SIMULTANEOUS ANALYSIS OF GLICLAZIDE, METFORMIN AND THEIR IMPURITIES BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Metformin (MET) and gliclazide (GCZ) are mostly used in patients with impaired insulin production (type II diabetes).Currently, both drugs are combined to improve patient compliance. Regulatory compendia of each drug are described in the United States and British Pharmacopeias. In tablet formulation,a significant impurity of GCZis gliclazide impurity F (GZF) whereas cyanoguanidine (CGN) is a major MET impurity. To date, methods for simultaneous analysis of both drugs are not available. For consumer protection, analytical methods of these drugs are urgently needed. The aim of this study was to optimize micellar electrokinetic chromatography (MEKC) conditionsfor the determination of MET and GCZ in a single run. Electrophoretic separation was carried out on the Beckman Coulter P/ACE MDQ instrument, equipped with a photodiode array at 225 nm and bare-fused silica capillary with a total length of 60.2 cm (to detector 50.0 cm).The temperature, voltage, and pressure were set at 25 ˚C, 15 kV, and 0.7 psi, respectively. The optimum conditions were obtained by varying type and concentration of buffer, surfactants, additives (i.e. ion pairing reagents, cyclodextrins (CD), and cetyl trimethylammonium bromide (CTAB)), organic modifiers, and the pH of the background electrolyte (BGE). Good separation of MET, CGN, GCZ, GZF andglibenclamide (GBM) used as an internal standard were found with 10 mM sodiumdihydrogen phosphate buffer(pH 7.0) containing 1.8 mg/mL carboxymethyl-beta-CD, 30 mM 2-hydroxypropyl-beta-CD, 3 mM CTAB, 15 mM propane sulfonate, 0.2 mg/mL Brij-35, and 15 % v/v methanol for 25 min. Due to the volatility of methanol, it was replaced by ethanol in method validation and application experiments. The method was validated and the results showed good linearity (r^2> 0.9974), precision (%RSD<3.35), and recovery (98.9-102.0%).The method can besuccessfully applied for quality control of combined MET and GCZ formulations.

KEY WORDS:CAPILLARY ELECTROPHORESIS/GLICLAZIDE/METFORMIN

64 pages

การวิเคราะห์กลัยคาไซด์, เมทฟอร์มิน และสารปนเปื้อนโดยแคปิลลารีอิเล็กโทรโฟรีซีส

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บทคัดย่อ

เมทฟอร์มิน และกลัยคาไซด์ นิยมใช้ในการรักษาโรคเบาหวาน ชนิดที่ 2 ซึ่งผู้ป่วยมีภาวะพร่องอินซูลิน ในปัจจุบันมีการนำยาทั้งสองชนิดมาผลิตในรูปแบบผสมเพื่อความสะดวกในการรับประทานยาของผู้ป่วย การควบคุมคุณภาพของยาแต่ละชนิด มีระบุในตำรายาของอเมริกาและอังกฤษ สำหรับเภสัชภัณฑ์ยาเม็ด เมทฟอร์มินมีสารปนเปื้อนที่สำคัญคือ ไซยาโนกัวนิดีน ในขณะที่กลัยคาไซด์ มีสารปนเปื้อนที่สำคัญคือ สารปนเปื้อนเอฟ (GZF) ปัจจุบันนี้ยังไม่มีวิธีวิเคราะห์ยาผสมสองชนิดนี้ จุดมุ่งหมายของงานวิจัยนี้ เพื่อศึกษาและพัฒนาวิธีวิเคราะห์สารเหล่านี้ในคราวเดียวโดยใช้เทคนิค ไมเซลลาร์ อิเล็กโทรไคเนติก โครมาโตกราฟี ทำการวิเคราะห์ โดยใช้เครื่องมือยี่ห้อ Beckman coulter P/ACE MDQ,วิเคราะห์การดูดกลืนคลื่นแสงด้วย โฟโต ไดโอด อาเรย์ ที่ ความยาวคลื่น 225 นาโนเมตร , หลอดซิลิกาแคปิลลารี มีความยาว ทั้งหมด 60.2 ซม. (ระยะถึงเครื่องตรวจวัด 50.0 ซม.) อุณหภูมิที่ใช้ 25 องศาเซลเซียส , ความต่างศักย์ 15 กิโลโวลต์, ใช้ความดันในการฉีด 0.7 ปอนด์ต่อตารางนิ้ว การพัฒนาวิธีวิเคราะห์ ทำโดยศึกษาปัจจัยต่างๆ เช่นการปรับสัดส่วนข องชนิดและความเข้มข้นของบัฟเฟอร์ , สารลดแรงตึงผิว, สารเติมแต่ง เช่นไอออนแพร์รีเอเจนต์ , ไซโคลเด็กทริน , ซีติลไตรเมทิลแอมโมเนียมโบรไมด์ , ตัวทำละลายสา รอินทรีย์ และค่าพีเอช ผลการศึกษา พบการแยกสารเมทฟอร์มิน ,ไซยาโนกัวนิดีน , กลัยคาไซด์ , สารปนเปื้อนเอฟและกลัยเบนคลาไมด์ (ใช้เป็นสารมาตรฐานภายใน) ทำได้ภายใน 25 นาที โดยใช้ 1.8 มิลลิกรัมต่อมิลลิลิตร คาร์บ็อกซีเมททิล- เบตา-ไซโคลเด็กทริน , 30 มิลลิโมลาร์ 2-ไฮดรอกซี โพรพิล-เบตา-ไซโคลเด็กทริน , 3 มิลลิโมลาร์ ซีทิล ไตรเมทิลแอมโมเนียมโบร์ไมด์ , 15 มิลลิโมลาร์ โพรเพน ซัลโฟเนต , 0.2 มิลลิกรัมต่อมิลลิลิตร บริท-35 และเมทานอล ร้อยละ 15 โดยปริมาตร ใน 10 มิลลิโมลาร์ ฟอสเฟต บัฟเฟอร์ , พีเอช 7.0 การประเมินวิธีและประยุกต์ในการวิเคราะห์ยา ใช้เอทานอลแทนเมทานอล เนื่องจากเมทานอลระเหยอย่างรวดเร็ว ทำให้ผลการวิเคราะห์คลาดเคลื่อน วิธีที่พัฒนาขึ้นให้ค่าความสัมพันธ์เส้นตรงที่ดี (r^2> 0.9974), ค่าความแม่นยำ ดี (%RSD < 3.35) และค่าความถูกต้องอยู่ในช่วงร้อยละ 98.9-102.0 ดังนั้นวิธีนี้สามารถนำมาใช้ควบคุมคุณภาพของยาผสมเมทฟอร์มินและกลัยคาไซด์อย่างมีประสิทธิภาพ

64 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

AR	analytical grade
ave	average
BGE	background electrolyte
BP	British Pharmacopoeia
CD	cyclodextrin
CE	capillary electrophoresis
CCE	chiral capillary electrophoresis
CE-UV	capillary electrophoresis UV/Vis spectroscopy
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CGN	cyanoguanidine
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
cm	centimeter
CMC	critical micelle concentration
CZE	capillary zone electrophoresis
DM	diabetes mellitus
E	applied electric field
EOF	electroosmotic flow
EtOH	ethanol
F_E	electric force
F_F	frictional force
g	gram
GBM	glibenclamide
GC	gas chromatography
GCZ	gliclazide
GDM	gestation diabetes mellitus

LIST OF ABBREVIATIONS (cont.)

GZF	gliclazide impurity F
HPLC-ECD	high pressure liquid chromatography-electrochemical detection
HPLC-UV	high pressure liquid chromatography-UV/Vis spectroscopy
i.d.	inner diameter
ICH	the international conference on harmonization
IDDM	insulin-dependent diabetes mellitus
kV	kilovoltage
l	effective length of capillary
L	total length of capillary
LC	liquid chromatography
LC-MS-MS	liquid chromatography tandem mass spectrometry
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MET	metformin
mg	milligram
mL	milliliter
mM	millimolar
N	number of theoretical plate
NIDDM	non insulin-dependent diabetes mellitus
o.d.	external diameter
pH	negative logarithm of hydrogen ion concentration
psi	pound per square inch
q	ion charge
r	ion radius
r^2	coefficient of determination
R_s	resolution
RSD	relative standard deviation
s	second

LIST OF ABBREVIATIONS (cont.)

SD	standard deviation
TLC-UV	thin layer chromatography-UV/Vis spectroscopy
t_m	migration time
USP	United State Pharmacopoeia
UV	ultraviolet
V	volt
v	ion velocity
v/v	volume by volume
°C	degree celcius
μ_a	apparent mobility of the analyte
μ_e	electrophoretic mobility
μ_{eof}	electro osmotic mobility
µg	microgram
µm	micrometer
µM	micromolar
η	viscosity of the BGE

CHAPTER I
INTRODUCTION

Diabetes is a metabolism disorder, which causes elevated blood glucose levels. Main types of diabetes are type I and type II (1), in which type 2 diabetes is now a major concern worldwide with an estimation of 366 million patients by the year 2030 (2). Metformin (MET) and gliclazide (GCZ) are drugs of choice for type 2 diabetes treatments and combination of both drugs in a single dosage form is popular to enhance patient compliance (3). Gilbenclamide (GBM) is also used in type 2 diabetics. However, quality control of the anti-diabetic drugs is a priority. Impurities of MET (i.e. cyanoguanidine (CGN)) and GCZ (i.e. impurity F (GZF)) are extremely harmful and toxic (4, 5).

The regulartory compendia, British Pharmacopoeia (BP) and United State Pharmacopoeia (USP) recommend high performance liquid chromatography (HPLC) for determination of MET in tablet formulation (6, 7). The assay of GCZ is official in BP using HPLC method. There are analytical techniques used for determination of MET and/or GCZ i.e. TLC-UV (8), HPLC-UV (9-18), LC-MS-MS (19-21), CE-UV (22-25), HPLC-ECD (26), spectrophotometry (27, 28), spectrofluorometry (28), GC (29). So far, there is no report on the simultaneous analysis of anti-diabetic drugs (MET and GCZ).

Currently, CE is an attractive technique for pharmaceutical analysis because it offers several advantages such as high efficiency and selectivity, flexibility of method development, separation speed, minimized organic waste, low costs and small sample requirements (30). Due to the different solubility of MET and GCZ and the similarities of the parent compounds with their impurities, micellar electrokinetic chromatography (MEKC) shows potential for the separation of these compounds (31). MEKC could be achieved by addition of surfactant into background electrolyte to form micelle, thus the separating mechanism attribute to electrophoresis and

chromatography. These effects enable neutral and closely related compounds are separated (30).

This aims of this work was to develop a simple and rapid method for the simultaneous analysis of 5 analytes (MET, CGN, GBM, GCZ, GZF) by capillary electrophoresis (CE). GBM acted as internal standard. CGN and GZF were for testing the purity of major analytes (MET and GCZ).

Method development was performed on mode of instrumentation and optimization by investigation of several factors such as types and concentrations of buffer, surfactant, organic solvents and additives (i.e. cyclodextrin, ion-pair reagent, CTAB). Method validation was studied in terms of linearity, accuracy, precision. Finally, the developed method was applied for the determination of MET and GCZ in pharmaceutical formulations.

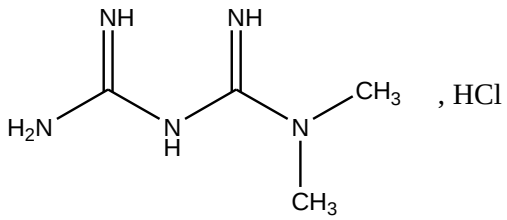
CHAPTER II
LITERATURE REVIEW

2.1 Diabetic mellitus and anti-diabetic drugs

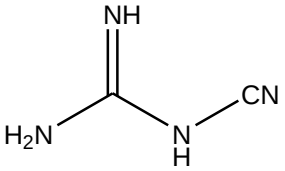
Diabetes can be classified into two major types. Type I diabetes or insulin dependent diabetes mellitus (IDDM) is found in patients who can no longer produce insulin due to destructions of beta-cells (insulin producing cells) and need injectable insulin to control blood sugar levels (1). Type II diabetes or non-insulin dependent diabetes mellitus (NIDDM) is patients who can still produce insulin, but have insulin tolerance, impaired insulin secretion or increased glucose production (1). In type II patients, beta-cells are gradually destroyed and the patients need anti-diabetes drugs, in some cases injectable insulin, to control their blood sugar levels. Other specific types include diabetes caused by genetic defects in beta-cell functions or in insulin actions, infections and drug or chemical induced. Another type of diabetes includes gestational diabetes mellitus (GDM), which glucose intolerance occurs during pregnancy due to metabolic changes (1). This specific diabetes is less common than type I and II diabetes.

