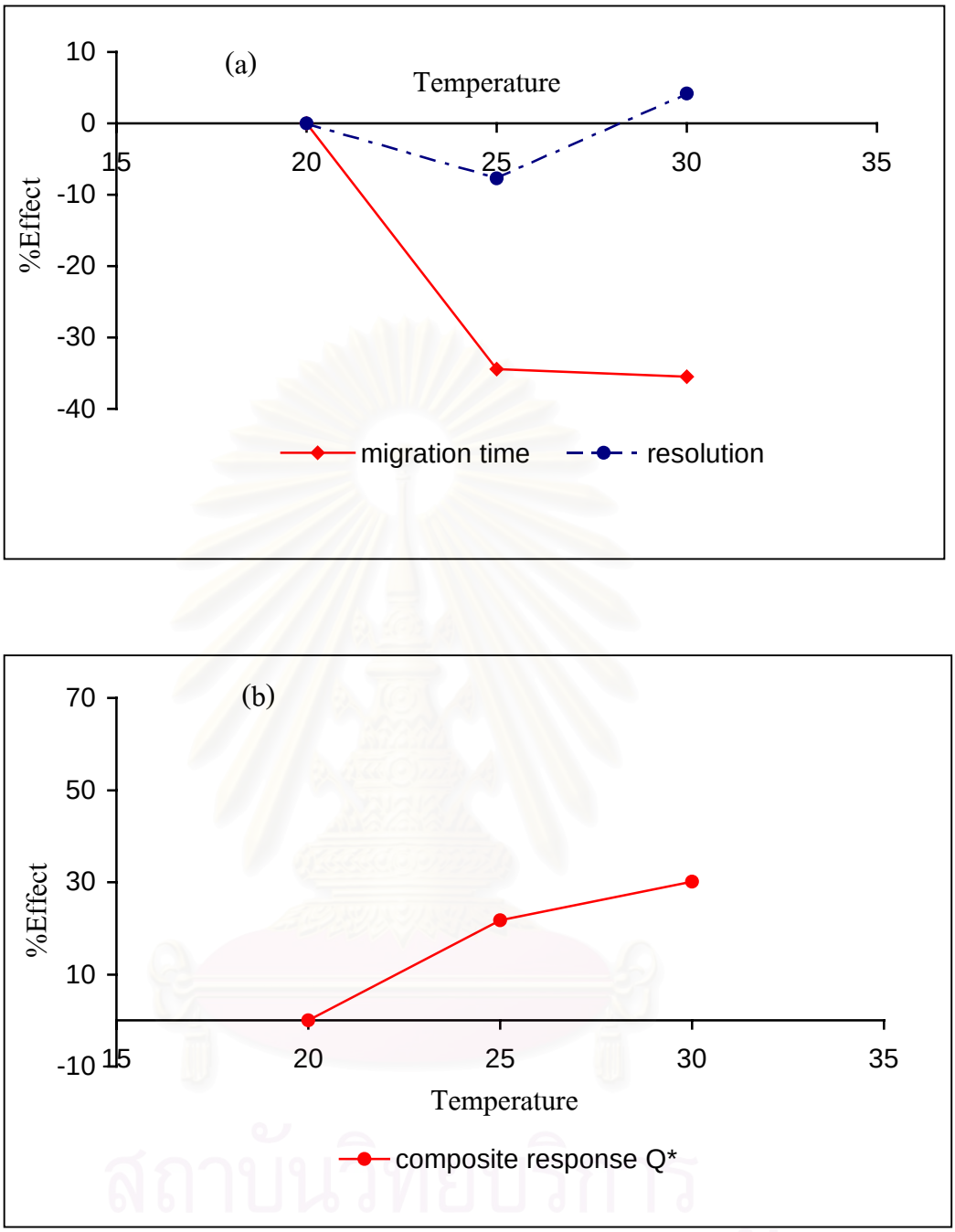


Figure 17 Effect – plots of temperature on (a) migration time and resolution and (b) composite response, Q*

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The optimum condition for enantioseparation of cis- and trans-tramadol was obtained using 8.5 mM of CM-β-CD in 65 mM phosphate buffer at pH 2.5, applied voltage of 18 kV and capillary temperature of 30$^{o}$C. The electropherogram of the optimized condition is shown in Figure 18.

**D. Selection of the internal standard**

Seven compounds (phenformin, d-norpseudoephedrine, pyrimethamine, procaine, levodopa, dopamine and metformin) were screened as the internal standard. Peaks of phenformin, d-norpseudoephedrine, pyrimethamine and procaine were found overlapping with either those of trans- or cis-tramadol enantiomers. Levodopa eluted after the cis-tramadol enantiomers, causing a rather long analysis time. Dopamine and metformin eluted before trans-tramadol enantiomers with baseline resolution of each compound. Both dopamine and metformin gave sharp and symmetrical peaks. Although metformin had shorter migration time, dopamine was more appropriate since it had a rather flat and smoother baseline. Thus, dopamine was selected as an internal standard according to the criteria that it was not present in the tramadol preparations studied, stable, nonreactive, protonated in phosphate buffer at pH 2.5, completely resolved from tramadol enantiomers and eluting before peaks of compounds studied.

The electropherogram of a standard mixture solution of trans- and cis-tramadol and the internal standard, dopamine, was shown in Figure 19.

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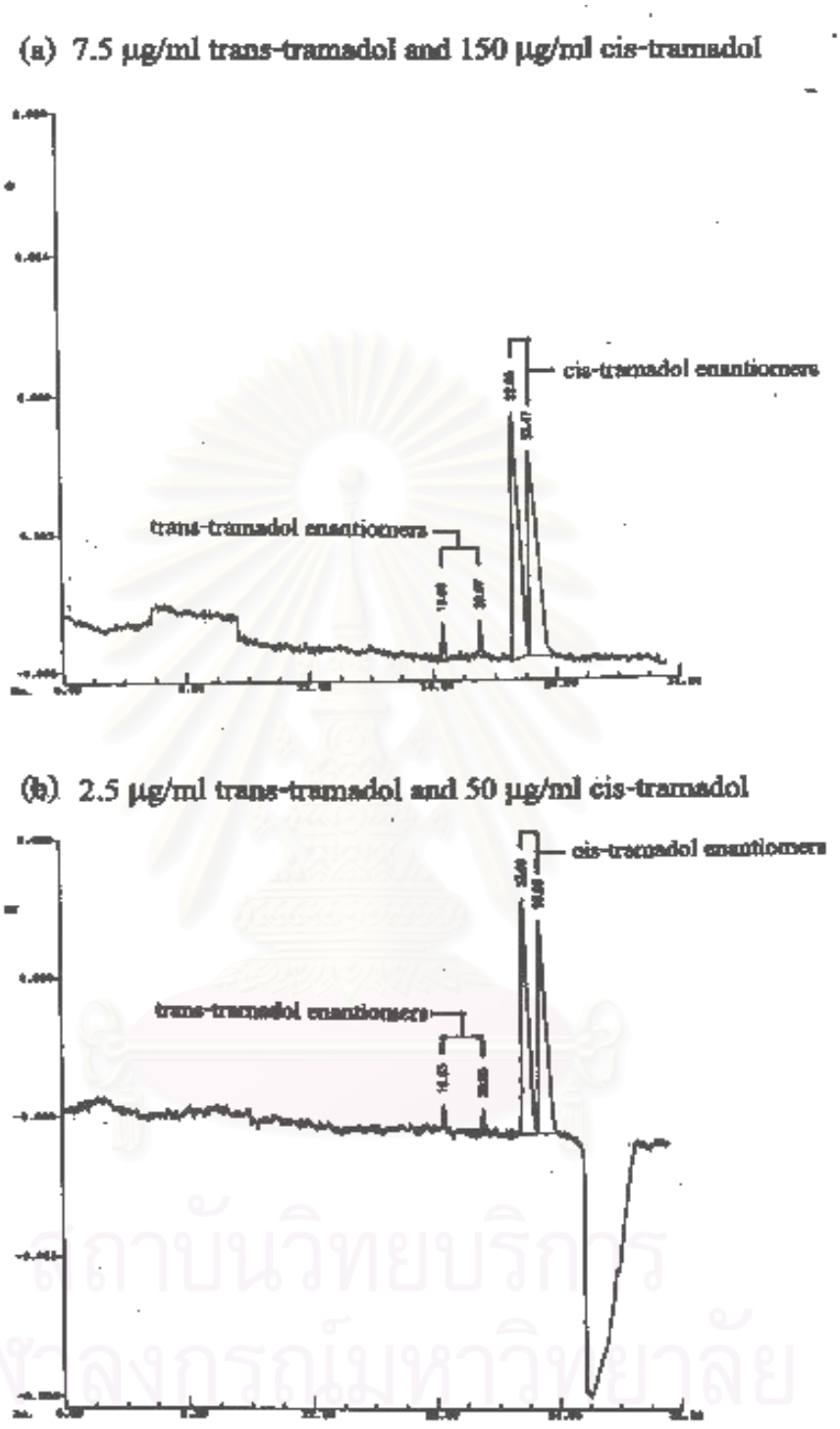
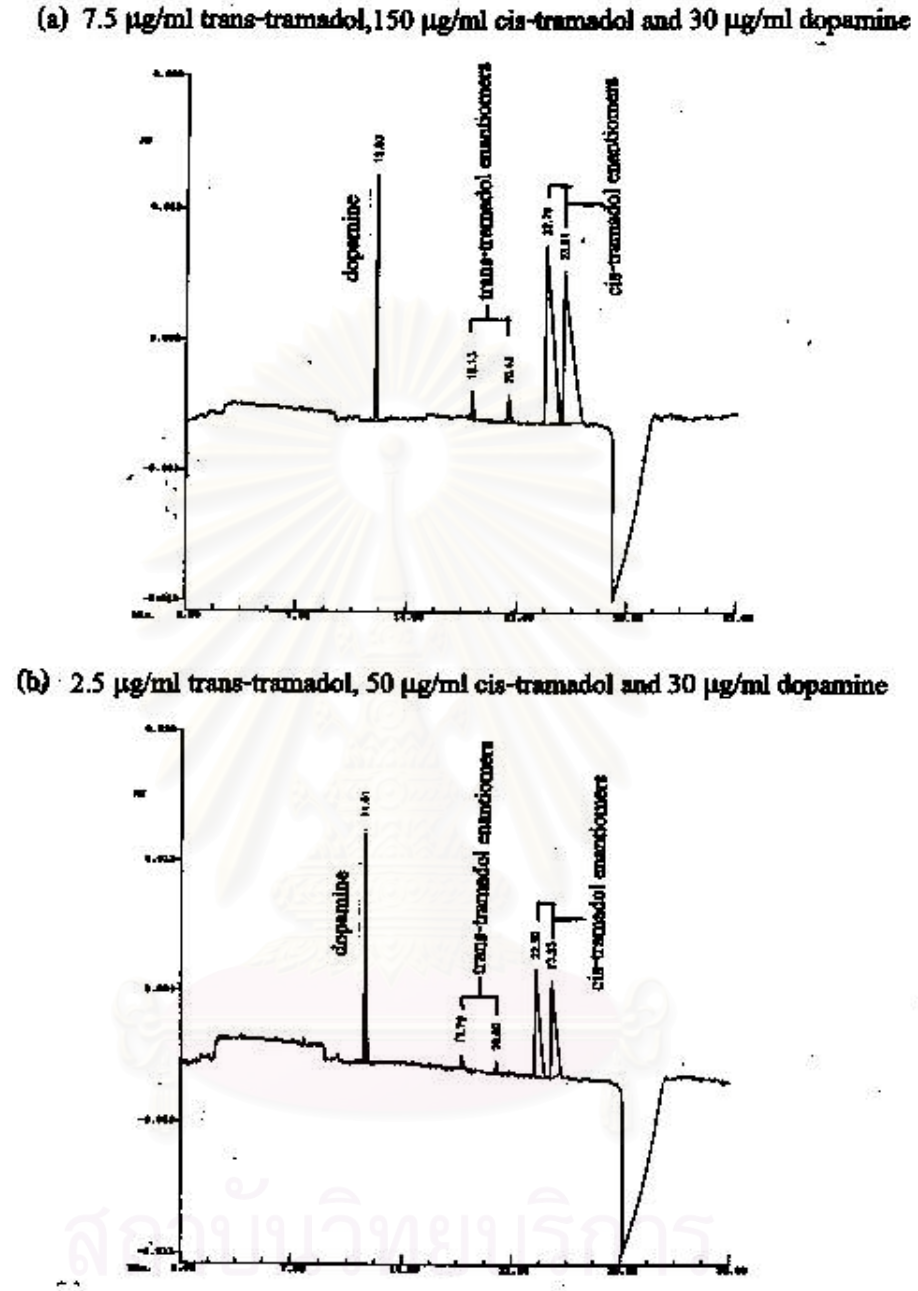



