รายงานการวิจัยฉบับสมบูรณ์

โครงการ **การศึกษาความคงสภาพของเมทฟอร์มินและกลัยคาไซด์ในภายใต้สภาวะเครียดโดยแคปปิลลารีอิเล็กโทรโฟรีซีส**

Stability study of metformin and gliclazide under stress conditions by capillary electrophoresis

โดย ศาสตราจารย์ ดร. ลีณา สุนทรสุข
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คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล

มิถุนายน 2556

สัญญาเลขที่ RMU5380012

รายงานการวิจัยฉบับสมบูรณ์

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

ACKNOWLEDGEMENT

The researcher would like to express her deepest thanks to the Thailand Research Fund and Office of the Higher Education Commission (RMU5380012) for providing the funding for this work. Thanks also go to Ms. Athiporn Doomkaew, Mr. Brompoj Pruthiwanasan and Ms. Pornpan Prapatpong from Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, and Ms. Nantana Nuchtavorn from Faculty of Pharmacy, Siam University for their technical assistances on this work.

บทคัดย่อ

แคปิลลารีอิเล็กโทรโฟรีซิสสองวิธีได้รับการพัฒนาขึ้นสำหรับการวิเคราะห์ยาเมทฟอร์มินและกลัยคาไซด์ และสารสลายตัวที่เกิดขึ้นจากยาทั้งสองชนิดนี้ ได้แก่ ไซยาโนกัวนิดีน สารเจือปนกลัยคาไซด์บี และเอฟ จากการศึกษาพบว่าสภาวะที่เหมาะสมสำหรับการวิเคราะห์ยาเมทฟอร์มินและไซยาโนกัวนิดีน คือ ซิเตรตบัฟเฟอร์ที่ความเข้มข้น 40 มิลลิโมลาร์ที่พีเอช 6.7 แคปิลลารียาว 60 เซนติเมตร เส้นผ่าศูนย์กลางภายใน 50 ไมโครเมตร ฉีดสารด้วยแรงดัน 50 มิลลิบาร์ 5 วินาที อุณหภูมิ 30 องศาเซลเซียส และความต่างศักย์ไฟฟ้าที่ 15 กิโลโวลล์ ตรวจหาการดูดกลืนรังสีอัลตราไวโอเลตที่ความยาวคลื่น 214 นาโนเมตร สภาวะที่เหมาะสมสำหรับการวิเคราะห์ยากลัยคาไซด์ และสารเจือปนกลัยคาไซด์บี และเอฟ ได้แก่ ฟอสเฟตบัฟเฟอร์ที่ความเข้มข้น 10 มิลลิโมลาร์ ซึ่งประกอบด้วย15 มิลลิโมลาร์ โซเดียมโดเดซิล ซัลเฟต ที่พีเอช 7.0 แคปิลลารียาว 40 เซนติเมตร เส้นผ่าศูนย์กลางภายใน 50 ไมโครเมตร ฉีดสารด้วยแรงดัน 50 มิลลิบาร์ 5 วินาที อุณหภูมิ 25 องศาเซลเซียส และความต่างศักย์ไฟฟ้า ที่ 20 กิโลโวลล์ ตรวจหาการดูดกลืนรังสัอัลตราไวโอเลตที่ความยาวคลื่น 225 นาโนเมตร วิธีวิเคราะห์ทั้งสองได้รับการประเมินในหัวข้อความสัมพันธ์เส้นตรง ความเที่ยงตรง ความถูกต้อง สภาพไว และความทนทานของวิธี พบว่าให้ความสัมพันธ์เส้นตรง (r^2 มากกว่า 0.99) ความเที่ยงตรง (%RSDs น้อยกว่า 2.00 เปอร์เซ็นต์) และความถูกต้อง (%recovery 98.3-100.9 เปอร์เซ็นต์) ที่ดีสำหรับสารทุกชนิด ลิมิตการตรวจหาและลิมิตการวิเคราะห์ปริมาณมีค่าน้อยกว่า 40 และ100 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ วิธีวิเคราะห์มีความทนทานของวิธีศึกษาจากการปรับเปลี่ยนปัจจัยบางอย่างที่มีผลต่อการแยกสารซึ่งยังคงให้ค่า %RSDs น้อยกว่า 1.76 เปอร์เซ็นต์

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

ABSTRACT

Two capillary electrophoresis (CE) methods were developed as stability indicating methods for metformin (MET) and gliclazide (GCZ). The optimum CE condition for the separation of MET and its degradation product (cyanoguanidine, CGN) was in 40 mM citrate buffer (pH 6.7) using a fused-silica capillary with an effective length of 60 cm and an inner diameter of 50 μm, injection at 50 mbar for 5 s, temperature of 30 ºC, applied voltage of 15 kV and diode array detection (DAD) at 214 nm. MET and CGN were well separated under these conditions with the migration times of 9.9 and 19.0 min, respectively, and a resolution (R_s) of 38.9. GCZ and its major degradation products (gliclazide impurity B, GCZ-B and gliclazide impurity F, GCZ-F) could be resolved in 10 mM phosphate buffer (pH 7.0) containing 15 mM sodium dodecyl sulfate using a fused-silica capillary with an effective length of 40 cm and an inner diameter of 50 μm, injection at 50 mbar for 5 s, temperature of 25 ºC, applied voltage of 20 kV and DAD detection at 225 nm. GCZ-B, GCZ-F and GCZ migrated at 3.82, 5.21 and 5.32 min, respectively with R_s of 11.52 (GCZ-B/GCZ-F) and 2.06 (GCZ-F/GCZ). Method validation showed good linearity (r^2 > 0.99), precision (%RSDs < 2.00%) and accuracy (%recovery between 98.3 and 100.9%) for all compounds. Limits of detection (LOD) and quantitation (LOQ) were of less than 40 and 100 μg/mL, respectively. The methods were robust upon alteration of factors affecting their separation with %RSDs of less than 1.76%.

The proposed CE methods could be used as stability indicating and assay methods for MET and GCZ. Stability study of MET and GCZ were performed under hydrolysis (acid, base and neutral conditions), oxidation and photolysis condition. MET was stable in neutral (water) and acid (0.1 N HCl) hydrolysis, but degraded to 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 GCZ-B under acid and neutral hydrolysis and oxidation. Applications of the methods for assays of MET and GCZ in raw material 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 requirements of pharmacopeias. No degradation products, CGN GCZ-B and GCZ-F, were observed in the investigated samples.

บทสรุปผู้บริหาร
Executive Summary

1. ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

Quality control of active pharmaceutical ingredients (e.g. assay, impurity testing, stability study, etc.) is essential from pharmaceutical industry and clinical point of views. Currently, drug substances and products are subjected to stability studies according to the International Conference on Harmonization (ICH) guidelines Q1AR2. The guidelines require a forced degradation or stress studies under hydrolytic, oxidative, dry heat and photolytic conditions. 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. Unfortunately, stability study remains unclear or misunderstood by many manufacturers.

This research focused on stability studies of metformin (MET) and gliclazide (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 or non-insulin dependent diabetes mellitus (NIDDM), in which can still produce insulin, but have insulin tolerance, impaired insulin secretion or increased glucose production. NIDDM is now a worldwide chronic disease due to carbohydrate metabolism disorders, which cause elevated blood glucose levels (> 110 mg/dL, fasting plasma glucose). The high blood glucose levels affect normal functions of eyes, kidney, blood vessels, nervous systems and wounds.

CE was chosen as a method of choice since it is rapid, efficient and cost effective. Optimization of CE condition for the analysis of MET and GCZ was investigated. Stability study of both drugs was carried out under various stress condition (e.g. acid, basic, oxidation, hydrolysis, heat and photodegradation). Degradation behavior, amounts of the drugs and/or degradation products from these conditions from different interval were determined. Outcomes of the research included simple and rapid stability indicating methods (SIMs) for the determination of MET and GCZ and their degradation products. In addition, protocols for stability study of both drugs and stability data/profile of the drugs under various stress conditions were established.

2. วัตถุประสงค์ของโครงการ

The ultimate goals of this research were to develop CE methods for the stability study of MET and GCZ, to establish stability study protocol and to obtain stability profile of both drugs.

3. ระเบียบวิธีวิจัย

3.1 Literature review

Physicochemical properties and stability data of both drugs and their impurities and degradation products were collected. Reports on analytical and stability indicating methods of MET and GCZ were searched and tabulated.

3.2 CE optimization

CE conditions for the separation of MET, GCZ and their degradation products were investigated by varying chemical (e.g. types, pH and concentrations of buffer, types and concentrations of surfactants and organic solvent) and instrumental factors (e.g. capillary dimension, temperature and voltage). The optimized conditions were evaluated based on analytical parameters including migration time, resolution, tailing factor, number of theoretical plates and percent relative standard deviations (%RSD) of migration time and peak area.

3.2 Method validation

The developed CE methods were validated for the analysis of MET, GCZ and their major degradation products. Analytical performance characteristics were evaluated in terms of linearity, precision, accuracy, limits of detections (LOD) and quantitation (LOQ) and robustness.

3.4 Stability study

Stability of MET and GCZ was investigated under various stress conditions. Hydrolysis was performed in acid (0.1 N HCl), alkaline (0.1 N NaOH) and neutral condition (water) at 80 $^{\circ}$C. Oxidation was carried out in 3% hydrogen peroxide at 80 $^{\circ}$C. Photolysis of the drug solutions in 0.1 N HCl, water and 0.1 N NaOH was also investigated. One set of the samples was exposed to sunlight and the other was kept in darkness. Effects of stress conditions on amounts of MET, GCZ and their degradation product formations were determined by the optimized CE condition. Amounts of the remained drugs and their degradation products were quantified and tabulated.

3.5 Pharmacokinetic profile

Pharmacokinetic profile on the stability of MET and GCZ was established. Plots of the remained drugs and degradation product under various stress condition and time were illustrated.

3.6 Application

The established CE method was applied to analyze commercial MET and GCZ raw material and tablets available on the markets.

3.7 Data analysis and report writing

4. แผนการดำเนินงานวิจัย (Research plan)

Schedule	Month								
	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36
1. Literature review									
2. CE optimization									
3. Method validation									
3. Stability study									
4. Pharmacokinetic profile									
5. Application									
6. Data analysis and writing a report									

5. งบประมาณโครงการ (ตามระยะเวลาโครงการที่ได้เสนอรับทุน)*

รายการค่าใช้จ่าย (Item)	จำนวนเงิน (บาท) (in Baht)		
	ปีที่ 1	ปีที่ 2	ปีที่ 3
ก. หมวดค่าตอบแทน (Salary and fringe benefit)	120,000	120,000	120,000
ข. หมวดค่าจ้าง (Subcontract work)	60,000	60,000	30,000
ค. หมวดค่าวัสดุ (Supply)	50,000	50,000	40,000
ง. หมวดค่าใช้สอยและอื่นๆ (Other expense)	50,000	30,000	10,000
จ. หมวดค่าเดินทางไปต่างประเทศ	-	-	60,000
รวม	280,000	260,000	260,000
รวมตลอดโครงการ		**800,000**	

*ทุกหมวดถัวเฉลี่ยกันได้

6. สรุปผลการวิจัย

CE methods were developed as SIMs for MET and GCZ. The optimum CE condition for the separation of MET and its degradation product (cyanoguanidine, CGN) was in 40 mM citrate buffer (pH 6.7) using a fused-silica capillary with an effective length of 60 cm and an inner diameter of 50 μm, injection at 50 mbar for 5 s, temperature of 30 ºC, applied voltage of 15 kV and diode array detection at 214 nm (method 1). MET and CGN were separated under these conditions with the migration times of 9.9 and 19.0 min, respectively, and a resolution (R_s) of 38.9. GCZ and its major degradation products (gliclazide impurity B, GCZ-B and gliclazide impurity F, GCZ-F) could be resolved in 10 mM phosphate buffer (pH 7.0) containing 15 mM sodium dodecyl sulfate using a fused-silica capillary with an effective length of 40 cm and an inner diameter of 50 μm, injection at 50 mbar for 5 s, temperature of 25 ºC, applied voltage of 20 kV and diode array detection at 225 nm (method 2). GCZ-B, GCZ-F and GCZ migrated at 3.82, 5.21 and 5.32 min, respectively with R_s of 11.52 (GCZ-B/GCZ-F) and 2.06 (GCZ-F/GCZ).

Validation of the CE method (method 1) on the analysis of MET and CGN was evaluated in terms of linearity, precision, accuracy, LOD and LOQ and robustness. 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 ($\pm$ 0.5 unit) and separating voltage ($\pm$ 2 kV), the method was still robust with %RSDs of migration time and peak area of less than 1.73%. The validated method was applied for analysis of MET and CGN in stress studies of MET in raw material and commercial tablets. Forced degradations of MET were performed under hydrolysis (acid, alkaline and neutral conditions), oxidation and photolysis condition. Results revealed that MET was stable under neutral hydrolysis at room (both protected from light and exposure to sunlight) and elevated temperature (80 ºC). Amounts of MET was constant (about 100.0%) throughout the investigated time in aqueous solutions. Under acid hydrolysis, MET was also rather stable, degradation was observed after 7 days upon exposure to sunlight. MET rapidly degraded by alkaline hydrolysis and oxidation at elevated and room temperature (both protected from light and exposure to sunlight). CGN and two other unknown products were observed as its degradation products. For assays of MET raw material and commercial tablets, content of MET in all samples (%labeled amount between 99.8 and 100.2% with %RSDs of less than 1.28%) met requirements of USP and BP pharmacopeias (98.5-101.0% for raw material and 95.0-105.0% for tablet). No degradation products, especially CGN, were observed in the investigated samples.