Anti-diabetes drugs are divided into seven groups: 1) aldose reductase inhibitors, 2) alpha-glucosidase Inhibitors, 3) biguanides, 4) meglitinides, 5) sulfonylureas, 6) thiazolidinediones and 7) miscellaneous. Among several classes of anti-diabetes drugs, biguanides (e.g. buformin, metformin and phenformin) and sulfonylureas (e.g. carbutamide, glibencamide, gliclazide, etc.) are drugs of choice for treatments of diabetes. Biguanides can reduce hepatic glucogenesis and increase peripheral glucose utilization whereas sulfonylureas enhance insulin secretion. Both drugs are effective in the presence of some endogenous insulin production (1). Metformin (MET) was firstly recommended for NIDDM because it dose not cause myocardial infarction and produces less hypoglycemia effect than sulfonylurea (32). Both glibencamide (GBM) and gliclazide (GCZ) (Fig 2.1) are also used in NIDDM. Hence, GCZ is improved to second-generation sulfonylurea which posseses lower risk

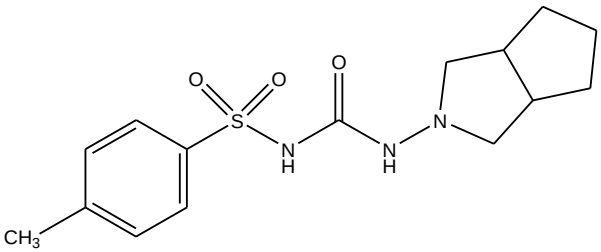
of hypoglycemia side effect than GBM. It is more suited for elderly patient (32). Additionally, GCZ is currently used to protect endothelial cells of blood vessels, which can reduce complications of diabetes on cardiovascular system (33). It is evident that for effective treatments of NIDDM, combinations of at least two classes of anti-diabetics are generally prescribed. The most commonly used combination is MET and GCZ (3). Nowadays, there are 7 brands of combined MET and GCZ launched in the market worldwide to enhance patient's compliance (32).



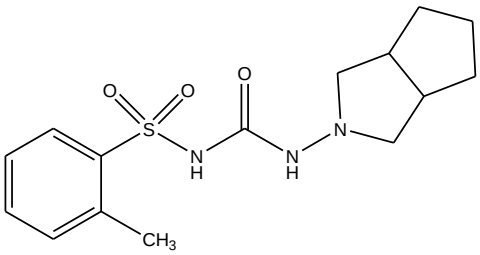
Metformin (MET)



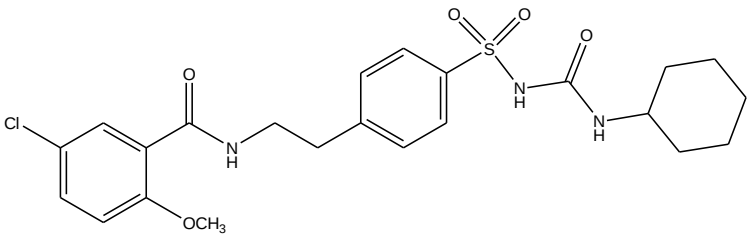
Cyanoguanidine (CGN)



Gliclazide (GCZ)



Gliclazide impurity F (GZF)



Glibenclamide (GBM)

Figure 2.1 Structures of MET, GCZ, GBM and their impurities.

Table 2.1 Chemical properties of MET, GCZ, GBM and their impurities.

Name	MET	CGN	GCZ	GZF	GBM
MW	165.62	84.08	323.4	323.4	494.0
pKa	2.8, 11.5	9.1	5.8	4.06, 14.14	5.3
Stability	Protect from light	Keep away from alkali	Protect from light	-	-

2.2 Determination of anti-diabetic drugs

British Pharmacopoeia (BP) (6) and United State Pharmacopoeia (USP) (7) recommend high performance liquid chromatography (HPLC) for the assay of MET in tablets. GCZ is official in BP, using HPLC for assay of tablet formulations. Analytical techniques used for determination of MET and/or GCZ were TLC-UV (8), HPLC-UV (9-18), LC-MS-MS (19-21), CE-UV (22-25), HPLC-ECD (26), spectrophotometry (27, 28), spectrofluorometry (28), GC (29)(Table 2.1). Limitations of these techniques include long analysis time, peak tailing and low sensitivity. TLC of these drugs is feasible and cheap, but sometime is time- and labor-consuming. LC-MS-MS is employed for monitoring of both drugs in plasma because of its high sensitivity. However, the instrumentation and maintenance is costly. Spectrophotometry and spectrofluorometry of MET and GCZ can be achieved, but derivatization is required prior to the measurement. GC is limited to heat stable compounds and capillary clogging can be problematic. Therefore, there is a need of an efficient analytical method for the simultaneous determination of these drugs in combined formulation.

Table 2.2 Analytical methods for the determination of anti-diabetic drugs.

Method	Analyte	Matrix	Significant finding	Reference
TLC-UV, HPLC-UV	MET, GCZ, pioglitazone	tablet	• Quantitative determination was performed by HPLC, TLC and column chromatography	(8)
LC/MS/MS	MET, GCZ	human plasma	• linearity range = MET : 7.8-4,678.9 ng/mL • linearity range = GCZ : 10-10,000 ng/mL • %recovery = 7.1-104% • LOQ : MET = 7.8 ng/mL • LOQ : GCZ = 10.0 ng/mL • Intra- and Inter-day precision < 15%	(19)
CE-UV	MET, phenformin, glyburide	human plasma	• monitoring down to 1 µg/mL of MET and phenformin • LOD : MET = 12 ng/mL • LOD : phenformin = 6 ng/mL	(22)

Table 2.2 Analytical methods for the determination of anti-diabetic drugs (Continued).

Method	Analyte	Matrix	Significant finding	Reference
CE-UV	MET, rosiglitazone	tablet	• LOD : MET = 1 µg/mL • LOD : rosiglitazone = 0.5 µg/mL	(23)
CE-AD	Aminoheterocycle, azabicycle (impurity of gliclazide)	raw material	• linearity range : aminoheterocycle = 1-1,000 µM • linearity range : azabicycle = 2-1,000 µM • LOD : aminoheterocycle = 0.5 µM • LOD : azabicycle = 1 µM	(24)
HPLC-UV	Dicyandiamide (impurity of MET)	standard	• Good resolution of dicyandiamide and main peak • The test results were reproducible	(9)
HPLC-UV	GCZ, GBM, glipizide, glimepiride, gliquidone, repaglinide	tablet	• The successful method was operated on isocratic mode, using Altima C18 column, mobile phase : methanol-phosphate buffer (pH 3.0; 0.01M)(70:30, v/v), flow rate 1.0 mL/min, wavelength 230 nm	(10)

Table 2.2 Analytical methods for the determination of anti-diabetic drugs (Continued).

Method	Analyte	Matrix	Significant finding	Reference
HPLC-UV	MET	human plasma	• linearity range = 30-4,000 ng/mL • %recovery = 93.7% • LOQ = 30 ng/mL • accuracy = 98.3% (intra-day), 94.0% (inter-day)	(11)
HPLC-UV	MET	urine	• Detection limit = 0.18 nM • Quantitation limit = 0.59 nM	(12)
HPLC-UV	MET, rosiglitazone	human plasma	• LOQ : MET = 250 ng/mL • LOQ : rosiglitazone = 100 ng/mL • %recovery : MET = 103.88-105.64% • %recovery : rosiglitazone = 100.02-105.0%	(13)
HPLC-UV	GCZ	human plasma	• LOD = 10 ng/mL • linearity range = 100-5,000 mg/mL	(14)

Table 2.2 Analytical methods for the determination of anti-diabetic drugs (Continued).

Method	Analyte	Matrix	Significant finding	Reference
LC-MS-MS	MET	human plasma	• linearity range = 10-1,000 ng/mL • accuracy = 100.3-105.0% (intra-day), 101.2-105.3% (inter-day) • precision = 0.8-1.9% (intra-day), 1.5-8.6% (inter-day) • %recovery = 67.0%	(20)
HPLC-ECD	GCZ	human plasma	• linearity range = 50 nM - 4 µM • LOD = 10 nM	(26)
HPLC-UV	GCZ	human plasma	• linearity range = 0.1-10 µg/mL	(15)
HPLC-UV	MET, GCZ, glipizide, GBM, glimepyride	human plasma	• %recovery = 76.3-101.9% • LOQ = 5-22.5 ng/mL • precision < 9% (inter-day and intra-day)	(16)

Table 2.2 Analytical methods for the determination of anti-diabetic drugs (Continued).

Method	Analyte	Matrix	Significant finding	Reference
LC-MS-MS	MET	human plasma	• linearity range = 1-2,000 ng/mL • Inter-day and Intra-day precision < 8.6% • accuracy = 91-110% • LOD = 250 pg/mL	(21)
Spectrophotometry	GCZ	human plasma, tablet	• linearity range = 0.5-4 µg/mL • LOD = 0.05 µg/mL	(27)
Spectrophotometry, Spectrofluorometry	GCZ	tablet	• linearity range = 2-20 µg/mL (spectrophotometry) • LOD = 0.2 µg/mL (spectrophotometry) • linearity range = 0.2-2.5 µg/mL (spectrofluorometry) • LOD = 0.02 µg/mL (spectrofluorometry)	(28)
HPLC-UV	GCZ	human serum	• LOD = 70 ng/mL • linearity range = 75-10,000 ng/mL (r^2 = 0.999) • %recovery = 84.5%	(17)

Table 2.2 Analytical methods for the determination of anti-diabetic drugs (Continued).

Method	Analyte	Matrix	Significant finding	Reference
HPLC-UV	MET, GCZ, glipizide	tablet	• The method was carried out on Inersil® C-18 column • mobile phase : 75 mM camphor sulphonic acid in a Mixture of acetonitrile-water	(18)
GC	GCZ	tablet	• linearity range = 0.1-10 mg/mL • %recovery = 96.5%	(29)

2.3 Capillary electrophoresis

The electrophoretic separation technique is based on the principle that under the influence of an applied potential field, different species in solution migrate at different velocities from the another. When the voltage is applied, the charged species move toward the electrode of opposite charge. The velocities of the migrating species also depend on the electric field, sizes and mass-to-charge ratio of species and their environmental. The basic component of CE instrument (Figure 2.2) are a sample vials, source and destination vials, a capillary tube, a high voltage power supply, a detector, and software for operation and data output (30, 34).

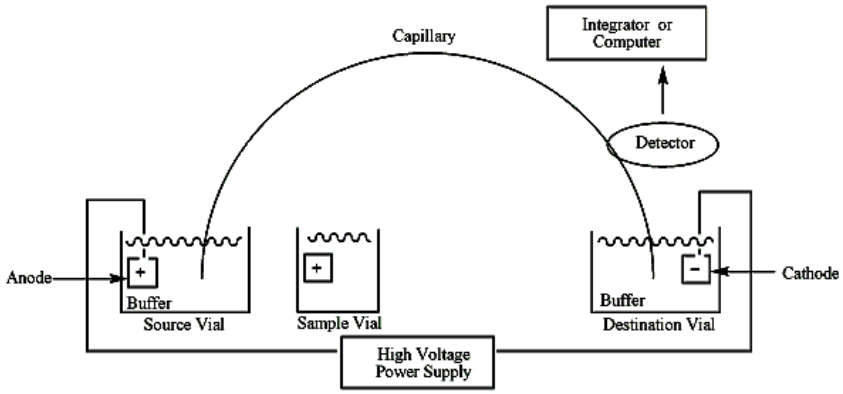


Figure 2.2 CE instrument (30).

2.3.1 Principle of CE

The CE separation is based on the different ion velocities in background electrolyte (BGE) under an applied electric field. The migration velocity of ions can be calculated by the following equation (34).

$$v = \mu_e E \quad (2.1)$$

$$\mu_e = \alpha \frac{F_E}{F_F} \quad (2.2)$$

$$F_E = qE \quad (2.3)$$

$$F_F = 6\pi\eta r \quad (2.4)$$

At equilibrium,

$$F_E = F_F \quad (2.5)$$

$$qE = 6\pi\eta r \quad (2.6)$$

$$\mu_e = q/6\pi\eta r \quad (2.7)$$

Where,

v = ion velocity (cm/s)

μ_e = electrophoretic mobility (cm^2/Vs)

E = applied electric field (V/cm)

F_E = electric force (N)

F_F = frictional force (N)

q = ion charge

η = the viscosity of the BGE (Ns/m^2)

r = ion radius (cm)

2.3.2 Electro osmotic flow (EOF)

The EOF results from the effect of the applied electric field on the solution double layer of the fused silica capillary wall. The ionized silanol groups (SiO^-) impart a layer of negative charge to the capillary wall and attract hydrated cations from the electrolyte solution and then arranged into two layers. As illustrated in Figure 2.3, the one layer is tightly bound by electrostatic forces (compact layer), and the other is more loosely bound (diffuse layer). When an electric field is applied, the diffuse layer breaks away (at the plane of shear) and move toward the cathode, dragging with it the bulk solution of the electrolyte, as a result of viscous drag. This flow of bulk solution is known as electro-osmosis. The EOF is dependent on a number of parameters including pH and ionic strength. An increase of the ionic strength undergoes a decrease of EOF (30, 34).

