METFORMIN, GLICLAZIDE AND GLIBENCLAMIDE ASSAY BY MICROEMULSION ELECTROKINETIC CHROMATOGRAPHY

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Abstract. A new microemulsion electrokinetic chromatography (MEEKC) method has been developed for the determination of metformin, gliclazide and glibenclamide, using a microemulsion consisting of 3.3% SDS, 6.6% n-butanol, 0.8% n-heptane and 89.3% 20 mM borate buffer (pH 9.0) as running electrolyte, 23 kV applied voltage at 30 °C, with direct UV detection at 208 nm. The method was found to be linear between 30 and 300 µg/mL for each drug. Quantitative results were calculated in respect to peak area and in respect to peak area *versus* time ratio, the latter providing more accurate results for the highly lipophilic sulfonylurea drugs.

Key words: glicazide, metformin, glibenclamide, microemulsion electrokinetic chromatography.

INTRODUCTION

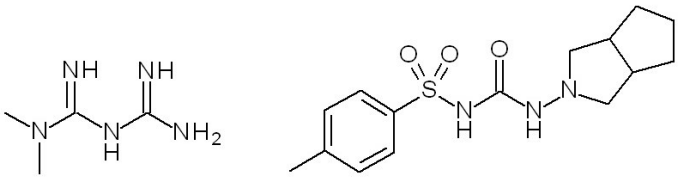
Electrokinetic chromatography (EKC) is a capillary electrophoretic separation technique introduced by Terabe in 1985. Instead of the simple buffer solution used in capillary zone electrophoresis, Terabe used an ionic micellar solution, the micelles acting as a pseudostationary phase. Separation in EKC is the result of a combination of chromatographic partitioning of solutes between the pseudostationary phase and the buffer phase (based on their hydrophobicity) and electrophoretic migration. This allows the separation of neutral as well as charged solutes, an advantage over the more frequently used capillary zone electrophoresis (CZE). The two most frequently used types of EKC are micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC).

Received: July 2016;
in final form August 2016.

ROMANIAN J. BIOPHYS., Vol. 26, No. 2, P. 93–106, BUCHAREST, 2016

MEEKC uses buffered microemulsions (ME) as the separation media, either oil in water (O/W) ME or water in oil (W/O) ME, the O/W type being the most frequently used.

The separation mechanisms are similar for both MEKC and MEEKC, but solutes are more easily able to penetrate the surface of a droplet rather than the rigid surface of a micelle. The MEs greater solubilizing power for both polar and non-polar compounds allows MEEKC to be applied to a wider range of solutes than MEKC. Also MEEKC provides a larger separation window resulting in greater separation capability for water-insoluble compounds. Thus MEEKC offers the possibility of resolving complex mixtures of solutes having very wide ranges of polarity and solubility. Its applications are equally wide in range: water solubility/hydrophobicity assessments, determination of various food, cosmetics and agricultural chemicals, drugs and excipients, bioanalysis, natural product analysis, environmental analysis, chiral separation [2, 10, 11, 13, 21].



Metformin

pKa1 = 2.8; pKa2 = 11.5

logP = 2.6

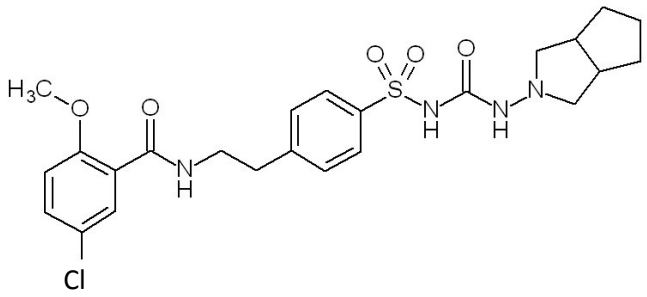
λmax = 233 nm

Gliclazide

pKa = 5.8

logP = 2.1

λmax = 230 nm



Glibenclamide

pKa = 5.3

logP = 4.8

λmax = 226 nm

Fig. 1. Chemical structures and characteristics of the three antidiabetics used.

Metformin (MT), a biguanide oral antidiabetic, glibenclamide (GB) and gliclazide (GZ), both oral sulfonylurea drugs, are frequently used separately or combined in type 2 diabetes mellitus. Structural formulas and basic physical-chemical characteristics of the three drugs are presented in Figure 1.

