SIMULTANEOUS ANALYSIS OF COMBINED ANTI-DIABETIC DRUGS BY CAPILLARY ELECTROPHORESIS AND ITS APPLICATION TO STABILITY STUDY

ATHIPORN DOOMKAEW

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ABSTRACT

Diabetes is a chronic disease, it is defined by a high blood sugar level. It is harmful to many organs such as eyes, kidneys, brain and heart. There are many groups of medicines to decrease the amount of blood sugar level. Biguanides (e.g. metformin (MET) and sulfonylureas (e.g. gliclazide (GCZ), glibenclamide GBM)) are the drug of choice for treatment of this disease. Currently, combinations of MET and GCZ, MET and GBM are commercially available to enhance patient compliance. Thus, quality control of these drugs and their impurities is a priority.

However, quality control methods for combined formulations of MET and GCZ, MET and GBM are not official in pharmacopeias. In this study, capillary electrophoresis (CE) is proposed due to the high efficiency, simplicity and environment friendliness of the method. The optimization condition analysis of MET, GCZ and GBM was in a 50 mM borate buffer (pH 9.0) using a capillary with a L_{total} of 64.5 cm, 50 μm i.d., injection at 50 mbar for 10 s, temperature at 25 °C and the applied voltage of 20 kV, all analysts migrated within 8.2 min. The validated method was applied for the determination of MET, GCZ and GBM in bulk and pharmaceutical formulations with the results found within USP and BP limits.

A stability indicating method is important to assure the quality of medicines, CE methods for a stability study of MET and GCZ were established and could be used for stability indicating and assay methods for MET and GCZ. A stability study of MET and GCZ were performed under hydrolysis (acid, base and neutral conditions), oxidation and photolysis conditions. MET was stable in neutral pH (water) and acidic (0.1 N HCl) hydrolysis, but degraded to cyanoguanidine (CGN) under alkaline hydrolysis (0.1 N NaOH) and oxidation (3% H_2O_2). Elevated temperature and exposure of MET to sunlight accelerated the degradation of MET. In contrast to MET, CGZ was stable under alkaline hydrolysis and rapidly degraded to GZB under acidic and neutral hydrolysis and oxidation. Applications of the methods for assays of MET and GCZ in raw materials and commercial tablets revealed that content of the drugs in all samples (%labeled amount between 99.1 and 100.2% with %RSDs of less than 0.95%) met the requirements of pharmacopeias. No degradation in the products, CGN GZB and GZF, were observed in the investigated samples.

KEY WORDS: CAPILLARY ELECTROPHORESIS/ METFORMIN/ GLICLAZIDE/ GLIBENCLAMIDE

102 pages

การวิเคราะห์ยาเบาหวานรูปแบบยาผสมในคราวเดียวกันโดยแคปิลลารีอิเล็กโทรโฟรีซีสและการศึกษาสภาพความคงตัวของยา

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บทคัดย่อ

เบาหวานเป็นโรคเรื้อรังซึ่งวินิจฉัยโดยระดับน้ำตาลในเลือดที่สูง ซึ่งทำให้เกิดอาการผิดปกติในหลายอวัยวะที่สำคัญในร่างกายเช่น ตา ไต สมอง หัวใจและหลอดเลือด การรักษาทำโดยการใช้ยาลดระดับน้ำตาลในเลือด ยาที่ใช้ในการลดระดับน้ำตาลในเลือดมีหลายชนิด กลุ่มยาที่เลือกใช้เป็นลำดับแรกคือ ยากลุ่มไบกัวไนด์ เช่น เมทฟอร์มิน ยากลุ่มซัลโฟนิลยูเรีย เช่น กลัยคาไซด์ และ กลัยเบนคลาไมด์ ปัจจุบันได้มีการพัฒนาสูตรตำรับยาผสมสองกลุ่มนี้เพื่อเพิ่มประสิทธิภาพในการรักษา และเพิ่มความร่วมมือในการใช้ยา ดังนั้นการควบคุมคุณภาพของยาทั้งสองกลุ่มนี้จึงเป็นสิ่งสำคัญ

อย่างไรก็ตามยังไม่มีวิธีวิเคราะห์ยาผสมในตำรายามาตรฐาน การศึกษานี้มีวัตถุประสงค์ที่จะวิเคราะห์ยาผสมด้วยเทคนิคแคปิลลารี เนื่องจากเป็นวิธีที่มีประสิทธิภาพสูง ง่ายและเป็นมิตรกับสิ่งแวดล้อม การพัฒนาเทคนิคแคปิลลารีอิเล็กโทรโฟรีซีสสำหรับการแยก เมทฟอร์มิน กลัยคาไซด์ และกลัยเบนคลาไมด์ ในคราวเดียวกัน สภาวะที่เหมาะสม ได้แก่ บอเรตบัฟเฟอร์ 50 มิลลิโมลาร์ที่พีเอช 9.0 แคปิลลารียาว 64.5 เซนติเมตร, เส้นผ่าศูนย์กลางภายใน 50 ไมโครเมตร ฉีดสารด้วยแรงดัน 50 มิลลิบาร์ 10 วินาที ที่อุณหภูมิ 25 องศาเซลเซียสและ ความต่างศักย์ไฟฟ้า 25 กิโลโวลต์ สภาวะดังกล่าวสามารถแยกสารได้อย่างสมบูรณ์ภายใน 8.2 นาที วิธีวิเคราะห์ที่ผ่านการประเมินได้ถูกนำไปประยุกต์ใช้ในการหาปริมาณของเมทฟอร์มิน กลัยคาไซด์ และกลัยเบนคลาไมด์ ทั้งในเภสัชเคมีภัณฑ์ในเภสัชภัณฑ์ทั้งยาเดี่ยวและยาผสม พบว่าปริมาณอยู่ในช่วงที่ตำรายากำหนดไว้

การศึกษาความคงสภาพของยาเป็นส่วนสำคัญในการประกันคุณภาพยาในงานวิจัยนี้ได้ศึกษาวิธีแคปิลลารีอิเล็กโทรโฟรีซีสสำหรับการศึกษาความคงสภาพของยาเมทฟอร์มิน และกลัยคาไซด์ วิธีที่พัฒนาขึ้นสามารถนำไปใช้เพื่อการศึกษาความคงสภาพของยาเมทฟอร์มินและกลัยคาไซด์ภายใต้สภาวะเครียดต่าง ๆ เช่น ไฮโดรลิซิสในสารละลายด่าง สารละลายกรด หรือสารละลายที่เป็นกลาง ออกซิเดชัน และแสง พบว่าเมทฟอร์มินคงตัวในสภาวะเป็นกลางและกรด แต่ไม่คงตัวในสภาวะด่างและสภาวะออกซิเดชันและให้ไซยาโนกัวนิดีนเป็นสารสลายตัว อัตราการสลายตัวของเมทฟอร์มินจะยิ่งเพิ่มมากขึ้นเมื่อให้ความร้อนหรือโดนแสง สำหรับกลัยคาไซด์มีความคงสภาพดีในสภาวะด่าง แต่สลายตัวอย่างรวดเร็วในสภาวะที่เป็นกลางและกรด และในสภาวะออกซิเดชัน ซึ่งสลายตัวเป็นสารเจือปนกลัยคาไซด์บี นอกจากนี้วิธีนี้ยังสามารถนำไปใช้ในการวิเคราะห์วัตถุดิบทางยาและยาเม็ดสำเร็จรูปของตัวยาทั้งสองชนิด ซึ่งพบว่าจากตัวอย่างที่วิเคราะห์ทั้งหมดมีปริมาณยาในช่วง 99.1-100.2 เปอร์เซ็นต์ (%RSDs น้อยกว่า 0.95 เปอร์เซ็นต์) ซึ่งเป็นไปตามมาตรฐานที่กำหนดในตำรายา และตรวจไม่พบสารสลายตัวใด ๆ ในตัวอย่างที่วิเคราะห์

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LIST OF ABBREVIATIONS

AR	analytical grade
BGE	back ground electrolyte
BP	British Pharmacopoeia
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CGN	cyanoguanidine
CIEF	capillary electrophoretic focusing
CITP	capillary isotechophoretic
cm	centimeter
CZE	capillary zone electrophoresis
DAD	photodiode array detector
dL	deciliters
DM	diabetes mellitus
E	applied electric field
EOF	electroosmotic flow
F_E	electric force
F_F	frictional force
g	gram
GBM	glibenclamide
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GCZ	gliclazide
GDM	gestation diabetes mellitus
GZB	gliclazide impurity B

LIST OF ABBREVIATIONS (cont.)

GZF	gliclazide impurity F
h	hour
HbA_{1c}	hemoglobin A1c
HPLC	high performance liquid chromatography
HPLC-UV	high pressure liquid chromatography-ultraviolet
HPTLC	high performance thin-layer chromatography
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
LOD	limit of detection
LOQ	limit of quantitation
M	molarity
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MET	metformin
mg	milligram
min	minute
mL	millimeter
mM	milimolar
MS	mass spectrometry
N	number of theoretical plates
NACE	non aqueous capillary electrophoresis

LIST OF ABBREVIATIONS (cont.)

NaOH	sodium hydroxide
NIDDM	non insulin-dependent diabetes mellitus
ng	nanogram
nm	nanometer
nM	nanomolar
o.d.	external diameter
pH	negative logarithm of hydrogen ion concentration
q	ion charge
r	ion radius
r^2	coefficient of determination
RH	relative humidity
R_s	resolution
RSD	relative standard deviation
s	second
SD	standard deviation
SDS	sodium dodecyl sulfate
SFC-MS-MS	super critical fluid chromatography tandem mass spectrometry
SIMs	stability indicatin methods
TF	tailing factor
TLC	thin layer chromatography
t_m	migration time
UPLC	ultra performance liquid chromatography
USP	United State Pharmacopeia
UV	ultraviolet
V	volt
v	ion velocity

LIST OF ABBREVIATIONS (cont.)

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 mellitus (DM) is a chronic disease that harms more than millions people in the world. Patients with DM have high blood sugar level which can lead to many symptoms such as heart disease, vascular disease, blindness, kidney failure, stroke and nerve disease (1, 2). There are four types of diabetes, type 1: insulin-dependent diabetes mellitus (IDDM), the patient's pancreas produces very little insulin, type 2: non-insulin dependent diabetes mellitus (NIDDM), the patient cannot use insulin effectively, type 3: other specific types of diabetes and type 4: gestation diabetes mellitus (GDM) (1-3), this type occurs with pregnancy but usually disappears after pregnancy. Most DM patients are type 2 (4). Treatment of NIDDM is commonly started with orally administration of biguanides and sulfonylureas. Metformin (MET) is a biguanide that is usually used for prevention and treatment of diabetes mellitus type 2. Gliclazide (GCZ) and Glibenclamide (GBM) are second generation of sulfonylurea that are employed to lower blood sugar levels (1-5). In order to enhance efficacy and patient compliance, combined formulations of the drug are commercially available, nowadays (6).

The quality control method of MET is official in both British pharmacopeia (7) and United State Pharmacopeia (8), while GCZ and GBM are official in British pharmacopeia. For MET raw material, non-aqueous titration is recommended, while HPLC is for MET tablets. GCZ is official in BP and non-aqueous titration is proposed for the assay of GCZ raw material, while HPLC is for GCZ tablets (7). Determination of MET, GCZ and GBM in bulk drugs, pharmaceutical products and biological fluids can be separately performed by various techniques such as HPLC-UV (9-26), ultra performance liquid chromatography (UPLC) (27), capillary electrophoresis (CE) (28-32), gas chromatography (GC) (33), GC-mass spectrometry (MS) (34), super critical fluid chromatography tandem mass spectrometry (SFC-MS-MS) (35), high performance thin-layer chromatography (HPTLC) (36-37), spectrophotometry (38-40)

spectrofluorometry (41) and LC-MS-MS (43-46). Analysis of the combined drugs are not reported. Therefore, there is a need of an efficient analytical method for the simultaneous determination of these drugs. CE was selected for this study because it provides high efficiency, selectivity and requires small amounts of organic solvent and samples. Additionally, it can be applied for analysis of anionic, cationic and neutral compounds. Optimization of MET, GBM and GCZ separation was studied by varying types of buffer, applied voltage, detector wavelength. The optimized method was validated according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use (ICH) (47) and applied to analyze the drugs in raw material, single and combined formulations.

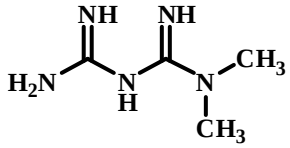
Stability indicated method is also important for quality assurance of pharmaceuticals. There were various techniques of stability tests of MET, GCZ and GBM (48-53). However, reported data is controversial. This research focused on stability studies of MET and GCZ under stress conditions using capillary electrophoresis (CE) as the analytical method. Both drugs were selected as models in this study since they are most commonly used for treatment of type 2 diabetes. The stress conditions were conducted according to the ICH guideline Q1A (R2) (54) and Bakshi M. and Singh S. (55). The forced conditions were hydrolysis (basic, acid and neutral), oxidation and photolysis. The CE condition for determination of MET under stress test was modified from Hamdan and co-workers (52). The CE condition for determination of GCZ under forced degradation was investigated by various modes of CE, different types, concentration and pH of back ground electrolyte (BGE), capillary length.

CHAPTER II
LITERATURE REVIEW

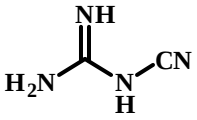
2.1 Diabetic mellitus and anti-diabetic drugs

Diabetic mellitus (DM) is carbohydrate metabolism disorders, it is defined as an elevated blood glucose associated with absent or inadequate pancreatic insulin secretion, with or without concurrent impairment of insulin action (1). Prolong high blood glucose level may cause of many harmful chronic diseases such as retinopathy, neuropathy and cardiovascular disease. The criteria for the diagnosis of DM are symptoms of diabetes plus random blood glucose concentration of ≥11.1 mM (200 mg/dL) or fasting plasma glucose of ≥7.0 mM (126 mg/dL) or two hour plasma glucose of ≥11.1 mM (200 mg/dL) during an oral glucose tolerance test, and the level of hemoglobin A_{1c} (HbA_{1c}) of ≥6.5% (2,3). Diabetes can be generally classified into four types, type 1 diabetes or insulin dependent diabetes mellitus (IDDM), type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM), type 3 other specific types of diabetes, type 4 gestation diabetes mellitus (GDM) (1-3). Ninety percents of the patients are type 2 diabetes and are in need of effective anti-diabetes drugs when dietary modification and exercise fail to maintain the normal glucose levels (4). There are many anti-diabetic drugs such as sulfonylureas (e.g. carbutamide, glibenclamide, gliclazide, etc.), biguanides (e.g. metformin, buformin, phenformin) and thiazolidinediones (e.g. rosiglitazone, pioglitazone) (3). Among several classes of anti-diabetic drugs, biguanides and sulfonylureas are drugs of choice for treatments of diabetes and are effective in the presence of some endogenous insulin production (5). Metformin (MET) is firstly recommended for NIDDM because it dose not cause myocardial infarction and produces less hypoglycemia effects than sulfonylurea (5). Gliclazide (GCZ) and glibenclamide (GBM) (Figure 2.1) are second generation sulfonylurea that are also used for treatments of NIDDM. Treatments of type 2 diabetes normally start with metformin, however, when functions of insulin-producing cells reduce, and weight gain occurs, additions of a second drugs (i.e. usually

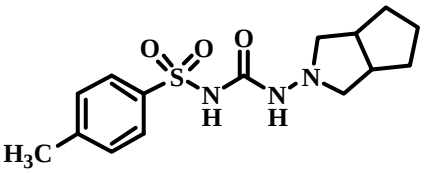
sulfonylureas) is required. Currently, combinations of MET and GCZ, MET and GBM are commercially available to enhance patient compliance (6).



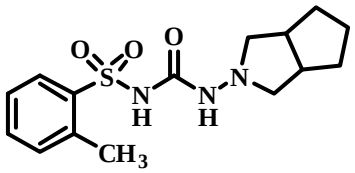
Metformin (MET)



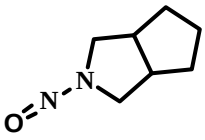
Cyanoguanidine (CGN)



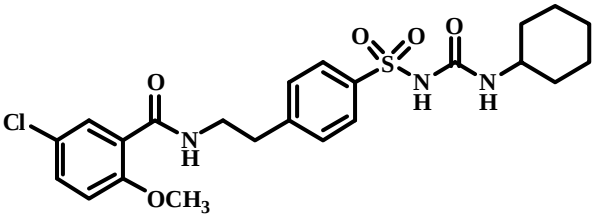
Gliclazide (GCZ)



Gliclazide impurity F (GZF)



Gliclazide impurity B (GZB)



Glibenclamide (GBM)

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

2.2. Determination of anti-diabetic drugs

British Pharmacopoeia (BP) (7) and United State Pharmacopoeia (USP) (8) recommend non-aqueous titration and high performance liquid chromatography (HPLC) for the assay of MET in raw material and tablets, respectively. GCZ is official in BP and non-aqueous titration is proposed for the assay of glicalzide raw material, while HPLC is for gliclazide tablets (7). GBM is also official in BP titration and HPLC method is suggested for raw material and tablets respectively (7). Determination of MET, GCZ and GBM in bulk drugs, pharmaceutical products and biological fluids can be performed by various techniques (Table 2.1) such as HPLC-UV (9-26), ultra performance liquid chromatography (UPLC) (27), capillary electrophoresis (CE) (28-32), gas chromatography (GC) (33), GC-mass spectrometry

(MS) (34), super critical fluid chromatography tandem mass spectrometry (SFC-MS-MS) (35), high performance thin-layer chromatography (HPTLC) (36-37), spectrophotometry (38-40) and spectrofluorometry (41). Some limitations of these techniques include long analysis time, peak tailing and low sensitivity. LC-MS-MS (42-46) 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 the measurement. Therefore, there is a need of an efficient analytical method for the simultaneous determination of these drugs in combined formulation.

2.3 Stability study of anti-diabetic drugs

Quality control of pharmaceutical products is essential from pharmaceutical industry and clinical point of views. The pharmaceutical products should be remained effectively and safety until consumed by the patient. Stability study is one of tools for insuring the quality of pharmaceutical products. Chemical (e.g. degradation of active pharmaceutical ingredient, increase of toxic degrades), physical (e.g. loss of acceptance, changes of polymorphism) and microbial stability (e.g. loss of sterility) are major concerns in pharmaceutical stability. Therefore, stability study of pharmaceuticals is now a restrict requirement for Food and Drug Administration (FDA) departments in most countries, prior the marketing of the drugs in either raw material or dosage forms. Currently, drug substances and products are subjected to stability studies according to the International Conference on Harmonization (ICH) guidelines Q1AR2 (54). The guidelines require a forced degradation or stress studies. Stress testing is carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance.

The study aims to investigate any susceptible changes that might occur during storage, which can affect efficacy, quality and safety of the drugs. Importantly, it is necessary to ensure that no adverse degradations are produced upon exposure of

the drugs to various severe conditions (e.g. hydrolysis (acid, alkaline, neutral), photolysis and oxidation) or during storage. Determination of the remained drug substances and/or degradation products under these conditions is important to establish degradation pathway and intrinsic stability of drug molecules. The degradation products can be interfered/reduced efficacy, harmful or even toxic. According to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q1AR2, significant degradation products must be well separated from the active pharmaceutical ingredients and accurately quantified, thus the analytical methods have to be validated and stability indicating methods (SIMs). Unfortunately, SIMs remains unclear or misunderstood by many manufacturers. So, there is an urgent need to have common stability study protocols and procedures for establishment of SIMs.

This research focuses of stability study of MET and GCZ under stress conditions (e.g. hydrolysis (acid, alkaline, neutral), photolysis and oxidation) using capillary electrophoresis (CE) and the analytical tool. Although several researchers reported the SIMs for both drugs, their results were inconsistent and controversial. Table 2.2 shows the various technique and stability test of these analytes.

CE was chosen as a method of choice since it is rapid, efficient and cost effective. Principle of CE is based on different migration of charged species under an electric field and it could provide distinct separation mechanism for a wide range of compounds. Thus, CE should enable the separation of MET, GCZ and their degradation products generated during forced degradation processes. Ultimately, the research outcomes could be used as a standard guideline to establish stability study protocols and SIMs for other pharmaceuticals. Assays of active pharmaceutical ingredient (API) content, testing of related substances and impurities are very important. Excess related substances or impurities can cause adverse side effects and even toxicity, thus it is mandatory to control limits of these substances (56).

Impurities of MET (i.e. cyanoguanidine, CGN) and GCZ (i.e. impurity B (GZB) and F (GZF)) are extremely harmful and toxic (57-59). These impurities can be associated with the synthesis route of MET (Figure 2.2) and GCZ (Figure 2.3) or they are degradation products under various stress conditions.

MET impurities include impurity A-F, but only testing of impurity A or CGN is required by both BP and USP using HPLC. Impurity A is carcinogen and mutagen (9) and determination of its contents by HPLC is recommended by BP and USP (7, 8). GCZ impurities are impurity A-G, but only quantitation of GZB and GZF is recommended by BP using HPLC. GZB and GZF cause irritation of eyes, respiratory and gastrointestinal tracts and skin, hypoglycemia and allergy, harm to unborn child and carcinogenicity (58, 59).

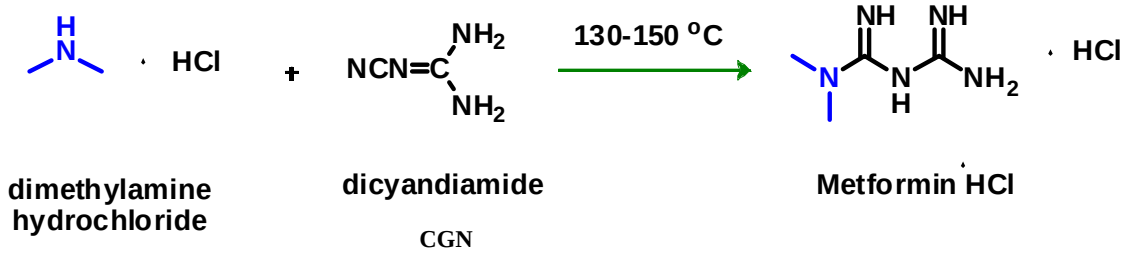


Figure 2.2 Synthesis of metformin.