The EOF can be calculated by the following equation,

$$\mu_a = \mu_e + \mu_{eof} \quad (2.8)$$

where, μ_a = apparent mobility (cm^2/Vs)

μ_{eof} = electro-osmotic mobility (cm^2/Vs)

The μ_{eof} can be measured using a neutral maker that moves at a velocity equal to the EOF.

$$\mu_a = IL/tV \quad (2.9)$$

where, l = effective capillary length (cm)

L = total capillary length (cm)

t = the migration time (s)

V = the applied voltage (volt)

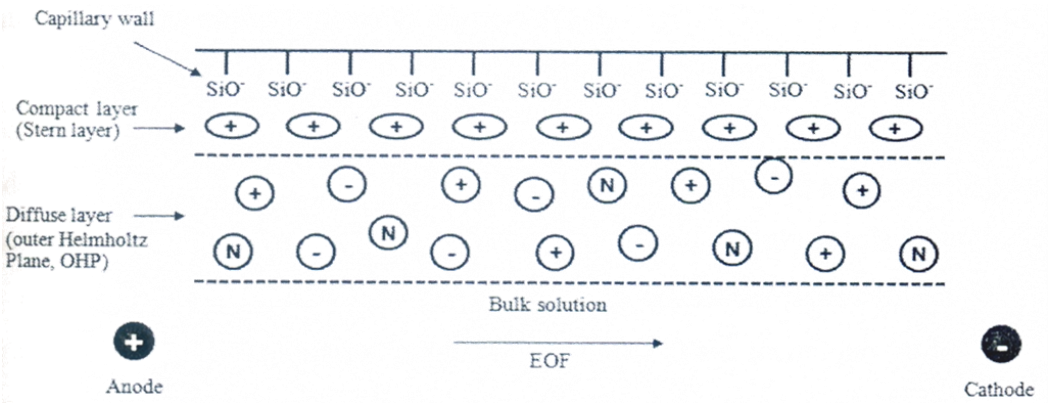


Figure 2.3 Electrical double layer at the ionized silica capillary wall and illustration of EOF (34).

EOF can control the migration behavior of eluted species in a capillary tube. When voltage is applies across the tube, in normal mode, cations move toward to cathode, anions move backward to anode and neutral compounds do not migrate. EOF enhances migrating behavior of all species to move to cathode. Cations move to cathode the fastest due to they migrate at the same direction with EOF, which EOF enhances the velocity of migration. Anions migrate the slowest, since they move backward to anode but EOF has sufficient force to carry anions to move to cathode and neutral compounds are carries by EOF at the same velocity but are not separated from each other. Thus in the presence of EOF, cations, anions and neutral compounds can be detected in a single run.

The magnitude of EOF is given by the following equations,

$$\mu_{EOF} = (\zeta/\eta) \qquad (2.10)$$

Where, μ_{EOF} = EOF mobility

ε = dielectric constant

ζ = zeta potential

η = solution viscosity

2.3.3 EOF modifiers

In capillary electrophoresis, when analytes migrate in the direction as same as the direction of EOF, this is called co-EOF separation. Conversely, if the direction of analytes is against the direction of EOF, this is called counter-EOF separation. EOF modifier is almost needed when counter-EOF flow is performed.

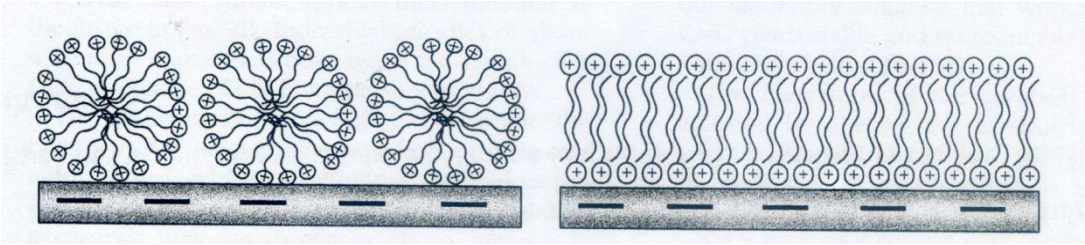
Table 2.3 Factors affecting the magnitude of EOF (36).

Parameter	Result
Electric field	• Proportional change in EOF
pH	• EOF is decreased when pH is reduced
Ionic strength of BGE concentration	• Increasing of ionic strength of BGE concentration results in decreasing of zeta potential, thus EOF decreased
Temperature	• EOF is proportional decreased due to the change of viscosity (2-3% per degree Celsius)
Organic modifier	• Changes zeta potential and viscosity, which decrease EOF
Surfactant	• Adsorbs to capillary wall by ionic or hydrophobic interactions • Anionic surfactants can increase EOF • Cationic surfactants can decrease or reverse EOF
Neutral hydrophilic polymer	• Adsorbs to capillary wall by ionic or hydrophobic interactions, which decrease EOF by shielding suface charge and increase viscosity
Covalent coating	• Chemical bonding to capillary wall, which affects to surface charge of capillary tube

Many strategies can be used in the modification of EOF direction (36), such as dynamic coating, permanent coating, covalent bonding and cross-linking of EOF modifier to the interior surface of capillary wall, however, the simplet method is belong to dynamic coating.

Dynamic coating is a method, in which an EOF modifier (e.g. cetyltrimethylammonium bromide (CTAB), tetradecyltrimethylammonium bromide (TTAB)) is added into background electrolytes to form transient layer at the interior surface of capillary wall (interacts to silanol group) (Figure 2.4). This results in a

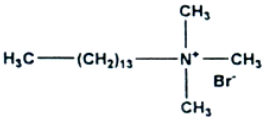
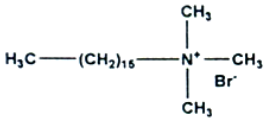
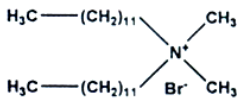
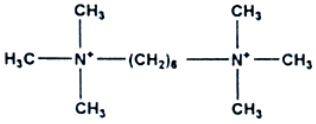
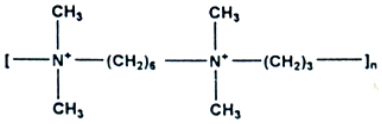
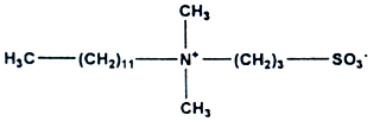
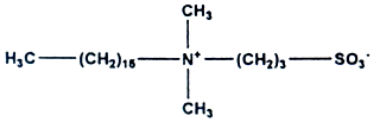
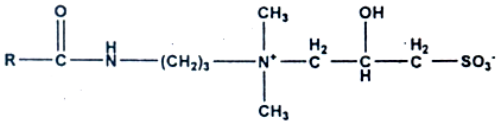
conversion of charge at the capillary surface from negative to positive and the direction of EOF is reversed. EOF modifiers, which are commonly used in dynamic coating, are cationic surfactants, polycations and zwitterionic surfactants (Table 2.3). These EOF modifiers can suppress or reverse EOF direction depending on the concentrations of EOF modifiers (37).



(a) (b)

Figure 2.4 The mechanism of dynamic coating by cationic surfactants (a) single-chain surfactants (b) double-chain surfactants (38).

Table 2.4 Common EOF modifiers for dynamic coating (38).

Name	Type	Structure
TTAB	Single-chain cationic surfactants	
CTAB	Single-chain cationic surfactants	
DDAB	Double-chain cationic surfactants	
HM	Polycations	
HDM	Polycations	
DDSA	Zwitterionic surfactants	
HDSA	Zwitterionic surfactants	
CAS U	Zwitterionic surfactants	

2.3.4 Electromigration dispersion (EMD)

The difference of conductivity between sample zone and the BGE causes electromigration dispersion (EMD) (39). This causes asymmetric peak shape in an electropherogram. When sample zone has higher conductivity than the running BGE (high mobility), tailing peak shape is obtained. Conversely, when sample zone has lower conductivity than the running BGE, in this case, fronting peak is obtained (Figure 2.5). Matching the mobilities of BGE or maintaining BGE concentration two

orders of magnitude of sample are typical approaches, which are used to minimize EMD phenomenon (39).

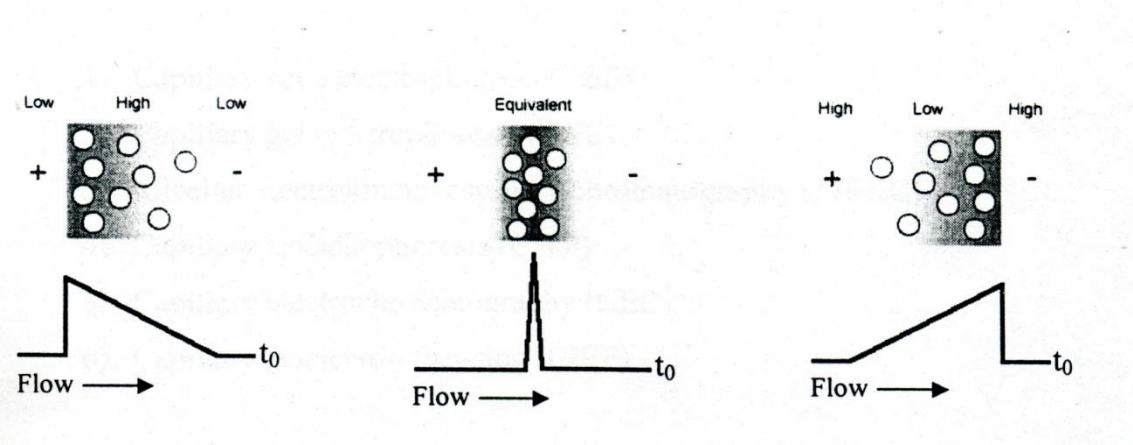


Figure 2.5 Electromigration dispersion (EMD) phenomenon due to the difference of conductivity between running BGE and solute (a) front peak (b) symmetrical peak (c) tailing peak (39).

2.3.5 Joule heating

When high electrical current is applied during electrophoresis, heat is generated, which is called "Joule heating", due to the passage of electrical current. Joule heating causes non-uniformity temperature gradient, local changes in viscosity, sample zone broadening and high baseline noise (39). These are detrimental effects in an analysis, thus joule heating should be minimized.

2.3.6 Stacking technique

The most recognized on-line pre-concentration method is "stacking technique". The advantage of the technique is increasing sensitivity of sample detection without alteration of CE instrumentation. The stacking phenomenon is illustrated in Figure 2.6. The sample ions are in lower conductivity zone than that of buffer zone. Under the applied voltage, electric field strength in sample zone (lower conductivity) is turned to higher than buffer zone. When analytes migrate with high velocity across the boundary, the length of sample zone is shortened by the stacking effect. The new arrangement of sample zone lead to higher sensitivity (40).

The length of stacking zone is given by,

$$l_{\text{stack}} = l_{\text{inj}} \frac{1}{\gamma} \qquad (2.11)$$

$$\gamma = E_{\text{s}} / E_{\text{BGS}} \qquad (2.12)$$

Where, l_{stack} = the length of the analyte zone after sample stacking

L_{inj} = the length of the analyte zone after injection

Es = electric field strength in the sample zone

E_{BGE} = electric field strength in the background electrolyte

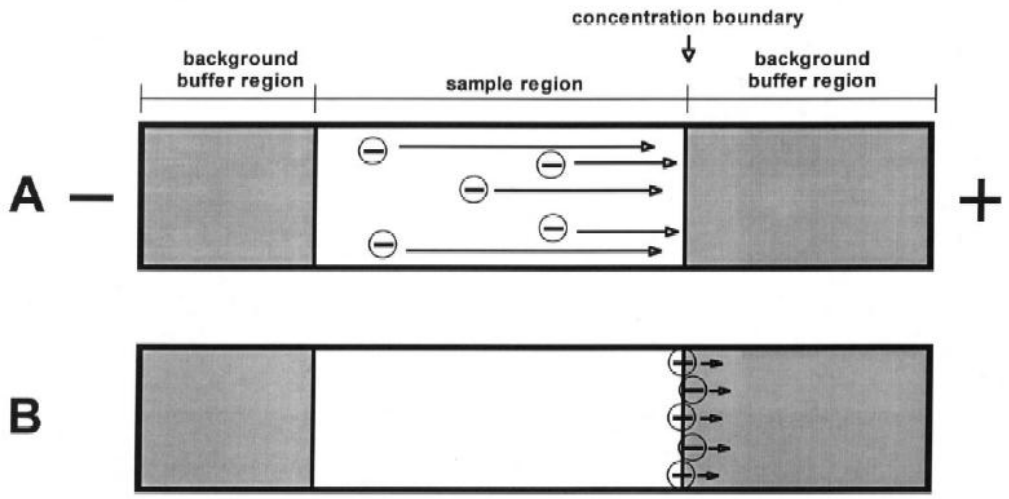


Figure 2.6 General sample stacking model (40).

2.3.7 Separation modes in CE

2.3.7.1 Capillary zone electrophoresis (CZE): CZE can be regarded as the basic mode of CE in which analytes are separated in free solution. Based on difference in their mobility or charge-to-mass ratio (41).

2.3.7.2 Micellar electrokinetic chromatography (MEKC): The mode is used for the separation of neutral and charged analytes. By additional of surfactant at higher concentration than critical micelle concentration (CMC), will form micelle in buffer. The analytes are separated by differential partitioning between micelle and buffer (41).