MT is the drug of first choice in overweight patients and it has also been studied in the treatment of gestational diabetes and polycystic ovary syndrome [16]. MT and GZ are the only two oral antidiabetics on the World Health Organization Model List of Essential Medicines, GZ having replaced GB, formerly on the list, since GB is not recommended for people over 60 years old [27]. Combinations of MT and GB, and of MT and GZ are commercially available. They have been studied in combination through a variety of techniques: near infrared spectroscopy [6], wavelet transforms [14], liquid chromatography-tandem mass spectrometry [5, 20, 23], high performance liquid chromatography [6, 13, 17, 24], micellar liquid chromatography [5], CZE [7, 15, 18], and MEKC [9, 12], but up to date there are no articles studying their behavior under MEEKC conditions. These analytes were chosen also because of the great difference in hydrophobicity between metformin and the sulfonylureas, and the similar hydrophobic character of the latter, which suites the applicability of MEEKC.

The purpose of this paper was to develop a MEEKC method for the simultaneous determination of these oral antidiabetics.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

All reagents used were of analytical grade: acetic acid glacial (Scharlau Chemie S.A.), sodium acetate anhydrous (Scharlau Chemie S.A.), disodium hydrogen phosphate (Merck), sodium dihydrogen phosphate (Merck), sodium tetraborate (Fluka), n-butanol (Chimactiv S.R.L.), n-heptane (Rotisolv, Carl Roth GmbH), sodium dodecyl sulphate (SDS) (Merck).

Glibenclamide was a gift from S.C. Laur Med S.R.L, gliclazide was a gift from S&D Chemicals and metformin hydrochloride was a gift from Chemicals Pharmaceuticals Manufacturing Impex S.R.L.

The buffers (10 mM acetate buffer solution pH = 4 and 10 mM phosphate buffer solution pH = 7, 10–40 mM borate buffer solution pH = 9) were prepared by adding the appropriate amounts of chemicals and ultrapure water to volumetric flasks [25]; pH was measured by a Metrohm 716 DMS Titrino pH-meter.

SDS-butanol-heptane-buffer microemulsions were prepared by weighing the appropriate amounts of SDS, butanol and heptane in volumetric flasks to yield 3.3% w/v SDS, 6.6% w/v butanol and 0.8% w/v heptane, followed by the addition of the buffer solution; the microemulsions were sonicated for 30 min.

Mixtures of gliclazide, glibenclamide and metformin were prepared by weighing amounts (corresponding to 0.3–0.5 mg/mL each) in volumetric flasks, followed by the addition of the working microemulsion and sonication for 30 min. Working standards and samples were obtained by diluting these mixtures with the working microemulsion to the desired concentration, followed by another sonication for 5 min.

The water used was ultrapure water obtained with Easypure (Rodi Barnstead) water purification system whenever necessary, an Elma S100H Elmasonic ultrasound bath was used for sonicating the solutions and microemulsions. All solutions and microemulsions were filtered through 0.45 μm syringe microfilters before use.

ELECTROPHORETIC SYSTEM AND CONDITIONS

MEEKC was carried on a G1600A system (Agilent Technologies), equipped with an on-column diode-array detector and a temperature control device. Data was acquired using Agilent Chemstation version B.0 2.0 software. A 50 μm internal diameter, 48 cm (40 cm effective length) fused-silica capillary was used.

The working microemulsion consisted of 3.3% SDS, 6.6% n-butanol, 0.8% n-heptane and 89.3% buffer solution.

At the start of each working day the capillary was rinsed with 1 M NaOH for 10 min, water for 15 min, and conditioned with the working microemulsion for 15 min. In between runs the capillary was flushed for 6 min with microemulsion.

The analytes were detected at 208 nm, full spectra being recorded between 200 and 600 nm. Measurements were performed at 30 °C. The applied voltage was 23 kV. Hydrodynamic injection, 5 s at 50 mbar was employed.

RESULTS

PRELIMINARY STUDIES

Initial measurements were performed at 25 °C, hydrodynamic injection at 20 kV and 50 mbar for 5 s, and maximum analysis time was set at 50 min. A higher voltage applied resulted in a better signal for small amounts of drug; therefore voltage was set at 23 kV.

The analytes were detected at 208, 226, 233 and 300 nm (Fig. 2), with full spectra being recorded between 200 and 600 nm; based on peak heights, 208 nm was selected as the optimum detection wavelength.