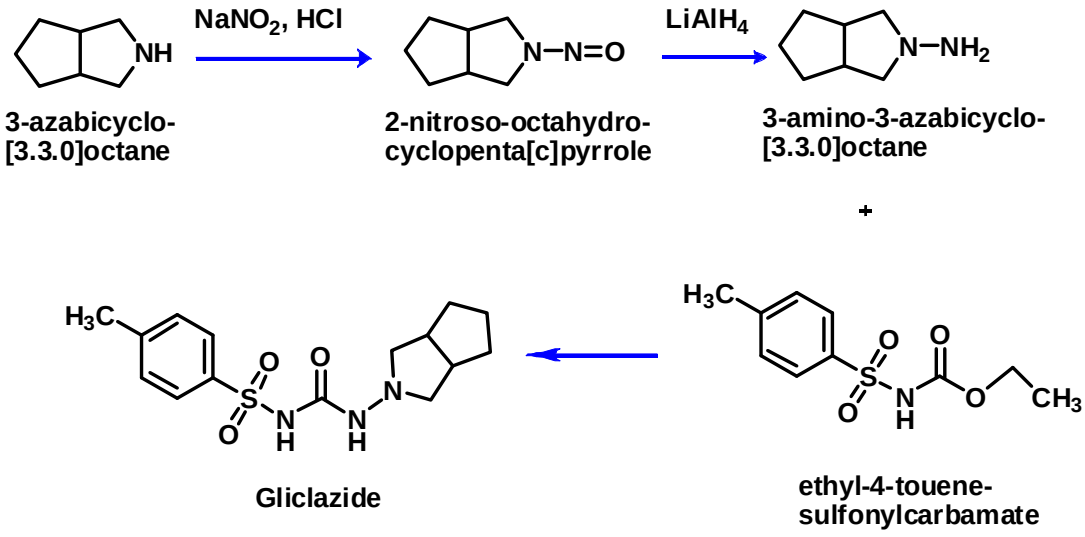


Figure 2.3 Synthesis of gliclazide

Table 2.1 Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
HPLC-UV	MET, 1-cyanoguanidine	tablets	· linearity range: 0.01-0.03 mg/mL of MET · precision: % RSD = 0.30 %	(9)
HPLC-UV	MET	human plasma	· linearity range: 30-4,000 ng/mL, r^2 = 0.9994 · LOQ = 30 ng/mL	(10)
HPLC-UV	MET, rosiglitazone	human plasma	· LOQ = 250 ng/mL(MET), 100 ng/mL(rosiglitazone) · extraction recovery : 105.64-103.88% (MET) 100.02-105.00% (rosiglitazone)	(11)
HPLC-UV	GCZ	human plasma	· linearity range: 10-5,000 ng/mL	(12)
HPLC-UV	GCZ	human plasma	· linearity range: 50 - 4,000 nM, r^2 > 0.9990 · precision: % RSD less than 5.3% , relative error 0.93% · LOD in plasma = 10 nM	(13)
HPLC-UV	GCZ	human plasma	· linearity range: 0.1-10 µg/mL · LOQ = 0.1 µg/mL	(14)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
HPLC-UV	MET, GCZ, glipizide, glibenclamide, glimperide	human plasma	· % recovery = 76.3-101.9% · LOQ = 5-22.5 ng/mL · precision: % coefficient of variation (%CV) < 9 % · accuracy: % relative error < 12 %	(15)
HPLC-UV	GCZ	human plasma	· linearity range: 75 - 10,000 nM, r^2 > 0.999 · % recovery = 84.5% · LOQ = 75 ng/mL	(16)
HPLC-UV	MET, CGN, melamine	tablets	· repeatability: % RSD :- MET = 1.02 % , CGN = 0.02% melamine = 0.36% · LOD: CGN =0.01 µg/mL, melamine = 0.04 µg/mL · LOQ: CGN =0.03 µg/mL, melamine = 0.12 µg/mL	(17)
HPLC-UV	MET, GCZ	human plasma	· linearity range: 0.05-5.00 µg/mL , r^2 > 0.99 · LOD = 50 ng/mL (MET), 49 ng/mL (GCZ)	(18)
HPLC-UV	MET, benfotiamine	bulk drug and formulation	• linearity range: 0.1-5 µg/mL (MET) 1-6 µg/mL (benfotiamine)	(19)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
HPLC-UV	MET, nateglinide, GCZ	pharmaceutical product	· linearity range: 0.4-16 µg/mL , r^2 = 0.999 (MET), 0.8-16 µg/mL , *r2* = 0.999 (nateglinide), 1-40 µg/mL ,*r2* = 0.999 (GCZ) · % recovery = 99.66% (MET), 100.08% (nateglinide), 100.31% (GCZ)	(20)
RP-HPLC	GCZ, GBM, glipizide, glimepirine, gliquidone, repaglinide	counterfeit anti-diabetic drugs	· linearity range: 1-100 µg/mL, r^2 > 0.9999 (GCZ, GBM, glipizide, glimepirine, gliquidone) 5-100 µg/mL , r^2 = 0.9999 (repaglinide) · LOD = 0.10-1.5 µg/mL, LOQ = 0.34-3.95 µg/mL	(21)
RP-HPLC	MET, rosiglitazone	pharmaceutical product	· linearity range: 0.03-0.50 µmole/mL, r^2 = 0.999 (MET) 0.03-0.50 µmole/mL, r^2 = 1.0 (rosiglitazone)	(22)
RP-HPLC	MET	standard	· linearity range: 1-250 µg/mL, r^2 = 0.9995 · % recovery = 100.25-101.13%	(23)
Ion-pair HPLC	MET, GCZ, glipizide	pharmaceutical product	· linearity range: 62.5-187.5 µg/mL (MET), 10-30 µg/mL (GCZ) 0.625-1.875 µg/mL (glipizide)	(24)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
Ion-pair HPLC	MET, related impurities	standard	· LOQ = 35-250 pg	(25)
Ion-pair HPLC	MET, GCZ	tablet	· r^2 > 0.999	(26)
UPLC	MET, sitabliptin	tablet	· LOD: MET =0.06µg/mL, sitagliptin = 0.2 µg/mL · LOQ: MET =0.2µg/mL, sitabliptin = 0.7 µg/mL	(27)
SPE-CE	MET, phenformin, GBM	human plasma	· LOD: MET =12 ng/mL, phenformin = 6 µg/mL · migration time 6.4 min for all analytes	(28)
CZE	MET, rosiglitazone	pharmaceutical product	· LOD: MET =1 µg/mL, rosiglitazone = 0.5 µg/mL	(29)
CZE-AD	aminoheterocycle, azabicycle	GCZ bulk	· linearity range: 1-1000 µM (aminoheterocycle), 2-1000 µM (azabicycle) · LOD : 0.5 µM (aminoheterocycle), 1.0 µM (azabicycle)	(30)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
MEKC, HPLC	GCZ, GBM, glipizide, tolbutamine, chlorpropamide	human plasma	· linearity : r^2 ³ 0.998 · % recovery ³ 80%	(31)
CZE	MET, sitagliptin	tablets human plasma	· linearity range: 50-500 µg/mL (MET), 10-100 µg/mL (sitagliptin) · LOD: MET =2.11 µg/mL, sitagliptin = 0.49 µg/mL · LOQ: MET =6.39 µg/mL, sitabliptin = 1.48 µg/mL	(32)
Capillary- GC	GCZ	pharmaceutical product	· linearity range: 0.1-10 mg/mL · precision: % RSD = 0.38-1.26% · LOD = 30 ng/mL	(33)
GC-MS	MET	human plasma	· linearity range: 100-3,000 ng/mL, r^2 = 0.996 · LOD = 40 ng/mL, LOQ = 100 ng/mL	(34)
SFC MS/MS	MET, GCZ	human plasma	· linearity range: 6-3,550 ng/mL (MET), 7.5-7500 ng/mL (GCZ)	(35)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
HPTLC	MET, pioglitazone, glimepiride	bulk drug pharmaceutical product	· R_F value: 0.83 (pioglitazone), 0.21 (MET), 0.89 (glimepiride) · linearity range: 10-40 µg/band, r^2 = 0.996 (MET) 0.3-1.2 µg/band, r^2 = 1.0 (pioglitazone) 0.04-0.16 µg/band, r^2 = 1.0 (glimepiride)	(36)
HPTLC	MET, repaglinide, glimepiride, sitagliptin pioglitazone	bulk drug pharmaceutical product	· R_F value: 0.10±0.02 (MET), 0.23±0.02 (repaglinide) 0.45±/0.02 (glimepiride), 0.54±/0.02 (pioglitazone), 0.66±0.02 (sitagliptin) · linearity range: 200-800 ng/spot (excepted sitagliptin) 2,000-8,000 ng/spot (sitagliptin) r^2 close to 0.999	(37)
spectrophotometry	MET, GCZ	pharmaceutical product	· linearity range: 2-14 µg/mL (MET), 2-24 µg/mL (GCZ) · % recovery = 98.94 % (MET), 99.12% (GCZ)	(38)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
spectrophotometry	GCZ	pharmaceutical product human urine human plasma	· linearity range: 0.5-5 µg/mL · minimum detectability (S/N=2) = 0.05 µg/mL · % recovery : 97.84 % ± 0.72 (urine), 97.43 % ± 0.83 (plasma)	(39)
spectrophotometry	MET, pioglitazone, glipizide	bulk drug pharmaceutical product	· linearity range: 5-50 µg/mL · assay : 99.95-102.10 % labled amount	(40)
spectrofluorimetry spectrophotometry	GCZ	pharmaceutical product	· spectrofluorimetry :- * range : 0.2-2.5 µg/mL * minimum detectability (S/N=2) = 0.02 µg/mL · spectrophotometry :- * range : 2-20 µg/mL * minimum detectability (S/N=2) = 0.2 µg/mL	(41)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
LC-MS-MS	MET	human plasma	· linearity range: 1-2,000 ng/mL · precision: % RSD < 8.6 % (intra and inter-day) · % recovery = 91-110% · LOD = 250 pg/mL in plasma · precision: % RSD = 0.30 %	(42)
LC-MS-MS	MET	human plasma	· linearity range: 10-1,000 ng/mL · precision: % RSD = 0.8-1.9% (Intra-day) = 1.5-8.6% (Inter-day) · % recovery = 100.3-105.0% (Intra-day) = 101.2-105.3% (Inter-day)	(43)
LC-MS-MS	MET, GCZ	human plasma	· linearity range: 10-1,000 ng/mL (GCZ), 7.8-4,678.9 ng/mL (MET), · precision : % RSD less than 15% · % recovery = 71-104% · LOQ: GCZ =7.8 ng/mL, MET = 10.0 ng/mL	(44)

Table 2.1 (Continued) Analytical methods for the determination of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
LC-MS-MS	MET, sitagliptin	human plasma	· linearity range: 25-3,000 ng/mL (MET), 5-800 ng/mL (sitagliptin), · % recovery: MET and its internal standard ³ 39% sitagliptin and its internal standard ³ 64%	(45)
LC-MS-MS	MET, pioglitazone, hydroxypioglitazone	human plasma	· linearity range: 25-3,000 ng/mL (MET), 15-2,500 ng/mL (pioglitazone) 10-1,500 ng/mL (hydroxypioglitazone)	(46)

Table 2.2 Stability study of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
HILIC	MET, CGN, melamine	tablet	· linearity range: 100-400 µg/mL (MET) · % recovery = 100.62 % (MET) · run time of all analytes less than 13 min	(48)
RP-HPLC	MET, linagliptin	bulk drug pharmaceutical product	· linearity range: 10-100 µg/mL (MET) 5-30 µg/mL (linagliptin)	(49)
RP-HPLC	MET, sitagliptin	pharmaceutical product	· linearity range: 50-450 µg/mL, r^2 = 0.9998 (MET) · linearity range: 10-150 µg/mL, r^2 = 0.9996 (sitagliptin) · precision: % RSD < 1.5 %	(50)
HPLC-UV	GCZ	tablet	· linearity range: 5-500 µg/mL · precision: % RSD < 1 % (intra-day), < 2 % (inter-day) · % recovery = 99.81-100.97 %	(51)

Table 2.2 (Continued) Stability study of anti-diabetic drugs

Method	Analyte	Sample	Significant finding	Reference
CE	MET	tablet	· linearity range: 0.2-2.0 mg/mL · precision: % RSD < 2 % (intra and inter-day) · LOD = 2.0 µg/mL, LOQ = 8 µg/mL	(52)
HPLC-MS	GBM	bulk drug	· linearity range: 0.4-10 µg/mL, r2 = 0.9982 (narrow) 0.4-500 µg/mL, r2 = 0.9993 (wide) · LOD = 0.1 µg/mL, LOQ = 0.4 µg/mL	(53)

2.4 Capillary electrophoresis

The electrophoretic separation technique is based on the principle that under the influence of an applied potential field, different species in solution will migrate at different velocities from on 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 components of CE instrument (Figure 2.4) 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 (60, 61).

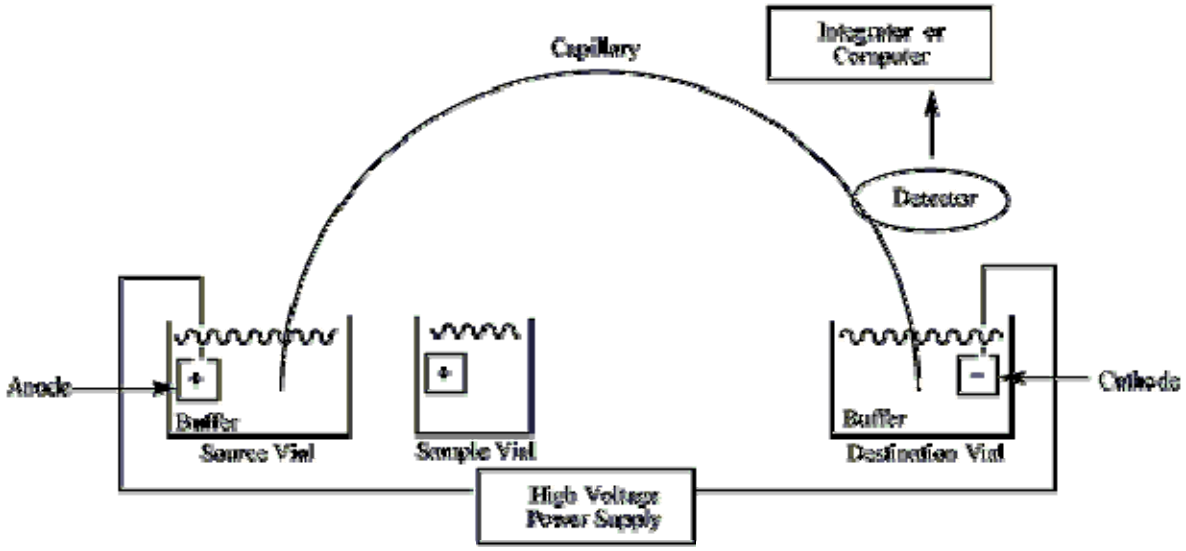


Figure 2.4 CE instrument (61)

2.4.1 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 following equation (60).

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

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

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

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

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)

2.4.1.2 Electro osmotic flow (EOF)

The EOF result from the effect of the applied electric field on the solution double layer of the fused silica capillaries wall. The ionized silanol groups (SiO^-) impart a layer of negative charge to the capillary wall attract hydrated cations from the electrolyte solution and then arranged into two layer. As illustrate in Figure 2.5, the one layer is tightly bound by electrostatic forces (compact layer), and the other is more loosely bound (diffuse layer). When an electric is applied, the diffuse layer breaks away (at the plane of shear) and moves 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 in EOF is led by increasing of pH, however, the increase of the ionic strength undergoes a decrease of EOF (60, 61).

The EOF can be calculated as 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 marker that moves at a velocity equal to the EOF.

$$\mu_a = lL/\ tV \quad (2.5)$$

Where l = effective capillary length (cm)

L = total capillary length (cm)

t = the migration time (s)

V = the applied voltage (volt)

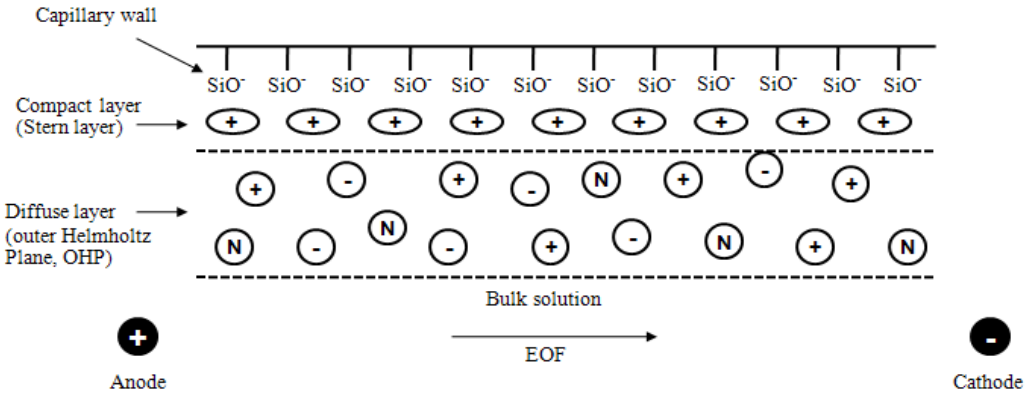


Figure 2.5 Electrical double layer at the ionized silica capillary wall and illustration of EOF (60)

2.4.2 Separation modes in CE

2.4.2.1 Capillary zone electrophoresis (CZE)

CZE is the most simple and widely use mode in CE. The separation mechanism is based on differences in the charge-to-mass ratio of the

analytes and relies principally on the pH controlled dissociation of acidic groups or the protonation of basic functions on the solute. Analytes migrate in the discrete zone with the differences in their electrophoretic mobilities. Neutral analytes are carried along by the EOF with un resolved peak but cations and anions can be separated from EOF with the higher and lower mobilities than EOF (60,61).

2.4.2.2 Micellar electrokinetic chromatography (MEKC)

MEKC is a combination technique of electrophoresis and chromatography. This mode can be separated analyze samples containing mixtures of charged and neutral compounds by adding the surfactants at a higher concentration than its critical micelle concentration (CMC) into the buffer for forming micelle. The migration velocity of the neutral analyte in MEKC depends on what portion of the analyte is incorporated into the micelle (60).

2.4.2.3 Capillary gel electrophoresis (CGE)

CGE is a technique that has been developed to aid in the separation of macromolecules. This mode provides a mechanism for separation on the basis of size differences. In CGE, the capillary is filled with gel or viscous polymer to create a molecular sieve. Large molecules are restricted and their velocities reduced but small molecules can pass through the pores (61).

2.4.2.4 Capillary isoelectric focusing (CIEF)

CIEF is used to separate zwitterionic compounds, such as peptides and proteins, by electrophoresis in a pH gradient which is generated between the cathode and anode by the use of carrier ampholytes. Analyte will migrate to a point where its net charge is zero. At the analyte isoelectric point (pI), migration stops and the sample is focused into a tight zone. This technique is commonly employed in protein characterization as a mechanism to determine a protein's isoelectric point (61).

2.4.2.5 Capillary isotachophoresis (CITP)

In the electrolyte system used in CITP, the sample zone migrate between a leading electrolyte and trailing electrolyte. CITP is used as a mode of separation for anions or cations, but not both simultaneously. When applied electric field, analytes having mobilities intermediate to those of the leading and trailing electrolytes stack into discrete zones. All analytes elute in order of a decreasing

mobility and a decreasing stepwise of conductivity versus time is recorded. The length of the analyte zone in CITP is a quantitative parameter relating to the concentration of analyte. The height of the step is qualitative parameter which is characteristic of the analyte and it is directly proportional to analyte mobility (61).

2.4.2.6 Microemulsion electrokinetic chromatography (MEEKC)

MEEKC is an electrodriven separation technique. It is similar to MEKC except microemulsion is used as a pseudo-stationary phase instead of micelle. Microemulsion are solutions of nanometer-sized droplets dispersed in liquid. Solutes are separated by a combination of the electrophoretic mechanism and by chromatographic partition of microemulsion droplets. The microemulsion droplets are usually formed by sonicating immicible organic solvent (heptanes or octane) with water. Surfactant is added at relatively high concentration to stabilize the emulsion. This technique is used effectively for analyzing both hydrophilic, hydrophobic compounds and pharmaceuticals products (62).

2.4.2.7 Non-aqueous CE (NACE)

NACE is alternatively technique of CZE by substitution of water by organic solvents. The physical and chemical properties of organic solvent are very different from each other and from water allowing the important characteristics of separations to be controlled on a wider scale than with water alone. The viscosity and dielectric constants of organic solvents affect both analyte mobility and the level of EOF. Solvent properties affect the acid-base behavior of analytes with pK_a values can differ up to many orders of magnitude. The use of non-aqueous medium allows enormous variations in electrophoretic mobilities and separations are not possible in aqueous CE can be performed with excellent selectivites. A further benefit of NACE is the increased solubility of hydrophobic compounds which extends the applicability of CE. Moreover, non-aqueous solvents are also fully compatible and even sometimes more appropriate than water, with most of the detectors hyphenated with CE (63).

2.4.2.8 Capillary electrochromatography (CEC)

In CEC, the capillary is packed with a stationary phase that is capable of retaining solutes as a manner like colume chromatography. However, there is no pump to force solutes through to the detector, the EOF is responsible to act as an

electropump. Because there is minimal backpressure, it is possible to use small-diameter packings and achieve very high efficiencies. The separation mechanism is depend on both the electrophoretic mobility of the analytes and the nature of the packing material. Application of this mode appears to be in the form of on-line analyte concentration that can be used to concentrate a given sample prior to separation by CZE (61).

CHAPTER III
MATERIALS AND METHODS

3.1 Material and instrument

Table 3.1 List of chemicals and reagents

Name	Grade	Source/Supplier
Metformin	RS	Sigma-Aldrich (Steinheim, Germany)
Glibenclamide	RS	Bureau of Drug and Narcotic (Nonthaburi, Thailand)
Gliclazide	RS	Sigma-Aldrich (Missouri, USA)
Cyanoguanidine	RS	Sigma-Aldrich (Missouri, USA)
Gliclazide impurity B	RS	EDQM (Strasbourg, France)
Gliclazide impurity F	RS	EDQM (Strasbourg, France)
Sodium dihydrogen phosphate	-	Merck (Darmstadt, Germany)
Sodium dedocyl sulphate	-	Sigma-Aldrich (Missouri, USA)
di-Sodium tetraborate dehydrate	-	QreC (New Zealand)
Citric acid	-	Sigma-Aldrich (Steinheim, Germany)
Methanol	HPLC	Labscan Asia (Bangkok, Thailand)
3% Hydrogen peroxide	-	Siribuncha Co,Ltd. (Nonthaburi, Thailand)
Hydrochloric acid	AR	Fluka (Bushs, Switzerland)
Sodium hydroxide	AR	Mallinckrodt Baker (Xalostoc, Mexico)
Sterile water for injection	-	Thainakornpattana Co., Ltd (Thailand)

RS= reference standard, HPLC = high performance liquid chromatography grade, AR= analytical grade

Table 3.2 List of instruments

Instrument	Source/Supplier
Capillary electrophoresis (^{3D} CE)	Agilent Technologies (Waldbronn, Germany)
Capillary; i.d.50 μm, o.d. 375 μm	Polymicro Technologies (Arizona, USA)
Autopipette	Gilson Pipetman (Middleton, USA)
pH meter	Consort model C830 (Turnhout, Belgium)
Analytical balance	Sartorius model AE 160 (Goettingen, Germany)
Diposable syringe 3mL	Nipro (Ayuddhaya, Thailand)
13-mm syringe filters nylon, 0.2 μm	Lubitech (Shanghai, China)
Centrifuge (Labfuge 200)	Heraeus (Hanau, Germany)
Ultrasonic sonicator (D-7700)	Elma (Singen, Germany)

3.2 Capillary electrophoresis general method

CZE was performed on a Hewlett-Packard instrument (^{3D} CE) system (model G1600A) and controlled by PC through Agilent ChemStation Plus software version A.08 (G1601A). The detector measured in the range of 190-600 nm (wavelength accuracy ± 2 nm) was a diode array detector, which was consisted of a deuterium. The regulation of high voltage was varied in a range of 0-30 kV (current 0-300 μA, power 0-6 W). The temperature control the capillary tube was varied from 5 to 60 °C (± 1 °C). The injection systems could be achieved by 1) applying pressure to sample vials (hydrostatic injection) and 2) applying voltage (electromigration injection).