2.3.7.3 Chiral capillary electrophoresis (CCE): CCE is used to separate enantiomers, that is isomeric compounds which are mirror images of each other. Because two enantiomers are sterically different, they interact differentially with the cyclodextrins. Cyclodextrins are molecules with a hydrophobic interior and a hydrophilic exterior. Alpha, beta and gamma cyclodextrins of 6, 7, 8 sugar molecules are in a ring (41).

2.3.7.4 Capillary electrochromatography (CEC): The analytes are passed through a capillary which is filled with LC-like packing materials. CEC provides higher efficiency than a comparable LC analysis because CEC produces equally velocity profile of analyte migration (41).

2.3.7.5 Capillary gel electrophoresis (CGE): CGE is used to separate long biomolecules. The slab gel electrophoresis is a prototype of CGE but CGE is developed for higher voltage applications. In CGE the capillary is filled with a polymer which forms on matrix. This acts as a molecular sieve and small molecules migrate more quickly through the polymer matrix than larger molecules (41).

2.3.7.6 Capillary isoelectric focusing (CIEF): CIEF is used to determine the isoelectric point of peptide and proteins. The analysis is done in two steps. In the first step, the capillary is filled with a solution containing a complex ampholyte mixture as well as the sample and marker protein. An electric field is applied where the background ampholytes establish a pH gradient. Proteins also start migrating depending on their net charge. When a protein reaches a point inside the capillary which the pH equals its isoelectric point, the protein becomes electrically

neutral and stop migrating. In the second step, the protein bonds are mobilized towards the detection window by applying an external pressure (41).

2.3.7.7 Capillary isotachophoresis (CITP): CITP uses a discontinuous buffer system. First, a leading electrolyte containing ions of high mobility is introduced into the capillary. Then a sample plug is injected and finally a tailing electrolyte containing low mobility ions is introduced. The leading electrolyte contains ions with mobility greater than any of the ions in the sample. Because of differences in the local electric field strength, sharp boundaries are established between leading electrolyte and the tailing electrolyte. Then the sample components elute in the order of their mobilities (41).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials and instrument

Table 3.1 List of chemicals and reagents.

Name	Grade	Source/Supplier
Metformin	RS	Bureau of Drug and Narcotic (Nonthaburi, Thailand)
Gliclazide	RS	Bureau of Drug and Narcotic (Nonthaburi, Thailand)
Sodium dihydrogen phosphate	-	Carlo erba (Calle Filadors, Spain)
SDS	-	Sigma-Aldrich (Missouri, USA)
Brij-35	-	Ubichem FC (Redditch, UK)
CTAB	-	Sigma-Aldrich (Missouri, USA)
Propane sulfonate	-	Sigma-Aldrich (Missouri, USA)
2HP-β-CD	-	Sigma-Aldrich (Missouri, USA)
CM-β-CD	-	Sigma-Aldrich (Missouri, USA)
Methanol	HPLC	Mallinckrodt Baker (Xalostoc, Mexico)
Ethanol	HPLC	Mallinckrodt Baker (Xalostoc, Mexico)
Sodium hydroxide	-	Carlo erba (Calle Filadors, Spain)
Phosphoric acid	-	Carlo erba (Calle Filadors, Spain)

RS = reference standard, HPLC = high performance liquid chromatography, SDS = Sodium dodecyl sulphate, Brij = Polyethylene glycol lauryl ether, CTAB = Cetyl trimethylammonium bromide, 2HP-β-CD = 2-Hydroxypropyl-beta-cyclodextrin, CM-β-CD = Carboxymethyl-beta-cyclodextrin

Table 3.2 List of instruments.

Name/Model	Source/Supplier
Capillary electrophoresis (P/ACE MDQ)	Beckman Coulter (California, USA)
Capillary tube (Bare fused silica, id. 50 µm)	Agilent Technologies (California, USA)
Micropipette (Calibra)	Socorex Swiss (Ecublens, Switzerland)
pH meter (FE20)	Mettler toledo (Ohio, USA)
Analytical balance (XP205)	Mettler toledo (Ohio, USA)
Nylon Syringe filter, 0.2 µm 13 mm (Vertipure)	Ligand (California, USA)
Centrifuges (Sorvall LYNX)	Thermo Scientific (Massachusetts, USA)
Ultrasonic bath (Tru-Sweep)	Crest Ultrasonic (New Jersey, USA)
Ultrapure water system (Milli-Q)	Merck Millipore (Massachusetts, USA)

3.2 Capillary electrophoresis general method

MEKC was performed on Beckman coulter P/ACE MDQ capillary electrophoresis system and data processing was by 32 Karat software version 7.0. The detector was a photodiode array detector scanning in a range from 190 to 300 nm (wavelength accuracy ±2 nm, bandwidth 10 nm). The voltage can be varied in a range of 0-30 kV (current 15-300 µA). The temperature in capillary cartridge is regulated by a liquid cooling system from 10-55 °C. The sample is injected by a hydrodynamic system.

The separation of MET, GCZ and GBM was performed by using a bare fused silica capillary with an internal diameter of 50.0 µm, a total length (L_{total}) of 60.2 cm and an effective length to detector (L_{eff}) of 50.0 cm. The pre-condition procedure for a new capillary for daily uses, between runs and storage was described in Table 3.3. Standard solutions were injected into the capillary inlet using pressure of 0.7 psi.

The optimum condition was investigated by varying; types and concentrations of buffer, surfactants, ion pairing agents, organic modifiers and cyclodextrins. Method development and optimization were studied as described in method development and optimization sections.

Table 3.3 Capillary conditioning procedures for the separation of MET, GCZ and GBM.

	Conditioning step	Time (min)
New use capillary	1. 1 N NaOH	15
	2. 0.1 N NaOH	15
	3. Water	15
Daily use	1. Ethanol	5
	2. Water	5
	3. 1 N NaOH	5
	4. 0.1 N NaOH	5
	5. Water	5
Between run	1. Water	4
	2. BGE	5
Overnight storage	1. Water	5
	2. 0.1 N NaOH	5
	3. Water	5
Long-time storage	1. Water	5
	2. 1 N NaOH	5
	3. Water	5
	4. 0.1 N NaOH	5
	5. Water	10
	6. Methanol	10
	7. Air	3

3.3 Assay of combined anti-diabetic drugs

3.3.1 Background electrolyte (BGE) preparation

Stock buffer solution 100 mM was prepared by dissolving 6.0 g sodium dihydrogen phosphate and 6.0 mL of 85% w/w phosphoric acid in water adjusted to 500 mL and kept in a refrigerator at 8 °C. Before daily used, the stock buffer solution was diluted 10-fold with water to obtain 10 mM phosphate buffer solution, and then adjusted to pH 7.0 with 0.1 N NaOH (as BGE). The 10 mM phosphate buffer solution (pH 7.0) was further diluted 5-fold with water to obtain 2 mM phosphate buffer solution (as standard and sample solvent).

3.3.2 Standard solution preparation

Standard solutions of MET, GCZ were separately prepared by weighing each standards equivalent to 10.0 mg into a 100-mL volumetric flask and dissolving with 50.0 mL EtOH. After shaking and sonication for 20 minutes, the solutions were adjusted to 100 mL with 2 mM phosphate buffer (pH 7.0) solution to obtain final concentrations of 100 µg/mL. The standard solutions were filtered through a 0.2 µm Nylon membrane and degassed for 10 minutes prior to injection.

3.3.3 Sample preparation

Twenty tablets of anti-diabetic drugs were finely ground to homogeneous powder, which was dissolved as described below.

Single drug : MET 500 mg /tablet

The accurately weighed powder equivalent to 500 mg MET was transferred into a 200- mL volumetric flask and dissolved with 100.0 mL EtOH. After shaking and sonication for 20 minutes, its volume was adjusted to 200 mL with 2 mM phosphate buffer solution. Two milliliters of the solution was pipetted to a 50- mL volumetric flask and adjust to volume with a mixture of EtOH and 2 mM phosphate buffer (pH 7.0)(50:50) to obtain the final concentration of 100 µg/mL.

Single drug : GCZ 80 mg /tablet

The accurately weighed powder equivalent to 80 mg GCZ was transferred into a 200- mL volumetric flask and dissolved with 100.0 mL EtOH. After

shaking and sonication for 20 minutes, its volume was adjusted to 200 mL with 2 mM phosphate buffer solution. Five milliliters of the solution was pipetted to a 20- mL volumetric flask and adjust to the volume with a mixture of EtOH and 2 mM phosphate buffer (pH 7.0)(50:50) to obtain the final concentration of 100 µg/mL.

Combined drug : MET 500 mg and GCZ 80 mg /tablet

The separately weighed powder equivalent to 500 mg MET and 80 mg GCZ was transferred into each 200- mL volumetric flask and dissolved with 100.0 mL EtOH. After shaking and sonication for 20 minutes, its volume was adjusted to 200 mL with 2 mM phosphate buffer solution. For assay of MET, two milliliters of the solution was pipetted to a 50- mL volumetric flask. For assay of GCZ, five milliliters of the solution was pipetted to a 20- mL volumetric flask. Adjust volume with a mixture of EtOH and 2 mM phosphate buffer (pH 7.0)(50:50) to obtain the final concentration of 100 µg/mL each sample.

3.3.4 Optimization

Method optimization was performed on a MEKC mode, which showed high efficiency and effectiveness in separation of structurally similar analytes (31). Types and concentrations of additives (buffer, surfactant, cyclodextrin, ion-pair reagent and organic modifier) were investigated to optimize analytical parameters such as migration time, resolution, tailing factor and number of theoretical plate, which were calculated by the following equations. Criteria for the optimum CE condition were shown in Table 3.4

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (3.1)$$

Where, R_s = Resolution

t_1 = Migration time of analyte 1

t_2 = Migration time of analyte 2

w_1 = Baseline peak width (in time) of analyte 1

w_2 = Baseline peak width (in time) of analyte 2

$$TF = \frac{w_{0.05}}{2f} \quad (3.2)$$

Where, TF = Tailing factor

$w_{0.05}$ = Width of peak at 5% of peak height

f = Width of line from leading edge of peak to the intercept of a perpendicular line dropped from the peak maximum to the base

$$N = 5.54\left(\frac{t}{w_{0.5}}\right)^2 \quad (3.3)$$

Where, N = Number of theoretical plates

t = Migration time of analyte

$w_{0.5}$ = Temporal peak width at half height

Table 3.4 Analytical parameter criteria.

Parameter	Criteria
Resolution	> 1.5
Migration time	< 30 min
Tailing factor	≤ 2.0
Number of theoretical plate	> 10,000

3.3.5 Method validation

The optimized MEKC condition was validated in terms of specificity, linearity, accuracy, precision according to the ICH guidline (42).

3.3.5.1 Specificity

Specificity was the ability to fluently evaluate the analyte in the presence of components, which may be expected to be present in the sample matrices. The specificity of an analytical method was tested by the peak purity test. The spectral data of standard solutions (100 µg/mL) were conducted to create spectral library. Under the same CE condition, the sample was analyzed.

The similarity index was calculated by comparing the current sample data with a standard spectrum stored in the library. Similarity index in a range of 0.9900 to 0.9999 was indicated a high similarity.

3.3.5.2 Linearity

Calibration curves of MET and GCZ were established on five different concentrations (50-150 µg/mL) in triplicate injections. Linear regression, coefficients of determination (r^2), slope and intercept were calculated by Microsoft Office Excel© (version 2007).

3.3.5.3 Precision

Precision of the method was determined from %RSD of t_m , peak area and peak height from injection, intra-day and inter-day precisions. Injection precision was performed by nine injections of the mid point of the calibration curves (100 µg/mL). For intra-day precision, three different concentrations (50, 100 and 150 µg/mL) of the standard solutions were injected in triplicates on the same day. For inter-day precision, three different concentrations (50, 100 and 150 µg/mL) of the standard solutions were analyzed on three different days and each concentration was injected in triplicates. %RSD was calculated by equation 3.4.

$$\%RSD = \left(\frac{SD}{X_{ave}}\right) \times 100 \qquad (3.4)$$

where SD = standard deviations

X_{ave} = mean values

3.3.5.4 Accuracy

Accuracy of the method was determined from % recovery by standard addition method. Standard solution was spiked into the sample to make the final concentration of 80-120% of the nominal concentration (100 µg/mL for MET and GCZ). Each concentration was injected in triplicates. Percent recovery was calculated from equation 3.5.

$$\%\ Recovery = \frac{Amount\ found - Amount\ added}{Amount\ added} \times 100 \qquad (3.5)$$

3.3.6 Application

The developed and validated method was applied to the assay of MET and GCZ tablet formulations. Two commercial brands of single drug and three different lots of combined drug were analyzed. Analyses were performed in triplicates and %labeled amounts of the drugs were calculated.

CHAPTER IV
RESULTS AND DISSCUSSION

4.1 CE method development for analysis of MET and GCZ

4.1.1 Wavelength selection

UV absorbance of the analytes (e.g. MET, CGN, GBM, GCZ, GCF) in a mixture of acetonitrile and water (40:60, v/v) was performed. Using a spectrophotometer scanning in a range of 200 to 400, the wavelength of 225 nm was selected because of being the most compromised wavelength.