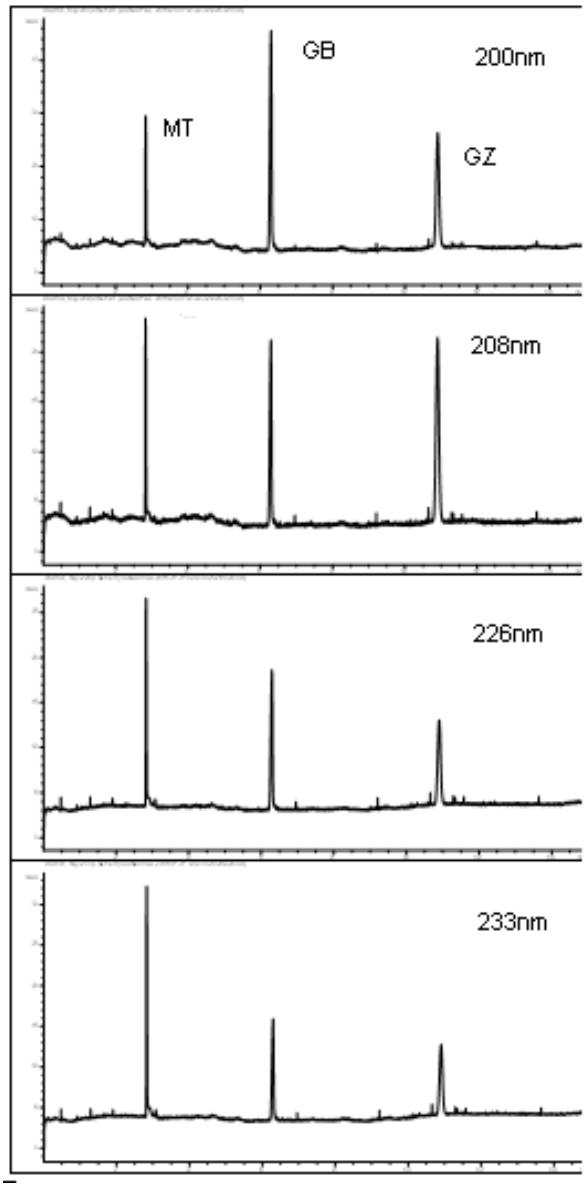


Fig. 2. Electrophoregrams of MT, GB and GZ, 200 μg /mL each, hydrodynamic injection (5 s, 23 kV, 50 mbar, 30 °C) detection at 200, 208, 226 and 233 nm.

METHOD OPTIMIZATION

Working microemulsion composition

Six microemulsions were prepared, with SDS as surfactant, and n-butanol as co-surfactant; the organic solvent and buffer used are given in Table 1. The proportions were 3.3% surfactant, 6.6% co-surfactant, 0.8% organic solvent and 89.3% buffer solution (proportions were taken from literature [2, 10, 11, 13, 21]). These MEs were studied as separation media under the same initial working conditions.

Effect of the buffer type and pH was investigated using 3 buffer systems: 10 mM acetate buffer solution pH 4.0; 10 mM phosphate buffer solution pH 7.0; 40 mM borate buffer solution pH 9.0 (Table 1).

Table 1

The composition of the microemulsions tested

Crt. No.	Organic solvent	Buffer solution
1	n-heptane	10 mM acetate buffer pH 4.0
2	n-heptane	10 mM phosphate buffer pH 7.0
3	n-heptane	40 mM borate buffer solution pH 9.0
4	ethyl acetate	10 mM acetate buffer pH 4.0
5	ethyl acetate	phosphate buffer solution pH 7.0 (10 mM)
6	ethyl acetate	borate buffer solution pH 9.0 (40 mM)

Due to the acidic pH, in the case of MEs with pH = 4.0 acetate buffer the electroosmotic flow (EOF) was reduced leading to increased migration time, GZ and GB would not reach the detector within 50 min of analysis time, whereas MT would have a migration time greater than 25 min.

In the case of the MEs with pH = 7.0 phosphate buffer, all the analytes would migrate to the detector, but within a long analysis time (30 min for GB which reached the detector last). Other pH values for phosphate buffer system were not investigated because even at low buffer concentration (10 mM) generated a rather high current.

Borate buffer is commonly used in capillary electrophoresis, as it generates lower current than other buffer systems. The MEs in pH = 9.0 borate buffer allowed the separation of MT, GB and GZ with good resolution in about 23 min under the initial conditions.

In the case of the three MEs using ethyl acetate as organic phase the background noise was higher than in the case of the three n-heptane MEs. Therefore, pH = 9.0 borate buffer and n-heptane were selected for further method development.

Figure 3 summarizes these observations.