The separation of MET, GCZ and GBM was performed by using a fused-silica capillary tube with a total length (L_{total}) of 64.5 cm, an effective length (L_{eff}) of 56 cm, an inner diameter (i.d.) of 50 μm and an outer diameter (o.d.) of 375 μm. The pre-conditioning procedure for a new capillary, for daily uses, between runs and storage was described in Table 3.3.

The separation conditions were optimized by varying the background electrolyte (BGE) types, pHs and concentrations, applied voltage and injection time. Standard solutions were injected into the anodic capillary inlet using 50 mbar pressures. The detection was performed using a diode-array detector at a wavelength of 210 nm with a bandwidth 8 nm.

For stability study of MET, a fused-silica capillary tube with a total length (L_{total}) of 68.5 cm, an effective length (L_{eff}) of 60 cm, an inner diameter (i.d.) of 50 μm and an outer diameter (o.d.) of 375 μm was used. The pre-conditioning procedure was the same as described in the separation of the combined anti-diabetic drugs (MET, GCZ and GBM) excepted that between runs were rinsed with 0.1 N NaOH, sterile water for injection and BGE for 1, 1, 2 minutes, respectively. The detection was performed using a diode-array detector at a detection wavelength of 214 nm with a bandwidth 8 nm.

For stability study of GCZ, a fused-silica capillary tube with a total length (L_{total}) of 48.5 cm, an effective length (L_{eff}) of 40 cm, an inner diameter (i.d.) of 50 μm and an outer diameter (o.d.) of 375 μm was employed. The pre-conditioning

procedure was the same as described in the stability study of MET. The detection was performed using a diode-array detector at a detection wavelength of 225 nm with a bandwidth 8 nm.

Table 3.3 Capillary conditioning procedures for the separation of anti-diabetic drugs (MET, GCZ and GBM)

	Conditioning Step	Time (min)
New capillary		
1) Rinse	1 N NaOH	15
2) Rinse	0.1 N NaOH	15
3) Rinse	Sterile water for injection	15
4) Rinse	BGE	15
Daily condition before use		
1) Rinse	1 N NaOH	5
2) Rinse	0.1 N NaOH	5
3) Rinse	Sterile water for injection	5
4) Rinse	BGE	5
Between run		
1) Rinse	0.5 M HCl	1
2) Rinse	Sterile water for injection	1
3) Rinse	0.1N NaOH	1
4) Rinse	BGE	2
Daily storage		
1) Rinse	1 N NaOH	5
2) Rinse	Sterile water for injection	5
3) Rinse	0.1 N NaOH	5
4) Rinse	Sterile water for injection	5
Storage (over night)		
1) Rinse	Sterile water for injection	5
2) Rinse	1 N NaOH	5
3) Rinse	Sterile water for injection	5
4) Rinse	0.1 N NaOH	5
5) Rinse	Sterile water for injection	10
6) Rinse	MeOH	10
7) Rinse	Air	10

3.3. Assay of combined anti-diabetic drugs

3.1 Standard solution preparation

Stock standard solutions of MET, GCZ, GBM were prepared separately by transferring an accurate weight equivalent to 10.0 mg of each standard in a 10-mL volumetric flask. Methanol was used as solvent to make the final concentration of 1,000 µg/mL. All stock standard solutions were kept in a refrigerator (at 4-8 °C) and were filtered through a 0.2-µm membrane and degassed for 15 minutes prior injection.

3.3.2 Sample preparation

Twenty tablets of anti-diabetic drugs tablets were grounded to make homogeneous powder, then accurate weights of the powder (equivalent to 1,000 mg of MET, 160 mg of GCZ and 10 mg of GBM) were transferred to a 100-mL volumetric flask. Methanol approximately 60 mL was added into the solution, sonicated for 30 minutes and it was adjusted to volume by methanol to make concentration of 10 mg/mL for MET, 1.6 mg/mL for GCZ and 0.1 mg/mL for GBM. For MET tablets and the combination formula of MET/GCZ tablets and MET/GBM tablets, the solutions were centrifuged for 10 minutes at 4500 rpm and the filtrate was diluted to make the final concentration of 1,000 µg/mL for MET, 160 µg/mL for GCZ, 10 µg/mL for GBM. For GCZ and GBM tablets the stock solutions of GCZ (1.6 mg/mL) and GBM (0.1 mg/mL) were filtered with Whatman paper and diluted with methanol to obtain the concentration of 160 µg/mL and 10 µg/mL for GCZ and GBM, respectively. All sample solutions were filtered through a 0.2-µm membrane and degassed for 15 minutes prior injection.

3.3.3 Optimization

The CE method for separation of MET, GCZ and GBM was modified from Jin Lv, et al. (33). The separations were carried out in borate buffer as back ground electrolyte (BGE). The factors such as capillary type of buffers, voltage and wavelength of detector were optimized to obtain the optimum condition. Analytical parameters, including migration time, resolution, tailing factor and number of theoretical plate were calculated by the following equations.

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

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 1

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

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

$$N = 5.54\left(\frac{t}{w_{0.5}}\right)^2 \quad (3)$$

Where N = Number of theoretical plates

t = Migration time of analyte

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

3.3.4 Method validation

The optimum CZE condition was validated by the following procedures.

3.3.4.1 Linearity

Linearity of system was determined by constructing calibration curves of reference standard that was established using seven different concentrations (50%-150% of the nominal concentrations (1,000 µg/mL for MET, 160 µg/mL for GCZ, 10 µg/mL for GBM)). Each concentration was injected in triplicate. Coefficients of determination and percent relative standard deviations (%RSD) of slope and intercept were calculated.

$$\% \text{ RSD} = \frac{SD}{\bar{X}} \times 100 \qquad (4)$$

Where SD = standard deviation of y-intercept

$\bar{X}$ = mean value

Linearity of method was established by spiking 5 different standard concentrations to the samples solution to make the final concentration of 80% - 120% of the nominal concentration (1,000 µg/mL for MET, 160 µg/mL for GCZ, 10 µg/mL for GBM). Each concentration was injected in triplicate. Plots of calibration curves were constructed. Coefficients of determination and %RSDs of slope and intercept were calculated.

3.3.4.2 Specificity

Specificity is 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 is tested by comparing results of samples containing impurities with the results of samples without the impurities.

3.3.4.3 Precision

Precision of an analytical method is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision is represented as %RSD. Repeatability precision was assessed by six determinations of 100% test concentration within one day.

Intermediate precision was done by analying three different concentrations (80%, 100% and 120%) of working standard and each concentration was injected in triplicates on the three different days.

3.3.4.4 Accuracy

Accuracy was determined using standard addition method. Standard solution was added into the solution to make the final concentration of 80% - 120% of the nominal concentration (1,000 µg/mL for MET, 160 µg/mL for GCZ, 10 µg/mL for GBM). Each concentration was injected in triplicates. Accuracy was calculated in term of percent recovery.

$$\% \text{ Recovery} = \frac{X_{found}}{X_{add}} \times 100 \qquad (5)$$

Where X_{found} = Concentration of found analytes

X_{add} = Concentration of added analytes

3.3.4.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection (LOD) and limit of quantitation (LOQ) were established based on singnal-to-noise ratio. The signal-to-noise ratio was 3 and 10 for LOD and LOQ, respectively.

3.3.5 Application

The developed and validated method were applied for the determination of thirteen samples of MET, GCZ, GBM in raw material, single and combined formulations. Each sample was analyzed in triplicate.

3.4. Stability study of MET and GCZ

3.4.1 Optimization

CE separation of MET and CGN was modified from Hamdan et al. (52). Citrate buffer was used as BGE. CE separation of GCZ, GZB and GZF were investigated by various modes of CE, different types, concentration and pH of BGE, capillary length (Table 3.4).

The key responses to determine the optimal CE conditions were the R_s N, TF and t_m which were calculated by equation (1) – (3) as previously described.

Table 3.4 Optimization of GCZ, GZB and GZF

Factor	Range
Mode of CE	CZE, MEKC
Type of buffer	Phosphate, citrate
Concentration of buffer	10, 15, 20 mM
pH of buffer	6.0-9.0
Capillary length	L_{eff} = 56 cm, 40 cm

3.4.2 Stress test

Stability studies of MET and GCZ were carried out under various forced condition as shown in Table 3.5. The remained drugs and known degrades were identified and quantified by the developed CE methods. The stability and pharmacokinetic profile were established.

Table 3.5 Stability study protocol

Condition	Duration	
Hydrolysis	Heated at 80 °C	Room temp (in a dark cabinet)
Alkaline (0.1 N NaOH)	8 h	30 d
Acid (0.1 N HCl)	8 h	30 d
Neutral (water)	8 h	30 d
Oxidation		
3% H_2O_2	8 h	7 d
Photolysis	Sunlight	
Alkaline (0.1 N NaOH)	30 d	
Acid (0.1 N HCl)	30 d	
Neutral (water)	30 d	

For MET stability study, MET raw material was dissolved in sterile water for injection to make the concentration of 12.5 mg/mL and was used as a stock solution in forced degradation studies. Two milliliters of the stock solution was transferred into a 50 mL volumetric flask, added with 4.2 mL of 0.1N NaOH, 0.1N HCl and water or 3% H_2O_2, 15 mL water and mixed (Table 3.5). The mixture was heat on the water bath at 80 °C, left at sun light or kept in dark at room temperature. The mixture was collected and adjusted to volume with water and filtered through 0.2 µm membrane prior CE analysis.

For GCZ stability study, GCZ raw material was dissolved in methanol to make the concentration of 4 mg/mL. Two milliliters of the stock solution was transferred into a 50 mL volumetric flask, added with 1.3 mL of 0.1N NaOH, 0.1N HCl and water or 3% H_2O_2, 15 mL 80% MeOH and mixed (Table 3.6). The mixture was heat on the water bath at 80 °C, left at sun light or kept in dark at room temperature. The mixture was collected and adjusted to volume with 80% MeOH and filtered through 0.2 µm membrane prior CE analysis.

Table 3.6 Preparation of Forced degradation solutions for MET

condition	stock (mL)	0.1N NaOH (mL)	0.1N HCl (mL)	SWI (mL)	3% H_2O_2 (mL)	SWI (mL)	volume (mL)
Initial	2					15	50
Alkaline (80 °C)	2	4.2				15	50
Acid (80 °C)	2		4.2			15	50
Water (80 °C)	2			4.2		15	50
Oxidation (80 °C)	2				4.2	15	50
Alkaline (dark cabinet)	2	4.2				15	50
Acid (dark cabinet)	2		4.2			15	50
Water (dark cabinet)	2			4.2		15	50
Oxidation (dark cabinet)	2				4.2	15	50
Alkaline (sunlight)	2	4.2				15	50
Acid (sunlight)	2		4.2			15	50
Water (sunlight)	2			4.2		15	50

Table 3.7 Preparation of Forced degradation solution for GCZ

condition	stock (mL)	0.1N NaOH (mL)	0.1N HCl (mL)	SWI (mL)	3% H_2O_2 (mL)	80% MeOH (mL)	volume (mL)
Initial	2					15	50
Alkaline (80 °C)	2	1.3				15	50
Acid (80 °C)	2		1.3			15	50
Water (80 °C)	2			1.3		15	50
Oxidation (80 °C)	2				1.3	15	50
Alkaline (dark cabinet)	2	1.3				15	50
Acid (dark cabinet)	2		1.3			15	50
Water (dark cabinet)	2			1.3		15	50
Oxidation (dark cabinet)	2				1.3	15	50
Alkaline (sunlight)	2	1.3				15	50
Acid (sunlight)	2		1.3			15	50
Water (sunlight)	2			1.3		15	50

3.4.3 Method validation

The optimized conditions obtained from the previous section were evaluated for its linearity, specificity, precision, accuracy, limits of detection (LOD) and quantitation (LOQ) and robustness

3.4.3.1 Linearity

Calibration curves of the investigated compounds were established for five different concentrations (n = 3) in the range of 400-600 µg/mL for MET, 2-40 µg/mL of CGN, 128-192 µg/mL for GCZ, 20-60 µg/mL of GZB and 10-50 µg/mL of GZF. Calibration curves were plotted between peak area versus standard solutions concentrations. Linear regression and coefficient of determination (r^2) were calculated.

3.4.3.2 Specificity

Specificity is 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 is tested by comparing results of samples containing impurities with the results of samples without the impurities.

3.4.3.3 Precision

- Repeatability (within day precision)

The precision of an analytical method is to express the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed condition. Repeatability precision was assessed using 6 determinations at 100% test concentration within one day and was presented as %RSD.

- Intermediate precision (day to day precision)

Intermediate precision was performed by analyzing three different concentrations (80%, 100% and 120%) of working standard and each concentration was injected in triplicates on the different 3 days. Intermediate precision was determined in term of %RSD.

3.4.3.4 Accuracy

Accuracy was determined by a standard addition method. Known amounts of three different concentrations of standard solution (80%-120%) were added into 50% concentration of samples and analyzed. Each concentration was injected in triplicates. Accuracy will be calculated in term of percent recovery

$$\% \text{ Recovery} = (X_{found} / X_{add}) \times 100 \quad (6)$$

where X_{found} was concentration of found analyte found and

X_{add} was concentration of analyte added.

3.4.3.5 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were established based on signal-to-noise ratios (S/N). The S/N ratios were 3 and 10 for LOD and LOQ, respectively.

3.4.3.6 Robustness

Chemical (i.e. pH) and physical parameters (i.e. voltage) were evaluated for the robustness test. The variation around the optimal values such as the optimal pH $\pm$ 0.5 units and the optimal voltage $\pm$ 2 kV were investigated. %RSDs of

peak area and migration time were then calculated to test for the robustness of the methods

3.4.4 Application

The developed and validated methods were applied for the determination of pharmaceutical products of anti-diabetic drugs including 5 lots of raw material and 5 lots of commercial tablets of MET and GCZ. The contents of the drugs and degradation products were quantified and evaluated in comparison to limits in pharmacopeias.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Assay of combined anti-diabetic drugs

4.1.1 CE optimization of MET, GBM and GCZ

4.1.1.1 Effect of applied voltage

Capillary zone electrophoresis (CZE) method was selected to separate the anti-diabetic drugs (MET, GBM and GCZ).The CZE method was modified from the work of Lv J. *et.al.*,(33). The condition was 50 mM borate buffer and applied voltage at 14 kV using amperometric detection. In this study, UV detection at the wavelength of 230 nm was employed due to the common UV absorption of MET, GBM and GCZ and the availability of the instrument. Under this condition, all analytes were well separated in 11.5 min. Increasing of separating voltage was attempted to reduce the migration time. Results showed that increasing of voltage from 14 kV to 20 kV reduced the migration time from 11.5 to 8.2 min (Figure 4.1).

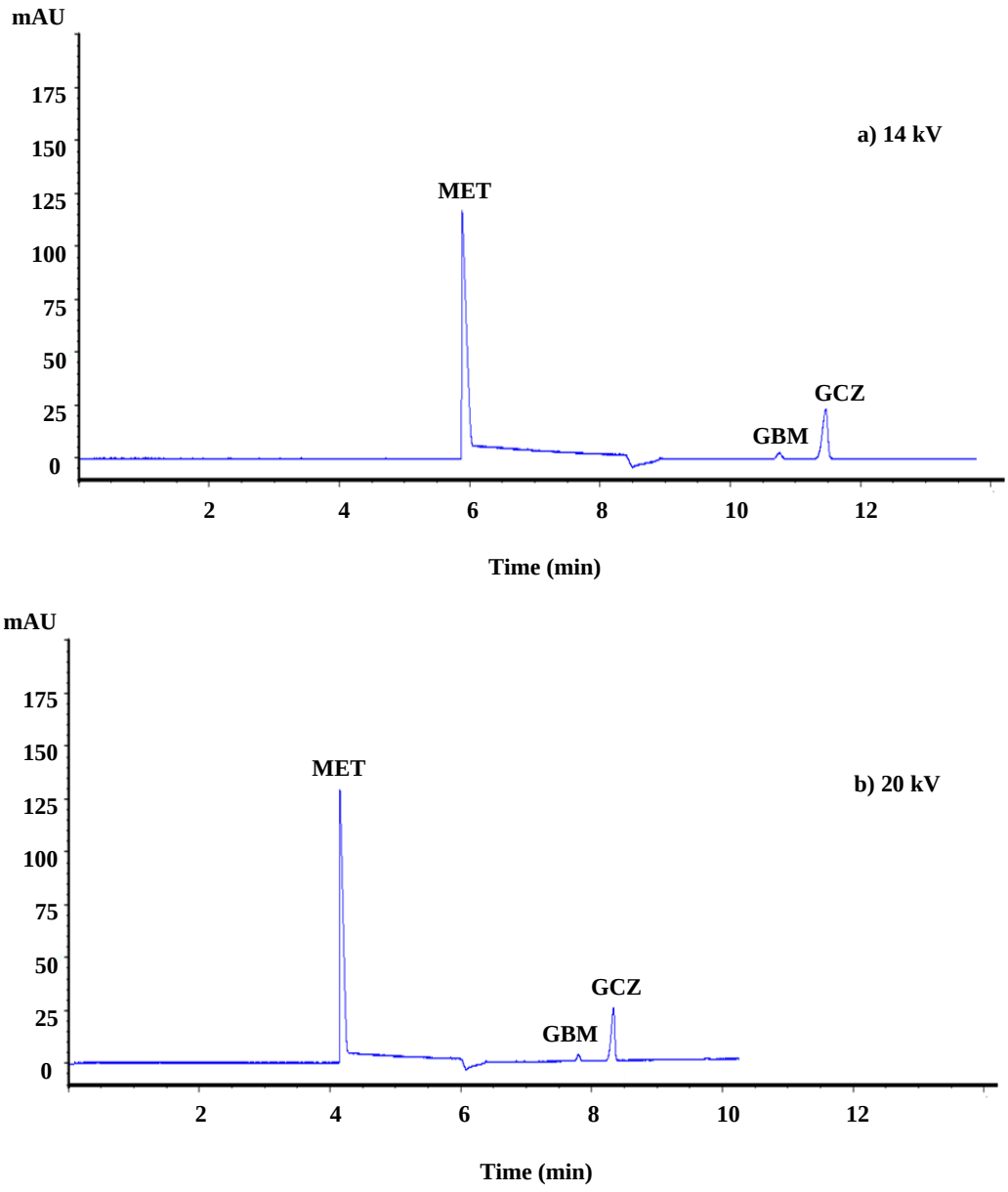


Figure 4.1 Electropherogram of applying voltage effects on the separation of MET, GBM and GCZ (concentration 1,000 µg/mL, 10 µg/mL, 100 µg/mL, respectively). Condition: BGE: 50 mM borate buffer pH 9.0; capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 10 s; temperature 25 ◦C; voltage a) 14 kV and b) 20 kV; detection by DAD absorbance at 225 nm.

4.1.1.2 Effects of type of buffer

Types of buffer was one of the factors that effects CE separation. Phosphate and borate buffer was investigated for the separation of MET, GBM and GCZ. Borate buffer offered the better separation with higher sensitivity and more symmetric peaks (Figure 4.2).

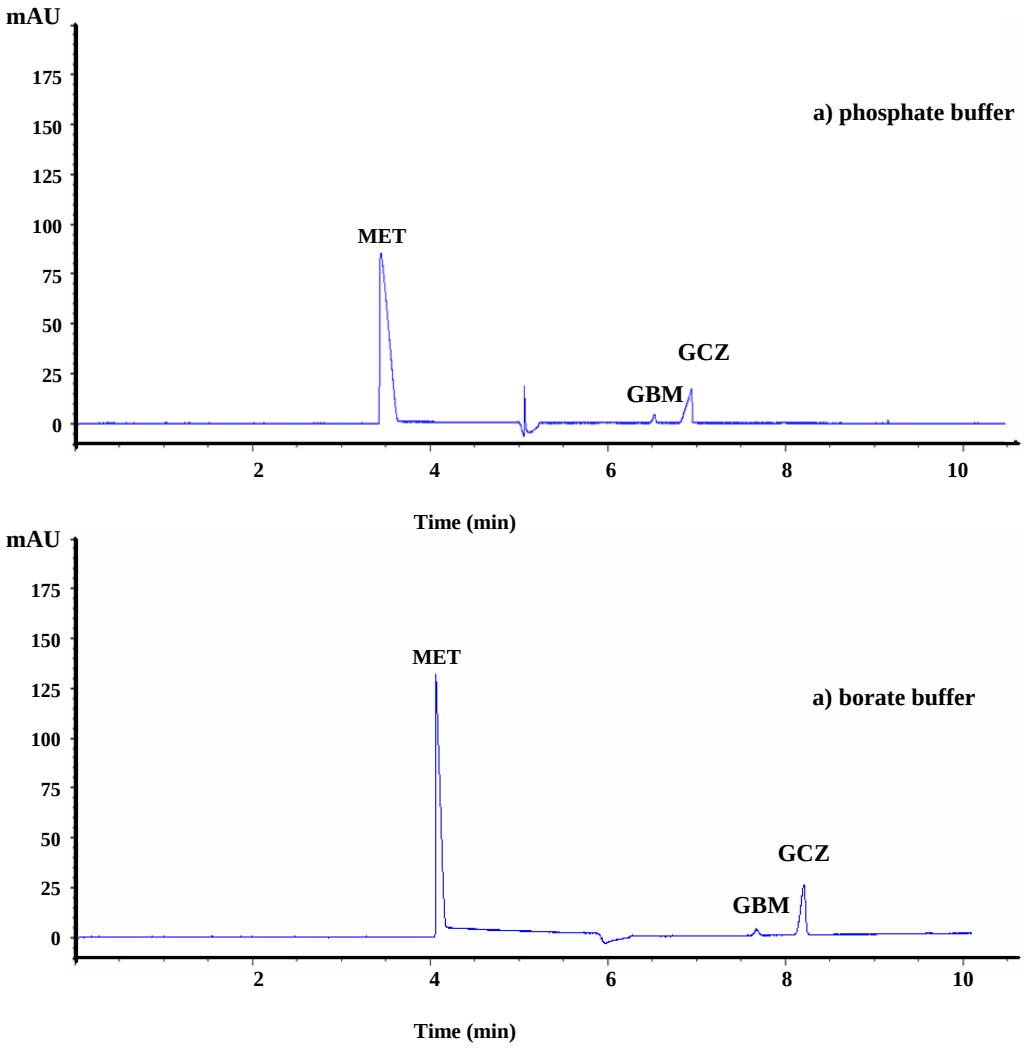


Figure 4.2 Effects of types of buffer on the separation of MET, GBM and GCZ (concentration 1,000 µg/mL, 10 µg/mL, 100 µg/mL, respectively). Condition: BGE: a) 10 mM sodium dihydrogen phosphate pH 7.0 and b) 50 mM borate buffer pH 9.0;capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 10 s; temperature 25 ◦C; voltage 20 kV; detection by DAD absorbance at 225 nm.