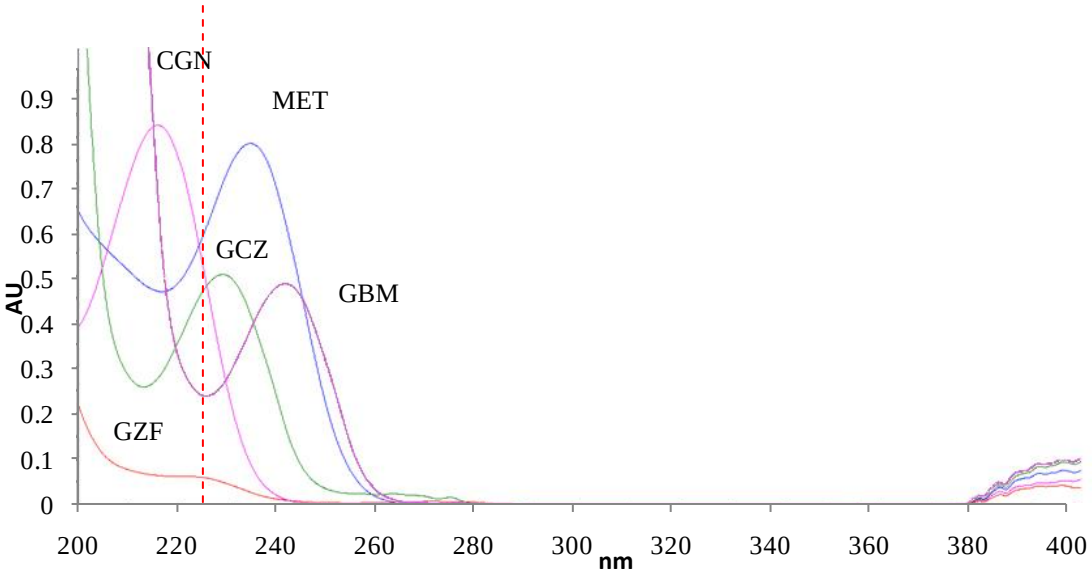
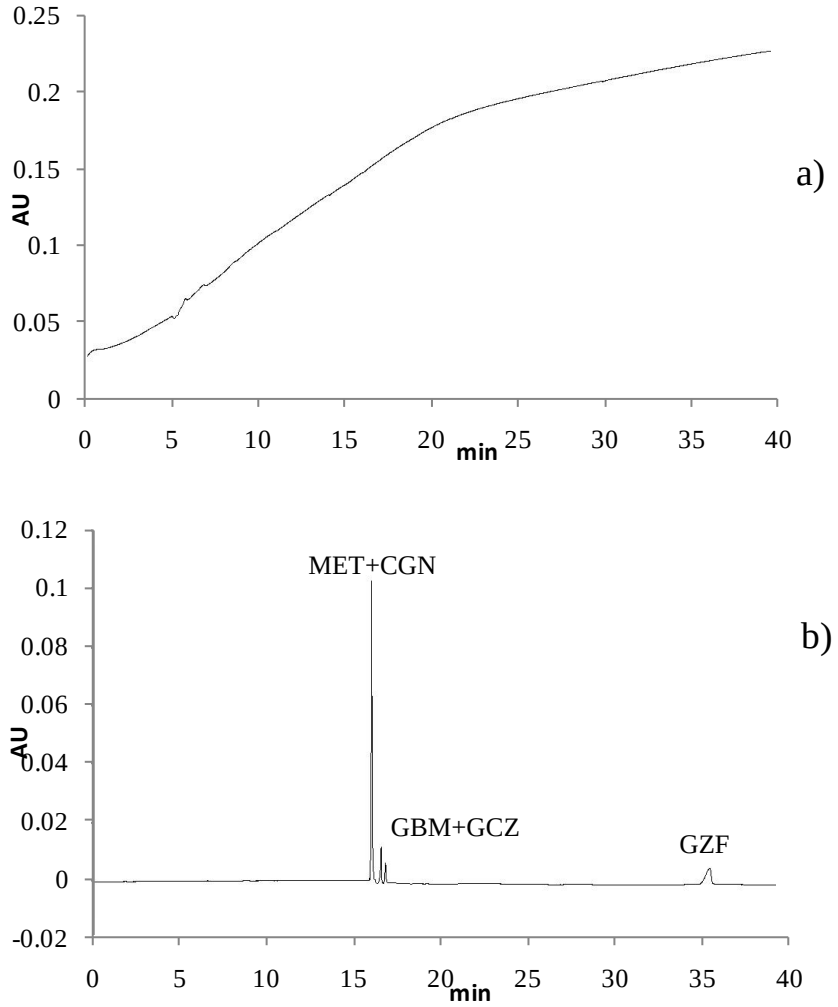


Figure. 4.1 UV spectra of MET, CGN, GBM, GCZ and GZF.

4.1.2 Modes of Instrument

In a preliminary study, the 100 µg/mL standard solutions were prepared in a mixture of ACN and 10 mM phosphate buffer (50:50). The standards were injected under pressure 0.7 psi for 30 seconds. The BGE was 10 mM phosphate buffer, pH 2.0. There were no any peaks to be detected as illustrated in Figure 4.2, (a). Therefore,

stacking technique was introduced in the experiment. According to the theory, using "diluted buffer" in analyte zone was led to the stacking phenomena. So, the new medium prepared was 100 µg/mL standard solutions in a mixture of ACN and 2 mM phosphate buffer (50:50). The new preparing standards was injected in the same manner into the same BGE, except using voltage at -15 kv in negative polarity, and +15 kv in positive polarity. The results were shown in Figures 4.2 (b) and (c), respectively. The negative polarity (Cathode at inlet) was rejected, because the last peak was too separated from the most peaks (RT > 30 min). While, the positive polarity (Anode at inlet) with stacking technique was selected to perform the CE optimization.



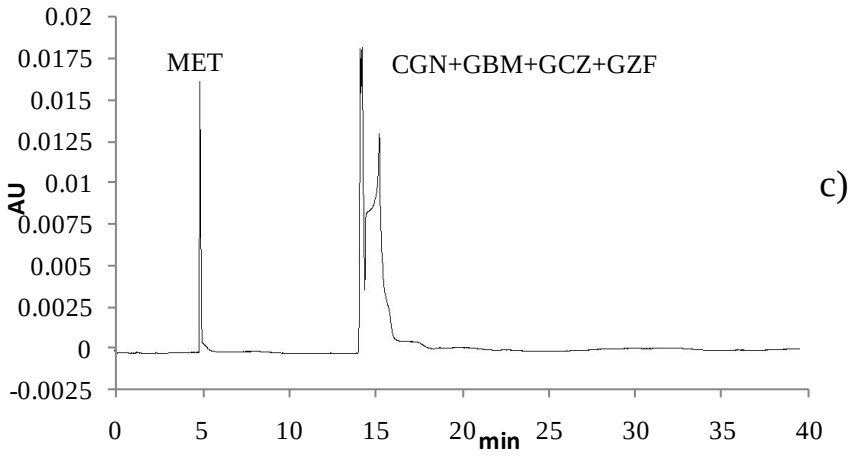


Figure 4.2 The separation of 100 µg/mL standard mixture (MET, CGN, GBM, GCZ and GZF). CE Condition: BGE: 10 mM phosphate buffer, pH 2.0; capillary 60.2 cm full length, 50.0 cm effective length, 50 µm ID, injection 0.7 psi, 30 s; temperature 25ºC; voltage ±15 kV; PDA detection at 225 nm. a) without stacking technique b) stacking technique with negative polarity c) stacking technique with positive polarity.

4.1.3 Types, concentrations and pH of buffer

Citrate, acetate and phosphate buffers were used in experiment with 10 mM, pH 2.0. Citrate buffer was generated too high Joule heating. Acetate buffer was excluded from experiment because of its very hygroscopic property. Phosphate buffer was selected to further experiment with varying of concentration (10, 20 and 30 mM) and pH (2.0, 3.0, 4.0, 5.0, 6.0, and 7.0). The concentrations at 20 and 30 mM were excluded because of too high Joule heating formation. In fact, using 10 mM phosphate buffer with various pH, (2.0 to 7.0) showed no significant separation of the analytes. Although, pH 7.0 was chosen for serving the solubility of GBM, the 10 mM phosphate buffer, pH 7.0 was used in further optimization.

From the CZE limitation, the MEKC showed a higher potential in separation with a wide range of additives (e.g. surfactant, cyclodextrins). So, 4.1.4 to 4.1.8 were the development in MECK technique.

4.1.4 Effect of nonionic surfactant and organic modifiers

Nonionic surfactant performed in MEKC was Brij-35 in 0.2, 0.3 and 0.4 mg/mL. The limit concentration found was 0.2 mg/mL, since the higher concentration gave the prolonged migration time of all analytes (> 40 min). Type and concentration of organic modifier were varied (ACN, MeOH, EtOH in 5, 10, 15 and 20%). In the experiment, the use of 15% MeOH was reasonable. Unfortunately, MeOH caused the high value of %RSD (t_m) in the injection precision. Hence, switching to the use of 15% EtOH was employed for method validation. Actually, EtOH caused precipitation of GBM (as internal standard). GBM was decided to exclude from experimental studies. Figure 4.3 shows the separation of the peaks in BGE comprised of 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0. This BGE combination was further optimization.

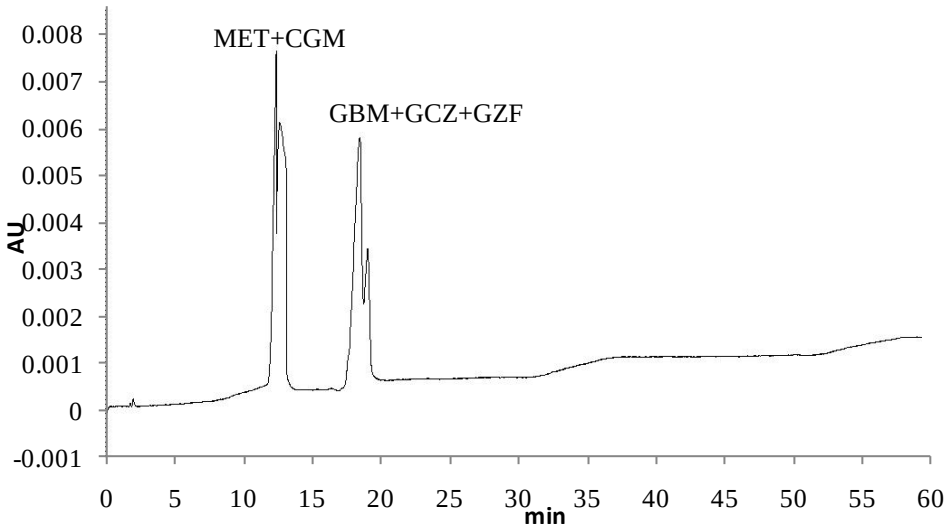


Figure 4.3 The separation of 100 µg/mL standard mixture (MET, CGN, GBM, GCZ and GZF). CE Condition: BGE: 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0; Operational condition : the same as Figure 4.2.

4.1.5 Effect of ion-pair reagents

MET and CGN are structurally related. Both can dissociate giving positive charges that can be chemically bonded with negative charges of sulfonated ion-pair reagents. Propane sulfonate was chosen to the experiment with various concentrations

of 5, 10, 15 and 20 mM. The concentration of 15 mM was the most suitable. Concentration of higher than 15 mM generated too much Joule heating. Therefore, BGE containing ion-pair reagent was used for the separation of MET and CGN. The BGE containing 15 mM Propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0 was further used for optimization study.

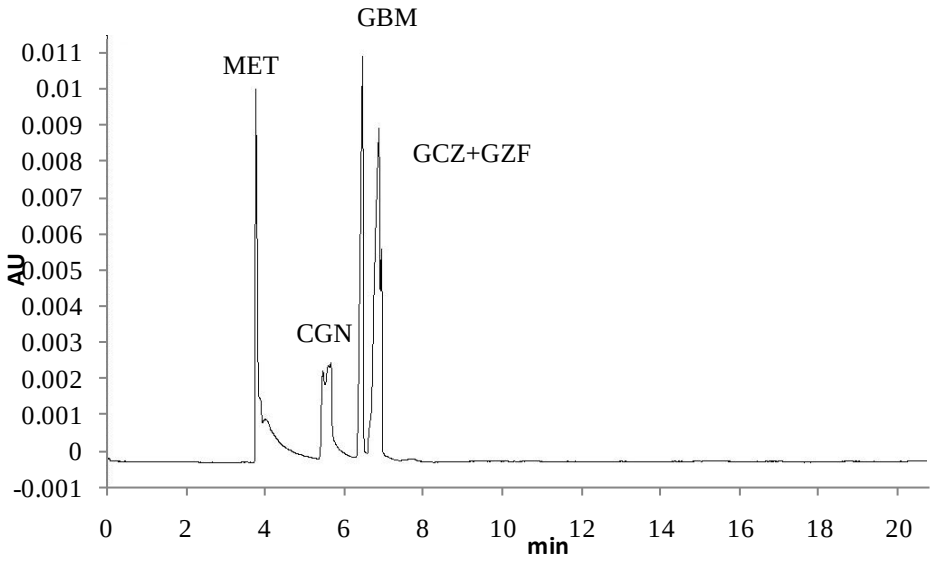


Figure 4.4 The separation of 100 µg/mL standard mixture (MET, CGN, GBM, GCZ and GZF). CE Condition: BGE: 15 mM Propane sulfonate , 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0; Operational condition : the same as Figure 4.2.