Figure 18 Electropherogram of standard mixture solutions of tramadol using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.




19 Electropherogram of a standard mixture of tramadol and 30 ug/ml

Figure 19 Electropherogram of standard mixture solutions of tramadol and dopamine as an internal standard using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30$^{o}$C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

## E. Analytical method validation

The procedure requires the assessment of selectivity, linearity and range, precision, accuracy, limit of detection (LOD) and quantification (LOQ), stability of standard and sample solutions and system suitability.

### 1. Selectivity

The representative electropherograms in figure 19-31 shown the identity of each separated peak and the separation between main peaks, impurity peaks and the internal standard. The relative migration time of the first ($1^{o}$) and the second ($2^{o}$) peaks of trans-tramadol enantiomers and $1^{o}$ and $2^{o}$ peaks of cis-tramadol enantiomers were 1.43, 1.57, 1.72 and 1.78, respectively, with respect to the migration time of internal standard.

Comparing the electropherogram of samples with that of the standard, the order of elution and migration times of the internal standard, trans-tramadol enantiomers and cis-tramadol enantiomers of samples corresponded with those of standard. No interferences from sample impurities were observed. Therefore, the developed CZE method has acceptable selectivity.

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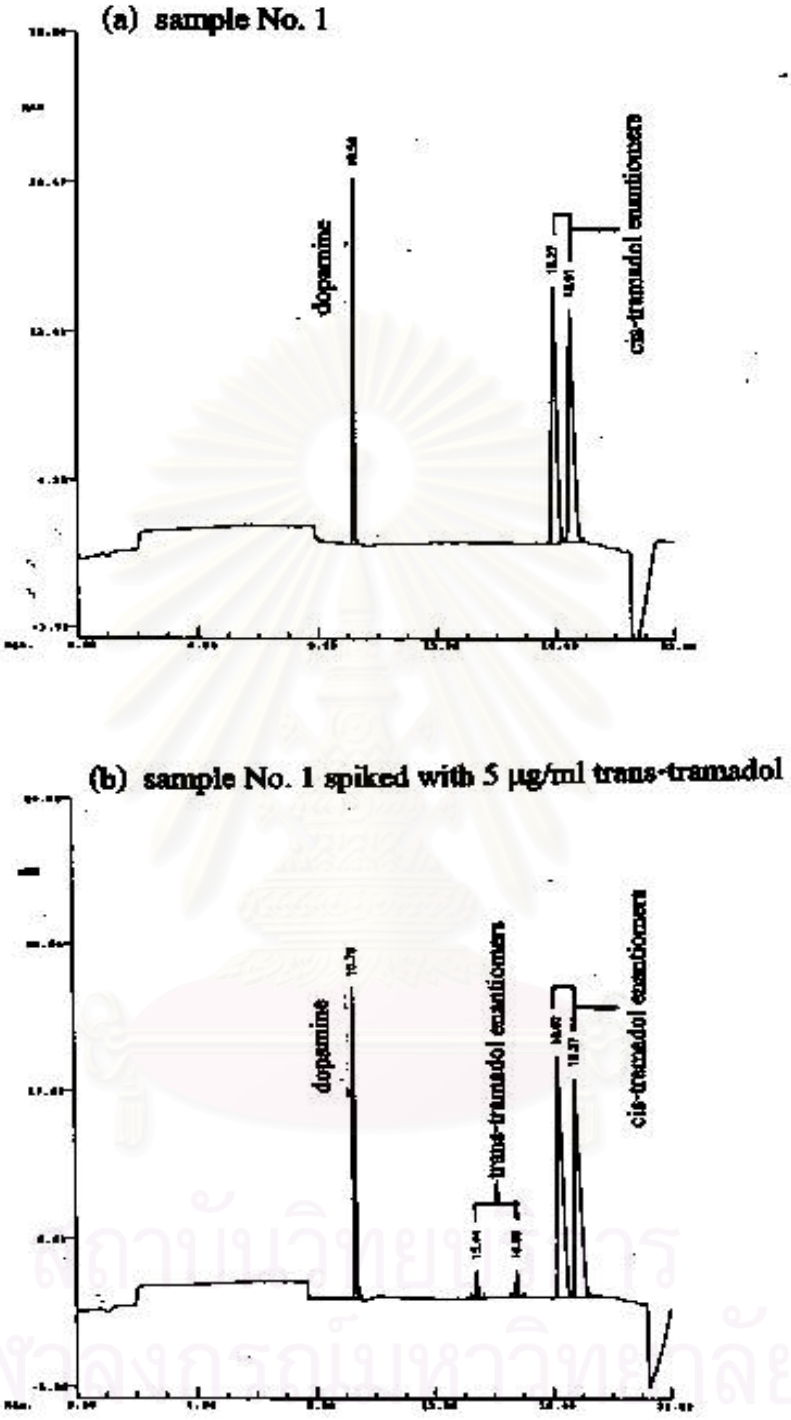


Figure 20 Electropherogram of cis-tramadol enantiomers in Tramadol Injection, sample No. 1 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

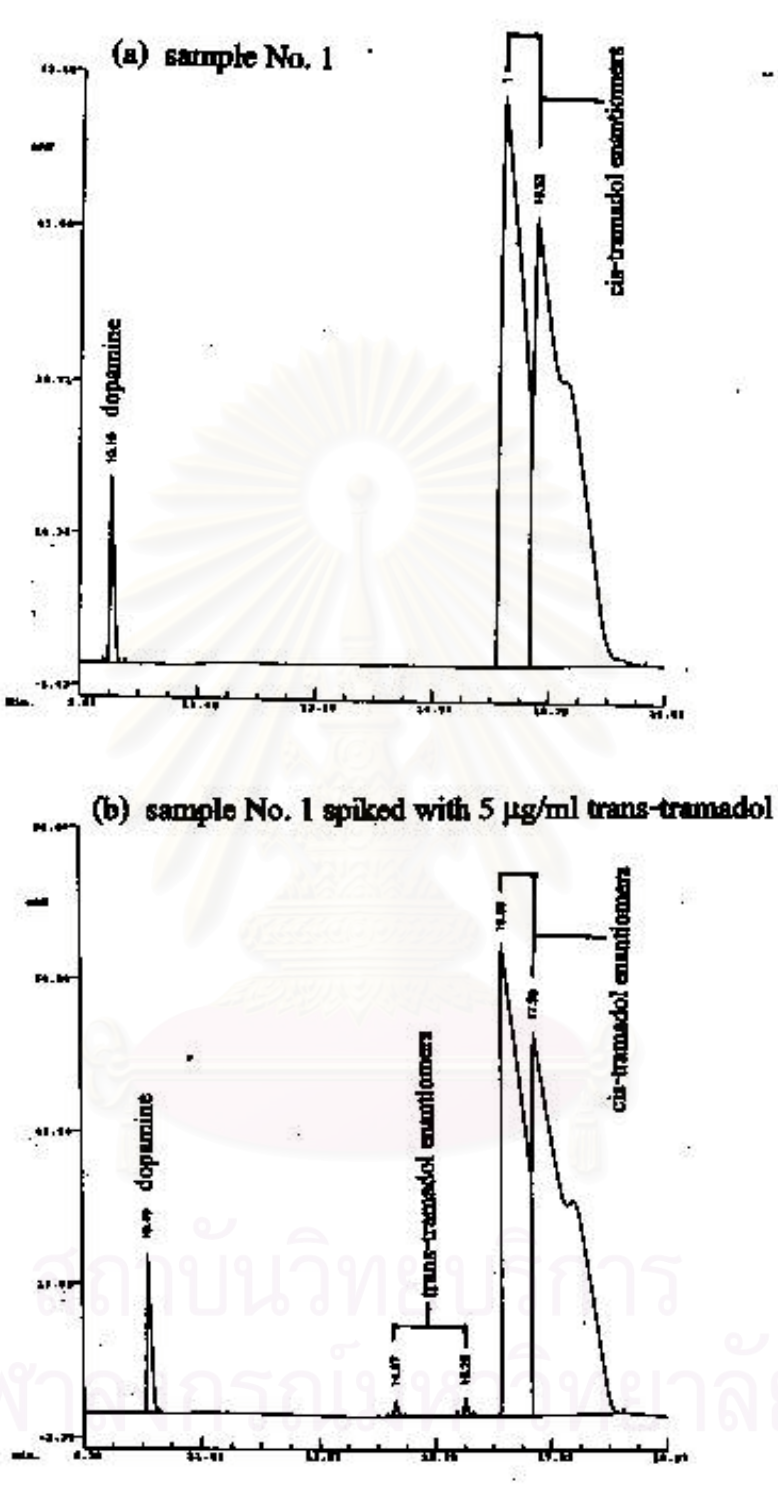


Figure 21 Electropherogram of trans-tramadol enantiomers in Tramadol Injection, sample No. 1 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