4.1.1.3 Effects of detection wavelength

Selection of appropriate detection wavelength is important, especially for a mixture of analytes. In this study, wavelengths at 210, 225 and 245 nm were chosen to simultaneous monitor MET, GCZ and GBM. The shorten the wavelength, the better is the sensitivity. In addition, the S/N of GBM was highest at 210 nm (S/N = 4.8) compared with 225nm (S/N = 4.0) and 245 nm (S/N = 2.9) (Figure 4.3).

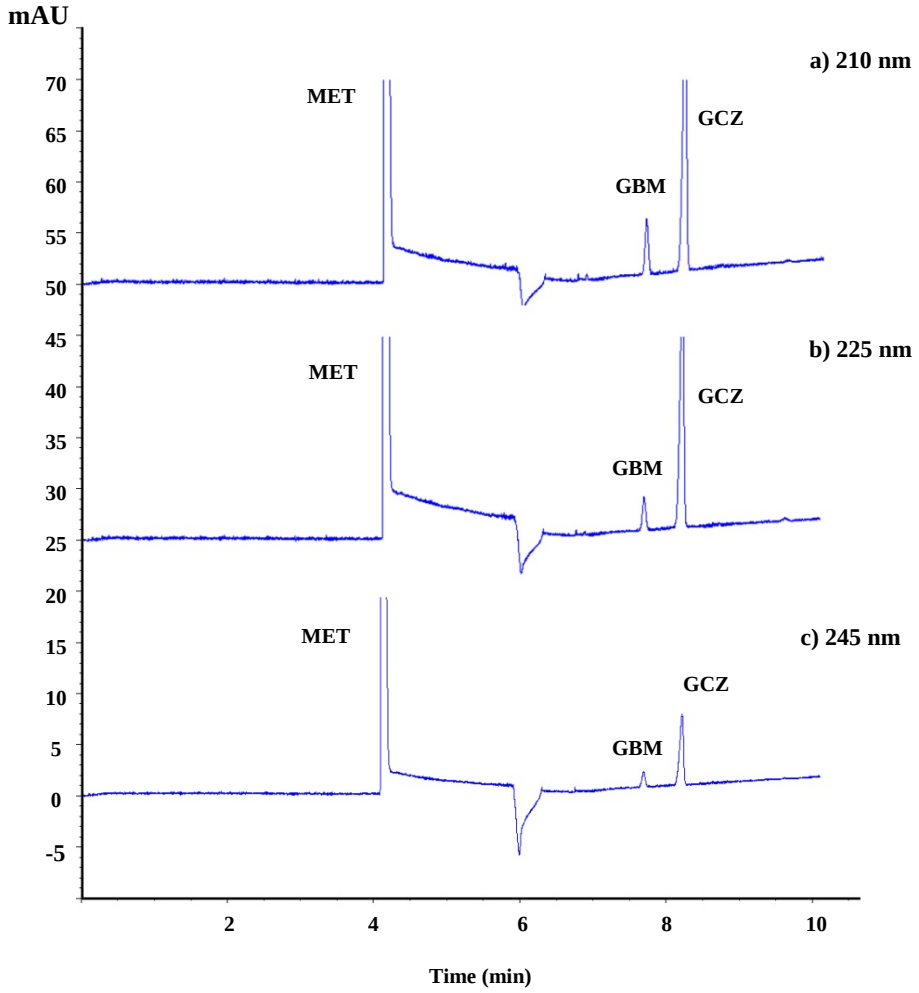


Figure 4.3 Effects of detection wavelengthon the separation of MET, GBM and GCZ (concentration 1,000 µg/mL, 10 µg/mL, 100 µg/mL, respectively); Condition: BGE: 50 mM borate buffer pH 9.0; capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 10 s; temperature 25 ◦C; voltage 20 kV; varied detection wavelength at a) 210 nm, b) 225 nm, c) 245 nm.

The optimized condition for the separation of MET, GBM and GCZ was in 50 mM borate buffer pH 9.0.The separation was performed using a fused-silica capillary tube with L_{total} of 64.5 cm, L_{eff} of 56.0 cm and i.d. of 50 µm, injection was at 50 mbar for 10 s, the temperature and applied voltage were 25 °C and 20 kV, respectively. The detection wavelength was at 210 nm (Figure 4.4 a). Figure 4.4 b shows the electropherogram of the drugs in the ratios that they are present in the combined formulations.

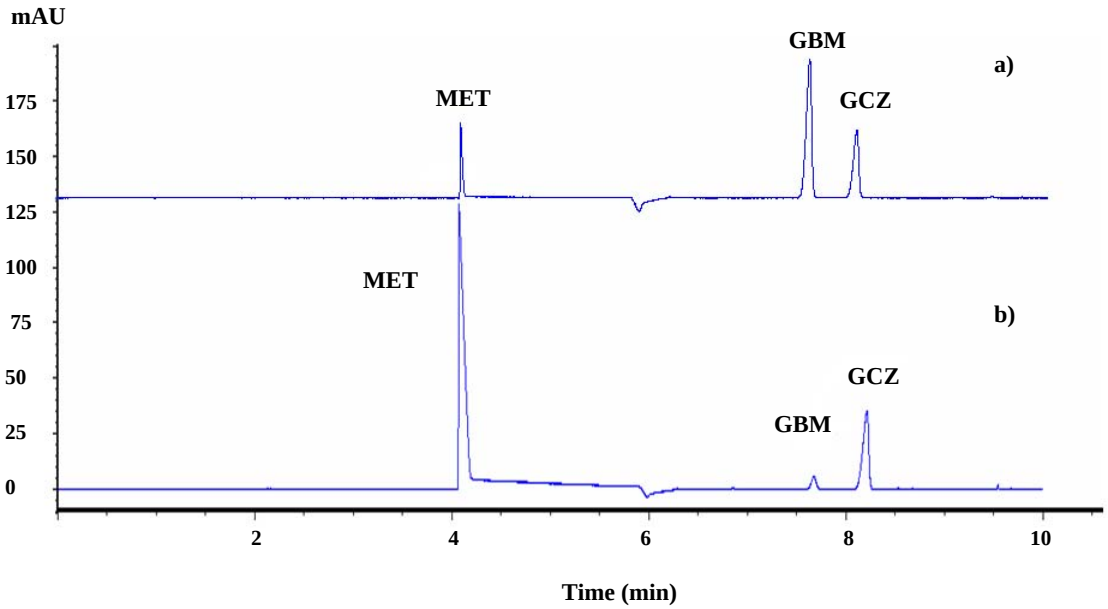


Figure 4.4 Electropherogram of optimum condition for the separation of MET, GBM and GCZ. a) A standard mixture of MET, GBM, GCZ (100 µg/mL) , b) standard mixture of MET, GBM, GCZ (1,000 µg/mL, 10 µg/mL, 100 µg/mL, respectively), condition; BGE: 50 mM borate buffer pH 9.0;capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 10 s; temperature 25 ◦C; voltage 20 kV; detection by DAD absorbance at 210 nm.

4.1.2 Method validation

The optimized CE condition for the separation of MET, GBM and GCZ was validated according to International Conference on Harmonization guidelines e.g. linearity, precision, accuracy, LOD and LOQ (47).

4.1.2.1 Linearity

For this research, linearity of system (Table 4.1) and linearity of method (Table 4.2) was investigated. In both cases, the method shows good linearity with r^2 of greater than 0.99.

Table 4.1 Linearity of system data

	Linearity	r^2
metformin (500-1,500 µg/mL)	$\bar{y}$ = 0.4054x+44.484	0.9940
glibenclamide (5-15 µg/mL)	$\bar{y}$ = 2.1112x-0.16023	0.9973
gliclazide (80-240 µg/mL)	$\bar{y}$ = 1.0520x+0.2918	0.9929

Table 4.2 Linearity of method data

	Linearity	r^2
metformin (800-1,200 µg/mL µg/mL)	$\bar{y}$ = 0.4077x+54.2597	0.9999
glibenclamide (8-12 µg/mL µg/mL)	$\bar{y}$ = 2.0524x+1.1473	0.9950
gliclazide (128-192 µg/mL µg/mL)	$\bar{y}$ =1.2670x+7.7836	0.9996

4.1.2.2 Specificity

Figure 4.5 shows specificity of MET, GBM and GCZ.

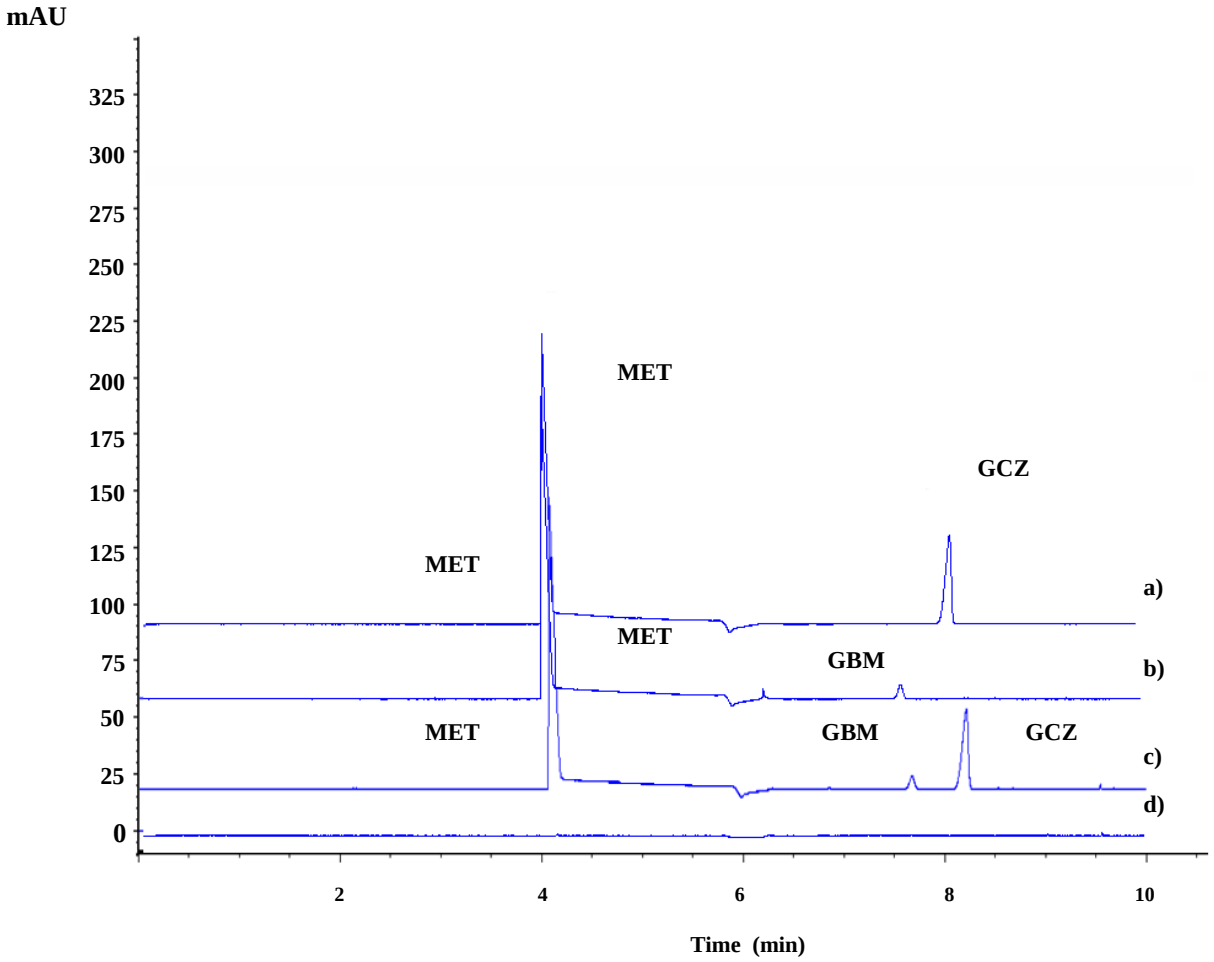


Figure 4.5 Electrochromatogram of a) sample MET (1000 µg/mL) and GCZ (160 µg/mL), b) sample MET (1000 µg/mL) and GBM (10 µg/mL), c) standard MET (1000 µg/mL), GBM (10 µg/mL), GCZ (160 µg/mL), d) BGE; Condition: 50 mM borate buffer pH 9.0 ; capillary 64.5 cm full length, 50 cm effective length, 50 µm ID, injection at 50 mbar, 10 s; 20 kV; 25 ◦C; detection by DAD absorbance at 210 nm.

4.1.2.3 Precision

Table 4.3 shows the repeatability of the method represented as %RSD of migration time and peak area. The % RSD of repeatability was less than 1.51%. Table 4.4 shows the intermediate precision of the methods, the % RSD was less than 1.90%.

Table 4.3 Repeatability data

		$\%RSD_{peak\ area}$	$\%RSD_{migration\ time}$
	metformin (1,000 µg/mL)	0.21	0.27
	glibenclamide (10 µg/mL)	1.17	0.31
day1	gliclazide (160 µg/mL)	0.47	0.32
	metformin (1,000 µg/mL)	0.25	0.10
	glibenclamide (10 µg/mL)	1.18	0.20
day2	gliclazide (160 µg/mL)	0.25	0.21
	metformin (1,000 µg/mL)	0.17	0.08
	glibenclamide (10 µg/mL)	1.51	0.18
day3	gliclazide (160 µg/mL)	0.39	0.20

Table 4.4 Intermediate precision data of MET, GBM and GCZ presented as % RSDs

	Concentration	Day 1		Day 2		Day 3	
	(µg/mL)	area	tm	area	tm	area	tm
	800 µg/mL	1.46	0.27	1.86	0.09	0.57	0.40
MET	1,000 µg/mL	0.21	0.27	0.25	0.10	0.17	0.08
	1,200 µg/mL	0.65	0.33	0.36	0.59	0.49	0.62
	8 µg/mL	1.37	0.35	0.96	0.12	1.66	0.56
GBM	10 µg/mL	1.17	0.31	1.18	0.20	1.51	0.18
	12 µg/mL	1.33	0.57	1.53	0.58	1.80	0.66
	128 µg/mL	1.90	0.35	1.62	0.13	1.06	0.57
GCZ	160 µg/mL	0.47	0.32	0.25	0.21	0.39	0.20
	192 µg/mL	0.60	0.34	0.67	0.58	1.45	0.43

4.1.2.4 Accuracy

Accuracy was evaluated by adding the known amount of five different concentrations of standard solution into 50% concentration of samples and the spiked samples were analyzed by the optimum condition. Table 4.5 shows the accuracy of the CE method in term of % RSD. The % recovery varied between 97.6 and 103.4% with % bias of less than 3.4%.

Table 4.5 Accuracy

conc. Added	analyte	% recovery				% bias			
		day 1	day 2	day 3	average	day 1	day 2	day 3	average
300 µg/mL	MET	97.6	99.6	100.4	99.2	-2.4	-0.4	0.4	-0.8
500 µg/mL		99.7	100.3	100.1	100.0	-0.3	0.3	0.1	0.0
700 µg/mL		100.4	100.0	100.2	100.2	0.4	0.0	0.2	0.2
3 µg/mL	GBM	103.4	102.2	103.1	102.9	3.4	2.2	3.1	2.9
5 µg/mL		98.0	101.7	100.5	100.0	-2.0	1.7	0.5	0.0
7 µg/mL		101.0	101.6	99.0	100.5	1.0	1.6	-1.0	0.5
48 µg/mL	GCZ	100.1	99.6	99.1	99.6	-0.1	0.4	0.9	0.4
80 µg/mL		100.4	100.2	100.3	100.3	-0.4	-0.2	-0.3	-0.3
112 µg/mL		99.9	100.1	100.2	100.1	0.1	-0.1	-0.2	-0.1

4.1.2.5 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were established based on singnal-to-noise ratio of 3 and 10 for LOD and LOQ, respectively. Table 4.6 shows LODs and LOQs of MET, GBM and GCZ. The overall LOQ is less than 12 μg/mL. The concentration is practical for determination of the drugs in dosage forms, which are usually exist in rather high concentrations.

Table 4.6 LOD and LOQ of MET, GBM and GCZ

	LOD (μg/mL)	LOQ (μg/mL)	%RSD (n = 6)
MET	2	5	1.28
GBM	2	5	1.46
GCZ	4	12	0.72

*%RDSs represent the %RSDs of LOQ

4.1.3 Applications

The developed and validated CZE method was used for analyzing of MET, GBM and GCZ in thirteen samples of pharmaceutical products, including raw material, single and combination formulations.The result showed that all samples had % labeled amount between 99.7 to 101.3 and % RDSs were less than 1.80% (Table 4.7), which comply to typical USP limit (% label amount between 95.0 and 105.0 %). Figure 4.6 shows eletropherograms of the samples comparing to the solvent and standard.

Table 4.7 Assay of samples (n = 3)[a]

Sample	%labeled amount	BP limit	USP limit
MET raw material	100.6 (0.59)	-	98.5-101.0%
GBM raw material	100.3 (0.51)	99.9-101.0	-
GCZ raw material	100.2 (0.90)	99.9-101.0	-
MET tablet lot A	101.3 (0.82)	-	95.0-105.0%
MET tablet lot B	100.7 (0.61)	-	95.0-105.0%
GBM tablet lot A	101.0 (0.61)	95.0-105.0%	-
GBM tablet lot B	101.0 (1.12)	95.0-105.0%	-
GCZ tablet lot A	100.4 (0.93)	95.0-105.0%	-
GCZ tablet lot B	99.9 (1.05)	95.0-105.0%	-
MET/GBM tablet lot A	MET: 101.0 (0.94), GBM: 100.8 (0.83)	-	-
MET/GBM tablet lot B	MET: 101.0 (0.70), GBM: 100.6 (1.59)	-	-
MET/GCZ tablet lot A	MET: 101.0 (1.80), GCZ: 100.9 (0.19)	-	-
MET/GCZ tablet lot B	MET: 100.1 (0.50), GCZ: 99.7 (0.42)	-	-

[a]: MET = metformin, GBM = glibenclamide, GCZ = gliclazide, number in parenthesis represents %RSD

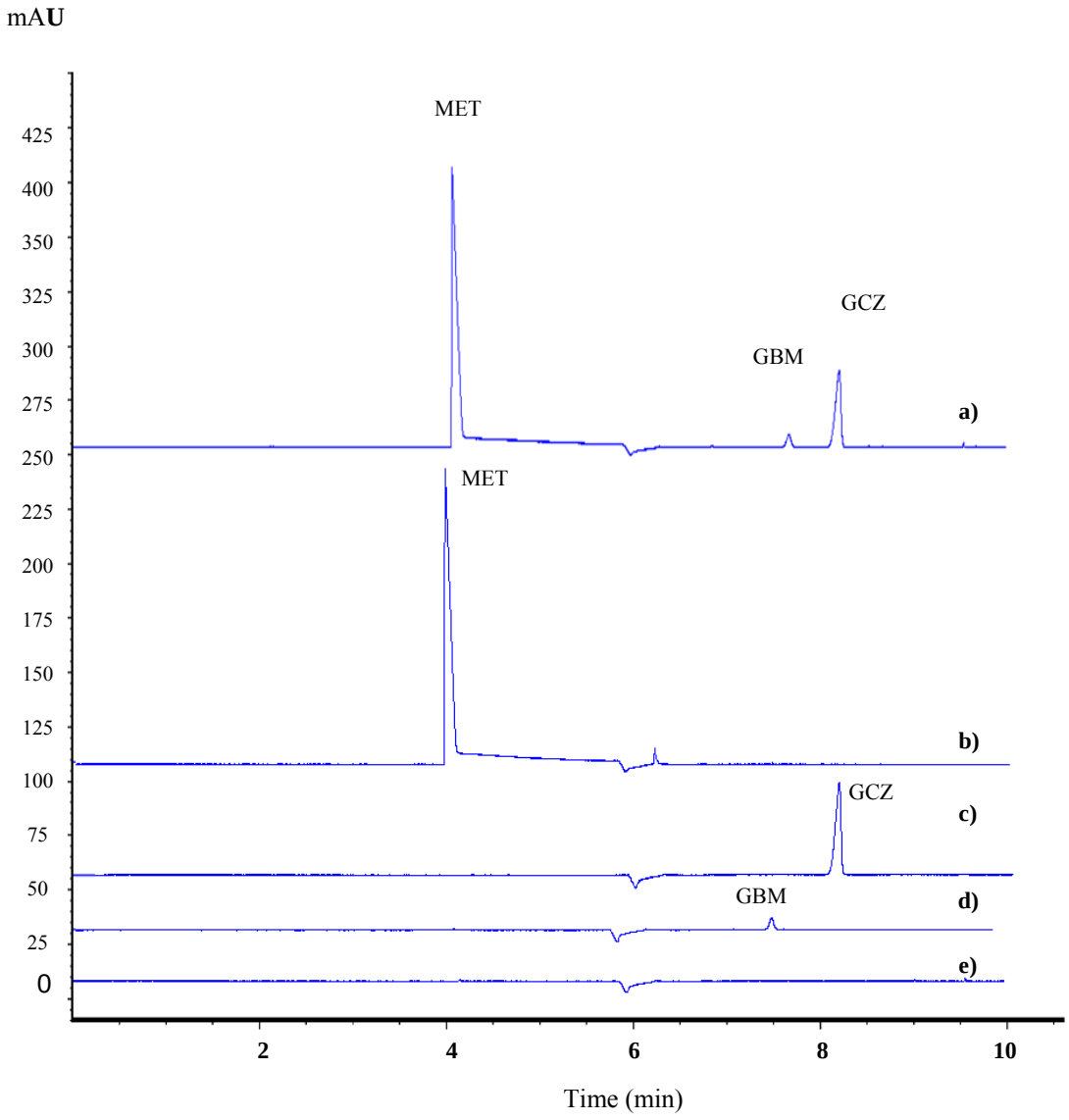


Figure 4.6 Electrochromatogram of a) standard MET (1000 µg/mL), GBM (10 µg/mL) and GCZ (160 µg/mL), b) sample MET (1000 µg/mL), c) sample GCZ (160 µg/mL), d) sample GBM (10 µg/mL), e) solvent (MeOH); Condition: 50 mM borate buffer pH 9.0 ; capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 10 s; temperature 25 ◦C; voltage 20 kV; detection by DAD absorbance at 210 nm.

4.2 Stability study of anti-diabetic drugs

4.2.1 CE optimization of MET and CGN

The CZE method for separation of MET and CGN was modified from Hamdanand et al.(52).The condition was in 40 mM citric buffer (pH 6.7), using a fused-silica capillary tube with a total length of 68.5 cm, an effective length of 60 cm and an inner diameter of 50 µm. The detection was at a wavelength of 214 nm with a bandwidth of 8 nm, applied voltage at 15 kV, temperature was at 30 °C and injection was by pressure at 50 mbar for 5 s. MET and CGN were well separated under these conditions (Figure 4.7).