4.1.6 Effect of 2HP-β-CD concentrations

2HP-β-CD was employed in the experiment for separation of Sulfonylureas (GBM, GCZ and GZF). 2HP-β-CD was varied in 10, 20, 30 and 35 mM. Concentration of higher than 30 mM caused unstable baseline. The concentration of 30 mM exhibited the most resolution of GBM and GCZ-GZF. The resolution was increased from 1.5 to 4.3, as illustrated in Figure 4.5. The BGE containing 30 mM 2HP-β-CD, 15 mM Propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0 was used for further study.

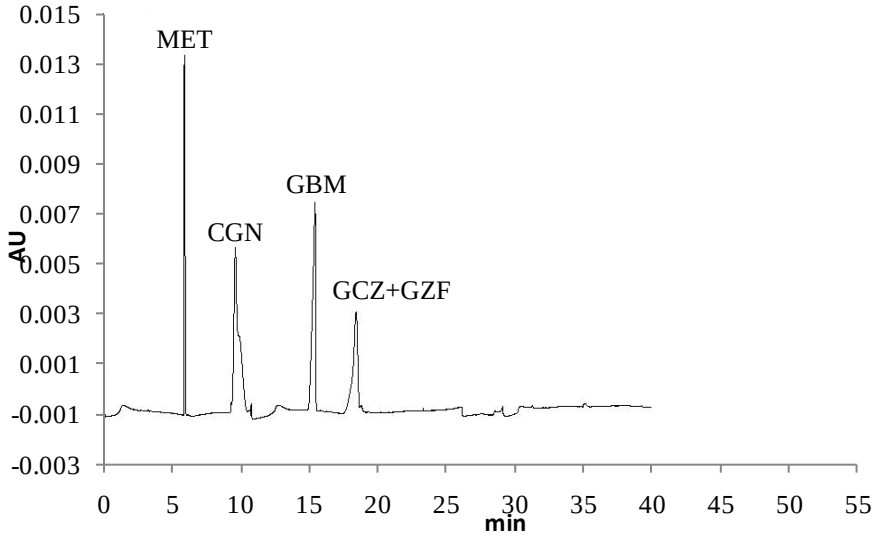


Figure 4.5 The separation of 100 µg/mL standard mixture (MET, CGN, GBM, GCZ and GZF). CE Condition: BGE: 30 mM 2HP-β-CD, 15 mM Propane sulfonate , 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0; Operational condition : the same as Figure 4.2.

4.1.7 Effect of cationic surfactant (CTAB) concentrations

CTAB can cause EOF reversal. The effect is able to extend migration time of all analytes that moved against EOF flow. The prolonged migration time of GCZ and GZF, allowed sufficiently interaction complex between 2HP-β-CD and GCZ-GZF. So, the separation of GCZ and GZF was occurred. CTAB was varied in concentration 1, 2 and 3 mM. Three mM concentration gave the highest of GCZ-GZF resolution with the value of 1.7. The separation was shown in Figure 4.6. The BGE containing 3 mM CTAB, 30 mM 2HP-β-CD, 15 mM Propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0, was further studied.

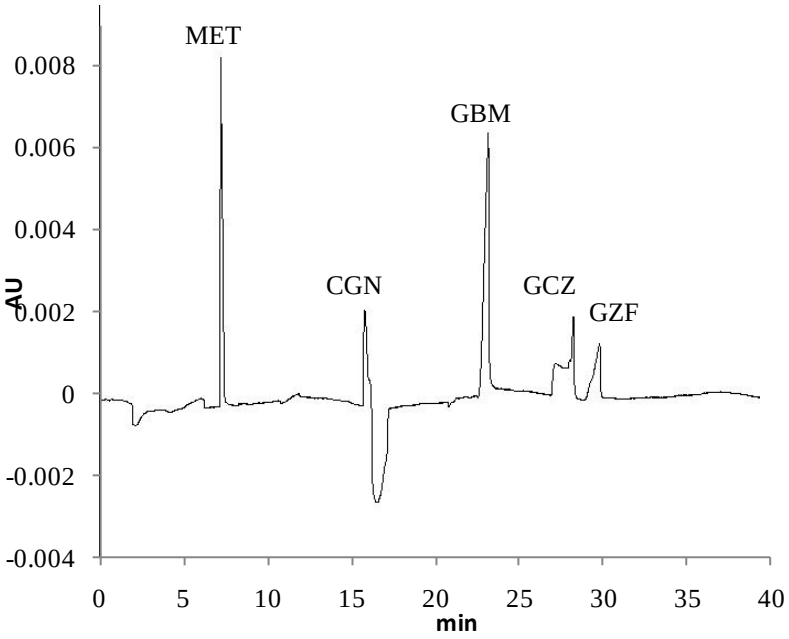


Figure 4.6 The increase of resolution between GCZ and GZF by additional of 3 mM CTAB. CE Condition: BGE: 3 mM CTAB, 30 mM 2HP-β-CD, 15 mM Propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0; Operational condition : the same as Figure 4.2.

4.1.8 Effect of CM-β-CD concentrations

GCF peak shape was still destroyed. The addition of second cyclodextrin (CM-β-CD) was beneficial. The formation of complexation between CM-β-CD and GCZ, reduced GCZ peak dispersion. So, GCZ peak shape will be restored. The various concentrations of CM-β-CD were performed at 1.8, 2.5 and 5.0 mg/mL. Concentration of higher than 1.8 mg/mL caused the prolonged migration time of GCZ and GZF (> 30 minutes). At 1.8 mg/mL CM-β-CD was suitable. The improved tailing factor of GCZ was 0.6. The baseline separation of all analytes was shown in Figure 4.7. The final BGE was a combination of 1.8 mg/mL CM-β-CD, 30 mM HP- β-CD, 3 mM CTAB, 15 mM propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0.

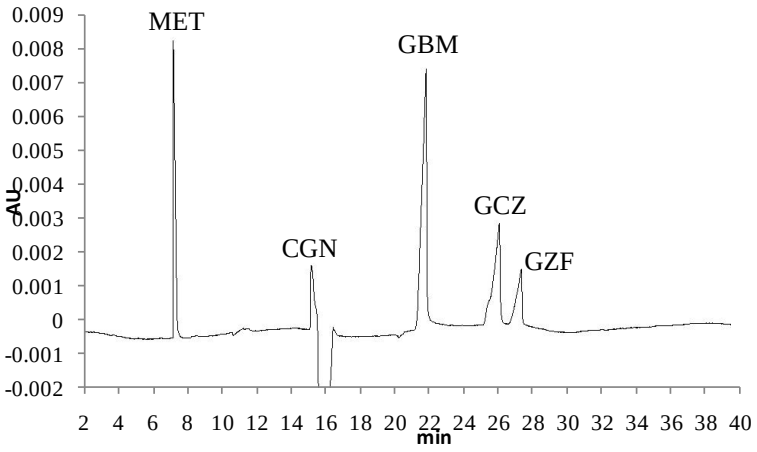


Figure 4.7 The separation by optimized MEKC. CE Condition: BGE: 1.8 mg/mL CM-β-CD, 3 mM CTAB, 30 mM 2HP-β-CD, 15 mM Propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in10 mM phosphate buffer, pH 7.0; Operational condition : the same as Figure 4.2.

The optimized factor of 5 analytes was demonstrated in Table 4.1. From the summarized table, MET was still out of the criterions because MET possessed tailing factor more than 2.0 (7). Therefore, in the method validation, the height of MET was used instead of the area. The separation of CGN was for testing of MET purity. So, the co-migration of CGN with EOF was acceptable. After using EtOH in place of MeOH in BGE, GBM was precipitated. So, GBM was excluded from method validation. The separation of GZF was for testing of GCZ purity. Hence, in method validation, MET and GCZ were the major analytes to quantify.

Table 4.1 The optimized analytical factors of 5 analytes (MET,CGN,GBM,GCZ and GZF).

Analyte	Time (min)	Theoretical plates	Resolution	Tailing factor
MET	7.3	19738	-	5.2
CGN*	15.5	-	-	-
GBM	21.1	30091	36.8 (MET/GBM)	0.6
GCZ	26.4	39282	6.3 (GBM /GCZ)	0.6
GZF	27.5	41058	1.7 (GCZ /GZF)	0.6

*co-elute with EOF

4.2 Method validation

The optimized condition for separation of MET and GCZ was evaluated in term of specificity, linearity, precision and accuracy according to ICH guidelines (42).

4.2.1 Specificity

One hundred µg/mL standard solutions (MET and GCZ) were separately injected to construct spectrum data. The sample solution was injected under the same condition. The similarity index was calculated by comparing the current sample data with a standard spectrum stored in the library. The results of matching spectra of MET and GCZ were illustrated in Appendix.

4.2.2 Linearity

Linearity of MET and GCZ was achieved by triplicate injections of individual analyte over five concentration levels, covering a range of 50-150 µg/mL. The least-square linear regression of the analytes was obtained by plotting peak area or peak height versus concentrations. The parameters were shown in Table 4.2, demonstrating the highest correlation coefficient of MET at r^2 = 0.9986 which was calculated from peak height. Meanwhile the best fit of GCZ linearity was obtained by peak area (r^2 = 0.9974). Therefore, peak height and peak area were selected to establish the linearity of MET and GCZ, respectively. The raw data and calibration curves were shown in Appendix.

Table 4.2 Linearity of MET and GCZ.

Analyte	Signal	Slope	y-intercept	r^2
MET	Peak area	1,499,764	14,290	0.9973
	Peak height	125,964	10,211	0.9986
GCZ	Peak area	2,040,720	32,554	0.9974
	Peak height	70,610	5,551	0.9858

4.2.3 Precision

The precision of the proposed method indicated the closeness of measurements derived from a series of sample analysis under test. Precision is calculated from injection, intra-day and inter-day precisions.

4.2.3.1 Injection precision

Injection precision was investigated at the middle point of the calibration curve (100 µg/mL) by nine replicate injections. The %RSD of peak area and peak height were calculated and data was shown in Tables 4.3 and 4.4. %RSDs calculated from peak areas and peak heights of MET were 1.24 and 0.63, respectively. Meanwhile, %RSDs calculated from peak areas and peak heights of GCZ were 1.48 and 3.35, respectively. According to the criteria of %RSD ≤ 2.0 (7), %RSD from both MET and GCZ were acceptable.

Table 4.3 Injection precision of MET at 100 µg/ml (n = 9).

Injection no.	MET		
	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	7.77	167940	23534
2	7.77	164281	23163
3	7.74	163483	23272
4	7.72	169487	23472
5	7.74	168241	23268
6	7.73	164432	23467
7	7.78	167283	23412
8	7.76	166481	23342
9	7.77	167485	23628
Average	7.75	166568	23395
SD	0.02	2057.66	147.01
%RSD	0.27	1.24	0.63

Table 4.4 Injection precision of GCZ at 100 µg/mL (n = 9).

Injection no.	GCZ		
	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	20.25	214790	11444
2	20.29	217997	11754
3	20.05	209905	12154
4	20.08	211947	12335
5	20.12	209255	12254
6	20.15	214128	12559
7	20.18	209083	12445
8	20.09	211074	12664
9	20.08	208921	12611
Average	20.14	211900	12247
SD	0.08	3141.65	410.37
%RSD	0.41	1.48	3.35

4.2.3.2 Intra-day precision

Intra-day precision was examined at three different concentrations (50, 100 and 150 µg/mL) of MET and GCZ on the same day. The %RSDs of peak area and peak height were determined and the results were shown in Tables 4.5-4.6. The %RSDs calculated from peak heights of the intra-day precision for MET were in a range of 0.44-1.60%. %RSDs of GCZ calculated from peak areas were in a range of 0.31-1.70%.

Table 4.5 Intra-day precision of MET (n = 3).

Injection no.	Conc (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	50	7.54	95238	15321
2		7.52	92237	15078
3		7.56	94325	15567
Average		7.54	93933	15322
SD		0.02	1538.36	244.50
%RSD		0.27	1.64	1.60
1	100	7.52	158230	24263
2		7.48	156495	24857
3		7.50	153274	24631
Average		7.50	156000	24584
SD		0.02	2514.86	299.82
%RSD		0.27	1.61	1.22
1	150	7.57	232748	28917
2		7.52	236891	29077
3		7.56	235720	29168
Average		7.55	235120	29054
SD		0.03	2135.75	127.07
%RSD		0.35	0.91	0.44

Table 4.6 Intra-day precision of GCZ (n = 3).