The CE method for the analysis of GCZ, GCZ-B and GCZ-F (method 2) was validated. The method showed good linearity (GCZ: y = 0.2544x – 0.6716, r^2 = 0.9981 (128-192 µg/mL), GCZ-B: y = 0.1749x + 0.0476, r^2 = 0.9986 (20-60 µg/mL) and GCZ-F: 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 GCZ-B and 99.4 and 99.6 for GCZ-F with %bias of less than 0.86%). LODs were 40, 20 and 10 µg/mL for GCZ, GCZ-B and GCZ-F, respectively. LOQs were 120, 50 and 50 µg/mL for GCZ, GCZ-B and GCZ-F, 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. The validated method was applied for analysis of GCZ, GCZ-B and GCZ-F in stress studies of GCZ, in raw material and commercial tablets. Stress studies of GCZ were investigated under various conditions as mentioned previously for MET. Results revealed that GCZ-F was not observed from all the stress conditions. GCZ was stable in alkaline hydrolysis both at room (both protected from light and exposure to sunlight) and elevated temperature (80 ºC). GCZ content was in a range of 94.5 to 100.0% throughout the investigated time in 0.1 N NaOH. Under acid hydrolysis at elevated temperature, GCZ rapidly degraded after 5 min. Under neutral hydrolysis and oxidation, GCZ also degraded at slower rates that those of acid hydrolysis. GCZ-B and another unknown impurity were obtained in acid hydrolysis and oxidation conditions. Furthermore, exposure of the GCZ in acid solution to sunlight accelerated its degradation and the formation of GCZ-B and another unknown impurity. Applications of the method for assays of GCZ in raw material and commercial tablets revealed that contents of CGZ in all samples (%labeled amount between 99.1 and 100.2% with %RSDs of less than 0.95%) met requirements of BP (99.0-101.0% for raw material and 95.0-105.0% for tablet). No degradation products, especially GCZ-B and GCZ-F, were observed in the investigated samples.

เนื้อหางานวิจัย

ความสำคัญและที่มาของปัญหา

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. 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 (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 metformin (MET) and gliclazide (GCZ) under stress conditions (e.g. hydrolysis (acid, alkaline, neutral), photolysis and oxidation) using capillary electrophoresis (CE) and the analytical tool. Both drugs were selected as models in this study since they are most commonly used for treatment of type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM), in which can still produce insulin, but have insulin tolerance, impaired insulin secretion or increased glucose production [1-5]. Although several researchers reported the SIMs for both drugs, their results were inconsistent and controversial. 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.

งานวิจัยที่เกี่ยวข้อง

Quality control of active pharmaceutical ingredients (API) and their finished products is a priority for consumer protection. United State Pharmacopoeia (USP), British Pharmacopoeia (BP) and European Pharmacopoeia (EP) have published guidelines for the quality control of various pharmaceuticals. In addition to assays of 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 [6].

Diabetes is a metabolism disorder, which causes elevated blood glucose levels. Main types of diabetes are type 1 and type 2, in which type 2 diabetes is now a major concern worldwide with an estimation of 366 million patients by the year 2030. MET and GCZ (Fig. 1) are drugs of choice for type 2 diabetes treatments and combination of both drugs in a single dosage form is popular to enhance patient compliance. However, quality control of the drugs and their impurity contents is a priority. Impurities of metformin (i.e. cyanoguanidine, CGN) and gliclazide (i.e. impurity B (GCZ-B) and F (GCZ-F)) are extremely harmful and toxic. These impurities can be associated with the synthesis route of MET (Fig. 2) and GCZ (Fig. 3) or they are degradation products under various stress conditions.

Table 1 summarizes limits of MET, GCZ and their impurities [7, 8]. Both BP and USP recommend non-aqueous titration and high performance liquid chromatography (HPLC) for the assay of metformin in raw material and tablets, respectively. Metformin impurities include impurity A-F, but only testing of impurity A or cyanoguanidine 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]. Gliclazide is official in BP and non-aqueous titration is proposed for the assay of glicalzide raw material, while HPLC is for gliclazide tablets [7]. Gliclazide impurities are impurity A-G, but only quantitation of impurities B and F (Fig. 1) is recommended by BP using HPLC. Impurity B and F cause irritation of eyes, respiratory and gastrointestinal tracts and skin, hypoglycemia and allergy, harm to unborn child and carcinogenicity [10, 11].

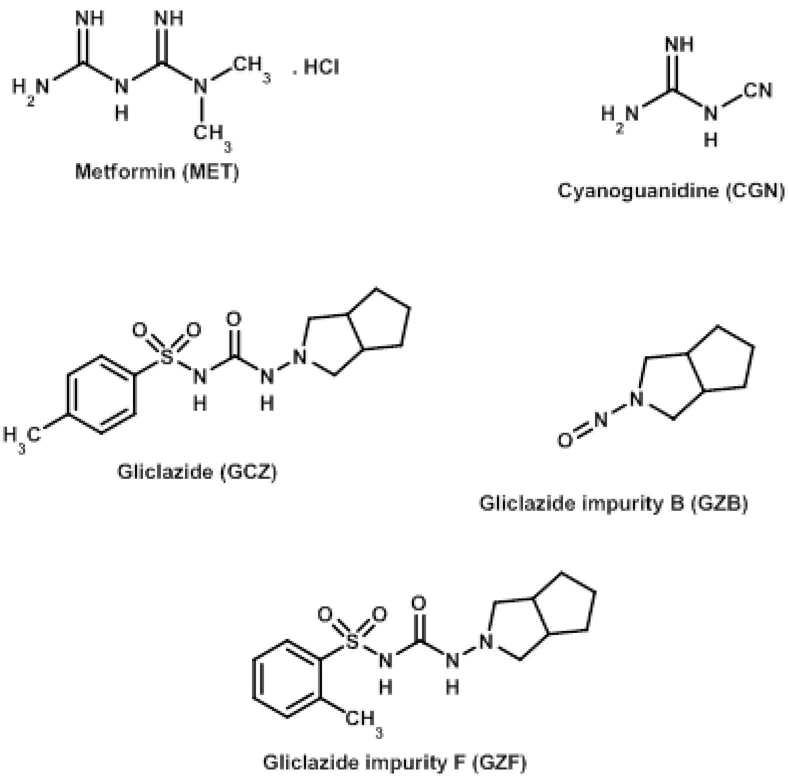

Figure 1 Structures of metformin, gliclazide, cyanoguanidine and gliclazide impurities B and F.

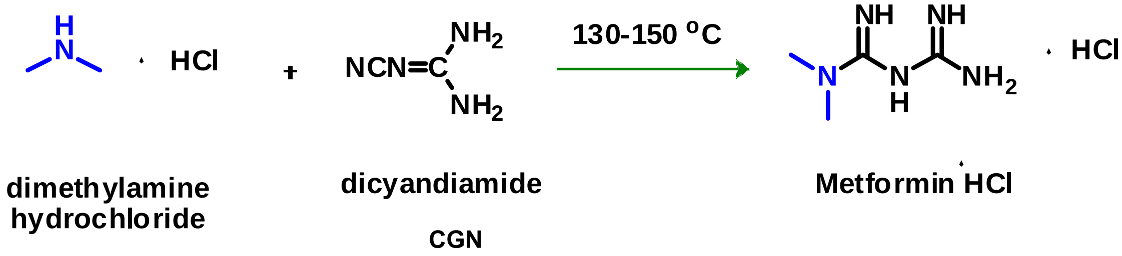

Figure 2 Synthesis of metformin.

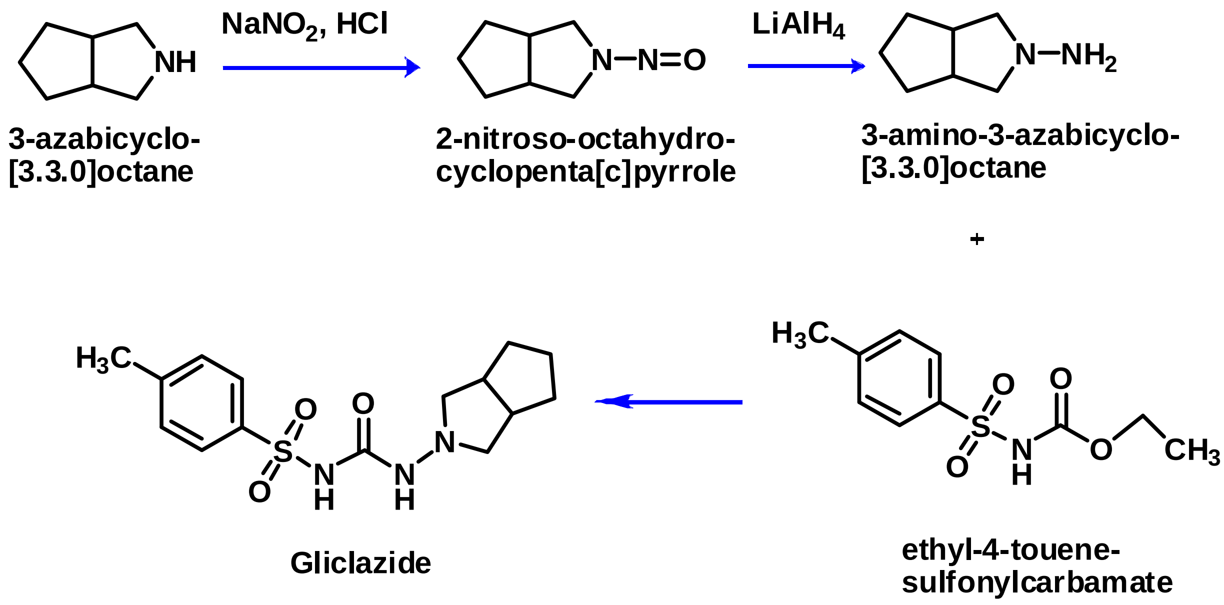

Figure 3 Synthesis of gliclazide.

Table 1 Limits of metformin, gliclazide and their impurities [7,8]*

	BP	USP
Metformin		
Raw material	98.5-101.0% la	98.5-101.1% la
Tablet	95.0-105.0% la	95.0-105.0% la
Impurity		
Impurity A	0.02% (raw material)	0.02% (raw material)
(cyanoguanidine)		0.1% (tablet)
Impurities B, C, D, E, F	NA	NA
Total impurities	0.1% (raw material)	0.6% (tablet)
Gliclazide		
Raw material	99.0-101.0% la	-
Tablet	95.0-105.0% la	
Impurity		
Impurity A, C, D, E, G	NA	
Impurity B	2 ppm	-
Impurity F	0.1% (raw material)	-
	0.2% (tablet)	-

la = labeled amount, NA = not applicable

From literature search, analyses of metformin can be performed by various techniques (e.g. CE, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), HPLC-mass spectrometry (MS), spectrophotometry and spectrofluorometry). Table 2 shows previous reports on the assays of metformin, gliclazide and other anti-diabetics in raw material, formulations and biological fluids.

HPLC-UV and HPLC-ECD are usually used for the analyses of MET and GCZ. Limitations of these techniques include long analysis time, peak tailing and low sensitivity. TLC of these drugs is feasible and cheap, but sometime is time- and labor-consuming. LC-MS-MS is employed for monitoring of both drugs in plasma because of its high sensitivity, however, the instrumentation and maintenance is costly. Spectrophotometry and spectrofluorometry of MET and GCZ can be achieved, but derivatization is required prior the measurement. GC is limited to heat stable compounds and capillary clogging can be problematic. Drawback of reported methods indicate that a novel method should be established for the analyses of MET and GCZ. Moreover, impurities of both drugs (i.e. CGN, GCZ-B and GCZ-F) should be determined according to USP and BP guideline. However, methods (i.e. HPLC) in both pharmacopoeias for testing of these impurities are laborious and time-consuming. From literature search, other methods for the quantitation of these impurities/degradation products have not been described.

Currently, CE is employed for investigating the stability of MET and GCZ under forced degradation. The established methods could be served as SIMs of both drugs. Since the remained drugs and degradation products could be well separated and accurately quantified. CE is highly efficient, rapid, inexpensive and environmentally friendly [34-36]. CE is a versatile method since various modes are available for different classes of analytes such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), microemulsion electrokinetic chromatography (MEEKC), non-aqueous capillary electrophoresis (NACE). Applications of CE for pharmaceutical analyses are ubiquitous and well described in literatures [34, 37-40].