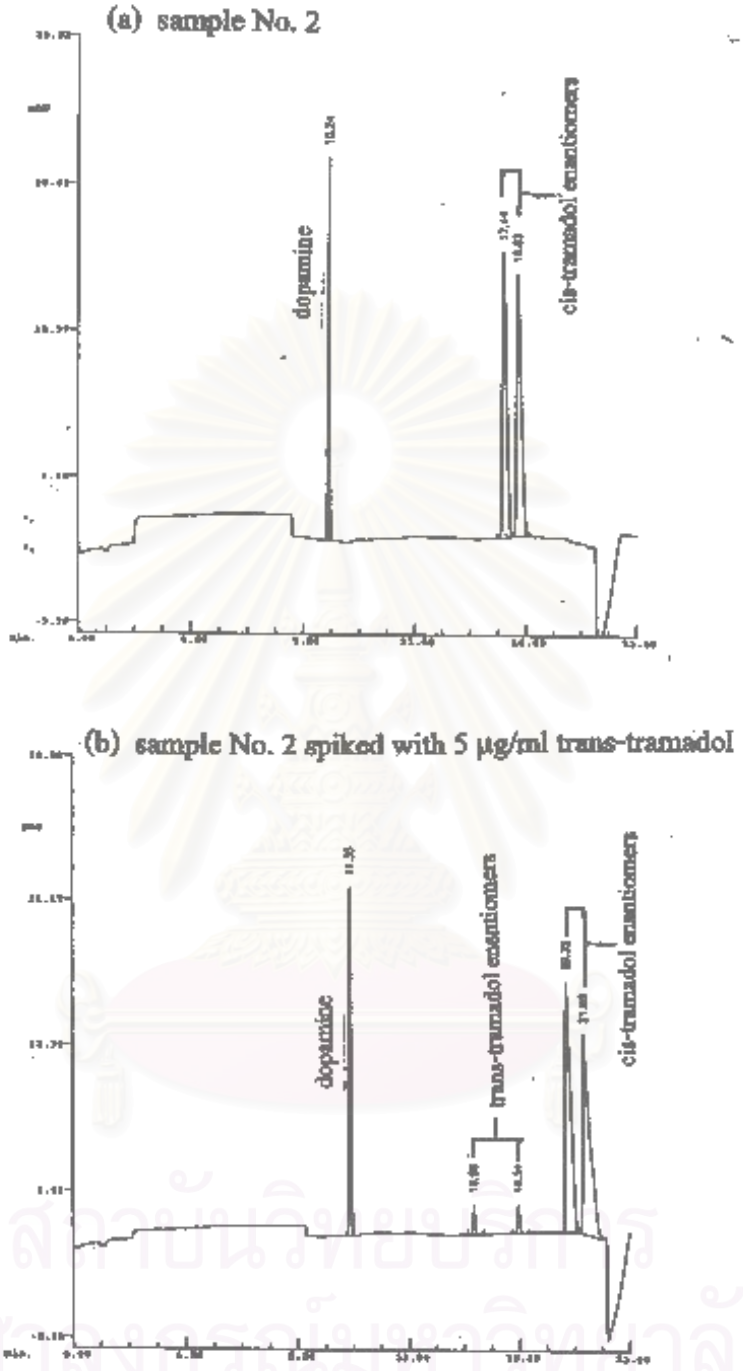


Figure 22 Electropherogram of cis-tramadol enantiomers in Tramadol Injection, sample No. 2 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30$^{o}$C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

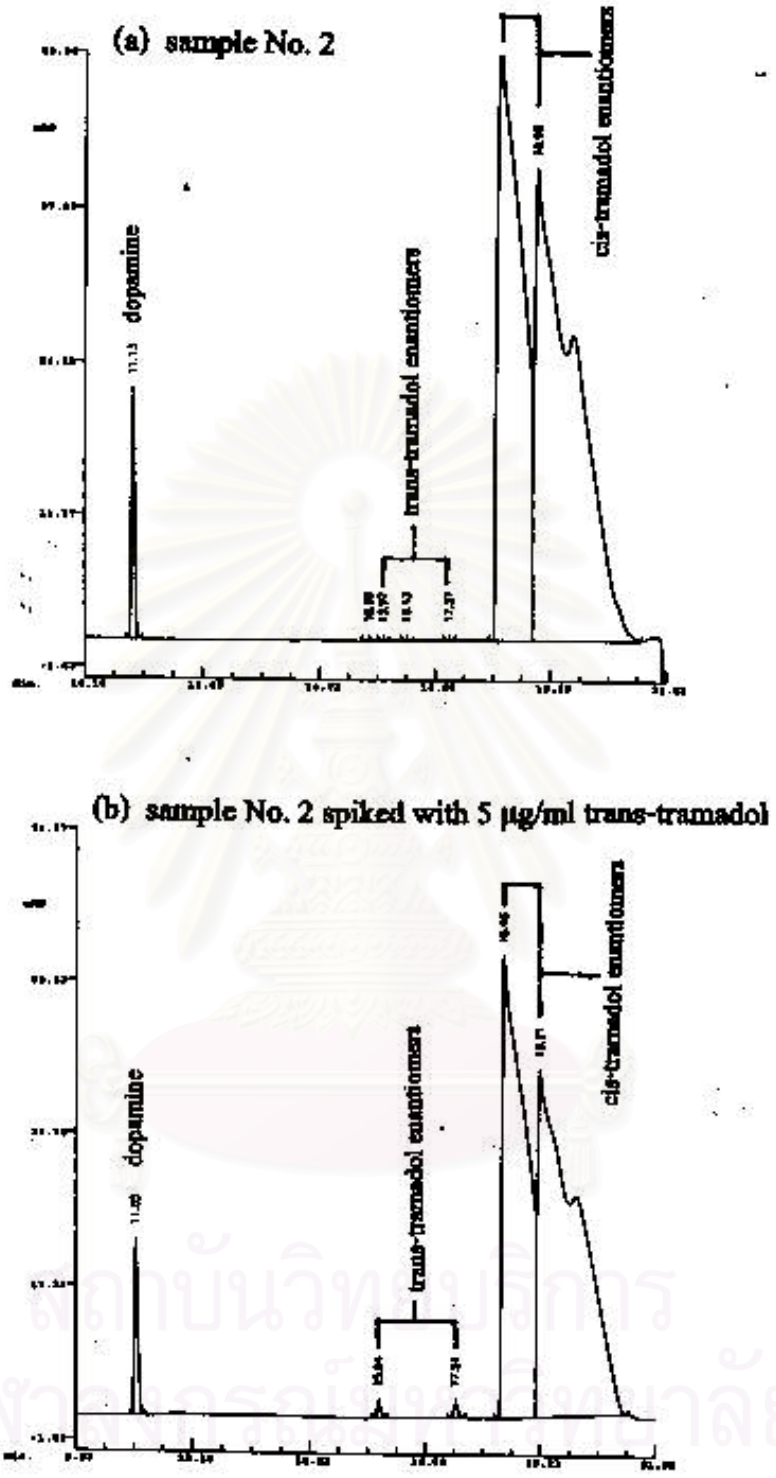


Figure 23 Electropherogram of trans-tramadol enantiomers in Tramadol Injection, sample No. 2 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

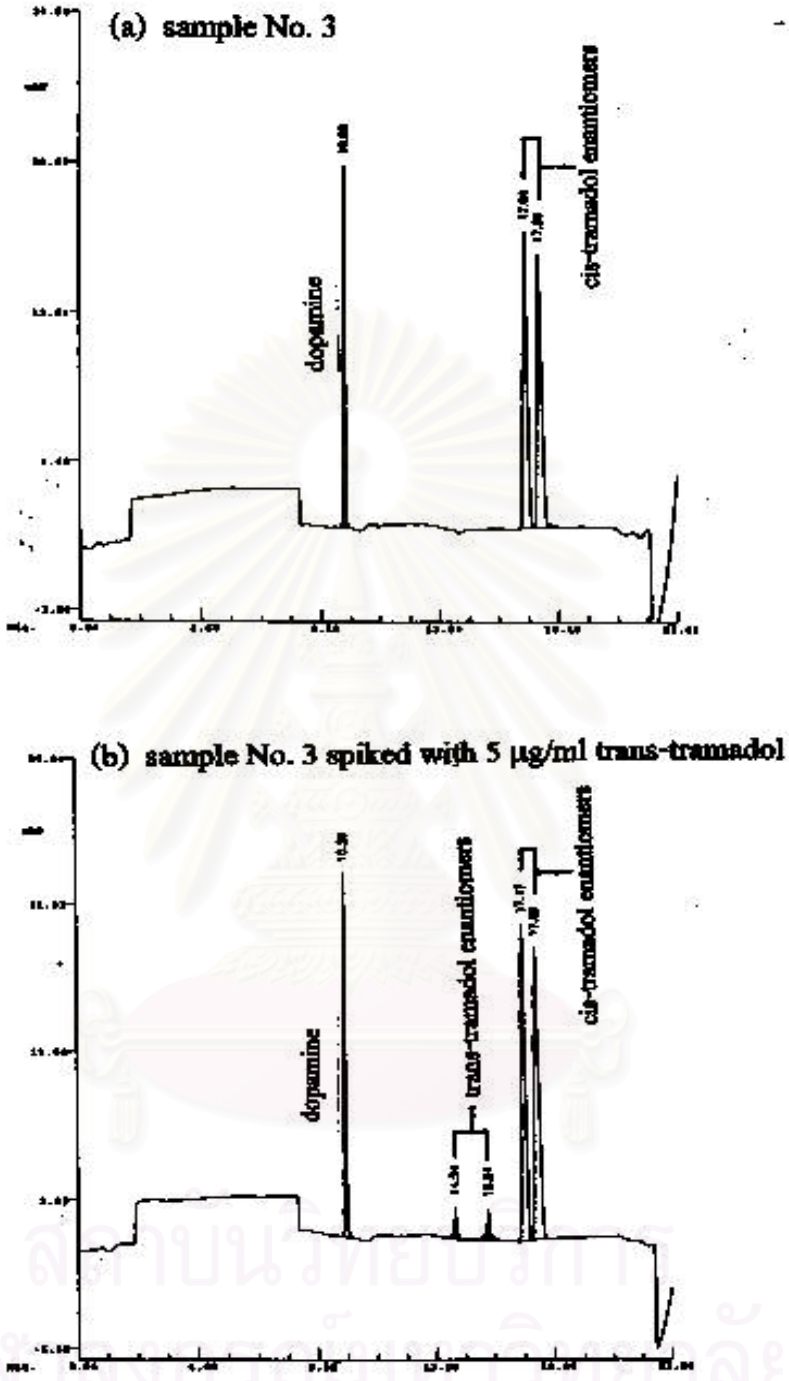


Figure 24 Electropherogram of cis-tramadol enantiomers in Tramadol Injection, sample No. 3 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