Injection no.	Conc (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	50	21.23	116280	8213
2		20.97	114256	7916
3		20.83	118203	8123
Average		21.01	116246	8084
SD		0.20	1973.72	152.29
%RSD		0.97	1.70	1.88
1	100	20.10	221987	12136
2		19.88	219684	12071
3		19.67	220456	11923
Average		19.88	220709	12043
SD		0.22	1172.16	109.16
%RSD		1.08	0.53	0.91
1	150	20.16	320252	15038
2		20.03	319823	15123
3		19.82	321741	14918
Average		20.00	320605	15026
SD		0.17	1006.64	103.00
%RSD		0.86	0.31	0.69

4.2.3.3 Inter-day precision

Inter-day precision was examined at three different concentrations (50, 100 and 150 µg/mL) of MET and GCZ on three different days. The %RSDs of peak area and peak height were determined and the results were shown in Tables 4.7-4.8. The %RSDs calculated from peak heights of the inter-day precision

for MET were in a range of 0.99-1.66%. %RSDs of GCZ calculated from peak areas were in a range of 1.61-1.97%.

Table 4.7 Inter-day precision of MET (n = 3).

Day	Conc. (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	50	7.31	92465	15307
2		7.60	91515	15534
3		7.50	94003	15657
Average		7.47	92661	15499
SD		0.13	1774.63	256.76
%RSD		1.70	1.92	1.66
1	100	7.32	156489	23323
2		7.59	155630	23248
3		7.51	160657	23608
Average		7.48	157592	23393
SD		0.12	3091.57	243.04
%RSD		1.62	1.96	1.04
1	150	7.36	244765	29412
2		7.61	245354	29189
3		7.50	251644	29394
Average		7.49	247254	29332
SD		0.11	3978.16	290.88
%RSD		1.44	1.61	0.99

Table 4.8 Inter-day precision of GCZ (n = 3).

Day	Conc. (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
1	50	23.61	116639	8150
2		22.67	118919	8302
3		22.79	118964	8045
Average		23.02	118174	8166
SD		0.46	2324.42	231.64
%RSD		2.00	1.97	2.84
1	100	23.48	229051	12011
2		22.65	230957	11851
3		22.83	232261	11891
Average		22.99	230756	11917
SD		0.41	3735.62	278.17
%RSD		1.80	1.62	2.33
1	150	21.10	317177	14627
2		21.77	323702	14991
3		21.77	313624	14954
Average		21.55	318168	14857
SD		0.34	5119.11	392.96
%RSD		1.60	1.61	2.64

4.2.4 Accuracy

Accuracy of the method was evaluated from percent recovery of MET and GCZ in pharmaceutical formulations. Using standard addition method in this study, the reference standard was separately weighted into the finely grounded tablets, covering three levels in a range of 80-120% of specified concentrations. Each concentration was quantified in three determinations under optimized CE condition.

Recoveries of MET and GCZ were shown in Tables 4.9-4.10 and the values were within the criteria of 98-102% (7).

Table 4.9 Recoveries of MET in pharmaceutical formulation (n = 3).

% added	Amount added (mg)	Total amount (mg)	Amount found (mg)	% Recovery
80	199.52	397.24	197.72	99.5
100	248.78	499.05	250.27	100.3
120	300.12	603.54	303.42	100.7
			Average	100.17
			SD	0.61
			%RSD	0.61

Table 4.10 Recoveries of GCZ in pharmaceutical formulation (n = 3).

% added	Amount added (mg)	Total amount (mg)	Amount found (mg)	% Recovery
80	33.78	68.22	34.44	102.0
100	39.71	78.98	39.27	98.9
120	47.56	95.83	48.27	101.5
			Average	100.78
			SD	1.65
			%RSD	1.64

4.3 Applications

The proposed method was applied for analysis of pharmaceutical preparations in single and combined formulations. In single drug, MET tablet was available in 500 mg, while GCZ was manufactured in 80 mg tablet. The fixed dose combination was composed of 500 mg MET and 80 mg GCZ. The results of assay

were shown in Table 4.11. All contents found were within the limit of 95.0-105.0% la. (6).

Table 4.11 Assay of MET and GCZ in tablet formulation.

Type	Content found (%la.)		Limits (BP 2013)
	MET	GCZ	
Single drug			
MET 500 mg			
Brand A	101.2		95.0-105.0%
Brand B	97.8		95.0-105.0%
GCZ 80 mg			
Brand C		96.3	95.0-105.0%
Brand D		97.8	95.0-105.0%
Combination drug			
MET 500 mg and GCZ 80 mg			
Lot E	100.3	98.3	-
Lot F	99.8	97.2	-
Lot G	101.6	99.4	-

* la = labeled amount

CHAPTER V
CONCLUSION

5.1 CE Development and optimization

CE method development was complied MEKC mode to simultaneous determination of 5 analytes (MET, CGN, GBM, GCZ, GZF). Since, the low sensitivity of CE instrumentation, the stacking technique was introduced by diluting buffer in analyte preparation. Because of their different chemical property (biguanides vs sulfonylureas) and closely similar structure (MET-CGN, GCZ-GZF), many strategies were conducted for its optimization. Mainly, BGE was developed by varying type and concentration of buffer, surfactant, organic modifier and additives. Buffer was studied by varying type (citrate, acetate and phosphate), concentration (10, 20 and 30 mM) and pH (2.0, 3.0, 4.0, 5.0, 6.0 and 7.0). Non-ionic surfactant (Brij-35) was used at various concentrations (0.2, 0.3 and 0.4 mg/mL). Organic modifier was varied by its type (ACN, MeOH and EtOH) and concentration (5, 10, 15 and 20%). The additives were added (e.g. cyclodextrins, ion-pair reagent and CTAB). Propane sulfonate was chosen as an ion-pair reagent using for MET-CGN separation. Propane sulfonate was varied in concentration of 5, 10, 15 and 20 mM. First cyclodexytrins using in BGE was 2HP-β-CD. The addition of 2HP-β-CD for separation of GBM and GCZ-GZF. 2HP-β-CD was varied in concentration of 10, 20, 30 and 35 mM. CTAB was used for separation of GCZ and GZF which was varied in concentration of 5, 10, 15 and 20 mM. Second cyclodextrins in BGE was CM-β-CD for improving GCZ tailing factor. CM-β-CD was varied in concentration of 1.8, 2.5 and 5.0 mg/mL. The optimized BGE was comprised of 1.8 mg/mL CM-β-CD, 30 mM 2HP-β-CD, 3 mM CTAB, 15 mM propane sulfonate, 0.2 mg/mL Brij-35 and 15% MeOH in 10 mM phosphate buffer, pH 7.0. Unfortunately, in method validation, BGE containing 15% MeOH caused %RSD (t_m) more than 2.0. So, 15% EtOH was used in BGE instead of MeOH. In fact, EtOH caused precipitation of GBM. GBM then was excluded from method validation.

Actually, the separation of CGN and GZF was for testing purity of MET and GCZ. Therefore, MET and GCZ were performed in method validation.

5.2 Method validation

From optimization, MET possessed tailing factor out of the criteria (not more than 2.0). So, peak height of MET was used in method validation and applied to determination for tablet formulation. Calibration curves of MET and GCZ were constructed in a range of 50-150 µg/mL. The data calculated for correlation coefficients (r^2) of MET were focused on peak height. Because peak height of MET gave higher correlation coefficient (r^2 = 0.9986) than peak area (r^2 = 0.9973). Whereas, the data_calculated for correlation coefficient (r^2) of GCZ were focused on peak area which gave higher correlation coefficients (r^2 = 0.9974) than peak height (r^2 = 0.9858). Precision was evaluated in terms of injection, intra-day, inter-day precisions. Peak height of MET gave the lower %RSD than peak area. For injection, intra-day, inter-day precisions, %RSDs were 0.63, 0.44 and 0.99, respectively. While, peak area of GCZ gave the lower %RSD than peak height. For injection, intra-day, inter-day precision, %RSDs were 1.48, 0.31 and 1.61, respectively. Recovery of MET (based on peak height) was 99.1-101.1%. Recovery of GCZ (based on peak area) was 98.9-102.0%

5.3 Determination of anti-diabetic drugs

The development and validated MEKC method was applied for analysis of MET and GCZ in tablet formulations. Two different brands of single-drug (MET and GCZ) and one brands (3 different lots) of combined drug (MET+GCZ) were complied in the determination. In single drug, the %label amounts of MET were 101.2 and 97.8 for brands A and B, respectively. The %label amounts of GCZ were 96.3 and 98.9 for brands C and D, respectively. In combined drug, %label amounts of MET were 100.3, 99.8 and 101.6 for lots E, F and G, respectively. The % label amounts of GCZ were 98.3, 97.2 and 99.4 for lot E, F and G, respectively. In conclusion, the great advantage of MEKC method was to reduce analysis time of combined anti-diabetic

drugs and was environmental friendly. The method can also be applied for routine analysis of single anti-diabetic drugs with excellent linearity, accuracy and precision.

REFERENCES

1. Powers AC, Diabetes mellitus. In: Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL, editor. Harrison's principles of internal medicine. 16th ed. New York: McGraw-Hill Companies, Inc.; 2005. p. 2152-3.
2. Shaw JE, Sicree R. Epidemiology of type 2 diabetes. In: Feinglos MN, Bethel MA, editor. Contemporary endocrinology type 2 diabetes mellitus [e-book]. Humana Press; 2008 [cited 2008 Nov 1]: 2-3. Available from: Spingerlink.com.
3. Nielsen HB, Henriksen JE. Antidiabetic combination therapy. In: Mogensen CE, editor. Pharmacotherapy of diabetes : new developments [e-book]. Springer; 2007 [cited 2008 Nov 1]: 105. Available from: Spingerlink.com.
4. U.S. national library of medicine. nlm.com. [homepage on the internet]. toxicity : cyanoguanidine; [cited 2008 Nov 1]. Available from: http://chem.sis.nlm.nih.gov/ chemidplus/jsp/common/Toxicity.jsp.
5. European directorate for the quality of medicines & healthcare. edqm.eu.[homepage on the internet]. safety data sheet: gliclazide impurity b; 2007 [updated 2007 Oct 30; cited 2008 Nov 1]. Available from: http://crs.edqm.eu/db/4dcgi/db/4 dcgi /msds?msds=g0326004.
6. British Pharmacopoeia Commission. British Pharmacopoeia 2008. London: The Stationery Office; 2004, pp 970-973.
7. The United State Pharmacopeial Convention, The United States Pharmacopoeia 31 The National Formulary 26 (Asian Eds), Rockville, 2008, pp.1320-1322.
8. Bhushan R, Gupta D, Jain A. TLC supplemented by UV spectrophotometry compared with HPLC for separation and determination of some antidiabetic drugs in pharmaceutical preparations. J Planar Chromatogr. 2006;19:288-96.

9. Wang LR, Huang MZ, Zhu SH, HPLC determination of metformin hydrochloride-related substances. J Zhejiang Univ. 2005;34(4):368-71.
10. Yao J, Shi YQ, Li ZR, Jin SH. Development of a RP-HPLC method for screening potentially counterfeit anti-diabetic drugs. J Chromatogr B. 2007;853:254-9.
11. Porta V, Schramm SG, Kano EK, Koono EE, Armando YP, Fukuda K, et al. HPLC-UV determination of metformin in human plasma for application in pharmacokinetics and bioequivalence studies. J Pharm Biomed Anal. 2008;46:143-7.
12. Skrzypek S, Mirceski V, Ciesielski W, Sokolowski A, Zakrzewski R. Direct determination of metformin in urine by adsorptive catalytic square-wave voltammetry. J Pharm Biomed Anal. 2007;45:275-81.
13. Yardimci C, Ozaltin N, Gurlek A. Simultaneous determination of rosiglitazone and metformin in plasma by gradient liquid chromatography with UV detection. Talanta. 2007;72:1416-22.
14. Foroutan SM, Zarghi A, Shafaati A, Khoddam A. Application of monolithic column in quantification of gliclazide in human plasma by liquid chromatography. J Pharm Biomed Anal. 2006;42:513-6.
15. Park JY, Kim KA, Kim SL, Park PW. Quantification of gliclazide by semi-micro high-performance liquid chromatography: application to a bioequivalence study of two formulations in healthy subjects. J Pharm Biomed Anal. 2004;35:943-9.
16. AbuRuz S, Millership J, McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. J Chromatogr B. 2005;817:277-86.
17. Rouini MR, Mohajer A, Tahami MH. A simple and sensitive HPLC method for determination of gliclazide in human serum. J Chromatogr B. 2003;785:383-6.
18. Vasudevan M, Ravi J, Ravisankar S, Suresh B. Ion-pair liquid chromatography technique for the estimation of metformin in its multicomponent dosage forms. J Pharm Biomed Anal. 2001;25:77-84.