Table 2 Analyses of metformin, gliclazide and various anti-diabetes drugs

Analyte	Matrix	Method*	References
Metformin, pioglitazone	Tablet	TLC- UV, HPLC -UV	[12]
Metformin, gliclazide	Tablet	TLC- UV, HPLC -UV	[12]
Metformin, gliclazide	Plasma	LC/MS/MS	[13]
Metformin, fenformin, glyburide	Plasma	CE-UV	[14]
Metformin, roziglitazone	Tablet	CE-UV	[15]
Aminoheterocycle, azabicycle (an impurity of gliclazide)	Raw material	CE-AD	[16]
Dicyandamine (a related compound of metformin)	Standard	HPLC-UV	[17]
Gliclazide, glybencamide, glypizide, tolbutamide, chlopropamide	Plasma and urine	CE- UV, HPLC -UV	[18]
Gliclazide, glybencamide, glypizide, glymipyride, glyquidone, repaglynide	Tablet	HPLC-UV	[19]
Metformin			
Metformin	Plasma	HPLC-UV	[20]
Metformin, roziglitazone	Urine	HPLC-UV	[21]
Gliclazide	Plasma	HPLC-UV	[22]
Metformin	Plasma	HPLC-UV	[23]
Gliclazide	Plasma	LC/MS/MS	[24]
Gliclazide	Plasma	HPLC-ECD	[25]
Metformin, glipizide, gliclazide, glibencamide, glimipyride	Plasma	HPLC-UV	[26]
	Plasma	HPLC-UV	[27]
Metformin			
Gliclazide	Plasma	LC/MS/MS	[28]
Gliclazide	Plasma, tablet	Spectrophotometry	[29]
	Tablet	Spectrophotometry	[30]
Gliclazide		Spectrofluorometry	
	Serum	HPLC-UV	[31]
Metformin, gliclazide, glipizide			
	Tablet	HPLC-UV	[32]
Gliclazide			
	Tablet	GC	[33]

*TLC = Thin layer chromatography, UV = UV-Visible spectrophotometer, HPLC = High performance liquid chromatography, LC/MS/MS = Liquid chromatography-mass spectrometry-mass spectrometry, CE = Capillary electrophoresis, AD = Amperometric detector, ECD = Electrochemical detector, GC = Gas chromatography

วัตถุประสงค์

1. To develop and validate capillary electrophoresis methods for stability study of metformin and gliclazide
2. To investigate stability of both drugs under various stress conditions
3. To establish pharmacokinetic profile on the stability of both drugs
4. To apply the developed method for the assay of both drugs in both raw material and commercial formulations

ขอบเขตการวิจัย

The current research focused on the method development of SIMs for the stability study of MET and GCZ. The methods were validated for the analysis of the drugs and their specified impurities/degrades. Forced degradations were performed by hydrolysis (acid, alkaline, neutral), photolysis and oxidation. The remained drugs and the known degrades were identified and quantified by the established method. The methods were applied to analysis of raw material and commercial tablets of both drugs.

วิธีดำเนินการวิจัย (Materials and Methods)

1. สารเคมีและรีเอเจนต์ (Chemicals and reagents)

Lists of chemicals and reagents are shown in Table 3.

Table 3 Chemicals and reagents

Name	Source/Supplier
Metformin	Sigma-Aldrich, Germany
Gliclazide and cyanoguanidine	Sigma-Aldrich, USA
Gliclazide impurity B and F	EDQM, France
Sodium dihydrogen phosphate	Merck, Switzerland
Sodium dodecyl sulphate	Sigma-Aldrich, USA
Citric acid	Sigma-Aldrich, Germany
Methanol (HPLC)	Labscan, Thailand
Sterile water for injection	Thainakornpattana Co., Ltd, Thailand

2. เครื่องมือ (Instruments)

List of instruments are shown in Table 4.

Table 4 Instruments

Name	Source/Supplier
Capillary electrophoresis instrument	Agilent Technologies, Germany
Capillary tube; ID 50 μm OD 375 μm	Polymicro Technologies, USA
pH meter	Consort model C380, Belgium
Analytical balance (AE 160)	Sartorius, Germany
Nylon syringe filters 13mm, 0.2 μm	Lubitech, China
Centrifuge (Labfuge 200)	Heraeus, Germany
Ultrasonic bath (D-7700)	Elma, Germany

3. Method

3.1 Instrumentation

Capillary zone electrophoresis was performed on a Hewlett-Packard instrument (^{3D}CE) system (model G1600A) and controlled by PC through Agilent ChemStation Plus software version A.09 (G1601A). The software was designed to run on a compatible computer HP Pentium 4 (500 MHz, RAM 256 MB, hard disk 20 GB) under Microsoft® Window NT 4.0 operating was used. An instrument was defined as running on a single time base, but could collect data from a number of different detectors simultaneously. The detector measured in the range of 190-600 nm (wavelength accuracy $\pm$ 2 nm) was a diode array detector, which was consisted of a deuterium a lamp and detected by continuous emission. 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 $^{\circ}$C ($\pm$ 1 $^{\circ}$C). The injection systems could be achieved by 1) applying pressure to sample vials (hydrostatic injection) and 2) applying voltage (electromigration injection).

3.2 Capillary conditioning

Separations of MET, GCZ and their degradation products were carried out using fused-silica capillaries with inner diameters of 50 μm and various lengths. Prior the use of a new capillary and between runs, the capillary needed to be pre-conditioned according to the procedures described in Table 5.

Table 5 Capillary conditioning procedure

Step	Duration (min)	Solvent
New capillary		
1) Rinse	15	1N NaOH
2) Rinse	15	Sterile water for injection
3) Rinse	15	0.1N NaOH
4) Rinse	15	Sterile water for injection
Between run		
1) Rinse	1	0.1 N NaOH
2) Rinse	1	Sterile water for injection
3) Rinse	2	Back ground electrolyte
Storage		
1) Rinse	5	Sterile water for injection
2) Rinse	5	1N NaOH
3) Rinse	5	Sterile water for injection
4) Rinse	5	0.1N NaOH
5) Rinse	10	Sterile water for injection
6) Rinse	10	MeOH
7) Rinse	10	Air

3.3 CE optimization

CE separation of MET and CGN was modified from Hamdan and co-workers [41]. Citrate buffer was used as back ground electrolyte (BGE) and other factors (e.g. capillary dimension, voltage and temperature) were optimized to obtain the optimum condition. CE separation of GCZ, GCZ-B and GCZ-F were investigated by various modes of CE, different types and pH of BGE, capillary dimension, voltage and temperature

The key responses to determine the optimal CE conditions were the R_s N, TF and t_m. The responses were calculated using the following equations

$$R_s = 2(t_{m2}-t_{m1})/(w_1+w_2) \quad (1)$$

$$N = 5.54\ (t_m/w_{0.5})^2 \quad (2)$$

$$TF = W_{5.0}/\ t_w \times 2 \quad (3)$$

where t_m, w, $w_{0.5}$, t_w were the migration time, the peak width, the peak width at half height, peak width at 5% of peak height, distance in min between peak front and retention time of peak, measured at 5% of peak height, respectively.

3.4 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

3.4.1 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 GCZ-B and 10-50 µg/mL of GCZ-F. Calibration curves were plotted between peak area versus standard solutions concentrations. Linear regression and correlation of determination (r^2) were calculated.

3.4.2 Precision

3.4.2.1 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.

3.4.2.2 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 Accuracy

Accuracy was determined by a standard addition method. Known amounts of three different concentrations of standard solution (80%-120%) were added into 100% concentration of samples and analyzed. Each concentration was injected in triplicates. Accuracy will be calculated in term of percent recovery

$$\% \text{ Recovery} = (X_{found} / X_{add}) \times 100 \qquad (4)$$

where X_{found} was concentration of found analyte found and X_{add} was concentration of analyte added.

3.4.4 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.

3.4.5 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

3.5 Stability study

Stability studies of MET and GCZ were carried out under various forced condition as shown in Table 6. The remained drugs and known degrades were identified and quantified by the developed CE methods. The stability and pharmacokinetic profile were established.

Table 6 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	30 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 7). 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 8). 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 7 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 8 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)	SWI (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

3.6. 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.

ผลการวิจัยและข้อวิจารณ์ (Results and Discussion)

1. CE optimization

1.1 Capillary zone electrophoresis (CZE) of MET and CGN

CZE was selected to determine MET and its degradation product since it was simple and provided high efficiency. CE 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 was of 15 kV, temperature was at 30 °C and injection was by pressure at 50 mbar for 5 s (method 1). MET and CGN were well separated under these conditions with the migration times of 9.9 and 19.0 min, respectively, and a resolution (R_s) of 38.9 (Table 9).

Table 9 Analytical parameters of MET and CGN under the optimized CZE condition (method 1)

	MET	CGN
Migration time (t_m, min)	9.87	19.03
Resolution	-	38.9
Tailing factor	0.28	0.78
Number of plate (x 10^4)	20	5.5
%$RSD_{peak\ area}$ (n = 6)	0.04	1.40
%RSD_{tm} (n = 6)	0.83	1.40

1.2 Micellar electrokinetic chromatography (MEKC) of GCZ, GCZ-B and GCZ-F

Resolution of GCZ, GCZ-B and GCZ-F was initially performed in CZE mode. Sodium dihydrogen phosphate buffer (10-20 mM) at different pH (pH 5.0-9.0) was selected since the buffer has wide ranges of pK_a that are suitable for analysis of acid, base and neutral compounds. Under these various conditions, GCZ and GCZ-F overlapped. Moreover, GZB remained un-charged and co-migrated with the EOF at all the investigated condition

MEKC was then considered as an alternative, by the addition of varied concentrations of SDS into 10 mM sodium dihydrogen phosphate buffer (pH 7.0). SDS at concentration of 50 mM could be firstly excluded from the investigation since no peaks were observed in 30 min. SDS concentrations significantly influenced resolution of MET/CGN and GZF/GCZ. Resolution of GZF/GCZ improved from 0.6 to 3.2 when SDS concentration increased from 10 to 20 mM, however resolution values dropped when SDS concentrations were higher than 20 mM. Presently, SDS at 15 mM was selected as a compromise for the resolution of GCZ/GZF. The optimal conditions were in 10 mM phosphate buffer and 15 mM SDS (pH 7.0) 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 be performed by a diode-array detector at a wavelength of 225 nm with a bandwidth of 8 nm., applied voltage was of 20 kV, temperature was at 25 °C, and injection was by pressure 50 mbar for 5 s (method 2). GCZ-B, GCZ-F and GCZ migrated at 3.82, 5.21 and 5.32 min, respectively with R_s of 11.52 (GCZ-B/GCZ-F) and 2.06 (GCZ-F/GCZ) (Table 10).

Table 10 Analytical parameters of GCZ, GCZ-B and GCZ-F under the optimized MEKC condition (method 2)

	GCZ-B	GCZ-F	GCZ
Migration time (t_m, min)	3.82	5.21	5.32
Resolution	-	11.52	2.06
Tailing factor	0.61	0.94	0.85
Number of plate (x 10^4)	0.76	14	1.4
%$RSD_{peak\ area}$ (n = 6)	1.14	1.40	0.43
%RSD_{tm} (n = 6)	1.98	1.63	1.76

2. Method validation

2.1 Method validation of method 1 (CZE of MET and CGN)

Linearity, precision, accuracy, LOD, LOQ and robustness data of MET and CGN under method 1 was presented in Table 11-15. Robustness data (Table 16) was evaluated from the variation around the optimal values of pH (6.7± 0.5) and the voltage (15± 2).

Table 11 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 12 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

Table 13 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 14 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 15 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 16 Robustness of MET and CGN (n = 6)

	%$RSD_{peak\ area}$	
	MET	CGN
pH of BGE effect		
pH 6.0	0.80	1.73
pH 6.7	0.04	1.40
pH 7.5	0.38	0.30
Voltage effect		
13 kV	0.09	1.44
15 kV	0.04	1.40
17 kV	0.48	0.71

Validation data indicated method 1 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%.

2.2 Method validation of method 2 (MEKC of GCZ, GCZ-B and GCZ-F)

Linearity, precision, accuracy, LOD, LOQ and robustness data of GCZ, GCZ-B and GCZ-F under method 2 was presented in Table 17-21. Robustness data (Table 22) was evaluated from the variation around the optimal values of pH (7.0± 0.2) and the voltage (20± 2).

Table 17 Linearity data of GCZ, GZB and GZF

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

Table 18 Repeatability data of GCZ, GZB and GZF

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

Table 19 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
GCZ-B	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
GCZ-F	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 20 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	GCZ-B	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	GCZ-F	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 21 LOD and LOQ data of GCZ, GZB and GZF (n = 6)

	GCZ	S/N	GCZ-B	S/N	GCZ-F	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 22 Robustness of GCZ, GCZ-B and GCZ-F (n = 6)

	$\%RSD_{peak\ area}$		
	GCZ	GCZ-B	GCZ-F
pH of BGE effect			
pH 6.8	0.92	0.21	0.63
pH 7.0	0.43	1.14	1.40
pH 7.2	0.34	0.10	0.65
Voltage effect			
18 kV	1.17	0.33	1.14
20 kV	1.76	1.98	1.63
22 kV	1.23	1.03	0.53

The CE method for the analysis of GCZ, GCZ-B and GCZ-F (method 2) 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), GCZ-B: y = 0.1749x + 0.0476, r^2 = 0.9986 (20-60 µg/mL) and GCZ-F: 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 GCZ-B and 99.4 and 99.6 for GCZ-F with %bias of less than 0.86%). LODs were 40, 20 and 10 µg/mL for GCZ, GCZ-B and GCZ-F, respectively. LOQs were 120, 50 and 50 µg/mL for GCZ, GCZ-B and GCZ-F, 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

3. Stability study

SIMs of MET and GCZ have been published in a few literatures [41-43], but they reports controversial and different results. Presently, stability studies of MET and GCZ were investigated and confirmed according to ICH guidelines [44] and guidance described by Singh and Bakshi [45].

3.1 Stability study of MET

MET was forced to degraded under various stress conditions. Amounts of the remained MET and CGN were determined and two other unknown degradation products were observed using CE method 1 and data were tabulated in Table 23-31. Pharmacokinetic profiles on stability of MET are demonstrated in Fig. 4-8 and electropherograms of MET and the degradation products are in Fig. 9. Mechanism for the formation of CGN from MET under the forced degradation in alkaline hydrolysis is proposed in Fig. 10.

Hydrolysis and oxidation of MET at elevated 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… 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 [41] and Ali's [42] 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 23 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 24 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 25 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 26 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 27 Degradation data of MET in water and 0.1 N HCl at room temperature (kept in a dark cabinet)*

Time (day)	%MET	
	Water	0.1 N HCl
0	100.0	100.0
1	100.9	99.
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 28 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 29 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	69.2	√	-
14	63.3	√	0.04
21	59.3	√	0.10
30	38.4	√	0.14

*Unknown 2 was not observed.