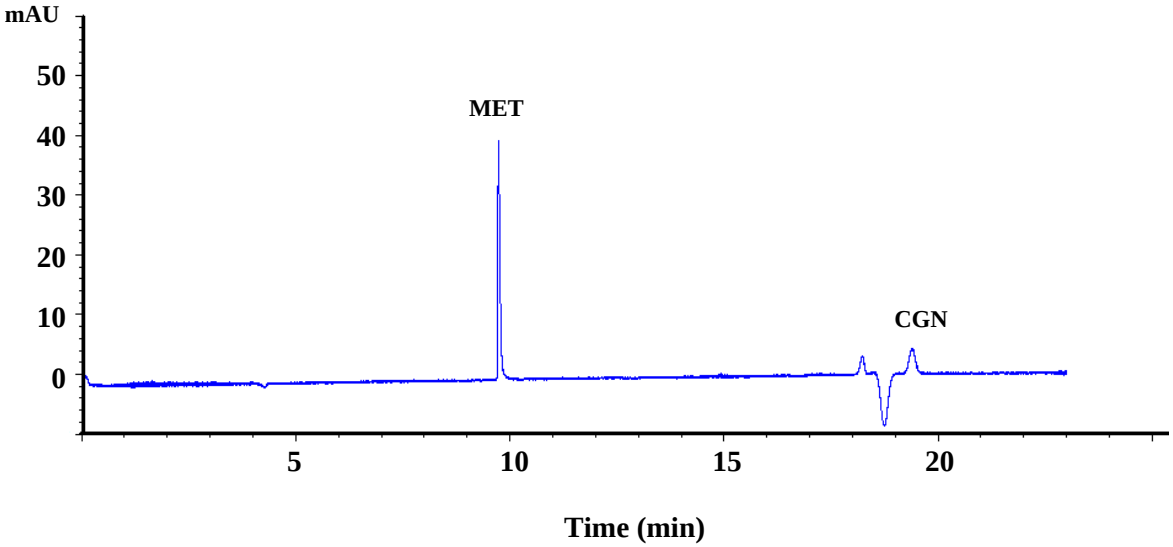


Figure 4.7 Electropherogram of the optimum condition on the separation of MET (600 µg/mL) and CGN (40 µg/mL). Condition: BGE: 40 mM citrate buffer pH 6.7 ; capillary 68.5cm full length, 60 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 15 kV; temperature 30 ◦C; detection by DAD absorbance at 214 nm.

4.2.2 CE optimization of GCZ, GZB and GZF

4.2.2.1 CZE method

The CZE condition in the previous section was applied for the separation of GCZ, GZB and GZF. Nevertheless, GCZ and its impurities were not separated, GZB was on EOF, GCZ was co-migrated with GZF (Figure 4.8).

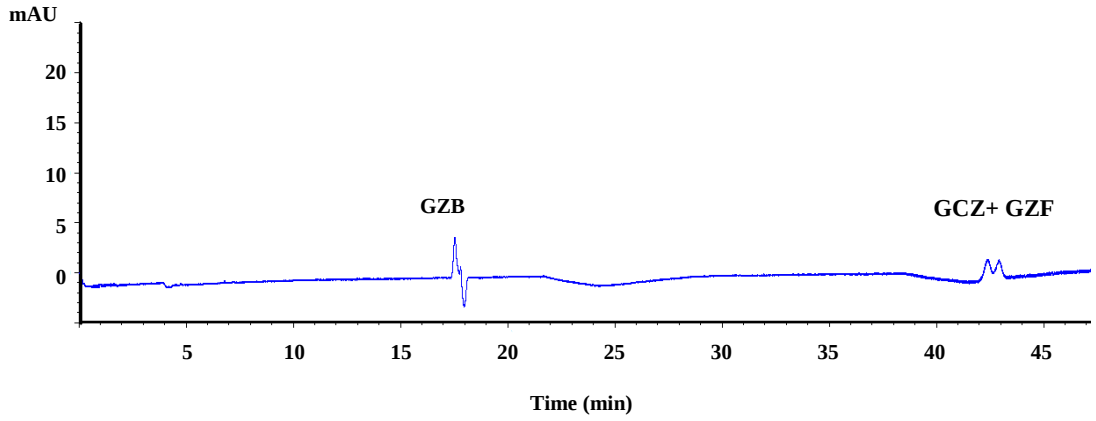


Figure 4.8 Electropherogram on the separation of GCZ (150 µg/mL), GZB and GZF (200 µg/mL) Condition: 40mMcitrate buffer pH 6.7 ; capillary 68.5cm full length, 60 cm effective length, 50µm ID, injection at 50 mbar, 5 s; 15 kV; 30 ◦C; detection by DAD absorbance at 225 nm.

Further optimization was required and phosphate buffer was selected to study because it has wide range of pH that suitable for analyze compounds that have several pK_a. In this investigation, MET and CGN were included as analytes. The result showed that CGN/GZB were co-migrated at EOF, and GCZ/GZF were overlapped (Figure 4.9).

CZE might not suitable for the separation of the investigated separations because some of them were electrically neutral. MEKC can be an alternative.

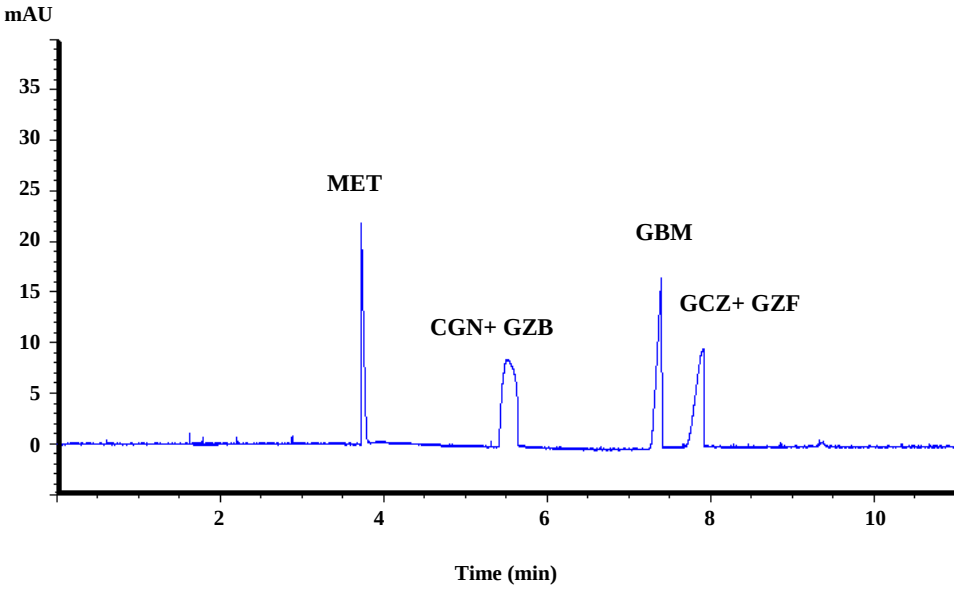


Figure 4.9 Electropherogram of the separation of MET, CGN, GCZ(100µg/mL), GBM, GZB and GZF (200µg/mL) Condition: BGE: 10 mM sodium dihydrogen phosphatebuffer pH 7.0 ; capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 20 kV; temperature 25 ◦C; detection by DAD absorbance at 225 nm.

4.2.2.2 MEKC method

MEKC method was selected since it benefitsthe separation neutral compounds. It may solve the co-overlapped peak of GCZ and GZF.

4.2.2.2.1 Effect of SDS concentration

Various concentrations of SDS (10, 15 and 20 mM) were added into 10 mM sodium dihydrogen phosphate buffer (pH 7.0) (Figure 4.10). Increasing SDS concentrations improved the resolution of GZF/GCZ, but reduced resolution of MET/CGN. Thus, 15 mM SDS was chosen as compromise.

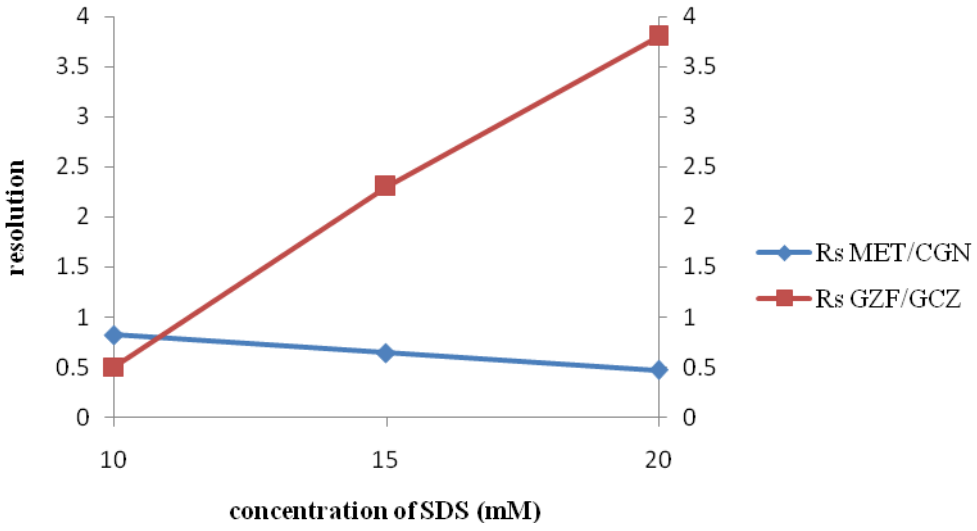


Figure 4.10 Plot of concentration of SDS vs. resolution of MET/CGN and resolution of GZF/GCZ. Conditions: BGE: various concentration of SDS (10, 15 and 20 mM) in 10 mM sodium dihydrogen phosphate buffer pH 7.0; capillary 64.5cm full length, 56 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 20 kV; temperature 25 ◦C; detection by DAD absorbance at 225 nm.

4.2.2.2.2 Effect of pH of BGE

Varying pH of BGE greatly affected the separation of GZF/GCZ. Increasing or lowering the pH from 7.0 reduced the resolution of GZF/GCZ and caused poor peak shapes of most analytes. Thus, pH 7.0 was selected as an optimal value (Figure 4.11).

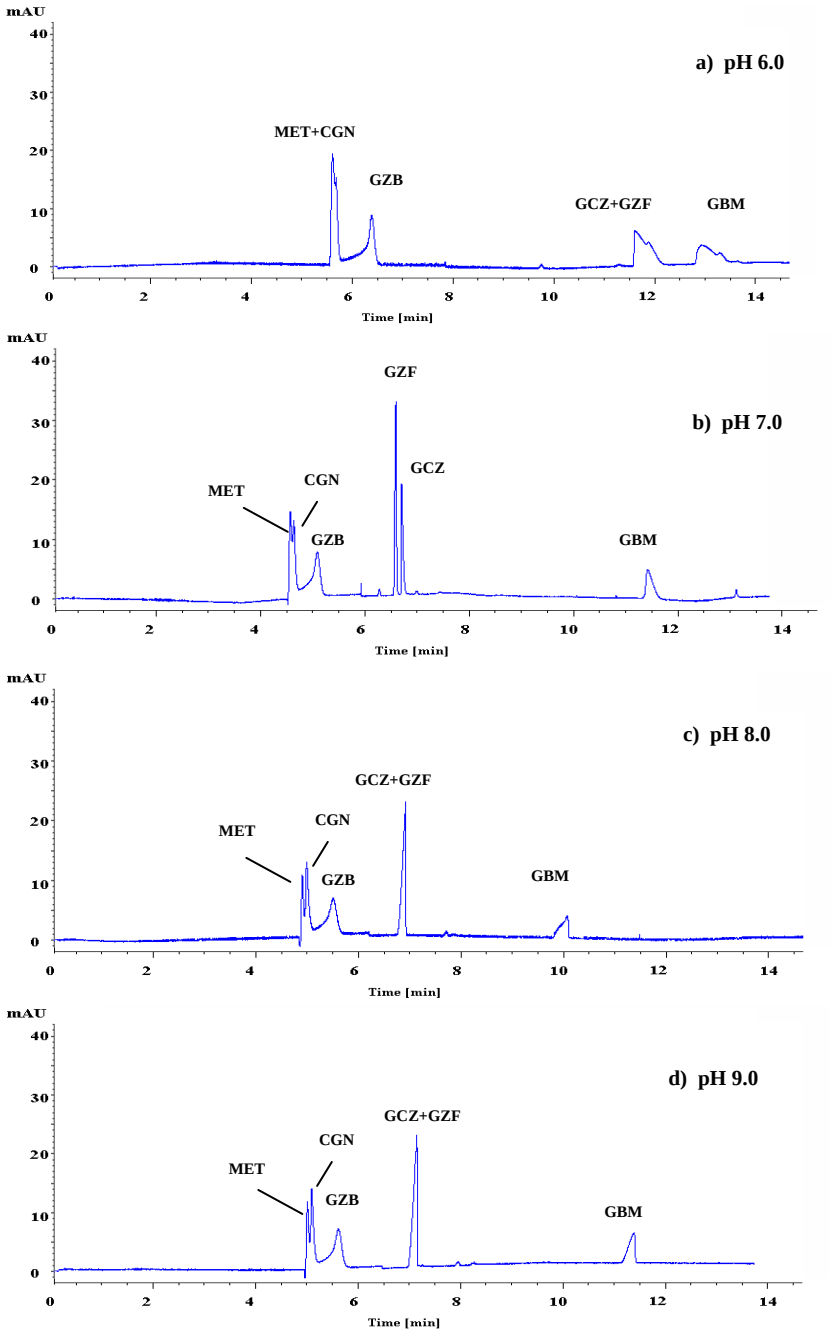


Figure 4.11 Electropherogram of pH effects on the separation of MET,CGN, GBM, GCZ (100µg/mL), GZB,GZF (200µg/mL) Condition: 10 mM sodium dihydrogen phosphate buffer containing 15 mM SDSwith varied pH: a) pH 6.0, b) pH 7.0, c) pH 8.0, d) pH 9.0 ; capillary 64.5cm full length, 50 cm effective length, 50µm ID, injection at 50 mbar, 5 s; 20 kV; 25 ◦C; detection by DAD absorbance at 225 nm.

Results from several experiments indicated that MET and CGN and GCZ and its impurities could not be separated using one CE

condition. In this section, separation of GCZ, GZB and GZF was focused in order to search for a method that can be applied for stability study of GCZ.

In order to reduce the migration time, the effective length of the capillary was shorten from 56 cm to 40 cm. The migration time of GCZ and its impurities reduced from 8 min to 5 min. Thus, the optimization condition for the separation of GCZ, GZB and GZF was in 10 mM phosphate buffer (pH 7.0) containing 15 mM SDS. The separation was performed using a fused-silica capillary tube with a total length of 48.5 cm, an effective length of 40 cm and an inner diameter of 50 µm. The detection was at a wavelength of 225 nm with a bandwidth of 8 nm, applied voltage at 20 kV, temperature was at 25 °C and injection was by pressure at 50 mbar for 5 s (Figure 4.12).

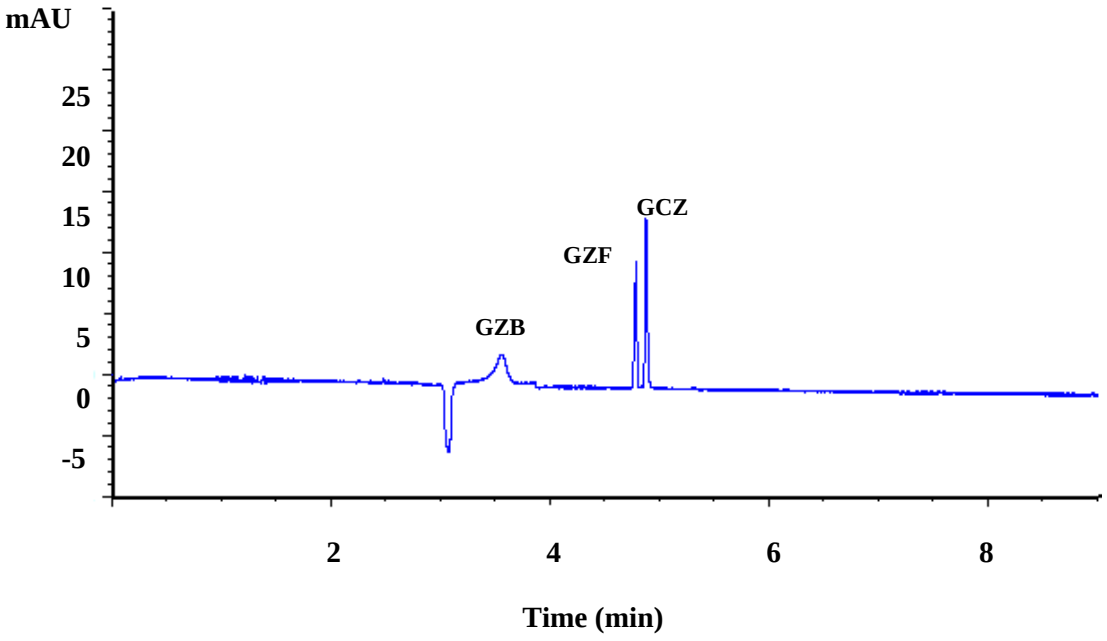


Figure 4.12 MEKC separation of GCZ, GZB and GZF (100µg/mL) Condition : 10 mM sodium dihydrogen phosphate buffer pH 7.0 adding 15 mM SDS ; capillary 48.5cm full length, 40 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 20 kV; temperature 25 ◦C; detection by DAD absorbance at 225 nm.

4.2.3 Method validation

4.2.3.1 Method validation of CZE method of MET and CGN

Linearity, precision, accuracy, LOD, LOQ and robustness data of MET and CGN of the CZE method (Figure 4.6) was presented in Table 4.8-4.13. Figure 4.13 shows specificity of CZE method. Robustness data (Table 4.13) was evaluated from the variation around the optimal values of pH (6.7± 0.5) and the voltage (15± 2).

Table 4.8 Linearity data of MET and CGN

	Range (µg/mL)	Linear regression	r^2
MET	400-600	y = 0.3881x - 4.0717	0.9985
CGN	2-40	y = 3.6108x + 2.9254	0.9968

Table 4.9 Repeatability data

		$\%RSD_{peak\ area}$	$\%RSD_{tm}$
Day 1	MET (500 µg/mL)	0.16	1.72
	CGN (10 µg/mL)	1.16	1.77
Day 2	MET (500 µg/mL)	0.15	1.93
	CGN (10 µg/mL)	0.86	1.95
Day 3	MET (500 µg/mL)	0.27	1.90
	CGN (10 µg/mL)	1.06	1.98

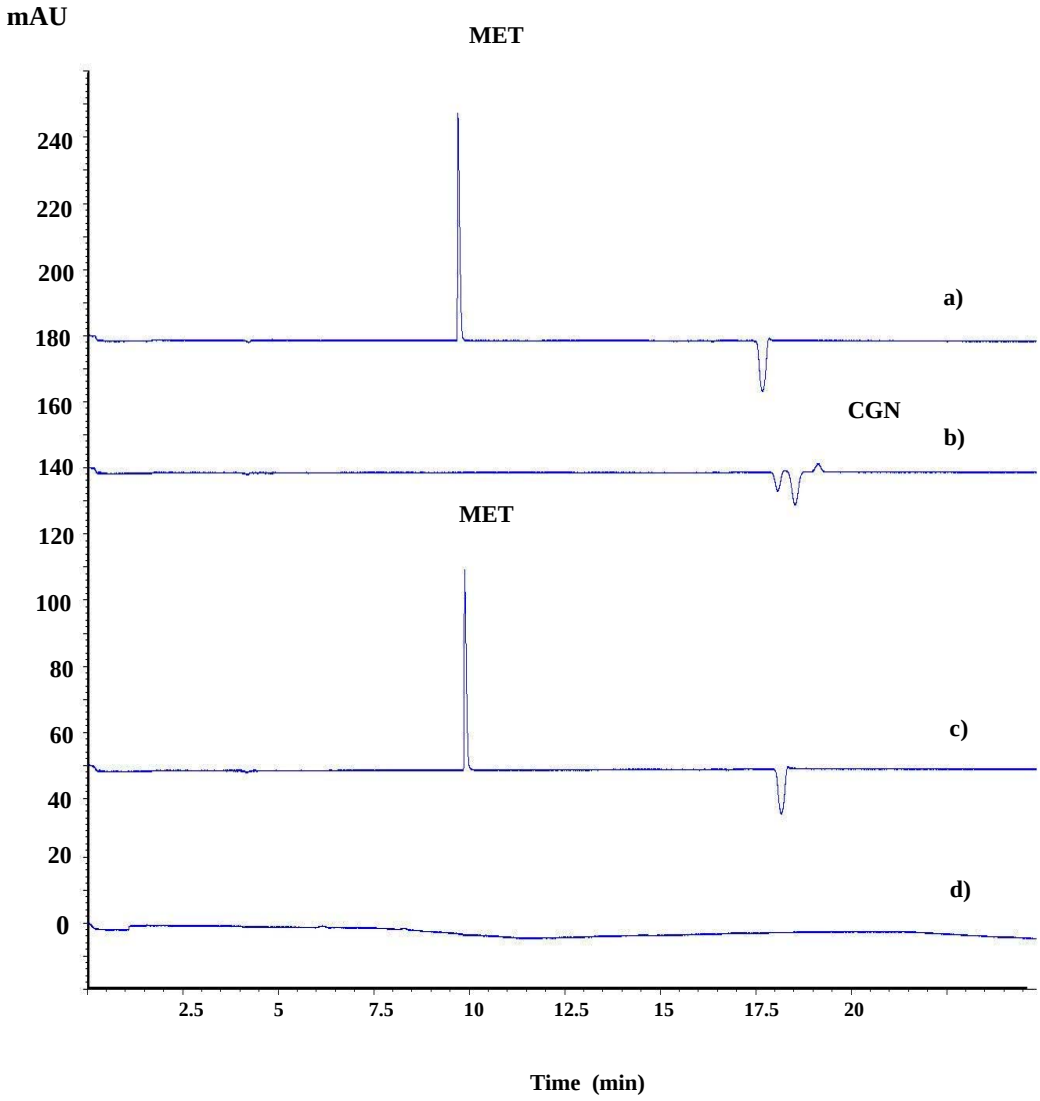


Figure 4.13 Electrochromatogram of a) standard MET (500 µg/mL), b) standard CGN (10 µg/mL), c) sample MET (500 µg/mL), d) BGE; BGE Condition: 40 mMcitrate buffer pH 6.7 ; capillary 68.5cm full length, 60 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 15 kV; temperature 30◦C; detection by DAD absorbance at 214 nm.