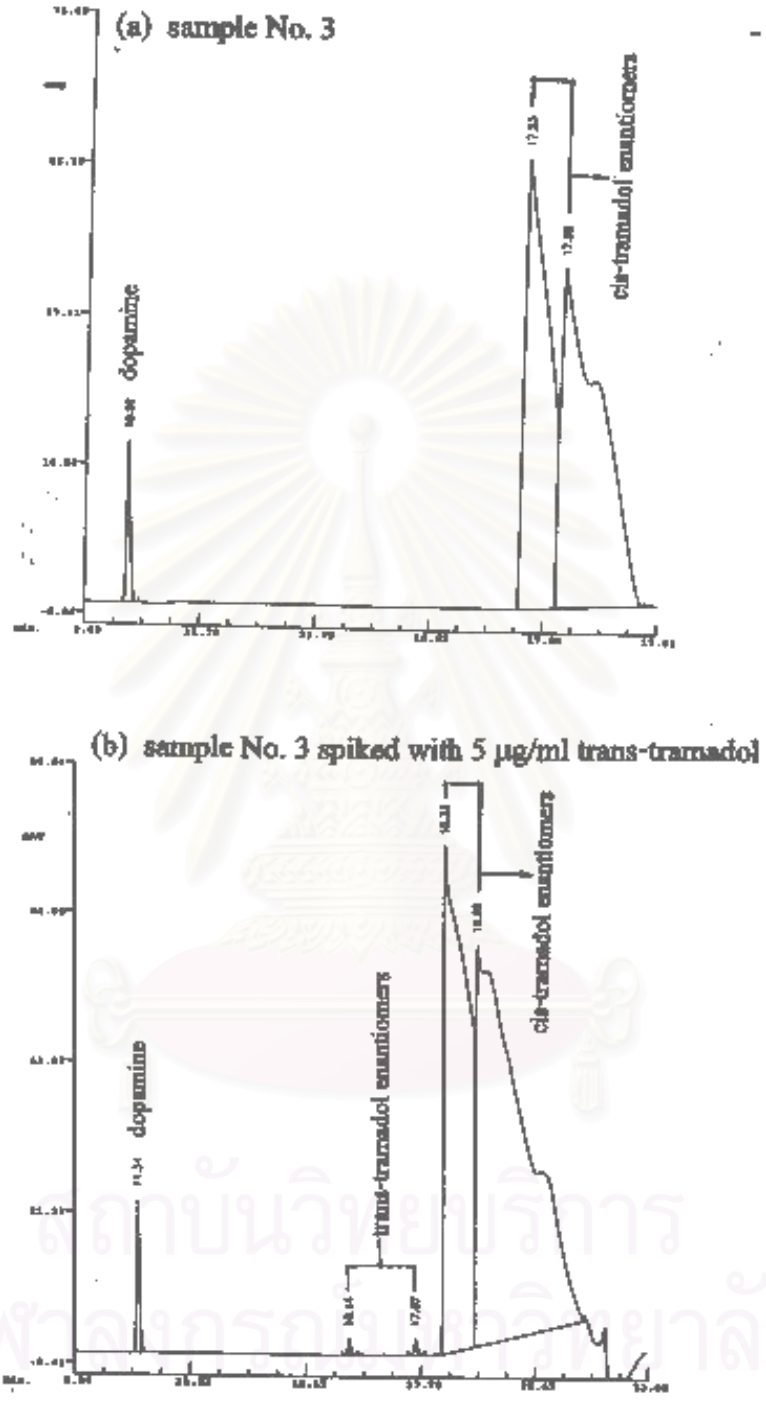


Figure 25 Electropherogram of trans-tramadol enantiomers in Tramadol Injection, sample No. 3 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

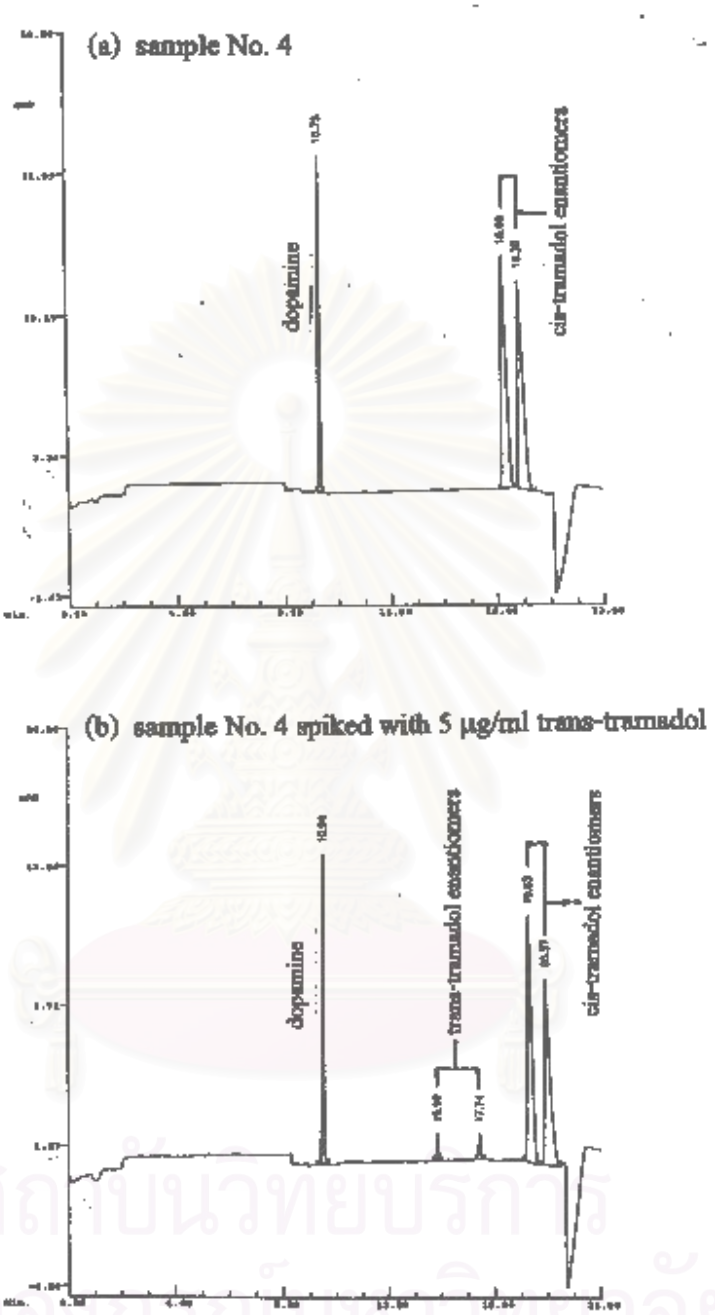


Figure 26 Electropherogram of cis-tramadol enantiomers in Tramadol Capsules, sample No. 4 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

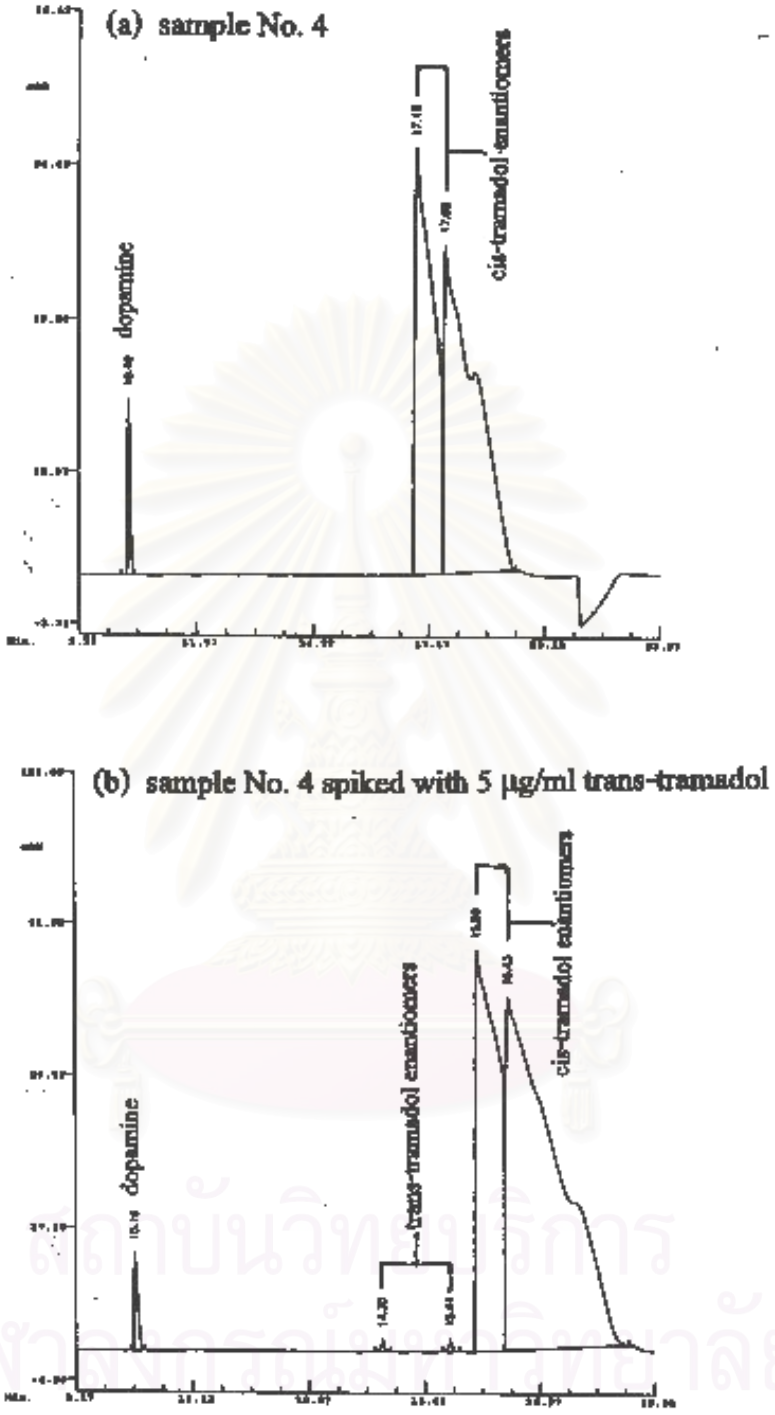


Figure 27 Electropherogram of trans-tramadol enantiomers in Tramadol Capsule, sample No. 4 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 μm i.d.