Table 30 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 31 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.

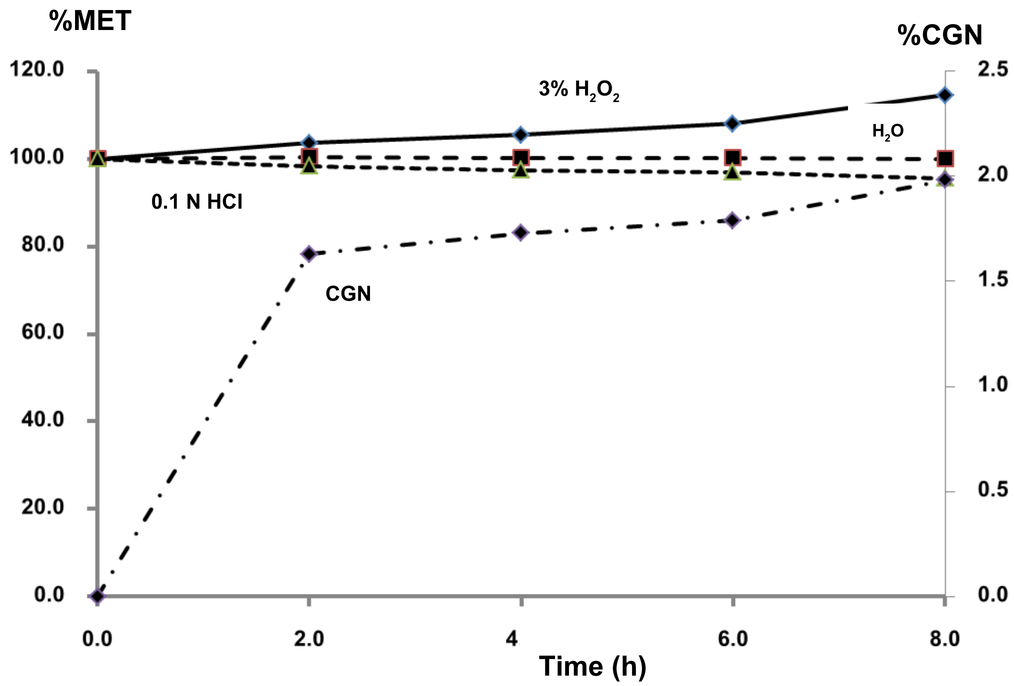

Figure 4 Pharmacokinetic profiles for the degradation of MET and CGN formation under neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at 80 ºC (CGN was observed only from oxidation condition)

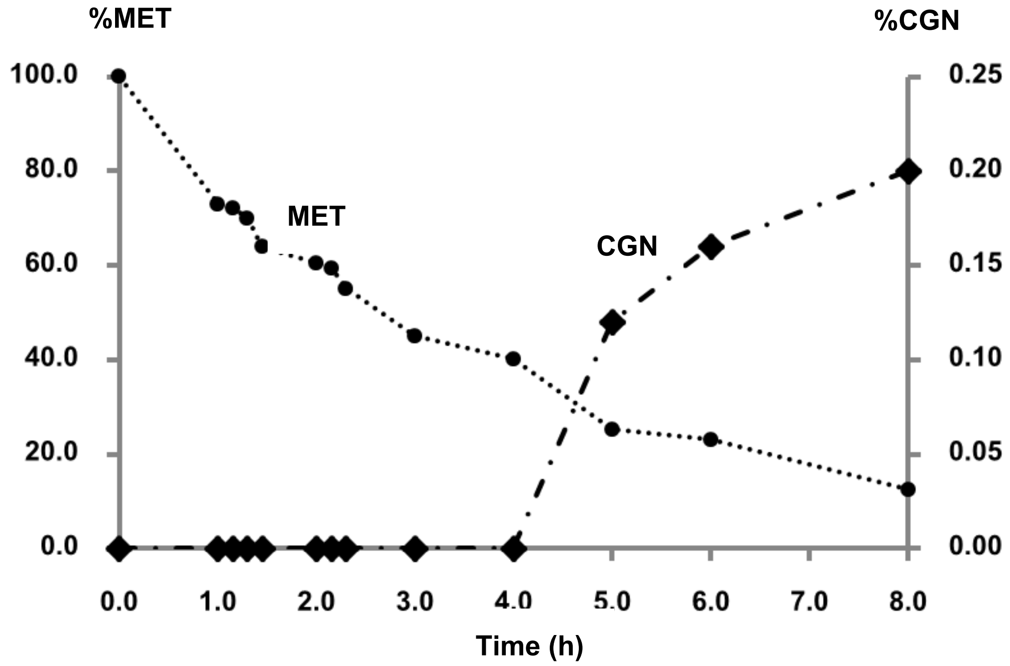

Figure 5 Pharmacokinetic profiles for the degradation of MET and CGN formation under alkaline hydrolysis (0.1 N NaOH) at 80 ºC.

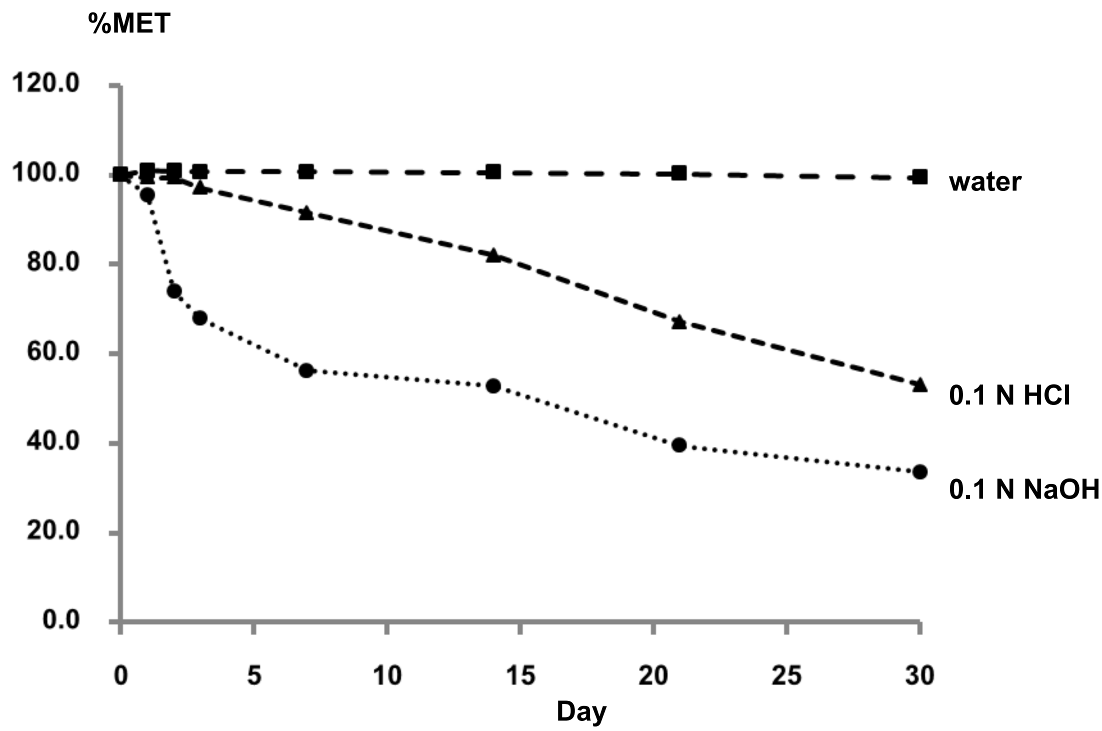

Figure 6 Pharmacokinetic profiles for the degradation of MET under neutral (water), acid (0.1 N HCl) and alkaline (0.1 N NaOH) hydrolysis at room temperature (kept in a dark cabinet) (CGN was not observed under these stress conditions).

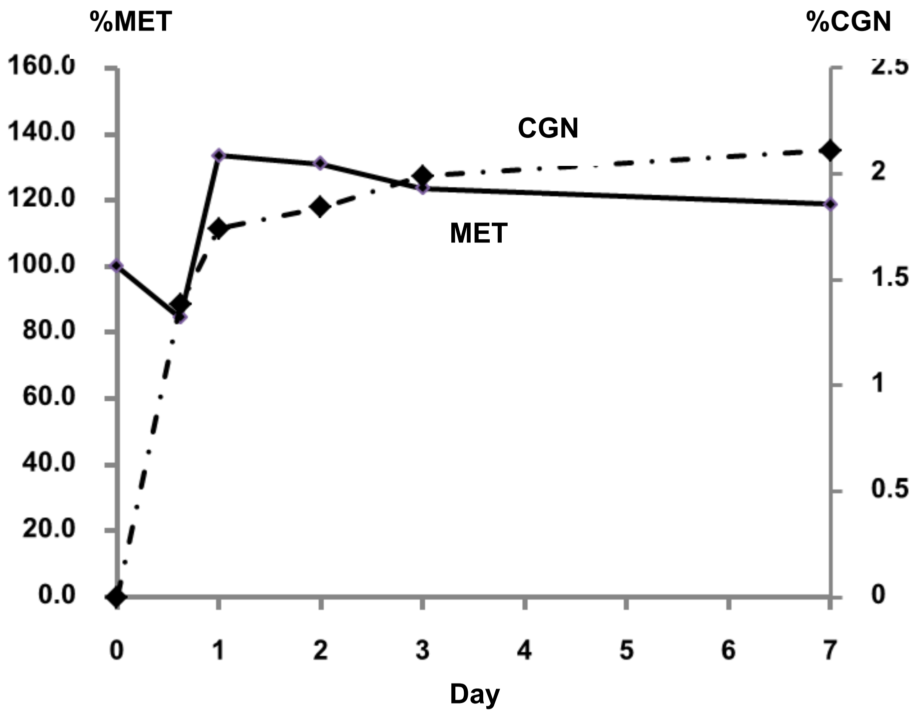

Figure 7 Pharmacokinetic profiles for the degradation of MET and CGN formation under neutral (water), acid (0.1 N HCl) and alkaline (0.1 N NaOH) hydrolysis at room temperature (kept in a dark cabinet).

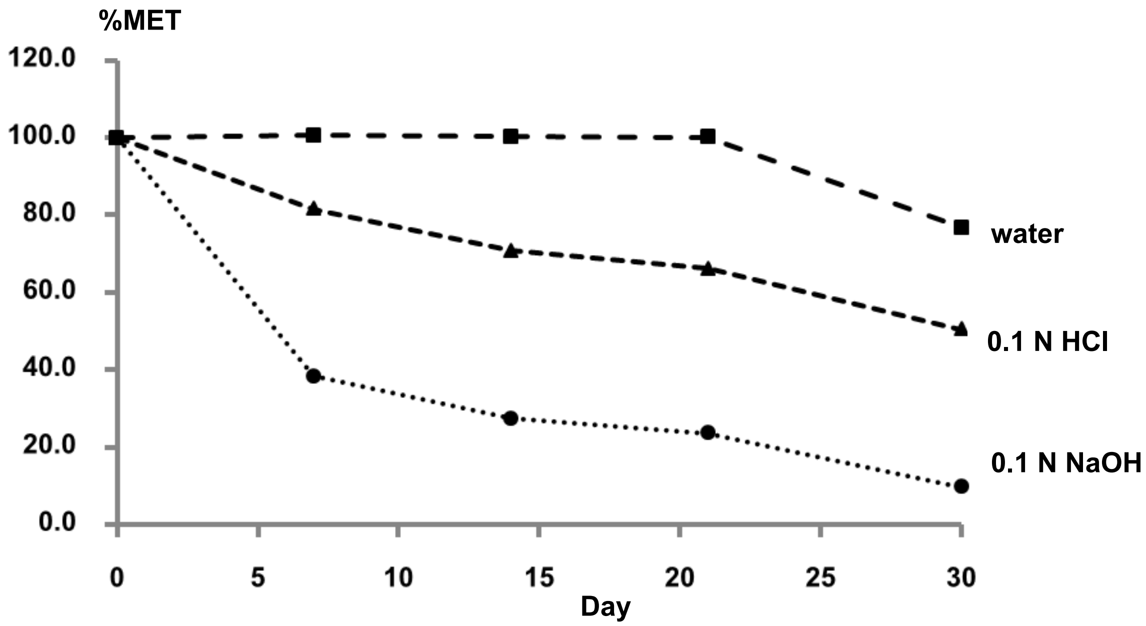

Figure 8 Pharmacokinetic profiles for the degradation of MET and CGN formation under neutral (water), acid (0.1 N HCl) and alkaline (0.1 N NaOH) exposed to sunlight.