Table 4.10 Intermediate precision data of MET and CGN presented as %RSDs

	Concentration (µg/mL)	Day 1		Day 2		Day 3	
		area	t_m	area	t_m	area	t_m
MET	400	0.55	1.77	0.86	1.54	0.63	1.39
	500	0.79	1.72	0.93	1.93	1.09	1.90
	600	0.38	1.52	0.24	1.34	0.22	1.51
CGN	2	1.01	1.68	1.37	1.94	0.60	1.90
	10	1.08	1.77	0.74	1.95	0.75	1.98
	40	0.89	1.64	0.74	1.95	0.94	1.75

Table 4.11 Accuracy data of MET and CGN

Conc. added (µg/mL)	Analyte	% Recovery				
		Day 1	Day 2	Day 3	Average	%RSDs
150	MET	98.0	98.5	98.4	98.3	0.28
250		100.1	100.0	100.1	100.1	0.09
350		99.1	98.9	99.1	99.0	0.12
Average		99.0	99.1	99.2	99.1	0.07
%RSD		1.08	0.77	0.89	0.91	
2	CGN	99.9	100.2	100.9	100.3	0.50
10		101.0	100.1	100.3	100.5	0.47
40		100.5	100.8	101.4	100.9	0.46
Average		100.5	100.4	100.9	100.6	0.26
%RSD		0.56	0.38	0.55	0.31	0.50

Table 4.12 LOD and LOQ data of MET and CGN (n = 6)

	MET	S/N	CGN	S/N
LOD (μg/mL)	30	3.04	2	2.79
LOQ (μg/mL)	100	9.92	8	9.63
$\%RSD_{peak\ area}$	1.75		1.91	
$\%RSD_{migration\ time}$	1.74		2.18	

LOD and LOQ were based on the signal to noise (S/N) ratios of 3 and 10, respectively

Table 4.13 Robustness of MET and CGN (n = 9)

	MET			CGN		
	1	2	3	1	2	3
pH 6.0	189.00	190.06	192.00	39.61	38.60	39.33
pH 6.7	188.53	188.43	188.37	39.79	40.64	40.15
pH 7.5	189.58	190.89	190.74	40.56	40.49	40.32
average	189.73			average	39.94	
%RSD	0.68			%RSD	1.99	

	MET			CGN		
	1	2	3	1	2	3
13 kV	188.92	189.11	189.28	39.46	40.32	39.23
15 kV	188.53	188.43	188.37	39.79	40.64	40.15
17 kV	189.12	190.28	188.50	39.86	39.59	40.15
average	188.95			average	26.68	
%RSD	0.32			%RSD	1.92	

Validation data indicated the method was valid for the analysis of MET and CGN. The method showed good linearity (MET: y = 0.3881x – 4.0717, r^2= 0.9985 (400-600 μg/mL) and CGN: y = 3.6108x + 2.9254, r^2 = 0.9968 (2-40 μg/mL)) and precision (%RSD for intra- and inter-day precision of less than 1.98%) for both MET and CGN. Accuracy represented as %recovery was between 98.3 and

100.1% for MET and 100.3-100.9% for CGN with %bias of less than 1.7%). LODs were 30 and 2 μg/mL for MET and CGN, respectively. LOQs were 100 and 8 μg/mL for MET and CGN, respectively. Upon altering of buffer pH (± 0.5 unit) and separating voltage (± 2 kV), the method was still robust with %RSDs of migration time and peak area of less than 1.73%.

4.2.3.2 Method validation of MEKC method of GCZ, GZB and GZF

Linearity, precision, accuracy, LOD, LOQ and robustness data of GCZ, GZB and GZFof theMEKC method(Figure 4.11) was presented in Table 4.14- 4.19. Figure 4.14 shows specificity of MEKC method. Robustness data (Table 4.18) was evaluated from the variation around the optimal values of pH (7.0± 0.2) and the voltage (20± 2).

Table 4.14 Linearity data of GCZ, GZB and GZF

	Range (μg/mL)	Linear regression	r^2
GCZ	128-192	y = 0.2544x - 0.6716	0.9981
GZB	20-60	y = 0.1749x + 0.0476	0.9988
GZF	10-50	y = 0.1968x + 0.0176	0.9999

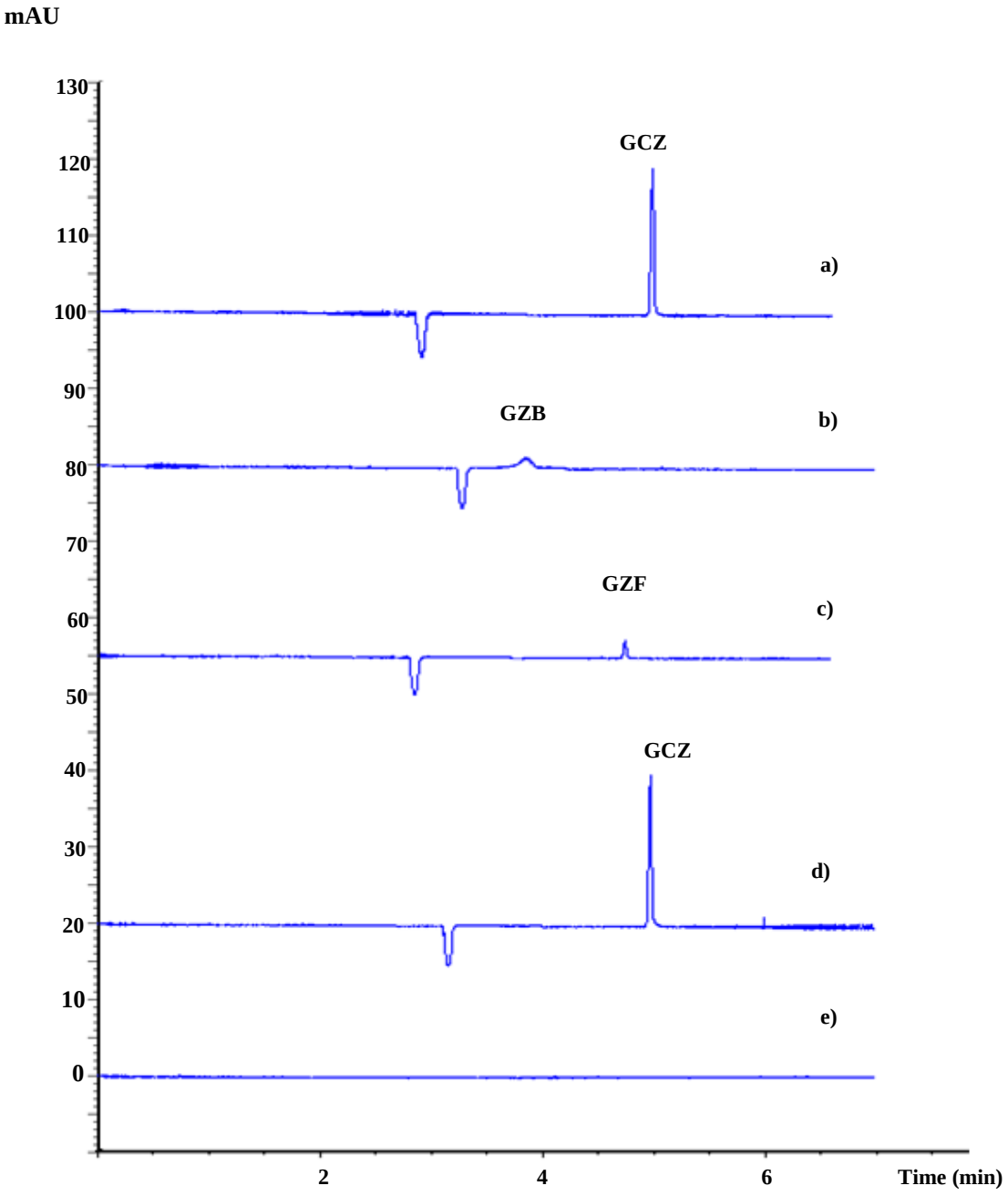


Figure 4.14 Electrochromatogram of a) BGE, b) sampleGCZ (160 µg/mL), c) standard GZF (30 µg/mL),d) standardGZB (40 µg/mL), e) standard GCZ (160 µg/mL), Condition: 10 mM sodium dihydrogen phosphate buffer pH 7.0 adding 15 mM SDS ; capillary 48.5cm full length, 40 cm effective length, 50µm ID, injection at 50 mbar, 5 s; voltage 20 kV; temperature 25 ◦C; detection by DAD absorbance at 225 nm.

Table 4.15 Repeatability data of GCZ, GZB and GZF

		$\%RSD_{peakrea}$	$\%RSD_{tm}$
Day 1	GCZ (160 µg/mL)	0.49	1.95
	GZB (40 µg/mL)	1.54	2.00
	GZF (30 µg/mL)	1.18	1.95
Day 2	GCZ (160 µg/mL)	0.71	1.39
	GZB (40 µg/mL)	1.42	1.89
	GZF (30 µg/mL)	1.64	1.40
Day 3	GCZ (160 µg/mL)	0.97	1.20
	GZB (40 µg/mL)	1.01	1.12
	GZF (30 µg/mL)	1.24	1.18

Table 4.16 Intermediate precision data of GCZ, GZB and GZF presented as %RSDs

	Concentration (µg/mL)	Day 1		Day 2		Day 3	
		area	t_m	area	t_m	area	t_m
GCZ	128	1.25	0.51	0.53	1.12	0.70	1.90
	160	0.49	1.95	0.71	1.39	0.97	1.20
	192	1.07	1.39	1.05	1.35	1.01	1.66
GZB	20	1.42	1.78	1.23	1.07	1.47	1.92
	40	1.52	2.00	1.42	1.89	1.01	1.12
	60	0.53	1.16	0.53	1.56	0.81	1.70
GZF	10	0.96	0.43	1.50	1.06	0.85	1.85
	30	1.18	1.95	1.64	1.40	1.24	1.18
	50	1.02	1.42	0.88	1.48	0.53	1.71

Table 4.17 Accuracy data of GCZ, GZB and GZF

Conc. added(µg/mL)	Analyte	% Recovery				
		Day 1	Day 2	Day 3	Average	%RSD
48		100.0	99.3	99.5	99.6	0.35
80	GCZ	99.6	100.6	100.0	100.1	0.50
112		99.5	99.7	100.6	99.9	0.59
Average		99.7	99.9	100.1	99.9	0.17
%RSD		0.28	0.66	0.53		
20		100.6	100.3	100.0	100.3	0.30
40	GZB	99.3	99.7	98.9	99.3	0.38
60		99.6	100.1	99.9	99.9	0.24
Average		99.9	100.0	99.6	99.8	0.21
%RSD		0.65	0.32	0.59		
10		99.3	100.1	99.9	99.8	0.46
30	GZF	99.5	99.5	99.5	99.5	0.04
50		99.5	99.2	98.7	99.1	0.41
Average		99.4	99.6	99.4	99.5	0.13
%RSD		0.12	0.47	0.63		

Table 4.18 LOD and LOQ data of GCZ, GZB and GZF (n = 6)

	GCZ	S/N	GZB	S/N	GZF	S/N
LOD(μg/mL)	40	3.6	20	3.26	10	2.56
LOQ (μg/mL)	120	9.86	80	8.6	50	8.76
$\%RSD_{peak\ area}$	0.86		0.69		0.95	
$\%RSD_{migration\ time}$	1.42		1.20		1.98	

LOD and LOQ were based on the signal to noise (S/N) ratios of 3 and 10, respectively

Table 4.19 Robustness of GCZ, GZB and GZF (n = 6)

	GCZ			GZB			GZF		
	1	2	3	1	2	3	1	2	3
pH 6.8	39.95	39.52	40.25	6.92	6.90	6.92	6.09	5.96	6.02
pH 7.0	39.73	40.01	39.99	6.93	7.05	6.90	6.01	5.88	5.99
pH 7.2	39.80	40.05	39.84	6.94	6.93	6.95	5.96	6.09	6.03
average	39.90			average	6.94		average	6.00	
%RSD	0.53			%RSD	0.65		%RSD	1.10	

	GCZ			GZB			GZF		
	1	2	3	1	2	3	1	2	3
18 kV	40.05	39.18	39.35	7.01	7.04	6.99	6.02	5.88	5.97
20 kV	39.73	40.01	39.99	6.93	7.05	6.90	6.01	5.88	5.99
22 kV	38.94	39.36	39.91	7.04	7.08	6.94	6.02	5.98	5.96
average	39.61			average	7.00		average	3.96	
%RSD	1.04			%RSD	0.89		%RSD	1.36	

The MEKC method for the analysis of GCZ, GZB and GZF was validated in terms of linearity, precision, accuracy, LOD and LOQ and robustness. The method showed good linearity (GCZ: y = 0.2544x – 0.6716, r^2= 0.9981 (128-192 μg/mL), GZB: y = 0.1749x + 0.0476, r^2 = 0.9986 (20-60 μg/mL) and GZF: y = 0.1968x + 0.0176, r^2 = 0.9999 (10-50 μg/mL)) and precision (%RSD for intra- and inter-day precision of less than 2.00%) for all compounds. Accuracy represented as %recovery was between 99.7 and 100.1% for GCZ, 99.0 and 100.0% for GZB and 99.4 and 99.6

for GZF with %bias of less than 0.86%). LODs were 40, 20 and 10 µg/mL for GCZ, GZB and GZF, respectively. LOQs were 120, 50 and 50 µg/mLfor GCZ, GZB and GZF, respectively. Modification of buffer pH (± 0.2 unit) and separating voltage (± 2 kV) showed that the method was robust with %RSDs of migration time and peak area of less than 1.76% for all compounds

4.2.4 Stability study

Stability indicating methods of MET and GCZ have been published in a few literatures (48-53), but they reports controversial and different results. Presently, stability studies of MET and GCZ were investigated and confirmed according to ICH guidelines (54) and guidance described by Singh and Bakshi (55).

4.2.4.1 Stability study of MET

MET was forced to degrade under various stress conditions. Amounts of the remained MET and CGN were determined and two other unknown degradation products were observed using CE method and data were tabulated in Table 4.20-4.29. Stability profiles on stability of MET are demonstrated in Fig. 4-15-4.19 and electropherograms of MET and the degradation products are in Fig. 4.20-4.23.

Hydrolysis and oxidation of MET at high temperature (80 ºC) in several solvents (e.g. water, 0.1 N HCl, 3% H_2O_2 and 0.1 N NaOH) show different stability profiles of the drug. MET was stable under neutral hydrolysis, but slowly degraded in acid hydrolysis from 100% to 96.6% during 2-8 h. Under chemical oxidation, MET slightly increased from 100% to 114.6% after 8 h, an extra peak at 19 min and CGN were found after the first 30 min. CGN linearly increased from 1.40 to 1.98% during the investigated duration. The drug rapidly degraded in alkaline hydrolysis, MET declined to 73.0% after 1 h and dramatically decrease to 12.5% after 8 h. Under this condition, an additional peak was monitored after 1.5 h, the second extra peak and CGN were detected after 5 h. Amounts of CGN increased from 0.12 to 0.2% from 5 to 8 h.

Stress test of MET under hydrolysis and oxidation of MET at room temperature (25 ºC, kept in a dark cabinet) in several solvents were also studied. MET remained stable in neutral hydrolysis under these conditions for 30 days with the amount found of 99.4%. Under acid hydrolysis, MET was stable up to 3 days, then

started to degrade after day 7 and 53.6% MET content was quantified after 30 days. In hydrogen peroxide solution, MET content fluctuated from 133.5 to 118.9% during day 1-7, an unknown peak and CGN were detected after day 1 and amounts of CGN increased from 1.7 to 2.1% during this period. Again, MET slowly degraded from 95.5% on day 1 to 33.6% on day 30 with the observation of an additional peak after day 14 and CGN was found at day 30 (0.11%).

Furthermore, stress studies of MET under hydrolysis with exposure to sunlight were investigated. MET was stable in neutral hydrolysis, but degraded in alkaline and acid solutions. CGN was detected in alkaline hydrolysis on day 14, but it was not observed in acid hydrolysis.

Current results are in good agreement with those of Haman's (52) and Ali's (48) reports in that MET degraded rapidly in alkaline hydrolysis. However, Ali reported occurrence of CGN only from photolysis and Haman did not identified any unknown degradation products. Presently, CGN was found in both from alkaline hydrolysis and chemical oxidation. In addition, two unknown degradation products were resolved from MET.

Table 4.20 Degradation data of MET in 0.1 N NaOH heated at 80 °C

Time (h)	%MET	Unknown 1	Unknown 2	CGN
0.00	100.0	-	-	-
1:00	73.0	-	-	-
1:15	72.2	-	-	-
1:30	70.0	√	-	-
1:45	64.0	√	-	-
2:00	60.6	√	-	-
2:15	59.4	√	-	-
2:30	55.1	√	-	-
3:00	45.1	√	-	-
4:00	40.2	√	-	-
5:00	25.3	√	√	0.12
6:00	23.1	√	√	0.16
8:00	12.5	√	√	0.20

Table 4.21 Degradation data of MET in water and 0.1 N HCl heated at 80 °C*

Time (h)	%MET	
	Water	0.1 N HCl
0.00	100.0	100.0
2.00	100.5	98.3
4.00	100.3	97.4
6.00	100.2	96.9
8.00	100.0	95.6

*Unknown 1 and 2 and CGN were not observed.

Table 4.22 Degradation data of MET in 3%H_2O_2 heated at 80 °C*

Time (h)	%MET	Unknown 2	%CGN
0.00	100.0	-	-
0.50	99.6	√	1.40
1.00	97.1	√	1.53
2.00	103.6	√	1.63
4.00	105.5	√	1.73
6.00	108.0	√	1.79
8.00	114.6	√	1.98

*Unknown 1 was not observed.

Table 4.23 Degradation data of MET in 0.1 N NaOH at room temperature (kept in a dark cabinet)*

Time (day)	%MET	Unknown 1	%CGN
0	100.0	-	
1	95.5	-	
2	73.9	-	
3	67.9	-	
7	56.3	-	
14	52.7	√	
21	39.5	√	
30	33.6	√	0.11

*Unknown 2 was not observed.

Table 4.24 Degradation data of MET in water and 0.1 N HCl at room temperature (kept in a dark cabinet)*

Time (day)	%MET	
	Water	0.1N HCl
0	100.0	100.0
1	100.9	99.4
2	100.9	99.4
3	100.7	97.1
7	100.7	91.5
14	100.5	82.1
21	100.2	67.2
30	99.4	53.1

*Unknown 1 and 2 and CGN were not observed.

Table 4.25 Degradation data of MET in in 3%H_2O_2 at room temperature (kept in a dark cabinet)*

Time (day)	%MET	Unknown 2	%CGN
0.00	100.0	-	-
0.625 (15 h)	84.6	√	1.38
1	133.5	√	1.74
2	131.0	√	1.84
3	123.6	√	1.99
7	118.9	√	2.11

*Unknown 1 was not observed.

Table 4.26 Degradation data of MET in 0.1 N NaOH (exposed to sunlight)*

Time (day)	%MET	Unknown 1	%CGN
0	100.0	-	-
1	85.0	-	-
2	81.2	-	-
3	77.9	-	-
7	38.4	√	-
14	27.4	√	0.04
21	23.6	√	0.10
30	9.6	√	0.14

*Unknown 2 was not observed.

Table 4.27 Degradation data of MET in 0.1 N HCl (exposed to sunlight)*

Time (day)	%MET	Unknown 1
0	100.0	-
7	81.6	-
14	70.9	-
21	66.2	√
30	50.5	√

*Unknown 2 and CGN were not observed.

Table 4.28 Degradation data of MET in water (exposed to sunlight)*

Time (day)	%MET
0	100.0
7	100.6
14	100.3
21	100.1
30	76.7

*Unknown 1 and 2 and CGN were not observed.

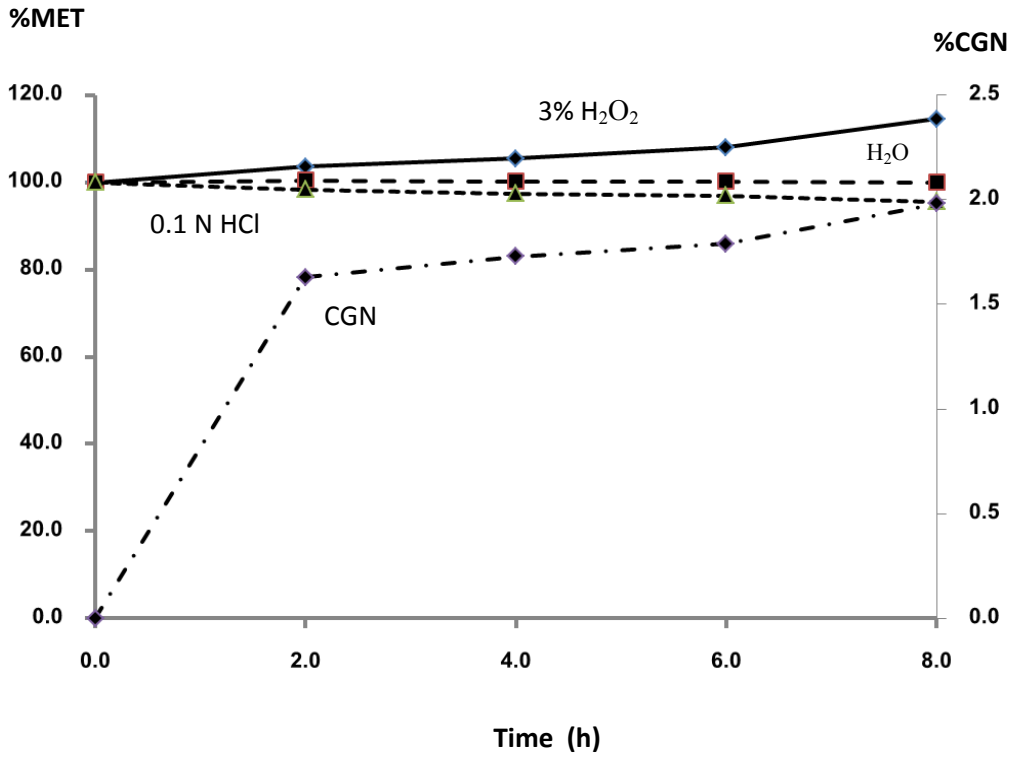


Figure 4.15 Stability profiles for the degradation of MET and CGN formation under neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at 80 ºC (CGN was observed only from oxidation condition)

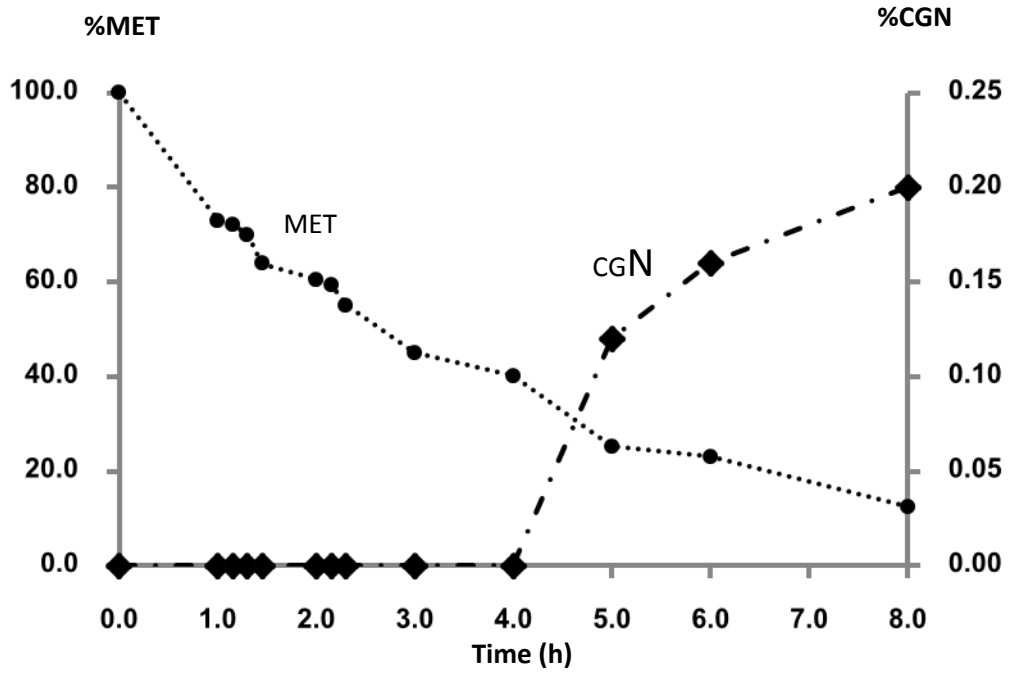


Figure 4.16 Stability profiles for the degradation of MET and CGN formation under alkaline hydrolysis (0.1 N NaOH) at 80 ºC.