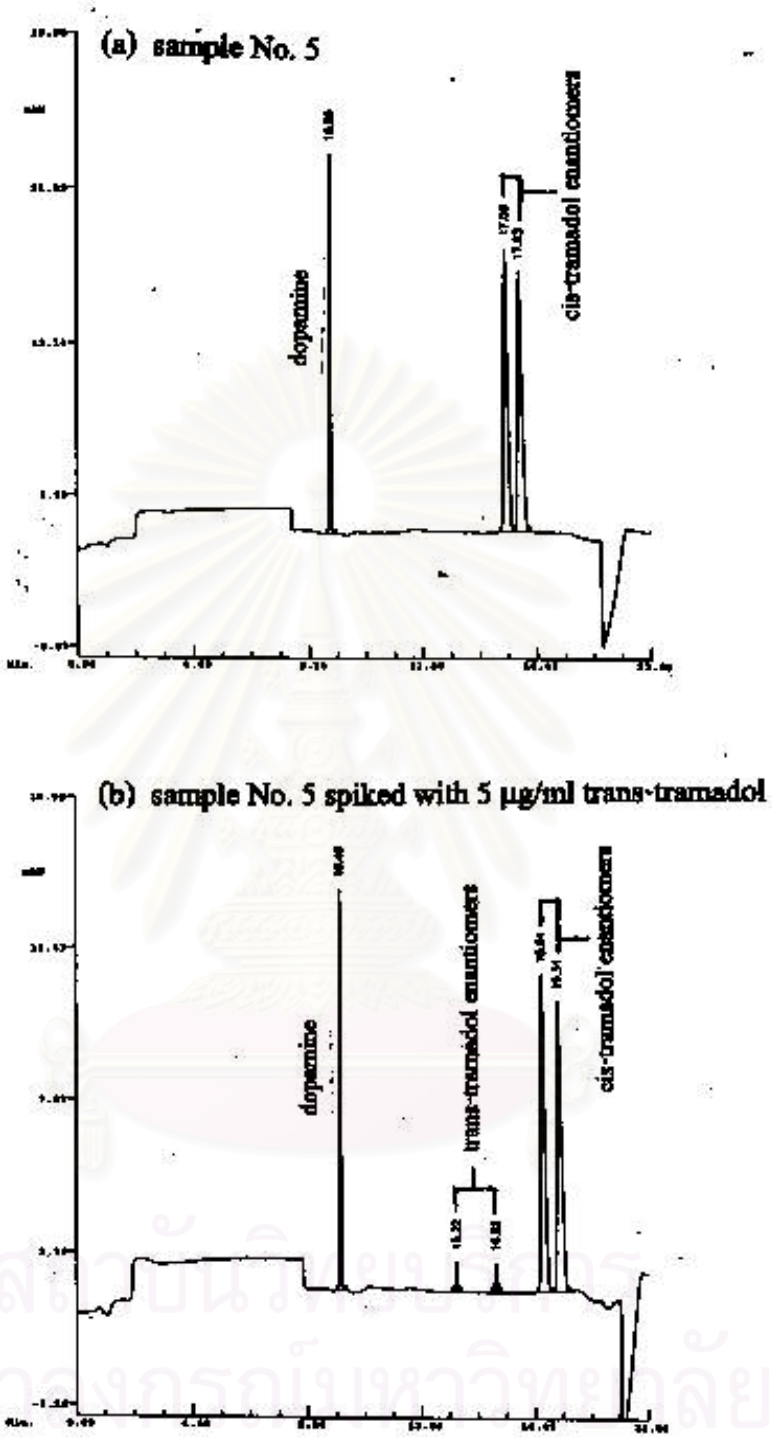


Figure 28 Electropherogram of cis-tramadol enantiomers in Tramadol Capsule, sample No. 5 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

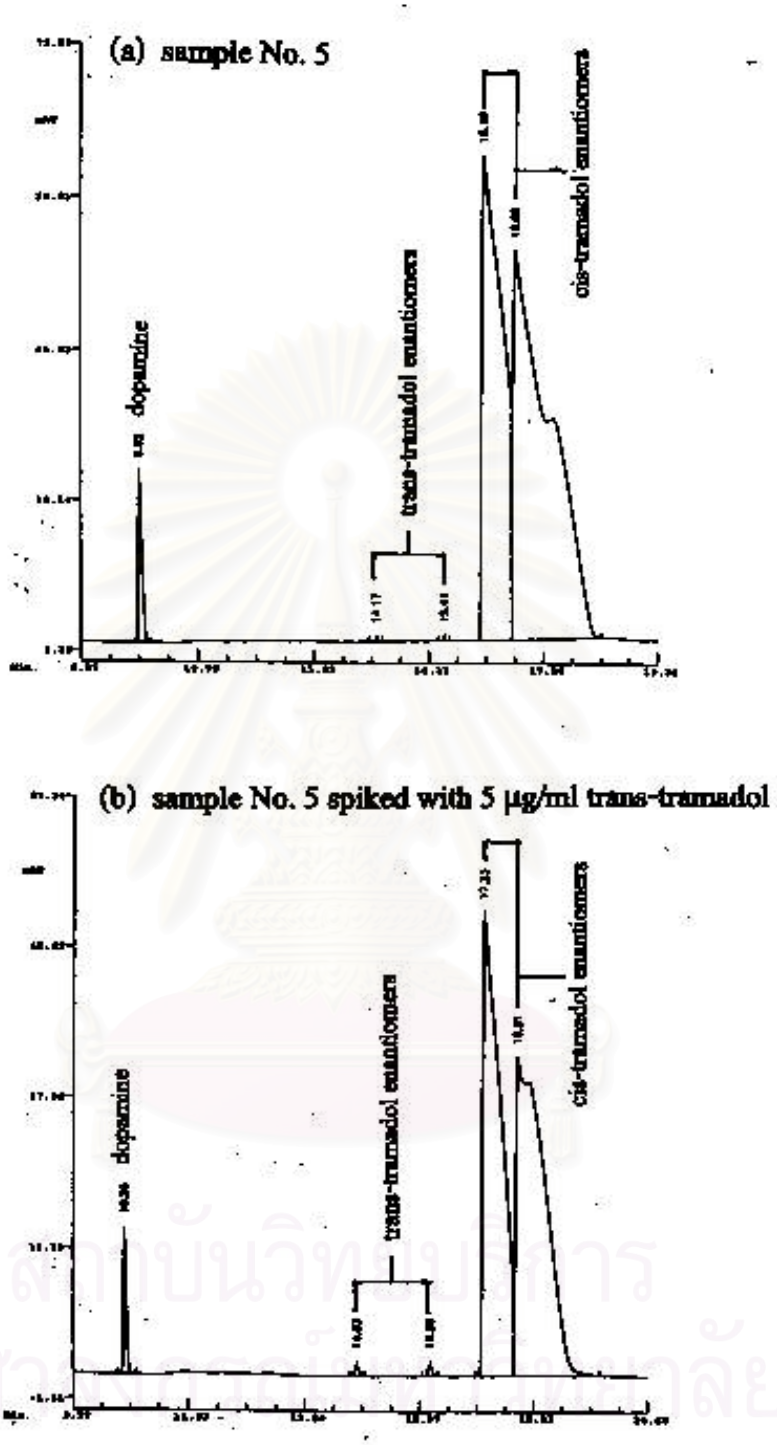


Figure 29 Electropherogram of trans-tramadol enantiomers in Tramadol Capsule, sample No. 5 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

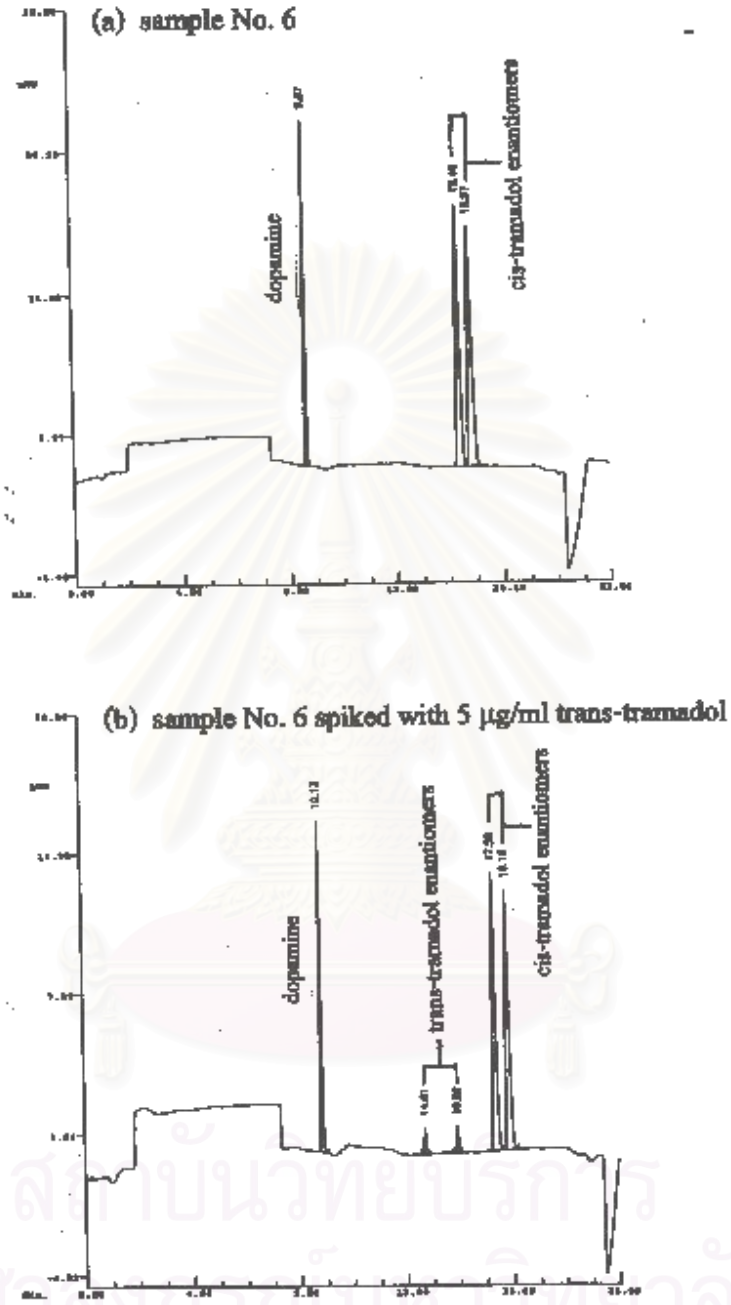


Figure 30 Electropherogram of cis-tramadol enantiomers in Tramadol Capsule, sample No. 6 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