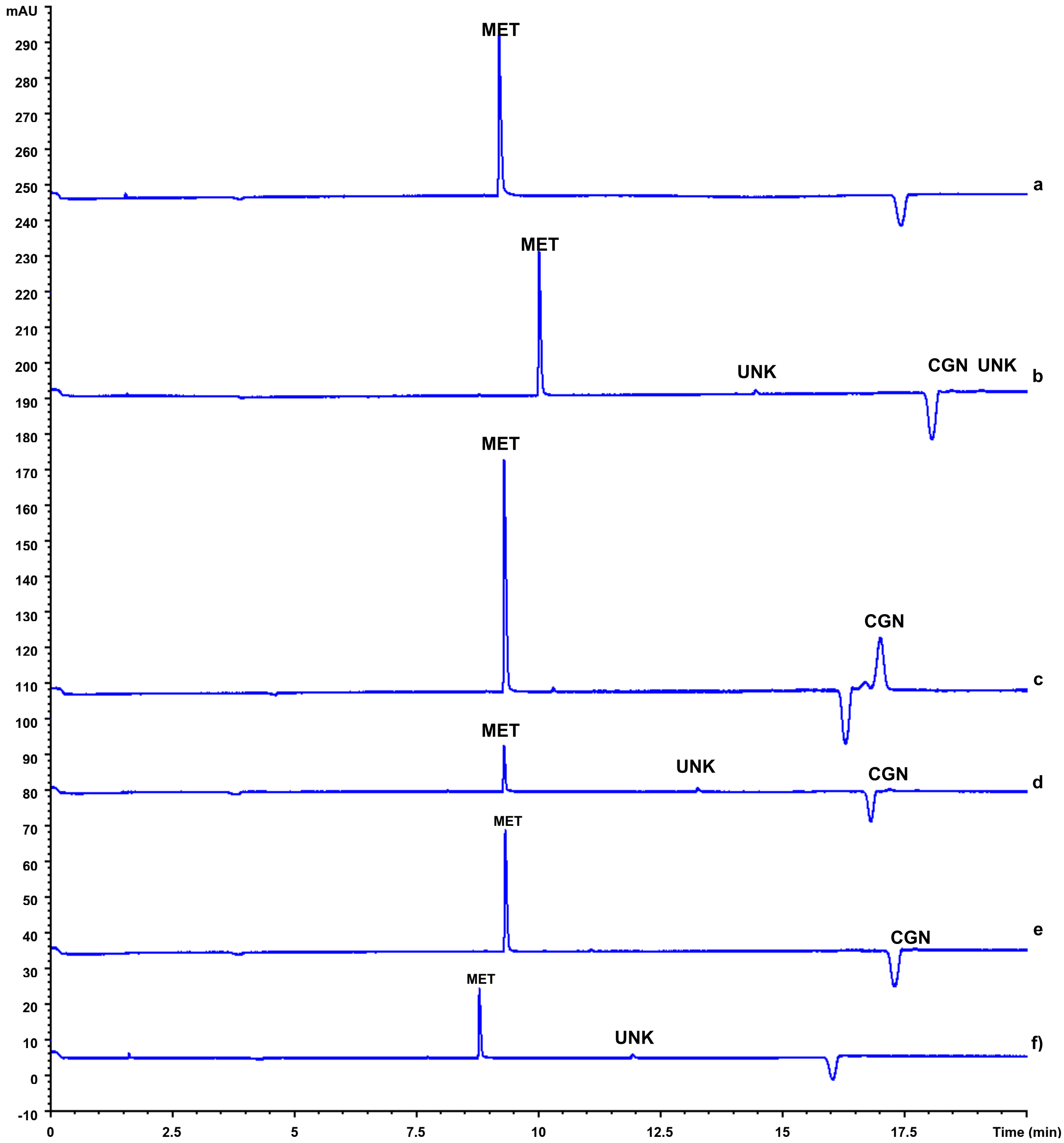

Figure 9 Electropherograms of MET degradation under various stress conditions a) Initial, b) in 0.1 N NaOH, heated at 80°C for 1.5 h, c) in 3%H_2O_2, heated at 80°C for 8 h d) in 0.1 N NaOH, at room temperature (kept in a dark cabinet for 14 days), e) in 0.1 N NaOH, exposed to sunlight for 7 days. CE conditions: method 1 (see Materials and Methods section).

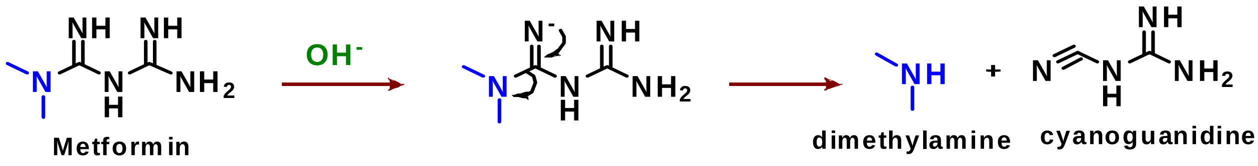

Figure 10 proposed mechanism for the formation of CGN during forced degradation of MET in alkaline hydrolysis.

3.2 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 GCZ-B, GCZ-F and other unknown degrades were shown in Table 32-36. Amounts of the remained GCZ, GCZ-B and GCZ-F were determined and another unknown degrades was detected from CE method 2. Pharmacokinetic profiles on stability of GCZ are demonstrated in Fig. 11-13 and electropherograms of GCZ and the degradation products are in Fig. 14-16. Under all stress conditions, GCZ-F was not detected. A proposed mechanism for the formation of GCZ-B from the forced degradation of GCZ under stress conditions is presented in Fig. 17

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. GCZ-B and GCZ-F 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. GCZ-B (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 GCZ-B was observed at 2 h under both neutral hydrolysis and oxidation. In addition, another unknown degrade was monitored at 10 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, GCZ-B 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 GCZ-B 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 GCZ-B was observed. In sunlight, GCZ degraded at faster rates from 96.0% on day 1 to 8.3% on day 30, GCZ-B 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. GCZ-B was not found, but another unknown degraded was detected since day 3.

Table 32 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 33 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	%GCZ-B	%GCZ	%GCZ-B	%GCZ	%GCZ-B	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 34 Degradation data of GCZ in 0.1 N NaOH at room temperature (kept in a dark cabinet) and exposed to sunlight*

Time	%GCZ	
(day)	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 35 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	%GCZ-B	%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, GCZ-B 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 36 Degradation data of GCZ in 0.1 N HCl and water exposed to sunlight*

Time	0.1 N HCl		water		
(day)	%GCZ	%GCZ-B	%GCZ	%GCZ-B	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

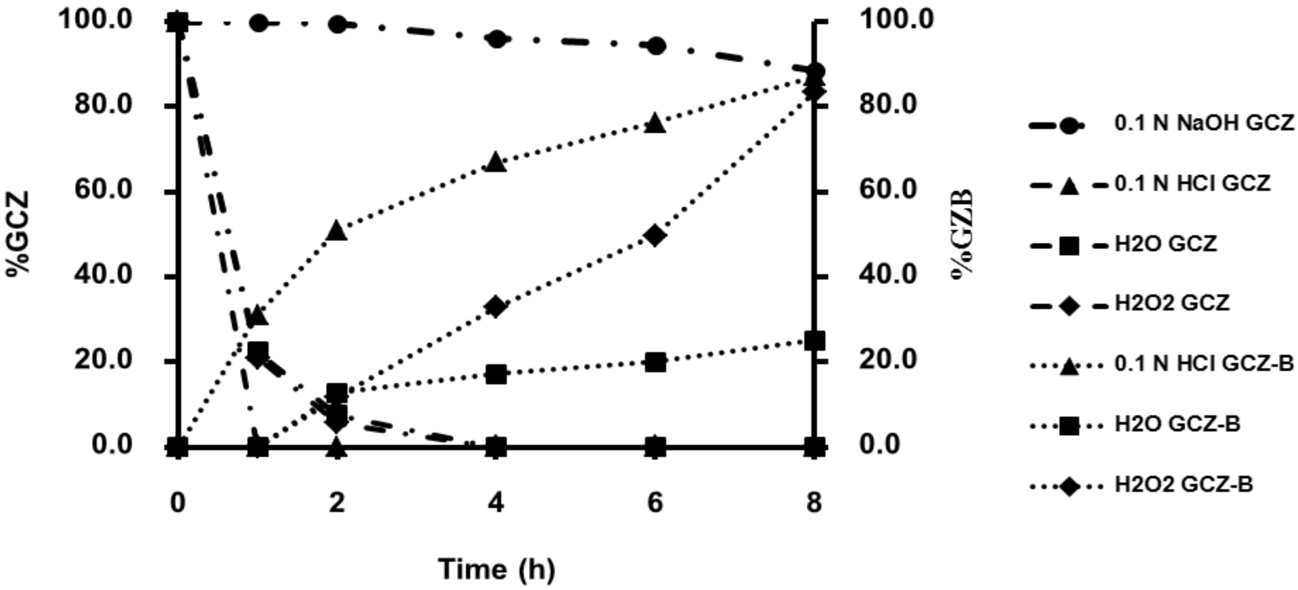

Figure 11 Pharmacokinetic profiles for the degradation of GCZ and GCZ-B formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at 80 ºC (GCZ-B was not observed in alkaline hydrolysis).

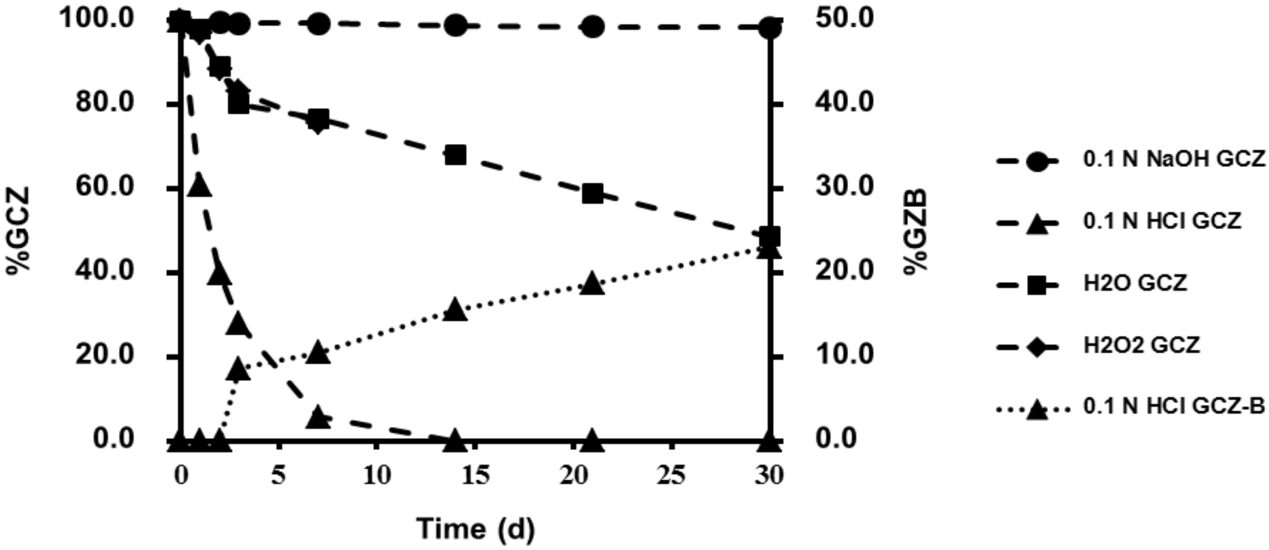

Figure 12 Pharmacokinetic profiles for the degradation of GCZ and GCZ-B formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis and oxidation (3% H_2O_2) at room temperature (kept in a dark cabinet) (GCZ-B was observed only in acid hydrolysis).

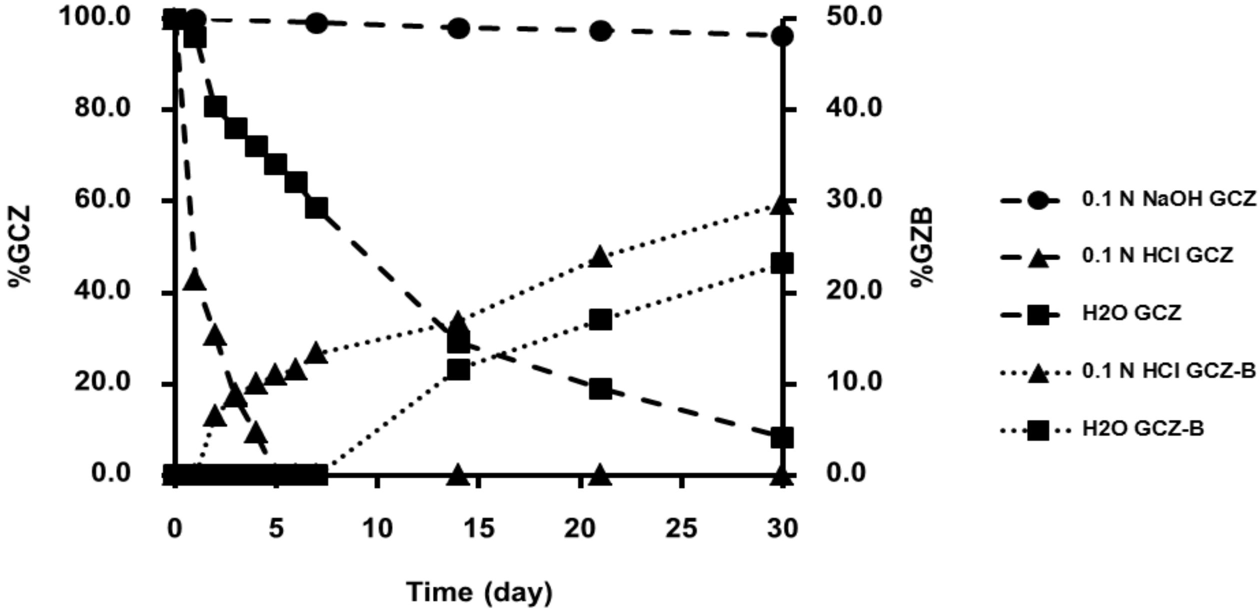

Figure 13 Pharmacokinetic profiles for the degradation of GCZ and GCZ-B formation under alkaline (0.1 N NaOH), neutral (water) and acid (0.1 N HCl) hydrolysis (exposed to sunlight) (GCZ-B was not observed in alkaline hydrolysis).