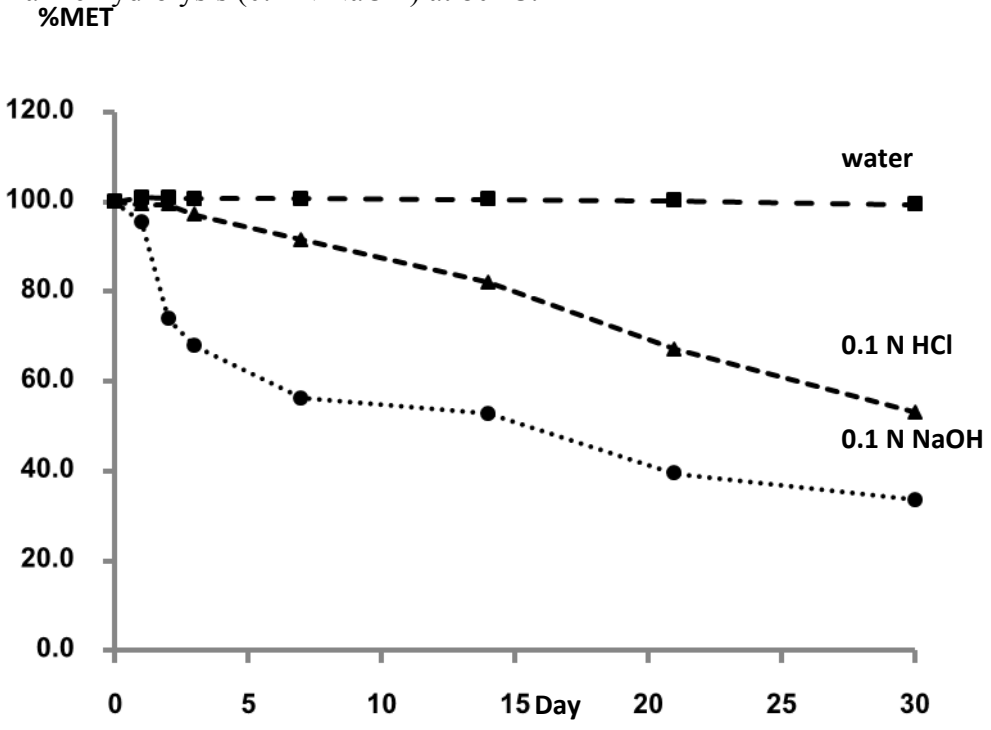


Figure 4.17 Stability profiles for the degradation of MET under neutral (water), acid (0.1 N HCl) and alkaline (0.1 N NaOH) hydrolysis at room temperature (kept in a dark cabinet) (CGN was not observed under these stress conditions).

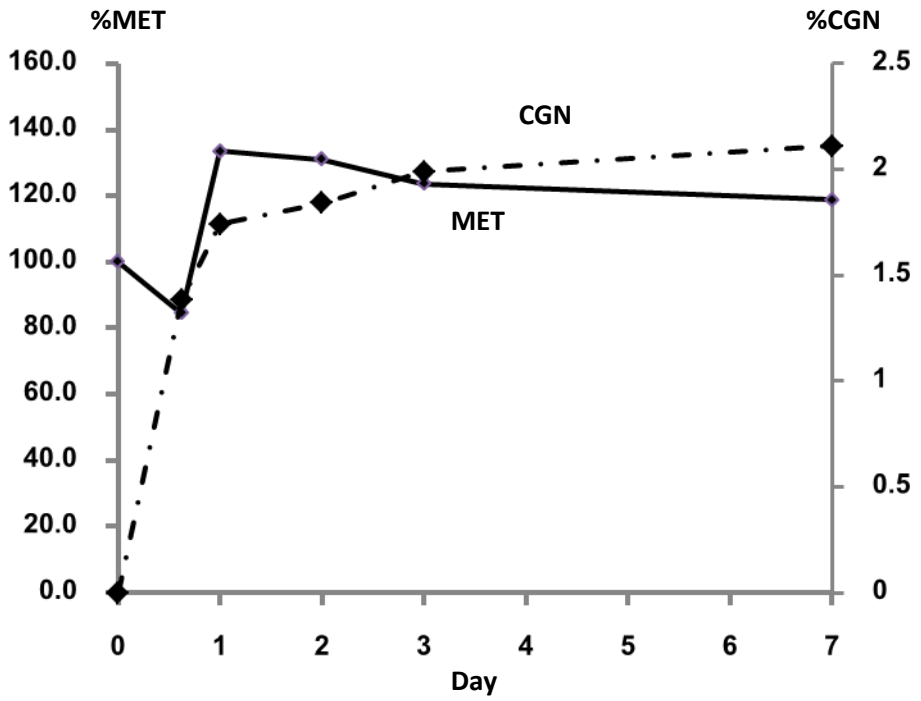


Figure 4.18 Stability profiles for the degradation of MET and CGN formation under oxidation (3% H_2O_2) at room temperature (kept in a dark cabinet).

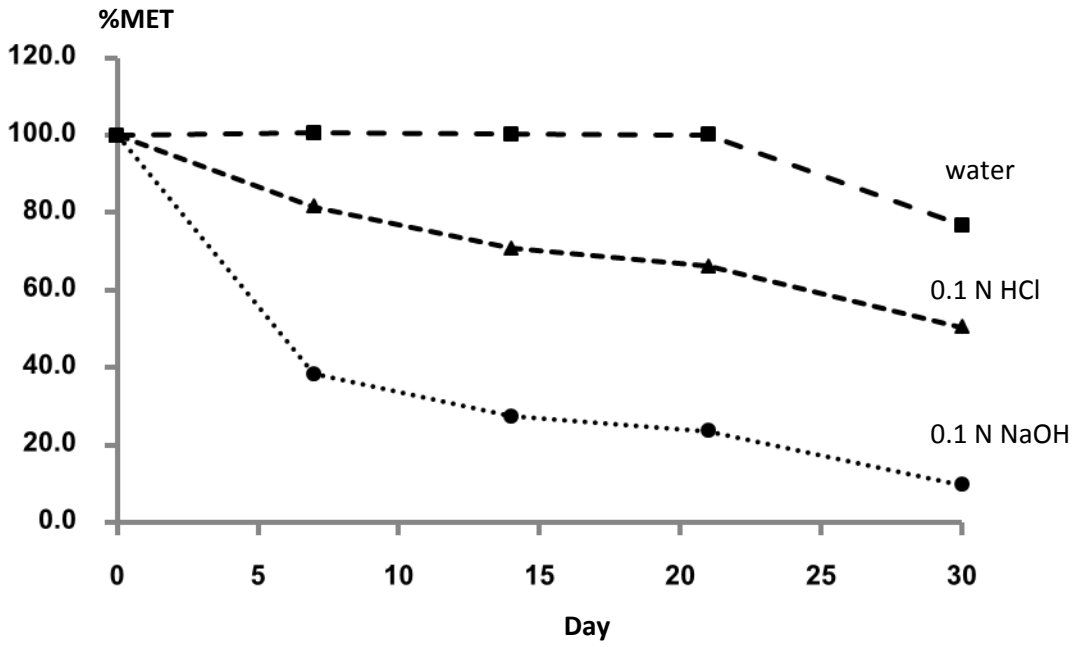


Figure 4.19 Stability profiles for the degradation of MET and CGN formation under neutral (water), acid (0.1 N HCl) and alkaline (0.1 N NaOH) exposed to sunlight.

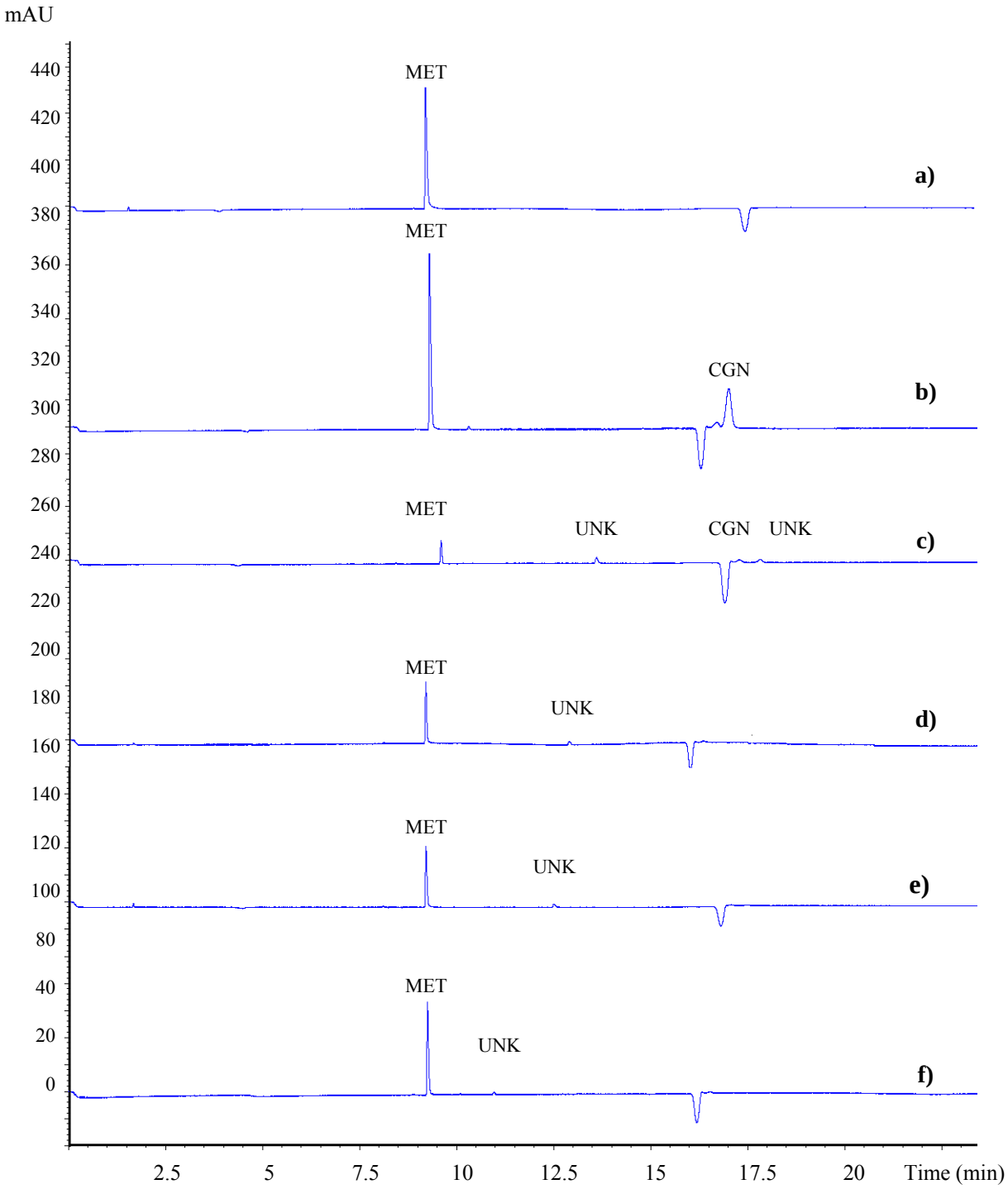


Figure 4.20 Electropherograms of MET degradation under various stress conditions a) Initial, b) in 3%H_2O_2, heated at 80°C for 8 h, c) in 0.1 N NaOH, heated at 80°C for 1.5 h d) in 0.1 N NaOH, at room temperature (kept in a dark cabinet for 30 days), e) in 0.1 N NaOH, exposed to sunlight for 7 days, f) in 0.1 HCl, exposed to sunlight for 30 days. CE conditions: as Figure 4.6.

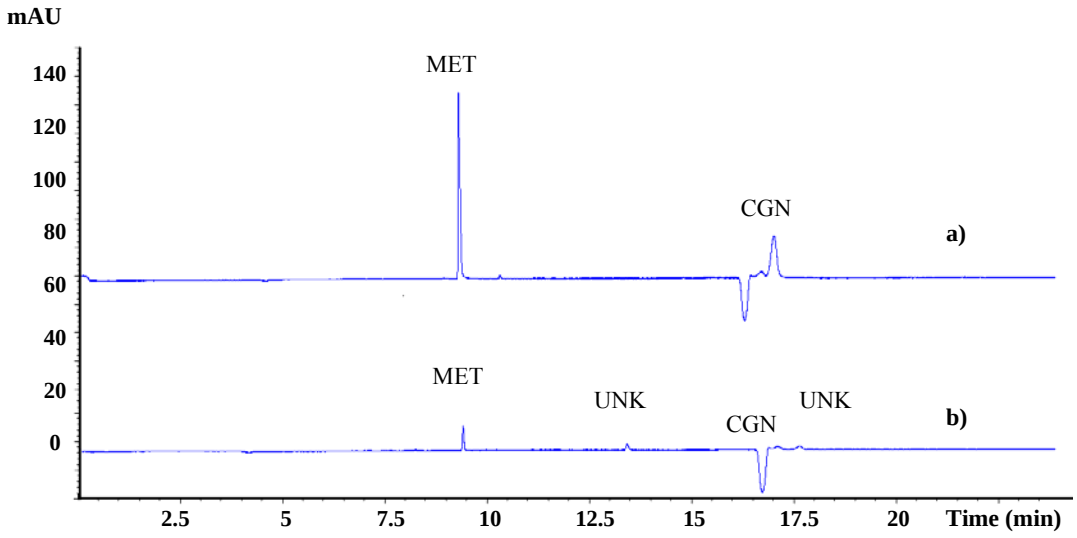


Figure 4.21 Electropherograms of MET degradation under heated at 80°C for 8 h, a) in 3%H_2O_2, b) in 0.1 N NaOH, CE conditions: as Figure 4.6.

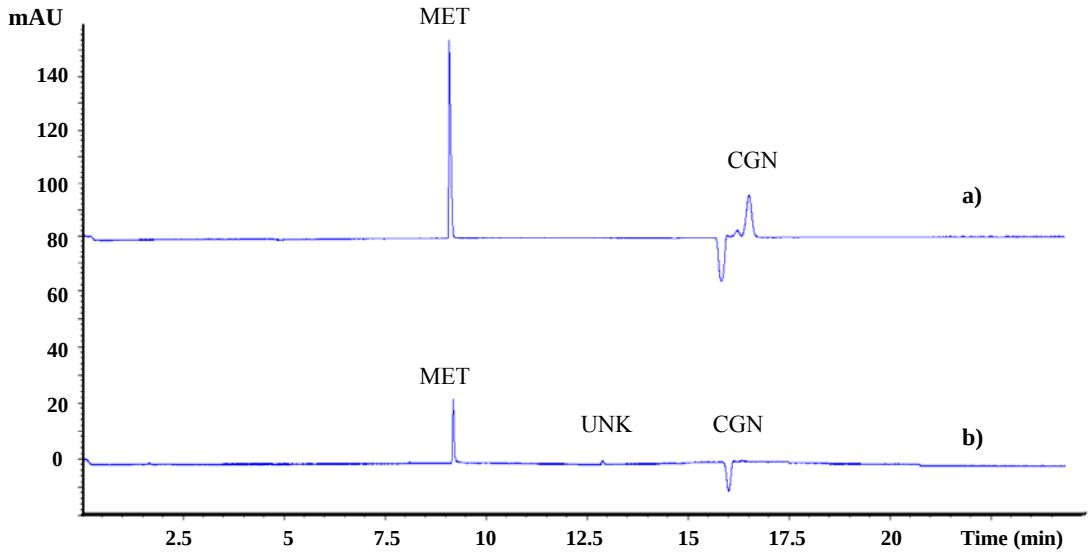


Figure 4.22 Electropherograms of MET degradation at room temperature (kept in a dark cabinet , a)in 3%$H_2O_2$for 1 day, b) in 0.1 N NaOH for 30 days, CE conditions: as Figure 4.6.

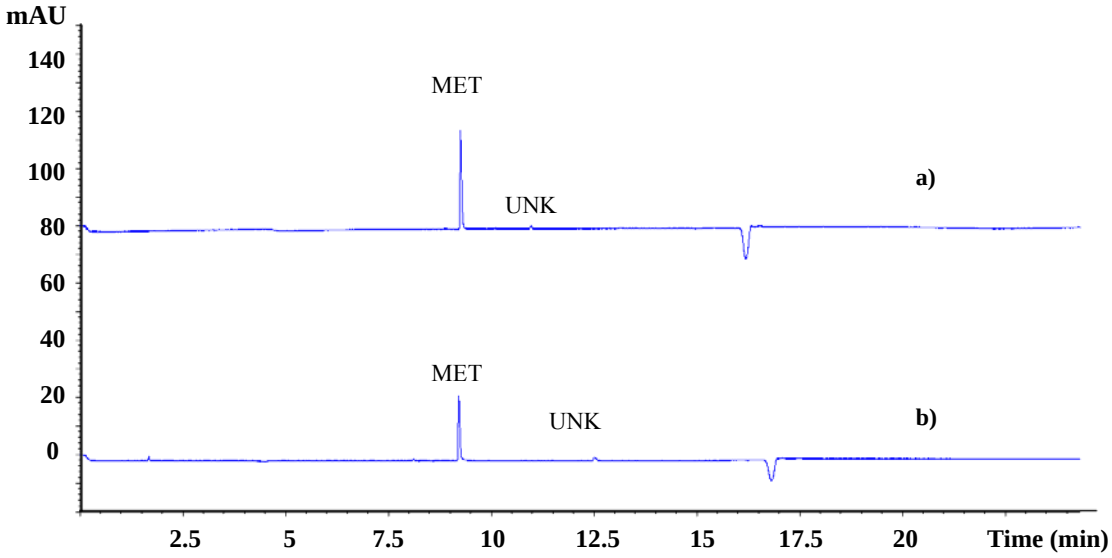


Figure 4.23 Electropherograms of MET degradation exposed to sunlight a)in 0.1N HClfor 30 days, b) in 0.1 N NaOH for 7 days, CE conditions: as Figure 4.6.

4.2.4.2 Stability study of GCZ

Stability of GCZ was investigated under hydrolysis (neutral, acid and alkaline conditions), oxidation and photolysis. Results on degradation of GCZ and formation of GZB, GZF and other unknown degrades were shown in Table 4.29-4.33. Amounts of the remained GCZ, GZB and GZF were determined and another unknown degrades was detected from MEKC method. Stability profiles on stability of GCZ are demonstrated in Fig. 4.24-4.26 and electropherograms of GCZ and the degradation products are in Fig. 4.27-4.29. Under all stress conditions, GZF was not detected.

At elevated temperature (80 ºC), GCZ was stable under alkaline hydrolysis (0.1 N NaOH) for 2 h (%GCZ ~ 99.6%). After 2 h, the drug slowly degraded and the remained GCZ was about 88.5% after 8 h. GZB and GZF were not observed in alkaline hydrolysis. However, GCZ rapidly degraded in 0.1 N HCl at this temperature. The amount of GCZ was about 76.5% during the acid hydrolysis for 5 min and completely degraded to 0% after 45 min. GZB (20.2%) was observed at 30 min and increased to 87.2% at 8 h. Similarly to acid hydrolysis, GCZ also degraded under neutral hydrolysis and oxidation at 80 ºC, but at slower rates. GCZ could not be detected after 3 h and GZB was observed at 2 h under both neutral

hydrolysis and oxidation. In addition, another unknown degrade was monitored at 6 min, in neutral and acid hydrolysis and oxidation at this temperature.

Stabilities of GCZ under alkaline, acid and neutral hydrolysis and oxidation at room temperature (kept in a dark cabinet) and upon exposure to sunlight were also performed. GCZ showed a good stability for 30 days in alkaline hydrolysis with the amount of GCZ in a range of 100.1-98.3% (room temperature, kept in a dark cabinet) and 100.0-96.4% (exposure to sunlight). In acid hydrolysis (at room temperature, kept in a dark cabinet), GCZ degraded from 100% to 61.0% during the first day and after that the drug rapidly degraded to 0% on day 14. Under this condition, GZB was observed at 8.6% on day 3 and increased to 23.1% on day 30. Another unknown degrade was detected in this condition since day 3. Exposure of GCZ in acid solution to sunlight accelerated its degradation to 0% and the formation of GZB increased to 29.8% on day 30. GCZ also degraded in neutral hydrolysis both at room temperature (kept in a dark cabinet) and upon exposure to sunlight. In the former condition, GCZ decreased from 97.9% on day 1 to 48.6% on day 30, another unknown degrade was detected but no GZB was observed. In sunlight, GCZ degraded at faster rates from 96.0% on day 1 to 8.3% on day 30, GZB was found since day 14 and another unknown degrade was monitored since day 1. Oxidation at room temperature (kept in a dark cabinet) could reduce stability of GCZ, its amount decreased from 96.7% on day 1 to 75.6% on day 7. GZB was not found, but another unknown degraded was detected since day 3.