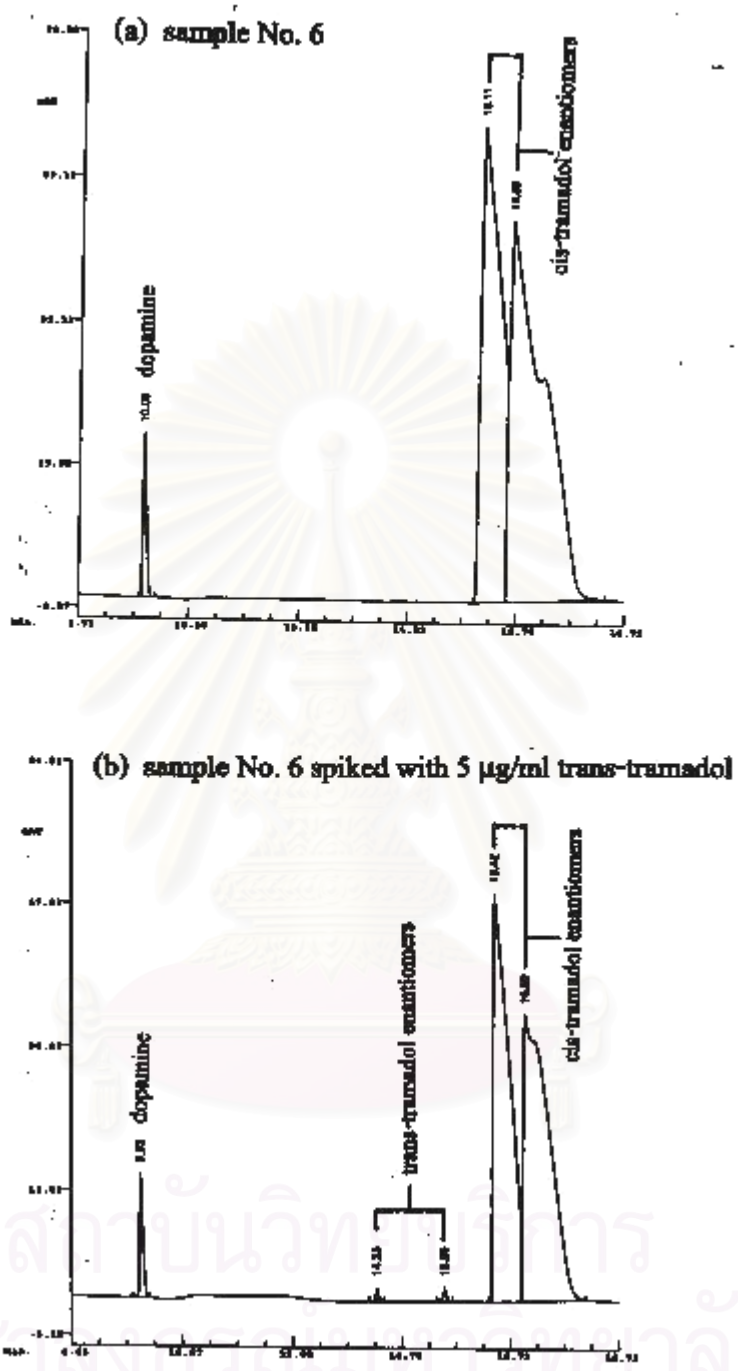


Figure 31 Electropherogram of trans-tramadol enantiomers in Tramadol Capsule, sample No. 6 using 8.5 mM CM-β-CD in 65 mM phosphate buffer pH 2.5, temperature 30°C, applied voltage 18 kV, UV detection 196 nm and fused silica capillary, 50 cm x 50 µm i.d.

**2. Linearity and range**

Calibration curves were established using five standard mixture solutions prepared with racemic trans- and cis-tramadol over the range of 56 - 150% and 70-130% of the nominal target of 5 and 100 μg/ml for trans- and cis-tramadol, respectively. 30 μg/ml of dopamine, an internal standard was included in standard mixture solutions. Calibration data calculated from peak height ratio, peak area ratio and normalized peak area ratio of cis- and trans-tramadol were shown in Table 13-15 and 16-18, respectively. The regression data calculated from peak height ratio, peak area ratio and normalized peak area ratio of cis- and trans-tramadol were respectively presented in Table 19 and 20. From Table 19 and 20, it is evident that the calibration curve calculated from normalized peak area ratio provided the best correlation. Figure 32 and 33 show calibration curves, calculating from normalized peak area ratio, of cis- and trans-tramadol, respectively. The calibration curves were found to be linear over the concentration ranges of 70-130 μg/ml for cis-tramadol (Figure 32) and 2.8-7.5 μg/ml for trans-tramadol (Figure 33).

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Table 13 Calibration data (peak height ratio) of cis-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Peak height | | | | Peak height ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 70 | 14479 | 13276 | 27755 | 21844 | 1.2706 |
| 2 | 85 | 15566 | 14118 | 29684 | 20495 | 1.4483 |
| 3 | 100 | 18903 | 17141 | 36044 | 21870 | 1.6481 |
| 4 | 115 | 19441 | 17625 | 37066 | 20984 | 1.7664 |
| 5 | 130 | 18157 | 16457 | 34614 | 20213 | 1.7124 |
| Slope | 0.0089 | | | | | |
| intercept | 0.6895 | | | | | |
| $r^2$ | 0.4227 | | | | | |

Table 14 Calibration data (peak area ratio) of cis-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Peak area | | | | Peak area ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 70 | 1619489 | 1666094 | 3285583 | 1078758 | 3.0457 |
| 2 | 85 | 1696791 | 1749254 | 3446045 | 937736 | 3.6748 |
| 3 | 100 | 2245910 | 2307729 | 4553639 | 1050837 | 4.3333 |
| 4 | 115 | 2337589 | 2421497 | 4759086 | 963182 | 4.9410 |
| 5 | 130 | 2596447 | 2683319 | 5279766 | 948849 | 5.5643 |
| Slope | 0.0421 | | | | | |
| intercept | 0.0961 | | | | | |
| $r^2$ | 0.9807 | | | | | |

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Table 15 Calibration data (normalized peak area ratio) of cis-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Normalized peak area | | | | Normalized Peak area ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 70 | 85574 | 85193 | 170767 | 99020 | 1.7245 |
| 2 | 85 | 94865 | 94792 | 189657 | 89497 | 2.1191 |
| 3 | 100 | 123226 | 122612 | 245838 | 98935 | 2.4848 |
| 4 | 115 | 128548 | 128952 | 257500 | 90113 | 2.8575 |
| 5 | 130 | 136358 | 136770 | 273128 | 85944 | 3.1779 |
| Slope | 0.0243 | | | | | |
| Intercept | 0.037 | | | | | |
| $r^2$ | 0.9961 | | | | | |

Table 16 Calibration data (peak height ratio) of trans-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Peak height | | | | Peak height ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 2.8 | 780 | 754 | 1534 | 20213 | 0.0758 |
| 2 | 4.0 | 1205 | 1163 | 2368 | 20984 | 0.1128 |
| 3 | 5.0 | 1628 | 1569 | 3197 | 21870 | 0.1462 |
| 4 | 6.0 | 1750 | 1692 | 3442 | 20495 | 0.1679 |
| 5 | 7.5 | 2400 | 2293 | 4693 | 21844 | 0.2148 |
| Slope | 0.0291 | | | | | |
| intercept | -0.004 | | | | | |
| $r^2$ | 0.9585 | | | | | |

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Table 17 Calibration data (peak area ratio) of trans-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Peak area | | | | Peak area ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 2.8 | 46148 | 49161 | 95309 | 948849 | 0.1004 |
| 2 | 4.0 | 68717 | 72593 | 141310 | 963182 | 0.1467 |
| 3 | 5.0 | 90597 | 97970 | 188567 | 1050837 | 0.1794 |
| 4 | 6.0 | 94964 | 104612 | 199576 | 937736 | 0.2128 |
| 5 | 7.5 | 142116 | 152798 | 294914 | 1078758 | 0.2733 |
| Slope | 0.0361 | | | | | |
| intercept | -0.0005 | | | | | |
| $r^2$ | 0.9902 | | | | | |

Table 18 Calibration data (normalized peak area ratio) of trans-tramadol (n=3)

| Standard No. | Concentration (μg/ml) | Normalized peak area | | | | Normalized Peak area ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$ enantiomer | $2^{o}$ enantiomer | racemate | dopamine | |
| 1 | 2.8 | 2927 | 2824 | 5751 | 85944 | 0.0669 |
| 2 | 4.0 | 4503 | 4351 | 8854 | 90113 | 0.0982 |
| 3 | 5.0 | 5949 | 5869 | 11818 | 98935 | 0.1194 |
| 4 | 6.0 | 6374 | 6413 | 12787 | 89497 | 0.1428 |
| 5 | 7.5 | 9007 | 8873 | 17880 | 99020 | 0.1805 |
| Slope | 0.0239 | | | | | |
| Intercept | 0.0008 | | | | | |
| $r^2$ | 0.9947 | | | | | |

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Table 19 Regression data of cis-tramadol

| Based on | Regression equation | Coefficient of determination ($r^2$) |
|---|---|---|
| Peak height ratio | y = 0.0089x + 0.6895 | 0.4227 |
| Peak area ratio | y = 0.0421x + 0.0961 | 0.9807 |
| Normalized peak area ratio | y = 0.0243x + 0.037 | 0.9961 |