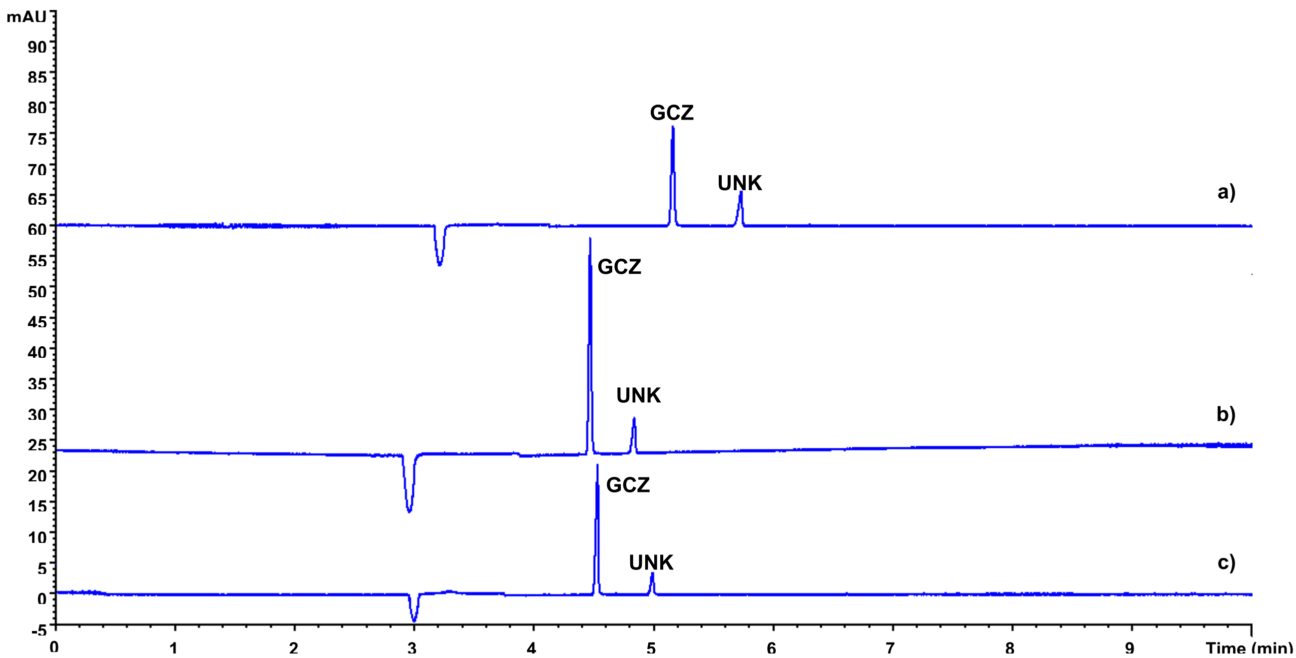

Figure 14 Electropherograms of GCZ degradation at 80°C a) in 0.1 N HCl for 5 min, b) in water for 10 min, c) in 3%H_2O_2 for 10 min. CE conditions: method 2 (see Materials and Methods section).

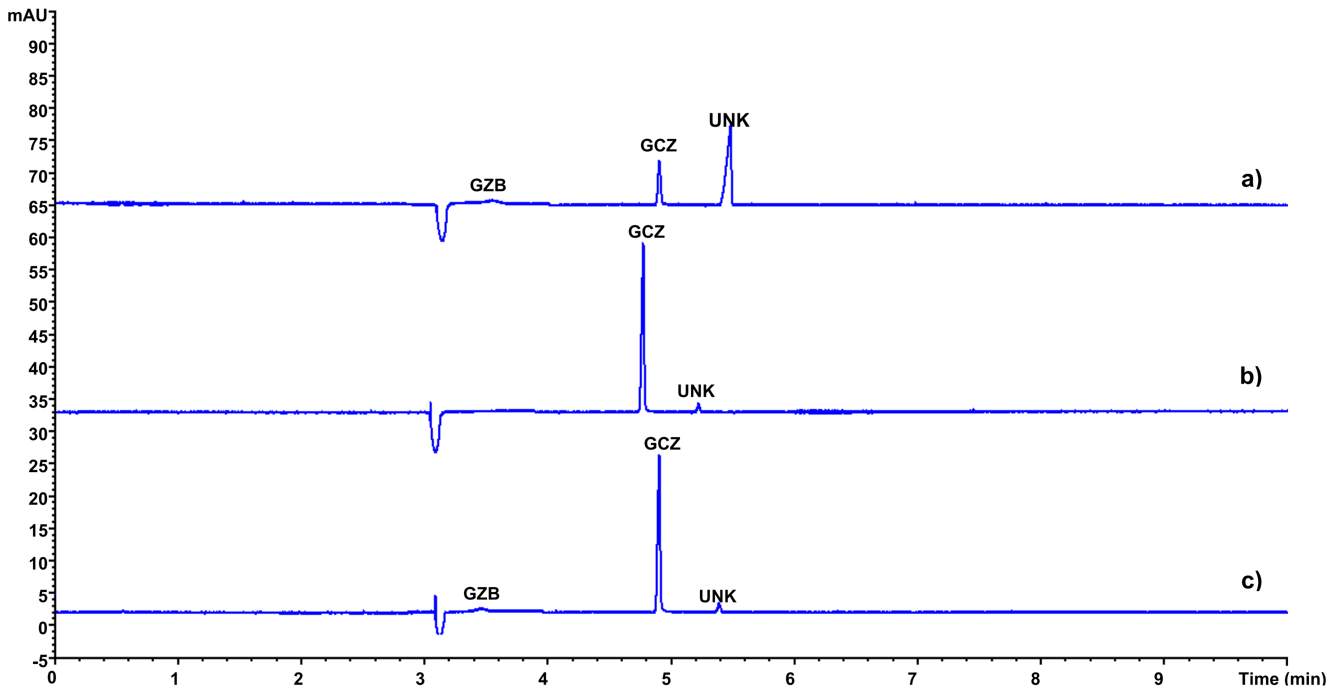

Figure 15 Electropherograms of GCZ degradation at room temperature (kept in a dark cabinet, after 3 days) a) in 0.1 N HCl, b) in water and c) in 3%H_2O_2. CE conditions: method 2 (see Materials and Methods section).

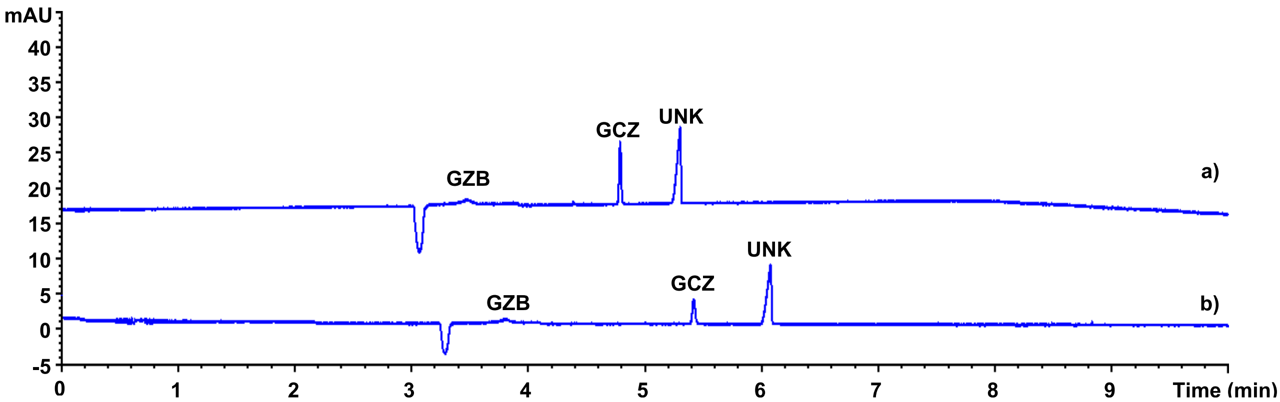

Figure 16 Electropherograms of GCZ degradation exposed to sunlight a) in water for 14 days and b) in 0.1 N HCl for 2 days. CE conditions: method 2 (see Materials and Methods section).

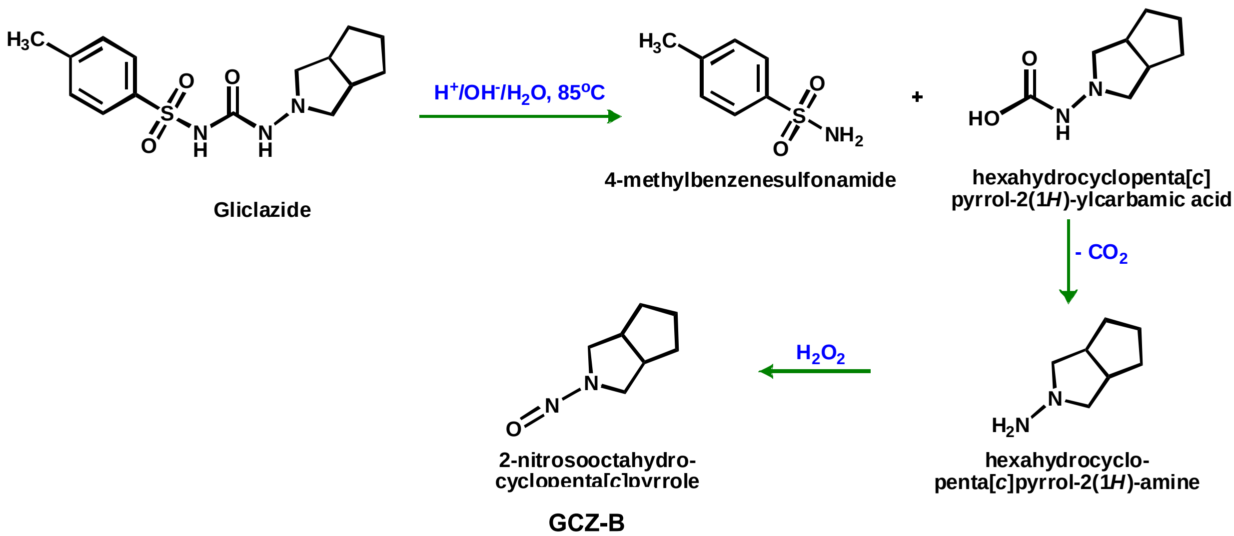

Figure 17 proposed mechanism for the formation of GCZ-B from the forced degradation of GCZ under stress conditions.

4. Application

The developed methods, CZE (method 1) and MEKC (method 2), 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 37. MEKC was utilized for assay of GCZ in two lots of raw material and three lots of tablets and results are shown in Table 38. 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 37 Assay of MET in samples by CZE (method 1)

	%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 38 Assay of GCZ in samples by MEKC (method 2)

	%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		

สรุปผลการวิจัย (Conclusion)

1. CE optimization

Two CE methods were developed 1) for the analysis of MET and CGN (a specified impurity of MET) and 2) for the analysis of GCZ, GCZ-B and GCZ-F (specified impurities of GCZ). The conditions are shown below.

CZE for the analysis of MET and CGN (Method 1)

Capillary	: 68.5 cm total length (8.5 cm to the detector), 50 µm
BGE	: 40 mM citrate buffer (pH 6.7)
Temperature	: 30 ºC
Voltage	: 15 kV
Injection	: pressure 50 mbar 5 s
Detector	: 214 nm

MEKC for the analysis of GCZ, GCZ-B and GCZ-F (Method 2)

Capillary	: 48.5 cm total length (8.5 cm to the detector), 50 µm
BGE	: 10 mM citrate buffer (pH 7.0) containing 15 mM sodium dodecyl sulfate
Temperature	: 25 ºC
Voltage	: 20 kV
Injection	: pressure 50 mbar 5 s
Detector	: 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 impurities.

2. Method validation

Method 1 and 2 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 39).

Table 39 Summary of validation data for MET, CGN, GCZ, GCZ-B and GCZ-F

	Method 1	Method 2
Regression	MET $y = 0.3881x - 4.0717$ ($r^2 = 0.9985$) CGN $y = 3.6108x + 2.9254$ ($r^2 = 0.9968$)	GCZ $y = 0.2544x - 0.6716$ ($r^2 = 0.9981$) GCZ-B $y = 0.1749x + 0.0476$ ($r^2 = 0.9986$) GCZ-F $y = 0.1968x + 0.0176$ ($r^2 = 0.9999$)
Precision (%RSD)	1.98	2.00
Accuracy (%R)	98.3-100.9	99.0-100.0
LOD (µg/mL)	30	40
LOQ (µg/mL)	100	50
Robustness (%RSD)	1.73	1.23

3. Stability study

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 40).

Table 40 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

4. Application

The developed methods, CZE (method 1) and MEKC (method 2), 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 41).