Table 4.29 Degradation data of GCZ in 0.1 N NaOH heated at 80 °C*

Time (h)	%GCZ
0	100.0
1	99.8
2	99.5
3	97.8
4	95.9
6	94.6
8	88.5

Table 4.30 Degradation data of GCZ in 0.1 N HCl, water and 3%H_2O_2 heated at 80 °C*

	0.1 N HCl		water		3%H_2O_2		
Time	%GCZ	%GZB	%GCZ	%GZB	%GCZ	%GZB	Unknown
0	100.0	-	100.0	-	100.0	-	-
5 min	76.5	-	NA	NA	NA	NA	√
10 min	49.0	-	97.7	-	80.03	-	√
15 min	21.5	-	74.5	-	56.97	-	√
20 min	16.6	-	51.8	-	48.23	-	√
30 min	4.9	20.2	39.1	-	40.17	-	√
40 min	0.0	22.6	35.7	-	35.21	-	√
1 h	0.0	31.1	22.4	-	21.00	-	√
2 h	0.0	51.0	7.6	12.7	5.68	11.76	√
4 h	0.0	67.0	0.0	17.1	0.00	32.76	√
6 h	0.0	76.3	0.0	20.0	0.00	49.66	√
8 h	0.0	87.2	0.0	25.0	0.00	83.39	√

*NA = not applicable, an unknown degrade was detected since 10 min in all conditions, except in 0.1 H HCl, it was detected since 5 min

Table 4.31 Degradation data of GCZ in 0.1 N NaOH at room temperature (kept in a dark cabinet) and exposed to sunlight*

Time (day)	%GCZ	
	At room temperature (kept in a dark cabinet)	Exposed to sunlight
0	100.0	100.0
1	100.0	NA
2	99.6	NA
3	99.3	NA
7	99.3	99.1
14	98.9	98.0
21	98.4	97.5
30	98.3	96.4

*NA = not applicable, degradation of GCZ in 0.1 N NaOH exposed to sunlight was monitored from day 7 to day 30

Table 4.32 Degradation data of GCZ in 0.1 N HCl, water and 3%H_2O_2 at room temperature (kept in a dark cabinet)

	0.1 N HCl		water	3%H_2O_2	
Time	%GCZ	%GZB	%GCZ	%GCZ	Unknown
0	100.0	-	100.0	100.0	-
2 h	96.9	-	NA	NA	√
4 h	81.6	-	NA	80.0	√
6 h	76.9	-	NA	57.0	√
8 h	73.1	-	NA	48.3	√
day 1	60.9	-	97.9	96.9	√
day 2	40.0	-	89.0	88.3	√
day 3	28.0	8.6	80.1	83.0	√
day 7	5.7	10.5	76.6	75.6	√
day 14	0.0	15.6	67.9	NA	√
day 21	0.0	18.7	59.0	NA	√
day 30	0.0	23.1	48.6	NA	√

*NA = not applicable, GZB was not observed in neutral hydrolysis (water) and oxidation condition, an unknown degrade was detected since day 3 in neutral hydrolysis (water) and oxidation condition, except in 0.1 H HCl, it was detected since 2 h

Table 4.33 Degradation data of GCZ in 0.1 N HCl and water exposed to sunlight*

Time (day)	0.1 N HCl		water		
	%GCZ	%GZB	%GCZ	%GZB	Unknown
0	100.0	-	100.0	-	-
1	42.9	-	96.0	-	√
2	30.9	6.5	80.8	-	√
3	17.2	8.9	76.1	-	√
4	9.3	10.0	72.1	-	√
5	0.0	11.0	68.3	-	√
6	0.0	11.6	61.3	-	√
7	0.0	13.3	58.5	-	√
14	0.0	16.8	29.2	11.6	√
21	0.0	23.9	19.0	17.0	√
30	0.0	29.8	8.3	23.2	√

*an unknown degrade was detected since day 1 in both conditions

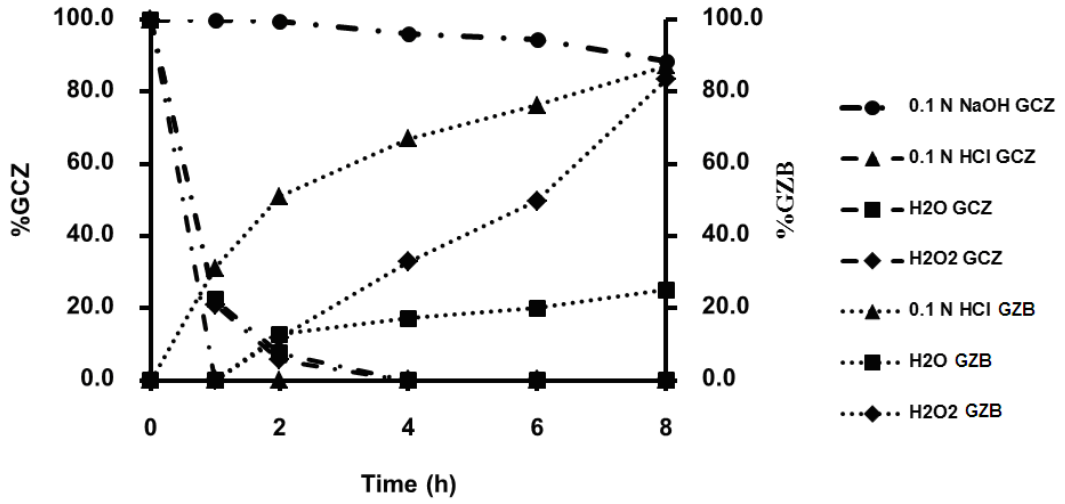


Figure 4.24 Stability profiles for the degradation of GCZ and GZB formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at 80 ºC (GZB was not observed in alkaline hydrolysis).

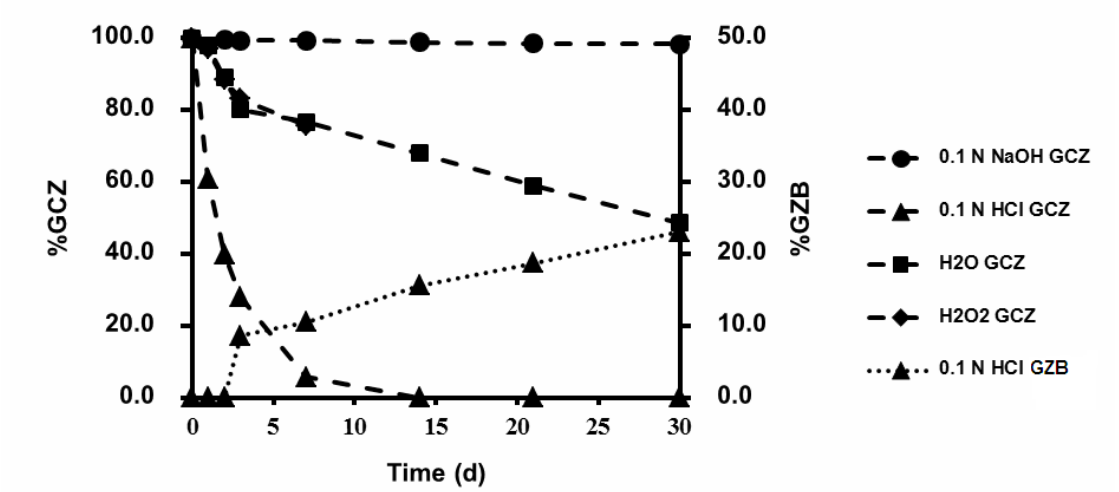


Figure 4.25 Stability profiles for the degradation of GCZ and GZB formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at room temperature (kept in a dark cabinet) (GZB was observed only in acid hydrolysis).

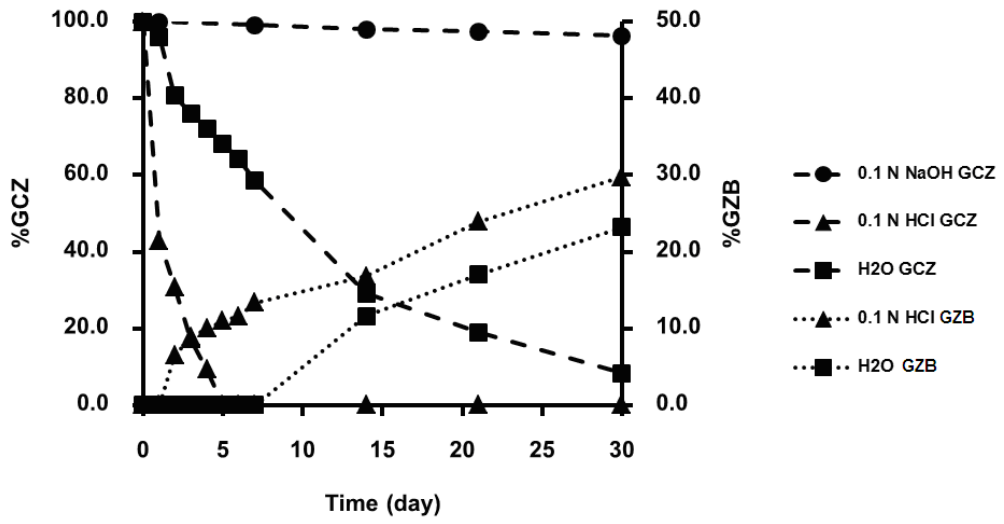


Figure 4.26 Stability profiles for the degradation of GCZ and GZB formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis (exposed to sunlight) (GZB was not observed in alkaline hydrolysis).

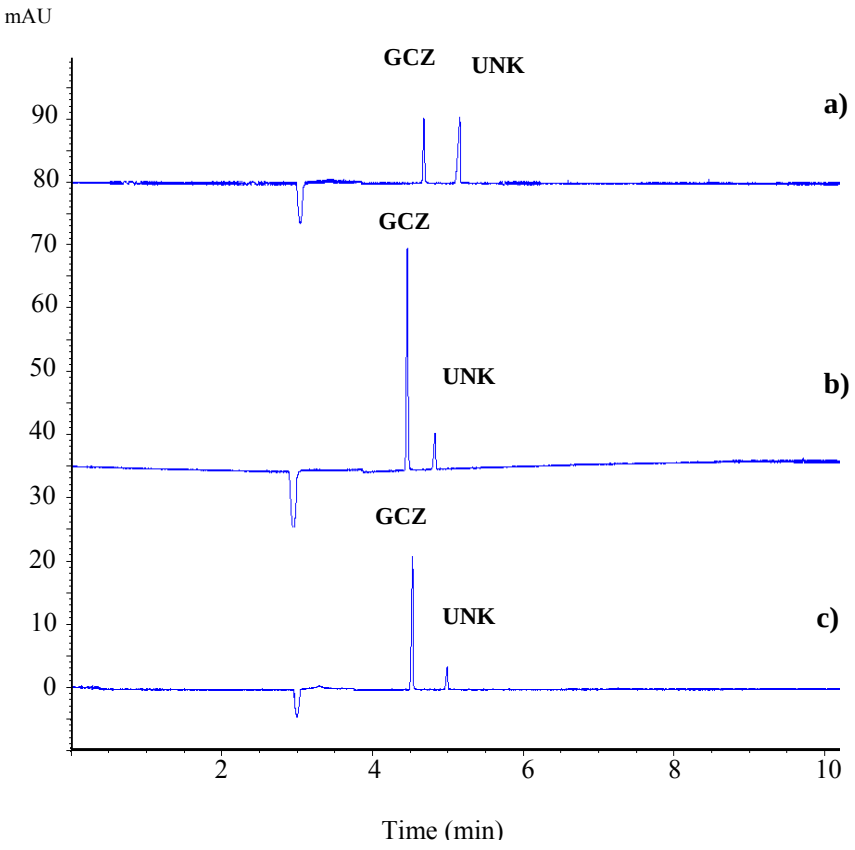


Figure 4.27 Electropherograms of GCZ degradation at 80°C a) in 0.1 N HCl for 10 min, b) in water for 10 min, c) in 3%H_2O_2 for 10 min. CE conditions: as MEKC method

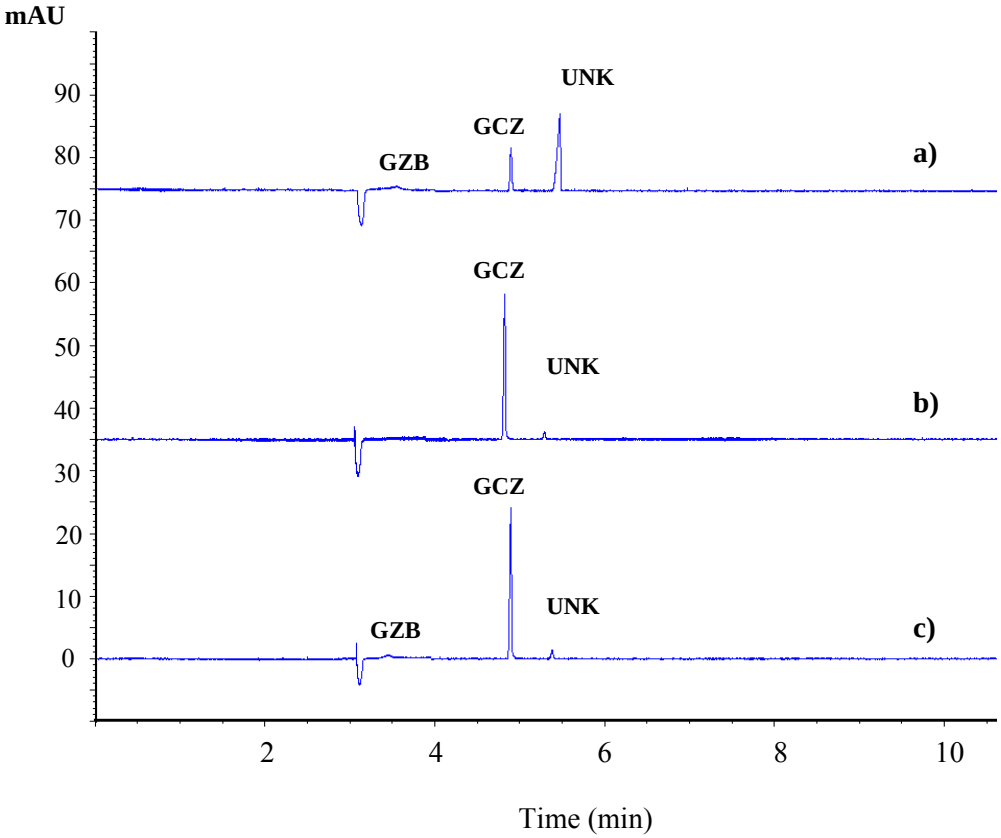


Figure 4.28 Electropherograms of GCZ degradation at room temperature (kept in a dark cabinet, after 3 days) a) in 0.1 N HCl, b) in water and c) in 3%H_2O_2. CE conditions: as MEKC method

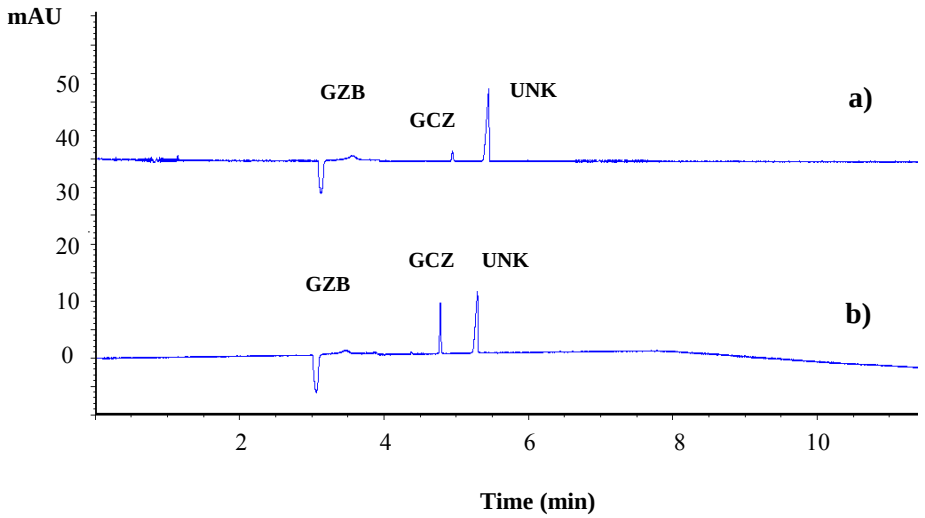


Figure 4.29 Electropherograms of GCZ degradation exposed to sunlight a) in water for 14 days and b) in 0.1 N HCl for 3 days. CE conditions: as MEKC method

4.2.5 Application

The developed methods, CZE and MEKC, were employed as stability indicating methods for MET and GCZ, respectively, as described in the previous section. Additionally, the methods could be applied for assay of the drugs in both raw material and formulations. CZE was used for analysis of MET in three lots of raw material and two lots of tablets and results are shown in Table 4.34. MEKC was utilized for assay of GCZ in two lots of raw material and three lots of tablets and results are shown in Table 4.35. Contents of the drugs in all tested samples met the limits required by both the USP and BP. No degradation products or impurities were found in the investigated samples.

Table 4.34 Assay of MET in samples by CZE method

	%Labeled amount	%RSD	BP limit	USP limit
Raw material			98.5-101.0	98.5-101.0
Lot 1	99.9	0.33		
Lot 2	100.2	0.46		
Lot 3	100.1	0.11		
Tablet			95.0-105.0	95.0-105.0
Lot 4	100.0	1.28		
Lot 5	99.9	0.79		

Table 4.35 Assay of GCZ in samples by MEKC method

	%Labeled amount	%RSD	BP limit	USP limit
Raw material			99.0-101.0	-
Lot 6	99.1	0.43		
Lot 7	99.3	0.07		
Tablet			95.0-105.0	
Lot 8	100.2	0.95		
Lot 9	99.8	0.42		
Lot 10	99.6	0.65		

CHAPTER V
CONCLUSION

5.1 Assay of combined anti-diabetic drugs

Simultaneous determination of MET, GCZ and GBM was achieved by CZE method. Optimization condition was in 50 mM borate buffer (pH 9.0). The separation was performed using a fused-silica capillary tube with L_{total} of 64.5 cm, L_{eff} of 56.0 cm and i.d. of 50 μm, injection was at 50 mbar for 10 s, the temperature and applied voltage were 25 °C and 20 kV, respectively, the detection wavelength was at 210 nm. The optimized CZE condition was validated in term of linearity, precision, recoveries, LOD and LOQ. Table 5.1 shows the summary of validation data for determination of MET, GCZ and GBM.

Table 5.1 Summary of validation data for MET, GCZ and GBM

	Result		
	MET	GBM	GCZ
Regression	y = 0.4054x+44.484 (r^2 = 0.9940)	y = 1.0520x+0.2918 (r^2 = 0.9929)	y = 2.1112x-0.16023 (r^2 = 0.9973)
Precision (%RSD)	1.86	1.66	1.90
Accuracy (%Recovery)	97.6-100.4%	98.0-103.1%	99.1-100.4%
LOD (μg/mL)	2	2	4
LOQ (μg/mL)	5	5	12

The validated method was applied for the determination of MET, GCZ and GBM in pharmaceutical formulations. The % label amounts of thirteen formulations were 99.7-101.3% (%RSD < 1.80) which were within limit of USP and BP. Table 5.2 shows assay of samples.

Table 5.2 Assay of samples (n = 3)[a]

Sample	%labeled amount	BP limit	USP limit
MET raw material	100.6 (0.59)	-	98.5-101.0%
GBM raw material	100.3 (0.51)	99.9-101.0	-
GCZ raw material	100.2 (0.90)	99.9-101.0	-
MET tablet lot A	101.3 (0.82)	-	95.0-105.0%
MET tablet lot B	100.7 (0.61)	-	95.0-105.0%
GBM tablet lot A	101.0 (0.61)	95.0-105.0%	-
GBM tablet lot B	101.0 (1.12)	95.0-105.0%	-
GCZ tablet lot A	100.4 (0.93)	95.0-105.0%	-
GCZ tablet lot B	99.9 (1.05)	95.0-105.0%	-
MET/GBM tablet lot A	MET: 101.0 (0.94), GBM: 100.8 (0.83)	-	-
MET/GBM tablet lot B	MET: 101.0 (0.70), GBM: 100.6 (1.59)	-	-
MET/GCZ tablet lot A	MET: 101.0 (1.80), GCZ: 100.9 (0.19)	-	-
MET/GCZ tablet lot B	MET: 100.1 (0.50), GCZ: 99.7 (0.42)	-	-

[a]: MET = metformin, GBM = glibenclamide, GCZ = gliclazide, number in parenthesis represents %RSD

5.2 Stability study of anti-diabetic drugs

Stability indicating methods for MET and GCZ were established. Determination of MET and its degradation product, CGN was performed by CZE method. The condition was in 40 mM citrate buffer (pH 6.7). The separation was performed using a fused-silica capillary tube with L_{total} of 68.5 cm, L_{eff} of 60.0 cm and i.d. of 50 μm, injection was at 50 mbar for 5 s, the temperature and applied voltage

were 30°C and 15 kV, respectively, the detection wavelength was at 214 nm. Determination of GCZ and its degradation products, GCB and GZF, was achieved by MEKC method. The condition was in 10 mM phosphate buffer (pH 7.0). The separation was performed using a fused-silica capillary tube with L_{total} of 48.5 cm, L_{eff} of 40.0 cm and i.d. of 50 μm, injection was at 50 mbar for 5 s, the temperature and applied voltage were 25°C and 20 kV, respectively, the detection wavelength was at 225 nm. Both methods provided baseline separation of all compounds with resolution values of greater than 11.5. System suitability test data revealed that the methods were efficient for the separation and analysis of the investigated drugs and their degradation products.

The CZE and MEKC method were validated according to ICH guidelines in terms of linearity, precision, accuracy, limits of detection and quantitation and robustness. Both methods were valid and reliable with good analytical parameter characteristics as shown below (Table 5.3).

Table 5.3 Summary of validation data for MET, CGN, GCZ, GZB and GZF

	CZE method	MEKC method
Regression	MET $y = 0.3881x - 4.0717$ ($r^2 = 0.9985$)	GCZ $y = 0.2544x - 0.6716$ ($r^2 = 0.9981$)
	CGN $y = 3.6108x + 2.9254$ ($r^2 = 0.9986$)	GZB $y = 0.1749x + 0.0476$ ($r^2 = 0.9968$)
		GZF $y = 0.1968x + 0.0176$ ($r^2 = 0.9999$)
Precision (%RSD)	1.98	2.00
Accuracy (%Recovery)	98.3-100.9	99.0-100.0
LOD (μg/mL)	30	40
LOQ (μg/mL)	100	50
Robustness (%RSD)	1.73	1.23

Stability study of MET and GCZ were performed under hydrolysis (acid, alkaline and neutral conditions), oxidation and photolysis condition. Experiments were conducted at elevated (80 ºC) and room temperature (kept in a dark cabinet) and exposed to sunlight. Stability of both drugs is summarized below (Table 5.4).

Table 5.4 Summary on the stability of MET and GCZ under various stress conditions

	MET	GCZ
At 80 ºC		
0.1 N NaOH	degrade	stable
0.1 N HCl	stable	degrade
Water	stable	degrade
3% H_2O_2	degrade	degrade
At room temperature (kept in a dark cabinet)		
0.1 N NaOH	degrade	stable
0.1 N HCl	stable	degrade
Water	stable	degrade
3% H_2O_2	degrade	degrade
Exposed to sunlight		
0.1 N NaOH	degrade	stable
0.1 N HCl	degrade	degrade
Water	stable	degrade

The developed methods, CZE and MEKC, were could be employed for assays of the MET and GCZ in pharmaceutical raw material and formulations. In all ten tested samples, contents of the drugs were within the limits required by both the USP and BP and no degradation products or impurities were found in the investigated samples (Table 5.5).

Table 5.5 Summary of assay data of MET and GCZ in raw material and tablet

	%Labeled amount	%RSD	BP limit	USP limit
MET				
Raw material			98.5-101.0	98.5-101.0
Lot 1	99.9	0.33		
Lot 2	100.2	0.46		
Lot 3	100.1	0.11		
Tablet			95.0-105.0	95.0-105.0
Lot 4	100.0	1.28		
Lot 5	99.9	0.79		
GCZ				
Raw material			99.0-101.0	-
Lot 6	99.1	0.43		
Lot 7	99.3	0.07		
Tablet			95.0-105.0	-
Lot 8	100.2	0.95		
Lot 9	99.8	0.42		
Lot 10	99.6	0.65		

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