Table 20 Regression data of trans-tramadol

| Based on | Regression equation | Coefficient of determination ($r^2$) |
|---|---|---|
| Peak height ratio | y = 0.0291x – 0.004 | 0.9585 |
| Peak area ratio | y = 0.0361x – 0.0005 | 0.9902 |
| Normalized peak area ratio | y = 0.0239x + 0.0008 | 0.9947 |

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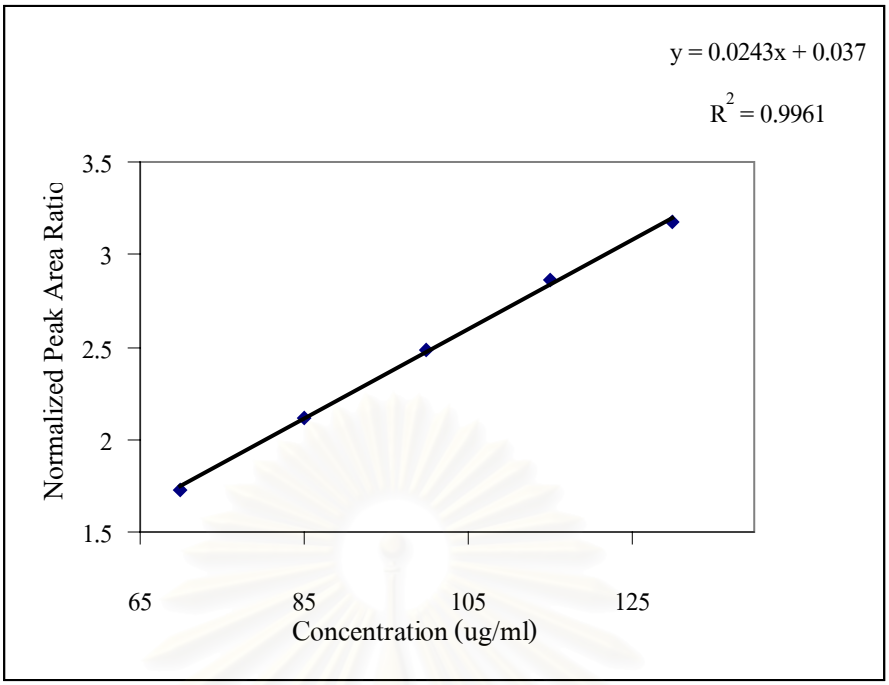


Figure 32 A calibration curve of cis – tramadol

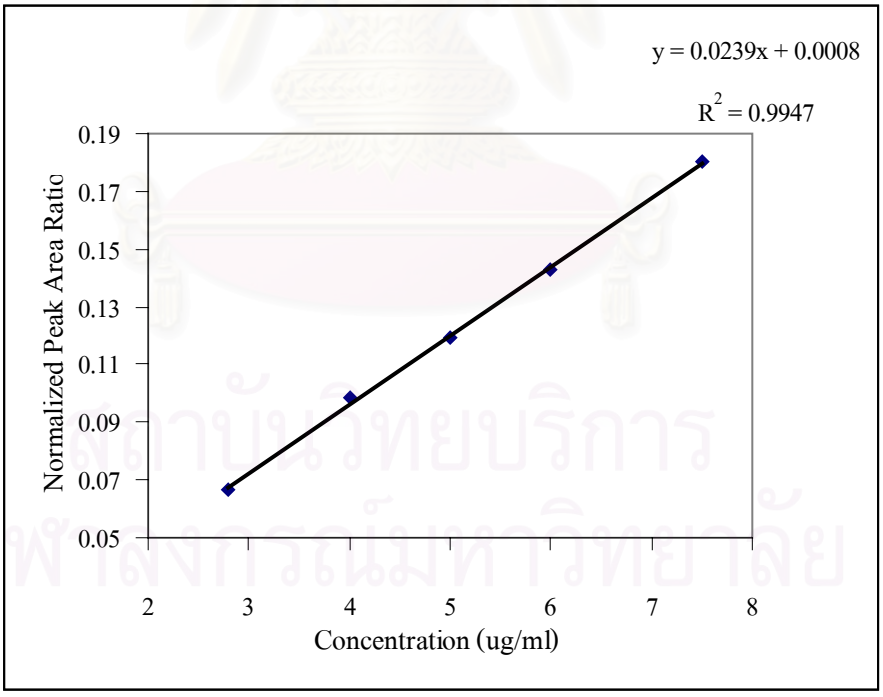


Figure 33 A calibration curve of trans – tramadol

**3. Precision**

The precision was determined by measuring the repeatability of the system and intermediate repeatability (intra-day and inter-day precision) of normalized peak area ratio.

3.1 System precision

The system precision was performed by triplicate injections of the standard mixture solution at three different concentrations. The relative standard deviation (% RSD) of normalized peak area ratio of cis- and trans-tramadol are shown in Table 21. The % RSD of cis- and trans-enantiomers were 1.40 - 2.03 and 0.59 – 3.95, respectively.

3.2 Intra-day precision

Six replicate analyses of tramadol injection and capsules sample were performed. The relative standard deviation of normalized peak area ratio of cis- and trans-tramadol enantiomers are shown in Table 22 and 23, respectively. The intra-day precision of all cis- and trans-tramadol enantiomers was less than 1.5%.

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Table 21 Analytical data of system precision (n = 3)

| Concentration (μg/ml) | cis-tramadol | | Concentration (μg/ml) | trans-tramadol | |
|---|---|---|---|---|---|
| | % RSD | | | % RSD | |
| | 1° enantiomer | 2° enantiomer | | 1° enantiomer | 2° enantiomer |
| 70 | 1.75 | 2.03 | 2.8 | 3.95 | 3.13 |
| 100 | 1.43 | 1.40 | 5.0 | 1.91 | 0.59 |
| 130 | 1.70 | 1.67 | 7.5 | 2.32 | 2.15 |

Table 22 Analytical data of intra-day precision of tramadol injection (n = 3)

| Analysis No. | Normalized peak area ratio | | | |
|---|---|---|---|---|
| | cis-tramadol | | trans-tramadol | |
| | 1° enantiomer | 2° enantiomer | 1° enantiomer | 2° enantiomer |
| 1 | 1.1784 | 1.1775 | 0.0623 | 0.0624 |
| 2 | 1.1705 | 1.1876 | 0.0618 | 0.0619 |
| 3 | 1.1509 | 1.1686 | 0.0616 | 0.0632 |
| 4 | 1.1808 | 1.1812 | 0.0617 | 0.0618 |
| 5 | 1.1825 | 1.1775 | 0.0619 | 0.0619 |
| 6 | 1.1695 | 1.1597 | 0.0639 | 0.0639 |
| Mean | 1.1721 | 1.1754 | 0.06215 | 0.0625 |
| SD. | 0.0116 | 0.0098 | 0.0007 | 0.0008 |
| % RSD | 1.00 | 0.84 | 1.21 | 1.37 |

Table 23 Analytical data of intra-day precision of tramadol capsules (n = 3)

| Analysis No. | Normalized peak area ratio | | | |
|---|---|---|---|---|
| | cis-tramadol | | trans-tramadol | |
| | $1^{o}$ enantiomer | $2^{o}$ enantiomer | $1^{o}$ enantiomer | $2^{o}$ enantiomer |
| 1 | 1.1679 | 1.1759 | 0.0623 | 0.0602 |
| 2 | 1.1646 | 1.1709 | 0.0624 | 0.0621 |
| 3 | 1.1741 | 1.1831 | 0.0620 | 0.0611 |
| 4 | 1.1699 | 1.1687 | 0.0618 | 0.0617 |
| 5 | 1.2036 | 1.1915 | 0.0605 | 0.0601 |
| 6 | 1.1843 | 1.1842 | 0.0607 | 0.0619 |
| Mean | 1.1774 | 1.1790 | 0.0616 | 0.0612 |
| SD. | 0.0145 | 0.0087 | 0.0008 | 0.0008 |
| % RSD | 1.23 | 0.74 | 1.33 | 1.42 |

3.3 Inter-day precision

Six replicate analyses of tramadol injection and capsules were performed on three non-consecutive days. The relative standard deviation of normalized peak area ratio of cis- and trans-tramadol enantiomers in tramadol injection and capsules are shown in Table 24 and 25, respectively. The inter-day precision of all cis- and trans-enantiomers was less than 1.0%.

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Table 24 Analytical data of inter-day precision of tramadol injection (n = 3)

| Day | Normalized peak area ratio | | | |
|---|---|---|---|---|
| | cis-tramadol | | trans-tramadol | |
| | $1^{o}$ enantiomer | $2^{o}$ enantiomer | $1^{o}$ enantiomer | $2^{o}$ enantiomer |
| 1 | 1.1689 | 1.1646 | 0.0622 | 0.0625 |
| 2 | 1.1721 | 1.1753 | 0.0611 | 0.0616 |
| 3 | 1.1872 | 1.1852 | 0.0617 | 0.0620 |
| Mean | 1.1761 | 1.1750 | 0.0617 | 0.0620 |
| SD. | 0.0097 | 0.0103 | 0.0005 | 0.0004 |
| % RSD | 0.83 | 0.88 | 0.89 | 0.73 |

Table 25 Analytical data of inter-day precision of tramadol capsules (n = 3)

| Day | Normalized peak area ratio | | | |
|---|---|---|---|---|
| | cis-tramadol | | trans-tramadol | |
| | $1^{o}$ enantiomer | $2^{o}$ enantiomer | $1^{o}$ enantiomer | $2^{o}$ enantiomer |
| 1 | 1.1774 | 1.1790 | 0.0616 | 0.0612 |
| 2 | 1.1630 | 1.1599 | 0.612 | 0.0608 |
| 3 | 1.1772 | 1.1754 | 0.0609 | 0.0611 |
| Mean | 1.1725 | 1.1714 | 0.0612 | 0.0610 |
| SD. | 0.0082 | 0.0101 | 0.0003 | 0.0002 |
| % RSD | 0.70 | 0.87 | 0.57 | 0.34 |

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**4. Accuracy**

Accuracy of the method was determined from percent recovery using standard addition method. Three different amounts of standard cis- and trans-tramadol were added to tramadol injection and capsule samples in a range of 80-120% of the expected assay concentration. The recoveries of cis- and trans-tramadol from spiked samples were within the range of 98.2 – 100.9.% and 101.2 – 106.2%, respectively

(Table 26 and 27). The mean recoveries of cis- and trans-tramadol in tramadol injection and capsules were 98.8% and 104.6%, and 99.6% and 101.7%, respectively.