Table 41 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		

Output ที่ได้จากโครงการ

1. Two new CE methods have been developed 1) for the analysis of MET and CGN (a specified impurity of MET) and 2) for the analysis of GCZ, GCZ-B and GCZ-F (specified impurities of GCZ). The methods show applications for 1) stability study of MET and GCZ and 2) assay of the drug. Unlike other reports, the proposed methods are novel, rapid, reliable and simple. In addition, they are cost effective in term of lower time- labor- and chemical-consuming.
2. Both methods are stability indicating, which can be applied for the stability study of MET and GCZ. The two methods provide baseline separation of the active drugs and their specified impurities/degrades and can accurately quantify amounts of the drugs and the degradation products. Consequently, stability data of MET and GCZ can be obtained and their stability pharmacokinetic profiles are firstly established using the developed methods
3. Three manuscripts are obtained from the current research.
    a. Doomkaew A, Prapatpong P, Buranphalin S, Suntornsuk L. Rapid and simultaneous analysis of combined anti-diabetic drugs by capillary zone electrophoresis. J. Pharm. Biomed. Anal. (submited).
    b. Doomkaew A, Prutthiwanasan B, Prapatpong P, Suntornsuk L. Stability indicating method of metformin by capillary zone electrophoresis. (in preparation).
    c. Doomkaew A, Nuchtavorn N, Prapatpong P, Suntornsuk L. Stability indicating method of gliclazide by micellar electrokinetic chromatography. (in preparation).
4. This work was presented in academic meetings and symposiums.
    a. Doomkaew A, Prapatpong P, Buranphalin S, Suntornsuk L. Capillary electrophoretic separation of metformin, gliclazide and their impurities. 20^{th} National Graduate Research Conference. 2-3 February 2011. Mahidol University.
    b. Suntornsuk L. Green Technology in Pharmaceutical Analysis: Capillary electrophoresis and microchip CE. The 7^{th} Pharma IndoChina. 14-16 December 2011, Bangkok, Thailand.
    c. Suntornsuk L. Highlights of a Decade Experience in Capillary Electrophoresis The 6^{th} Asia-Pacific Symposium on Ion Analysis. 26-28 November 2012. Padang, Indonesia.

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ภาคผนวก

Manuscript no. JPBA-D-B-00874

Title:	Rapid and simultaneous analysis of combined anti-diabetic drugs by capillary zone electrophoresis

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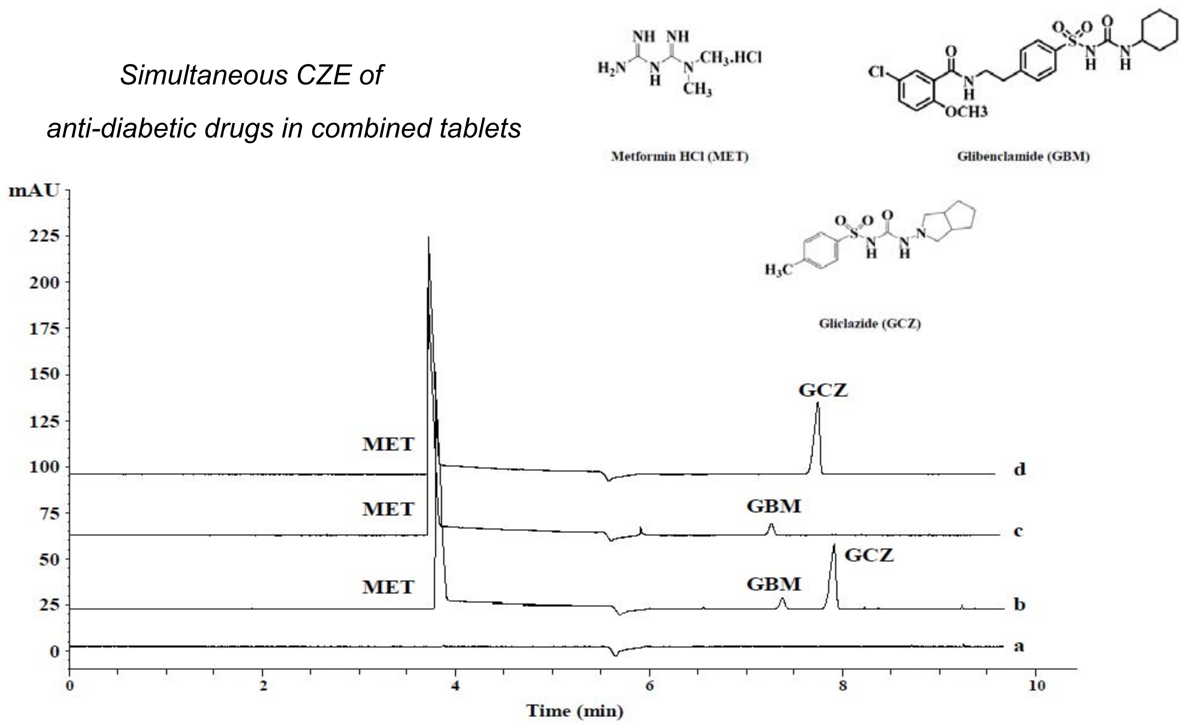
Abstract

A rapid capillary zone electrophoretic method coupled with a photodiode array detector (CZE-DAD) was established and validated for assays of commonly prescribed anti-diabetic drugs (metformin (MET), glibenclamide (GBM) and gliclazide (GCZ)) in thirteen samples including raw material, single and combined tablets. A simple CZE condition using 50 mM borate buffer (pH 9.0) as the background electrolyte, a capillary with an effective length of 56.0 cm and an inner diameter of 50 μm, a voltage of 20 kV, temperature of 25 °C and a detection wavelength at 210 nm was optimized. The conditions allowed separation of all drugs in 8 min. This work demonstrates the first simultaneous analysis of combined anti-diabetic drugs in formulations by CZE. The method provides high resolution of 5.39 (GBM/GCZ) with excellent validation data in terms of linearity (r^2 > 0.99), precision (%RSDs < 1.2%), recovery (99.2-102.9%) and low detection and quantitation limits (< 4 and 12 μg/mL, respectively). The procedure was fast (7 samples per hour) and cost effective, since no organic solvent, sample pre-treatments or clean-up procedures were required. The method was accurate, sensitive and reliable for routine quality control of MET, GBM and GCZ in pharmaceutical products.

Graphical abstract



Keyword: Capillary zone electrophoresis, Combined drugs, Metformin, Gliclazide, Glibenclamide

1. Introduction

Diabetes is a metabolic disorder, resulting in elevated blood glucose levels (> 110 mg/dL, fasting plasma glucose), which affect functions of eyes, kidney, blood vessels, nervous systems and wounds. Diabetes can be generally classified as type 1diabetes or insulin dependent diabetes mellitus (IDDM) and type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM). IDDM is found in patients who no longer can produce insulin and need injectable insulin to control blood sugar levels, while in NIDDM, patients can still produce insulin, but have insulin resistance, impaired insulin secretion or increased glucose production [1]. Ninety percent of the patients is type 2 diabetes and are in need of effective anti-diabetes drugs when dietary modification and exercise fail to maintain the normal glucose levels [2]. Among several classes of anti-diabetic drugs, biguanides (e.g. buformin, metformin (MET, Fig. 1) and phenformin) and sulfonylureas (e.g. carbutamide, glibenclamide (GBM), gliclazide (GCZ) (Fig. 1), *etc.*) are drugs of choice for treatments of diabetes and are effective in the presence of some endogenous insulin production.[3] Metformin is firstly recommended for NIDDM because it does not cause myocardial infarction and produces less hypoglycemia effects than sulfonylurea [3]. GBM and GCZ are second generation sulfonylureas that are also used for treatments of NIDDM. Treatments of type 2 diabetes normally start with MET, however, when functions of insulin-producing cells reduce, and weight gain occurs, additions of a second drugs (i.e. usually sulfonylureas) is required. Presently, combinations of biguanides and sulfonylurea such as MET/GCZ and MET/GBM are commercially available to enhance patient compliance [4]. However, analytical methods for the assays of the combined formulation are not yet available in pharmacopeias and literatures.

British Pharmacopoeia (BP) [5], and The United State Pharmacopeia (USP) [6] recommend non-aqueous titration and high performance liquid chromatography (HPLC) for the assay of MET in raw material and tablets, respectively. GBM raw material and tablet monographs are available in BP and titration and HPLC, respectively, are recommended for their assays. GCZ is official in only BP and non-aqueous titration is proposed for the assay of GCZ raw material, while HPLC is for tablets [5]. Researchers report analyses of MET, GBM and GCZ in raw material, formulations and biological fluids can be performed by various techniques such as HPLC-UV [7-17], HPLC-mass spectrometry (MS) [18-20], capillary electrophoresis (CE) [21-25], spectrophotometry [26,27] and spectrofluorometry [26].

From literature searches, several analytical methods for quantitation of MET, GBM and GCZ are time-, labor-, or solvent-consuming. Importantly, no analytical method has been described analyses of MET/GBM or MET/GCZ in the same formulation (s). Thus, there is a need for a practical method that would facilitate the assay of these drugs in pharmaceutical products. The main focuses of this work were to develop and validate a simple capillary zone electrophoretic (CZE) method for the simultaneous determination of MET, GBM and GCZ in a single run and to apply the method for assays of the drugs in raw material, single and combined formulations. From economics and practical aspects in pharmaceutical industry, it is desirable to have a common method that can be used for quality control of several active pharmaceutical ingredients (API), which might exist in pure forms, single or combined dosage forms.

2. Materials and methods

2.1 Chemicals and reagents

MET standard was purchased from Sigma-Aldrich (Steinheim, Germany). GBM standard was from Bureau of Drug and Narcotic (Nonthaburi, Thailand). GCZ standard and thiourea (as an electro-osmotic flow (EOF) marker) were obtained from Sigma-Aldrich (Missouri, USA). Disodium tetraborate decahydrate was from QRec (Auckland, New Zealand) and boric acid was from Riedel-de Haen (Seelze, Germany). Methanol and acetonitrile were HPLC grade. Water was sterile water for injection.

MET, GBM and GCZ raw materials were gifted from Siam Bheasach (Bangkok, Thailand). MET (500 mg/tablet), GBM (5 mg/tablet) and GCZ (80 mg/tablet) tablets and combined tablets (MET/GBM (500/5 mg/tablet) and MET/GCZ (500/80 mg/tablet) were purchased from local drugstores.

2.2 Instrumentation and electrophoresis procedure

Electrophoresis was performed on a ^{3D}CE instrument model G1600A (Agilent Technologies, Waldbronn, Germany) and controlled by a PC through Agilent ChemStation Plus software version A.08 (G1601A). A fused-silica capillary with a total length of 64.5 cm, an effective length of 56.0 cm and an internal diameter of 50 μm was used. The instrument was equipped with a photodiode-array detector (DAD) and monitoring wavelength was at 210 nm with a bandwidth of 8 nm. Temperature was at 25 ºC and applied voltage was 20 kV. Samples were hydrodynamically injected using pressure at 50 mbar for 10 s.

A new capillary was pre-conditioned with 1 M NaOH, 0.1 M NaOH, water and background electrolyte (BGE) for 15 min, respectively. Between run, the capillary was rinsed with 0.1 M NaOH, water and BGE for 1, 1 and 2 min, respectively.

2.3 Background electrolyte preparation and standard solution preparation

CZE separation of MET, GBM and GCZ was achieved by using 50 mM borate buffer (pH 9.0) as the BGE. The buffer was prepared by dissolving 0.5034 g of disodium tetraborate decahydrate in sufficient amounts of water, adjusted to pH 9.0 with 0.65 M boric acid and adjusted the final volume to 50 ml with water. The buffer was stable for 2 days after preparation.

Stock standard solutions were prepared by separately dissolving 500 mg of MET, 10 mg of GBM and 80 mg of GCZ in 50, 100, and 50 ml methanol, respectively. The stock standard solutions (10 mg/mL for MET, 0.1 mg/mL for GBM and 1.6 mg/mL for GCZ) were protected from light and stored in a refrigerator at 2-8 ºC, which were stable up to one month. The stock standard solutions were diluted ten times with methanol to obtain the working standard solutions (1,000 µg/mL for MET, 10 µg/mL for GBM and 160 µg/mL for GCZ). All solutions were filtered through 0.2 µm membranes and sonicated for 10 min before uses.

2.4 Method validation

Method validation was performed according to ICH guidelines [28]. Linearity was performed at five different concentrations in ranges of 50-150% of nominal concentrations (1,000, 10 and 160 µg/mL for MET, GBM and GCZ, respectively) of each drug. Each standard solution was injected in triplicate at 50 mbar for 10 s and analyzed on three different days using the previously specified conditions. Peak areas were then plotted against the corresponding concentrations to obtain the calibration curves. Precision was determined at 80% (n = 3), 100% (n = 6) and 120% (n = 3) of nominal concentrations of each drug. Intra-day precision was evaluated from three assays within the same day and inter-day precision was performed on three different days. Inter-day and intra-day precision was represented as percent relative standard deviations (%RSDs) of peak area and migration time. Accuracy was established by standard addition method, in which known amounts of the standards at varied concentrations were added into commercial tablets. The experiments were performed on three different days. The standard added samples were analyzed and the mean percent recoveries (%R), %RSDs and %bias were calculated. Specificity of the method was tested by injecting a standard drug mixture, containing 1,000 µg/mL for MET, 10 µg/mL for GBM and 160 µg/mL for GCZ, in comparison with BGE, solvent (methanol) and commercially tablets, both single and combined formulations. Their electropherograms were recorded and investigated for any extra peaks or interferences from the BGE, solvent or sample matrices. Limits of detection and quantitation were based on the 3 and 10 times of the standard deviations of responses over the slope of calibration curves. The concentrations at LOQ levels were analyzed in triplicates and %RSDs were calculated.

2.5 Applications

Applications of the developed CZE method on assays of MET, GBM and GCZ in raw material, single and combined formulations were performed in thirteen samples (i.e. three lots of raw material, six and four lots of single and combined tablets, respectively). For raw material, the drug powder was weighed, dissolved and diluted with methanol to obtain the concentrations of 1,000 µg/mL for MET, 10 µg/mL for GBM and 160 µg/mL for GCZ. For tablet dosage forms, both single and combined products, ten tablets from each sample were grounded, accurately weighed, dissolved with methanol, sonicated for 30 min and centrifuged at 4,500 rpm (x 906 *g*) for 10 min. The filtrate was then diluted with methanol to obtain the nominal concentrations (1,000 µg/mL for MET, 10 µg/mL for GBM and 160 µg/mL for GCZ). Each sample was injected three times into the CE using the proposed CZE conditions.