19. Zhong GP, Bi HC, Zhou S, Chen X, Huang M. Simultaneous determination of metformin and gliclazide in human plasma by liquid chromatography-tandem mass spectrometry: application to a bioequivalence study of two formulations in healthy volunteers. J Mass Spectrom. 2005;40(11): 1462-71.
20. Koseki N, Kawashita H, Niina M, Nagae Y, Masuda N. Development and validation for high selective quantitative determination of metformin in human plasma by cation exchanging with normal-phase LC/MS/MS. J Pharm Biomed Anal. 2005;36:1063-72.
21. Wang Y, Tang Y, Gu J, Fawcett JP, Bai X. Rapid and sensitive liquid chromatography-tandem mass spectrometric method for the quantitation of metformin in human plasma. J Chromatogr B. 2004;808: 215-9.
22. Lai EPC, Feng SY. Solid phase extraction-non-aqueous capillary electrophoresis for determination of metformin, phenformin and gliburide in human plasma. J Chromatogr B. 2006;843(1):94-9.
23. Yardimci C, Ozaltin N. Method development and validation for the simultaneous determination of rosiglitazone and metformin in pharmaceutical preparations by capillary zone electrophoresis. Anal Chim Acta. 2005;549(1-2):88-95.
24. Lv J, Wang Q, Chen X, He P, Fang Y. Determination of aminoheterocycle and azabicycle in gliclazide bulk by capillary zone electrophoresis with amperometric detection. J Pharm Biomed Anal. 2005;39:843-7.
25. Paroni R, Comuzzi B, Arcelloni C, Brocco S, Kreutzenberg SD, Tiengo A, et al. Comparison of capillary electrophoresis with HPLC for diagnosis of facitious hypoglycemia. Clin Chem. 2000;46(11):1773-80.
26. Kuo CY, Wu SM. High-performance liquid chromatography with electrochemical detection for analysis of gliclazide in plasma. J Chromatogr A. 2005;1088:131-5.
27. El-Enany N. Spectrophotometric determination of gliclazide in pharmaceuticals and biological fluids through ternary complex formation with eosin and palladium(II). Farmaco. 2004;59:63-9.

28. El-Enany N. Spectrofluorimetric and spectrophotometric determination of gliclazide in pharmaceuticals by derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole. J AOAC Int. 2003; 86(2):209-14.
29. Krzek J, Czekaj J, Moniczewska M, Rzeszutko W. Determination of gliclazide in pharmaceutical preparations by capillary gas chromatography with cool on-column injection and elimination of the matrix effect. J AOAC Int. 2001;84(6):1695-702.
30. Frank S, editor. Handbook of Instrumental Techniques for Analytical Chemistry: Capillary Electrophoresis. New Jersey: Prentice Hall PTR; 1997. p. 167-8.
31. Suntornsuk L, Capillary electrophoresis in pharmaceutical analysis: A survey on recent applications. J Chromatogr Sci. 2007; 45:559-577.
32. Antidiabetics:biguanide,sulfonylurea. In: Sweetman SC, editor. Martindale. 35th ed. London: Pharmaceutical press; 2007 : 396, 419.
33. Ceriello A. Effects of gliclazide beyond metabolic control. Metabolism. 2006; 55: 10-5.
34. ลีณา สุนทรสุข. แคปิลลารีอิเล็กโทรโฟรีซิส. กรุงเทพ: บียอนด์ เอ็นเทอร์ไพรซ์; 2550.
35. Weston A, Brown PR. HPLC and CE Principals and Practice. Utah: Academic Press; 1997.
36. Heiger D. High performance capillary electrophoresis: An introduction: Agilent Technologies; 2000.
37. Becker JL, Book P. Multiple effect of surfactants used as additives in background electrolytes in capillary zone electrophoresis: Cetyltrimethylammonium bromide as example of model surfactant. Electrophoresis. 2002; 23(12): 1947-52.
38. Melanson JE, Baryla NE, Lucy CA. Double-chained Surfactants for Semipermanent wall Coatings in Capillary Electrophoresis. Anal Chem. 2000; 72: 4110-4.
39. Weston A, Brown PR. HPLC and CE: Principles and practice; Academic press limited, California, USA. 1997.
40. Quirino JP, Terabe S. Sample stacking of cationic and anionic analytes in capillary electrophoresis. J. Chromatogr. A. 2000; 902: 120-1.

41. Camilleri P. Capillary Electrophoresis Theory and Practice. 2nd ed. London: CRC Press; 1997.
42. ICH. Validation of analytical procedure: text and methodology Q2 (R1). International Conference on Harmonization, International Federation of Pharmaceutical Manufacturers and Associations (IFPMA), Geneva. 2003.

APPENDIX

Table A1 Calibration data of MET.

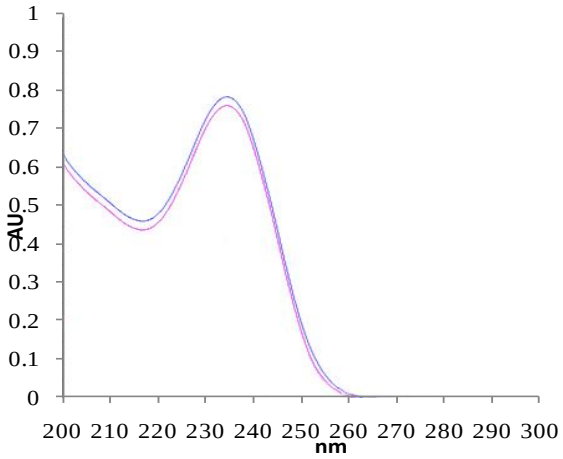
Conc. (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
50	7.59	92132	16538
	(0.72)	(1.66)	(1.76)
75	7.75	125776	19593
	(0.68)	(1.18)	(0.51)
100	7.82	160571	23070
	(0.75)	(1.40)	(0.97)
125	7.63	202386	26320
	(0.84)	(1.87)	(0.20)
150	7.54	244241	29055
	(0.79)	(1.31)	(0.64)
Slope		1,499,764	125,964
y-intercept		14,290	10,211
r^2		0.9973	0.9986

Number in parenthesis represent %RSD

Table A2 Calibration data of GCZ.

Conc. (µg/mL)	t_m (min)	Peak area (AU*s)	Peak height (AU)
50	22.32	129063	8682
	(0.92)	(1.60)	(1.34)
75	22.33	183146	10890
	(0.87)	(1.60)	(1.40)
100	22.57	237275	12791
	(1.02)	(1.23)	(1.07)
125	22.50	285533	14442
	(0.99)	(1.07)	(1.29)
150	22.19	327908	15558
	(1.08)	(1.19)	(1.42)
Slope		2,040,720	70,610
y-intercept		32,554	5,551
r^2		0.9974	0.9858

Number in parenthesis represent %RSD



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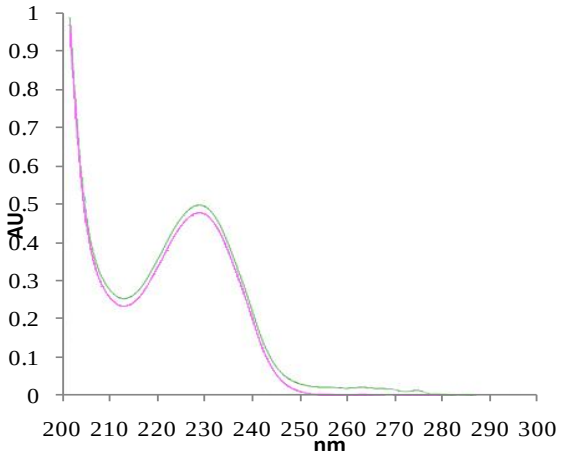

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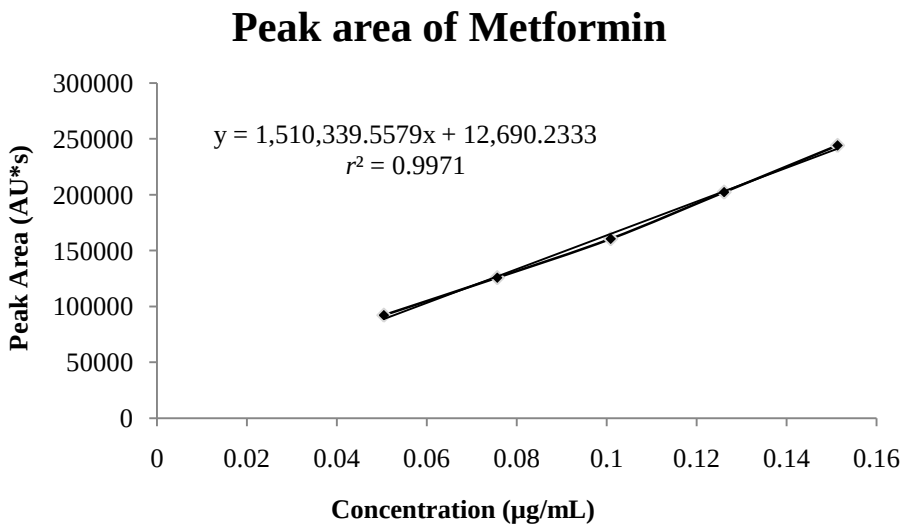
Figure A1 The matching spectra of sample solution (MET and GCZ) to standard spectrum library.



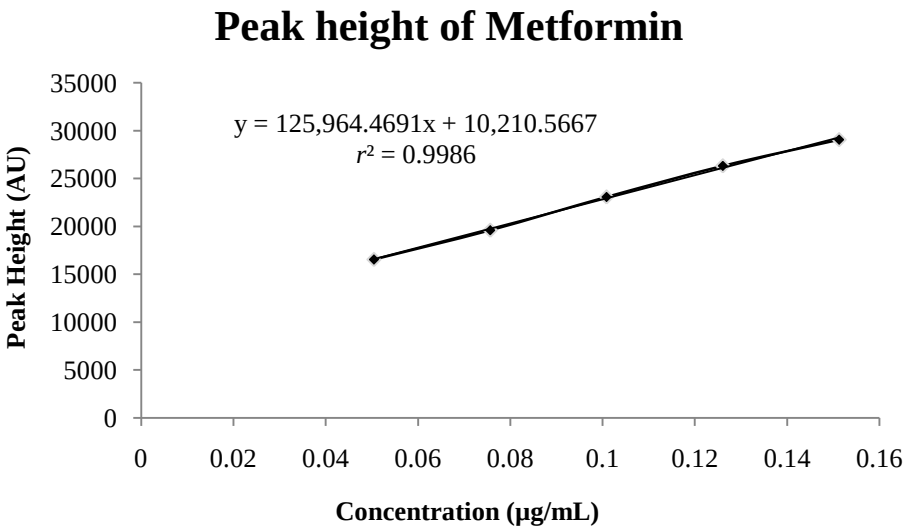
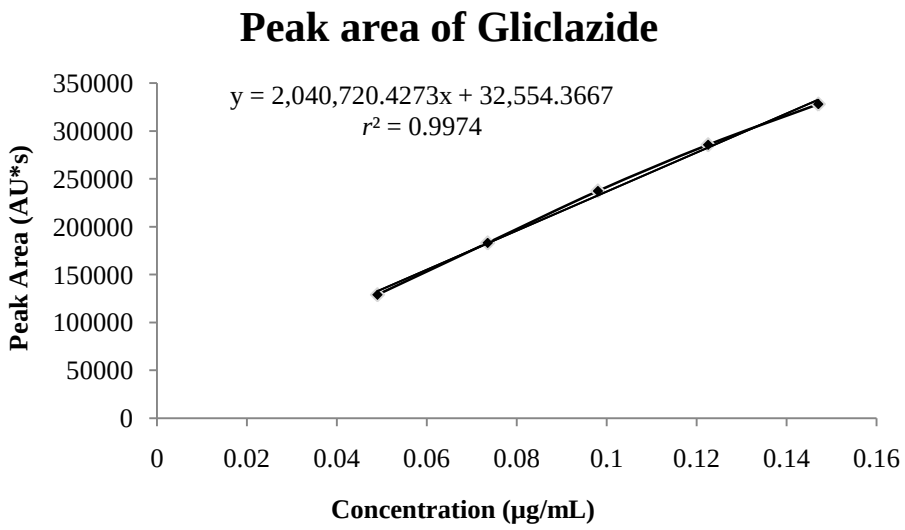


Figure A2 Calibration curve of MET (peak area and peak height VS concentration).



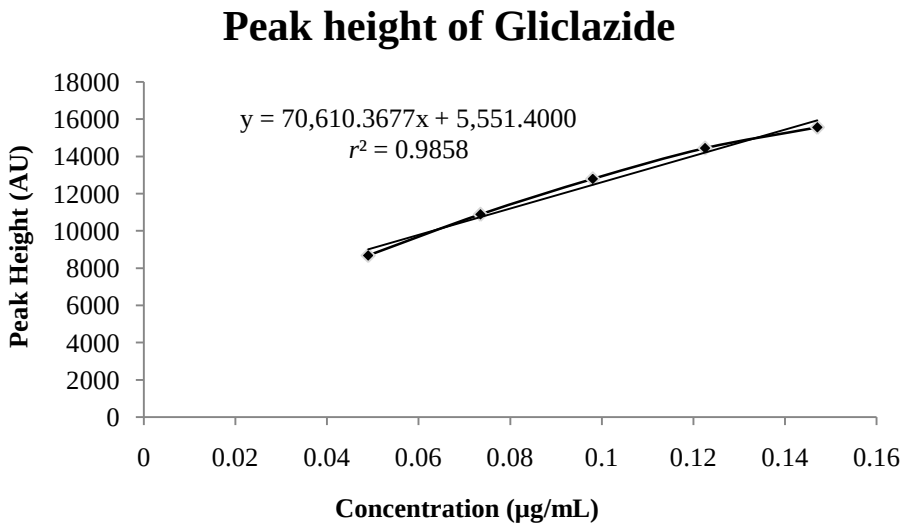


Figure A3 Calibration curve of GCZ (peak area and peak height VS concentration).

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