Table 26 Recoveries of cis- and trans-tramadol from tramadol injection (n = 3)

| Compound | Expected Concentration (%) | Amount added (mg) | Amount found (mg) | Recovery (%) |
|---|---|---|---|---|
| cis-tramadol | 80 | 40.57 | 39.83 | 98.2 ± 0.49 |
| | 100 | 49.45 | 49.11 | 99.3 ± 0.10 |
| | 120 | 59.62 | 58.89 | 98.8 ± 0.92 |
| trans-tramadol | 80 | 0.2196 | 0.2264 | 103.1 ± 0.30 |
| | 100 | 0.2745 | 0.2915 | 106.2 ± 0.86 |
| | 120 | 0.3294 | 0.3443 | 104.5 ± 0.50 |

Table 27 Recoveries of cis- and trans-tramadol from tramadol capsules (n = 3)

| Compound | Expected Concentration (%) | Amount added (mg) | Amount found (mg) | Recovery (%) |
|---|---|---|---|---|
| cis-tramadol | 80 | 40.70 | 41.07 | 100.9 ± 0.29 |
| | 100 | 50.90 | 48.52 | 98.4 ± 0.69 |
| | 120 | 60.50 | 60.15 | 99.4 ± 0.34 |
| trans-tramadol | 80 | 0.2196 | 0.222 | 101.2 ± 0.60 |
| | 100 | 0.2745 | 0.2808 | 102.3 ± 0.40 |
| | 120 | 0.3294 | 0.3347 | 101.6 ± 0.23 |

### 5. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of trans-tramadol were obtained with seven-point calibration curve, covering the concentration range of 1.85 – 5.0 μg/ml. The calibration data calculated from normalized peak area ratio was shown in Table 28.

Table 28 Calibration data for calculating LOD and LOQ of trans-tramadol

| Standard No. | Concentration (μg/ml) | Normalized peak area | | | | Normalized peak area ratio |
|---|---|---|---|---|---|---|
| | | $1^{o}$enantiomer | $2^{o}$enantiomer | racemate | dopamine | |
| 1 | 1.85 | 2952 | 3151 | 6130 | 90834 | 0.0672 |
| 2 | 2.0 | 3692 | 3050 | 6742 | 86769 | 0.0777 |
| 3 | 2.5 | 3761 | 3574 | 7335 | 87371 | 0.0840 |
| 4 | 3.0 | 4746 | 4284 | 9030 | 96550 | 0.0935 |
| 5 | 3.5 | 4960 | 4609 | 9569 | 90340 | 0.1059 |
| 6 | 4.0 | 5737 | 5057 | 10794 | 94696 | 0.1140 |
| 7 | 5.0 | 6440 | 6250 | 12690 | 94437 | 0.1344 |
| intercept | | 0.0333 | | | | |
| intercept SD, (σ) | | 0.0029 | | | | |
| slope, (S) | | 0.0203 | | | | |
| $r^2$ | | 0.9903 | | | | |

The LOD and LOQ were determined as 3.3 σ/S and 10 σ/S, respectively. For trans-tramadol, the LOD of 0.48 μg/ml and LOQ of 1.46 μg/ml (%RSD $<$ 10%) were found in this study.

**6. Stability of the standard mixture and sample solution**

6.1 Standard mixture and sample solution stability

The stability of a standard mixture and sample solution of 100 μg/ml cis- and 5 μg/ml trans-tramadol were assessed by analyzing at 0, 1, 2, 3 and 4 hours after left standing at room temperature. The concentrations of cis- and trans-tramadol in a standard mixture and sample solutions were found to be in the range of 98.7-101.9% and 98.6 - 101.2% of those at the zero hour, respcetively. The concentration of the tramadol did not change significantly ($p > 0.05$) as presented in Table 29 and 30. Therefore standard mixture and sample solutions of cis- and trans-tramadol could be assumed stable after being left at room temperature for at least four hours.

Table 29 Stability of cis- and trans-tramadol in a standard mixture solution stored at room temperature

| Hours at room temperature | concentration (μg/ml)[a] | |
|---|---|---|
| | trans-tramadol | cis-tramadol |
| | racemate | racemate |
| 0 | 4.74 | 99.42 |
| 1 | 4.68 (98.7%) | 101.10 (101.7%) |
| 2 | 4.83 (101.9%) | 100.53 (101.1%) |
| 3 | 4.77 (100.6%) | 99.87 (100.4%) |
| 4 | 4.81 (101.5%) | 99.23 (99.8%) |

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

Table 30 Stability of cis- and trans-tramadol in a sample solution stored at room temperature

| Hours at room temperature | concentration (μg/ml)[a] | |
|---|---|---|
| | trans-tramadol | cis-tramadol |
| | racemate | racemate |
| 0 | 4.89 | 89.43 |
| 1 | 4.92 (100.6%) | 88.15 (98.6%) |
| 2 | 4.91 (100.4%) | 88.88 (99.4%) |
| 3 | 4.95 (101.2%) | 89.20 (99.7%) |
| 4 | 4.87 (99.6%) | 89.37 (99.9%) |

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

6.2 Autosampler stability

The stability of processed standard mixture and samples in the autosampler were assessed by analyzing at 0, 6 and 14 hours. The concentrations of cis- and trans-tramadol in standard mixture and samples were found to be between 101.7-102.3% and 100.7-102.7% of those at the zero hour, respectively (Table 31 and 32). Therefore, processed samples could be left in the autosampler and assumed to be stable for at least 14 hours ($p > 0.05$).

Table 31 Stability of cis- and trans-tramadol in a standard mixture solution stored in the autosampler

| Hour in autosampler | concentration (μg/ml)[a] | |
|---|---|---|
| | trans-tramadol | cis-tramadol |
| | racemate | racemate |
| 0 | 4.78 | 99.63 |
| 6 | 4.87 (101.9%) | 101.34 (101.7%) |
| 14 | 4.89 (102.3%) | 101.94 (102.3%) |

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

Table 32 Stability of cis-and trans-tramadol in a sample solution stored in the autosampler

| Hour in autosampler | concentration (μg/ml)[a] | |
|---|---|---|
| | trans-tramadol | cis-tramadol |
| | racemate | racemate |
| 0 | 5.91 | 118.2 |
| 6 | 5.96 (100.8%) | 120.32 (101.7%) |
| 14 | 5.95 (100.7%) | 121.45 (102.7%) |

[a] Value in the parenthesis represented the percentage of the analyte at the specified time comparing to that of freshly prepared sample.

**7. System suitability**

System suitability was evaluated by performing six replicate injections of a standard mixture solution of 100 μg/ml cis-tramadol, 5 μg/ml trans-tramadol and 30 μg/ml internal standard. The % RSD of normalized peak area ratio of cis- and trans-tramadol enantiomers was less than 3%. Resolution of 8.85, 1.49 and 6.06, respectively were found between trans-tramadol enantiomer, cis-tramadol enantiomers and the second enantiomer of trans-tramadol and the first enantiomer of cis-tramadol. The system suitability data were summarized in Table 33.

Table 33 System suitability data of tramadol enantiomeric separation (n = 6)

| Parameter | trans-tramadol | | cis-tramadol | |
|---|---|---|---|---|
| | $1^{o}$enantiomer | $2^{o}$enantiomer | $1^{o}$enantiomer | $2^{o}$enantiomer |
| % RSD of normalized peak area ratio | 2.48 | 2.74 | 2.65 | 2.89 |
| resolution | 8.85 | 6.06 | 1.49 | |

**8. Determination of cis- and trans-tramadol in pharmaceutical preparation**

With the optimum CZE condition, three commercial brands of tramadol injection and capsule dosage forms were analysed in duplicate with duplicate injections. The obtained electropherograms were shown in Figure 20 -31.

The results of analyses of tramadol injection and capsules were shown in Table 34 and 35, respectively.

The manufacturer's specification limit of cis-tramadol was 90.0 – 110.0% of the labeled amount and the limit of trans-tramadol was not more than 0.25% (sample No.1 and 3) or not more than 0.5% (sample No.2). The results showed that all tramadol injection and capsules studied met the requirements of the manufacturers.

Table 34 Analysis of cis- and trans-tramadol in tramadol injection

| Sample No. | %trans-tramadol racemate (w/w) | % labeled amount[b] cis-tramadol racemate |
|---|---|---|
| 1 | ND[c] | 95.2 ± 0.52 |
| 2 | 0.04[a] | 95.1 ± 0.27 |
| 3 | ND[c] | 97.7 ± 1.02 |

[a] relative to cis-tramadol

[b] mean ± SD of duplicate analyses

[c] ND was not detected

Table 35 Analysis of cis- and trans-tramadol in tramadol capsules

| Sample No. | %trans-tramadol racemate (w/w) | % labeled amount[b] cis-tramadol racemate |
|---|---|---|
| 1 | ND[c] | 97.3 ± 0.08 |
| 2 | 0.06[a] | 93.6 ± 0.15 |
| 3 | ND[c] | 98.2 ± 0.25 |

[a] relative to cis-tramadol

[b] mean ± SD of duplicate analyses

[c] ND was not detected

# CHAPTER IV

# CONCLUSION

A capillary zone electrophoresis (CZE) method using cyclodextrin as a chiral selector, was developed for the enantiomeric separation of cis-and trans-tramadol. Both neutral and negatively charged β-cyclodextrins were investigated and the negatively charged carboxymethyl-β-cyclodextrin (CM-β-CD) proved to be the best chiral selector for these analytes. The method was optimized using the Plackett-Burman design involving the influences of buffer pH and concentration, chiral selector concentration, capillary temperature and the applied voltage. The optimum method gave a baseline resolution of the compounds within 25 minutes. It was achieved on an uncoated fused silica capillary (50 cm x 50 μm, i.d.) at 30$^{o}$C with background electrolyte consisting of 8.5 mM CM-β-CD in 65 mM phosphate buffer at pH 2.5, applied voltage of 18 kV and detected at 196 nm.

The optimum method was validated according to the ICH guidelines to determine the suitability of the method, with results confirming that the method is highly suitable for its intended purpose.

The studies were performed to evaluate the use of the developed CZE method as an analytical tool in analysis of cis-and trans-tramadol in Tramadol Injection and Tramadol Capsules. The results demonstrate that the CZE method is highly suitable for quantitative drug analysis due to its advantages, such as high resolution, high efficiency, simple instrumentation, low consumption of chiral selector and small sample and electrolyte volumes.

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