3. Results and Discussion

3.1 Capillary zone electrophoresis condition

It is our objective to establish a method for the simultaneous analysis of three major anti-diabetic drugs (MET, GBM and GCZ), since combined formulations of these drugs are commercially available, but no official methods are reported. CZE-DAD, using 50 mM borate buffer (pH 9.0), was a system of choice due to its simplicity and availability. The capillary was a standard bare fused silica capillary with an effective length of 56.0 cm and an inner diameter of 50 µm. The separating voltage of 20 kV and temperature of 25 ºC were chosen as a compromise of the resolution and run time. A detection wavelength at 210 nm was selected to ensure the maximum UV absorption of GBM, which exists in much lower amounts in the combined formulations. Under these conditions, MET migrated as the first peak, prior the EOF, since it was protonated and remained in cationic form. GBM and GCZ were deprotonated and migrated as anionic species after the EOF, indicated by the negative sign of their electrophoretic mobilities (Table 1). Table 1 reveals the analytical parameters and system suitability test data of the analytes under the developed CZE conditions, which confirmed that the method is suitable for the intended purpose. The conditions could provide baseline separation of the drugs in 8.2 min (Fig. 2) with resolution higher than 5.39, tailing factor close to 1.0 and number of plates greater than 2.5 x 10^4. The separation was repeatable with %RSDs of migration time and peak area of less than 1.36%.

3.2 Method validation

Method validation data in terms of linearity, specificity, precision, accuracy, limits of detection and quantitation is shown in Table 2 and 3. The method was linear over the studied ranges with high coefficients of determination (r^2 > 0.99) in all cases (Table 2). Specificity test revealed that buffer composition, solvent and sample matrices both from the single and combined formulations did not interfere the analysis, indicating that the method was specific for the investigated drugs (Fig. 2 and 3). Within- and between-day precision offered %RSDs of less than 1.51% for peak area and 0.32% for migration time (Table 3), thus the method is highly precise. Method accuracy is good, represented by %recoveries between 99.2 to 102.9% for all drugs with %bias of less than 2.9% (Table 3). Limits of detection and quantitation were in low levels of less than 4 and 12 µg/mL, respectively (Table 2), which were sensitive for assays of MET, GBM and GCZ in raw material and drug products. Validation results show that the method is efficient, precise and accurate for assays of MET, GBM and GCZ in pharmaceutical products.

3.3 Applications

Thirteen samples including MET, GBM and GCZ raw material, single and combined tables were analyzed using the proposed method. All assays could be achieved with minimal sample preparation by only dissolving and diluting the samples. Typical electropherograms of the analyzed samples are presented in Fig. 3b-3d and 4c-4d. Assay results show percent label amounts of all samples between 99.7 and 101.3 with %RSDs of less than 1.80% (Table 4). Percent label amounts of the studied raw material and single tablet samples meet either the BP or USP limits. There is no limit for the drugs in combined tablets since the formulations are not official in both pharmacopeias. However, percent label amounts of the investigated combined products were within 95.0-105.0%.

4. Conclusion

The developed CZE-DAD method offers convenience for accurate simultaneous analysis of MET, GBM and GCZ in pharmaceutical raw material, single and combined formulations. The uncomplicated sample preparation procedures, simple BGE containing no organic solvent and short analysis time allow the rapid quantitation of the drugs in samples, which is suitable for routine analytical work in quality control departments. The method is cost- and time-effective with high throughput.

Acknowledgements

Funding for this work was from Thailand Research Fund and Office of the Higher Education Commission (RMU5380012) and Nation Research Council of Thailand through Mahidol University (NRC) (NG P 0812555).

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Table 1 Analytical parameters and system suitability test data[a]

Analytical parameter	MET (1,000 µg/mL)	GBM (10 µg/mL)	GCZ (160 µg/mL)
Electrophoretic mobility ($x10^{-8} m^2/V.s$)	2.31	-1.16	-1.42
Migration time (min)	4.06	7.64	8.17
%RSD of t_m (n = 6)	0.15	0.23	0.24
Resolution	-	36.8	5.39
Tailing factor	0.83	1.00	0.91
Number of plates ($x10^5$)	0.25	1.14	3.63

[a]: MET = metformin, GBM = glibenclamide, GCZ = gliclazide, t_m = migration time

Table 2 Linearity, limits of detection and quantitation data[a]

	Linear equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
MET (500-1,500 μg/mL)	y = 0.4054x + 56.88	0.9940	2	5 (1.28)
GBM (5-15 μg/mL)	y = 2.1112x + 0.4152	0.9973	2	5 (1.46)
GCZ (80-240 μg/mL)	y = 1.0520x + 9.1478	0.9929	4	12 (0.72)

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

Table 3 Precision and recovery data[a]

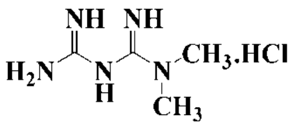
	Precision						%Recovery		
	Day 1		Day 2		Day 3		Day 1	Day 2	Day 3
	$\%RSD_{PA}$	$\%RSD_{tm}$	$\%RSD_{PA}$	$\%RSD_{tm}$	$\%RSD_{PA}$	$\%RSD_{tm}$			
MET	0.21	0.27	0.25	0.10	0.17	0.08	99.2 (1.47)	99.9 (0.35)	100.2 (0.15)
GBM	1.17	0.31	1.18	0.20	1.51	0.18	100.8 (2.68)	101.8 (0.34)	100.9 (2.06)
GCZ	0.47	0.32	0.25	0.21	0.39	0.20	100.0 (0.0)	100.0 (0.0)	100.0 (0.0)

[a]: PA = peak area, tm = migration time, MET = metformin, GBM = glibenclamide, GCZ = gliclazide, number in parenthesis represents %RSD

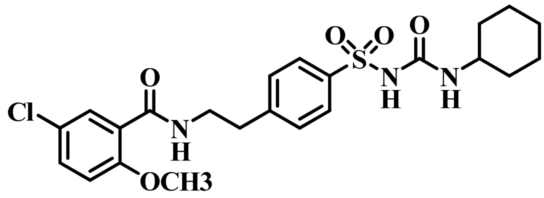
Table 4 Application (n = 3)[a]

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)	-	-

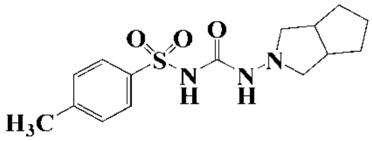
[a]: MET = metformin, GBM = glibenclamide, GCZ = gliclazide, number in parenthesis represents %RSD



Metformin HCl (MET)



Glibenclamide (GBM)



Gliclazide (GCZ)

Fig. 1 Structures of the investigated compounds.

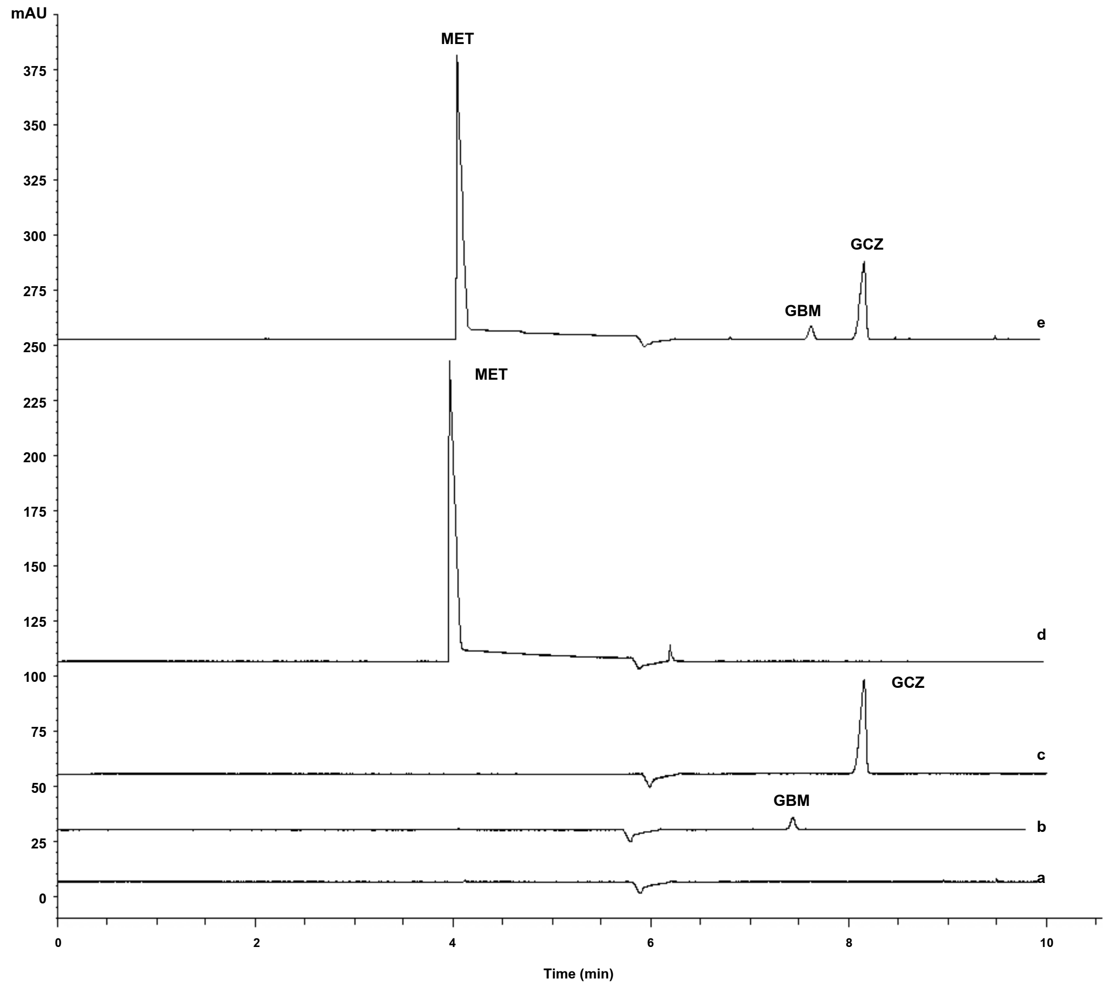


Fig. 2 Electropherograms of a) solvent (MeOH), b) GBM tablet (10 µg/mL), c) GCZ tablet (160 µg/mL), d) MET tablet (1,000 µg/mL), e) standard MET (1,000 µg/mL), GBM (10 µg/mL) and GCZ (160 µg/mL); Condition: 50 mM borate buffer pH 9.0; capillary 64.5 cm total length, 56.0 cm effective length, 50 µm ID, injection at 50 mbar, 10 s; 20 kV; 25°C; detection by DAD absorbance at 210 nm.

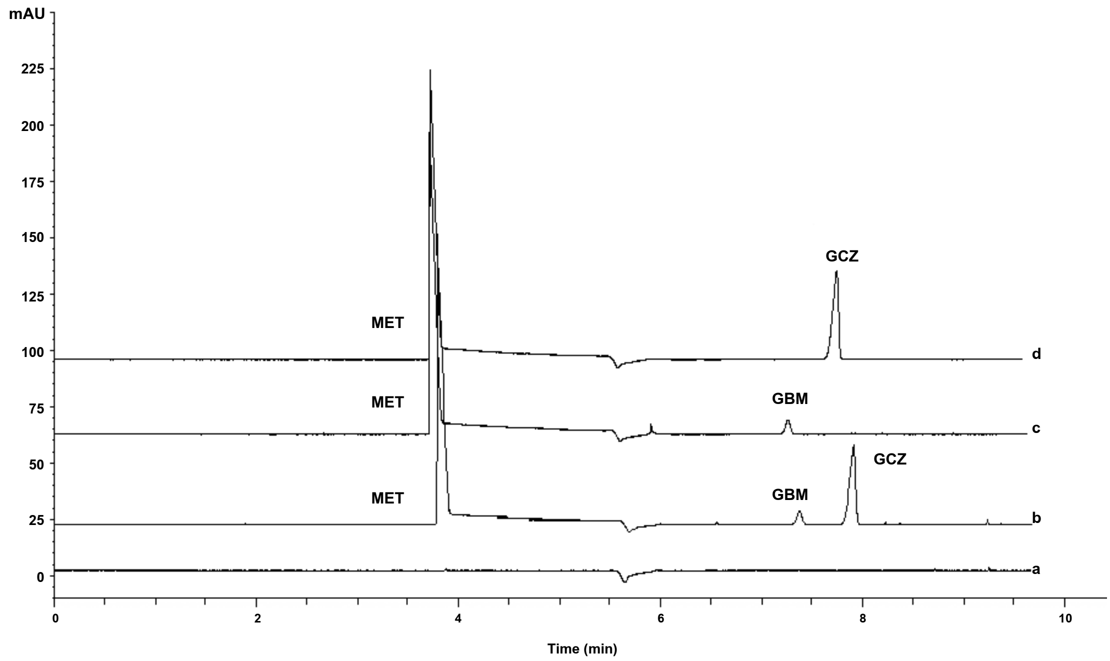


Fig. 3 Electropherograms of a) solvent (Me OH), b) standard MET (1,000 µg/mL), GBM (10 µg/mL), GCZ (160 µg/mL), c) combined MET (1,000 µg/mL) and GBM tablet (10 µg/mL), d) combined MET (1,000 µg/mL) and GCZ tablet (160 µg/mL); Condition: 50 mM borate buffer pH 9.0; capillary 64.5 cm total length, 56.0 cm effective length, 50 µm ID, injection at 50 mbar, 10 s; 20 kV; 25 $^{\circ}$C; detection by DAD absorbance at 210 nm.