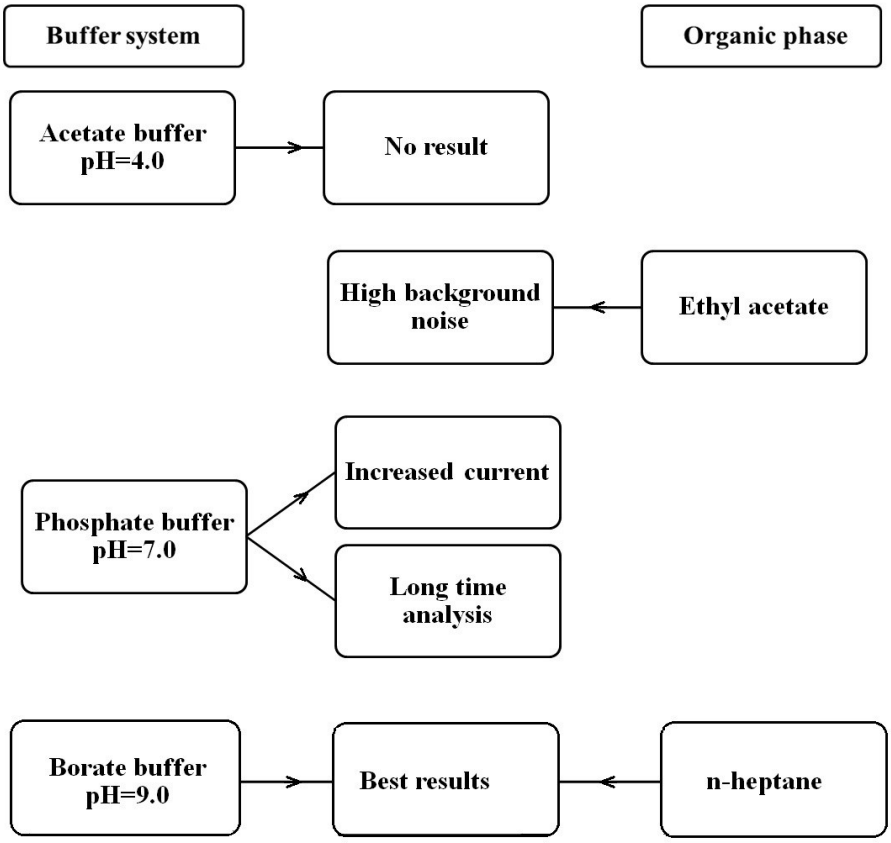


Fig. 3. The influence of buffer system and of the organic phase of the microemulsion on separation.

Sample preparation

Microemulsion was selected for the sample preparation as it dissolved all three drugs.

During the method development we tried to use a hydroalcoholic mixture of stock solutions of GB, GZ in methanol, and MT in water, but after mixing the stock solutions GB and GZ would precipitate in time. We also tried to use a solution of the analytes in borate buffer, which resulted in low peak heights.

Effect of buffer concentration

The concentration of the borate buffer was varied from 40 mM to 10 mM. The electrophoretic mobility slightly decreased with increasing concentration of the buffer for GB and GZ, while remaining rather constant for MT (Fig. 4), whilst the generated current increased (Fig. 5); 20 mM was selected for further studies.

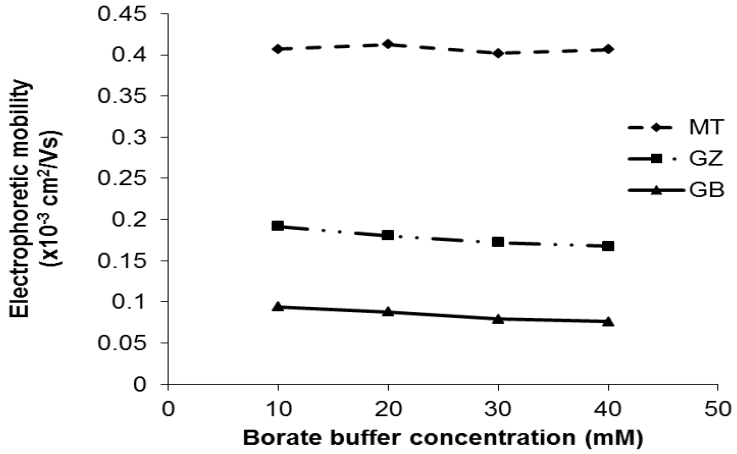


Fig. 4. Effect of borate buffer concentration on electrophoretic mobility, 20 kV, 25 °C.

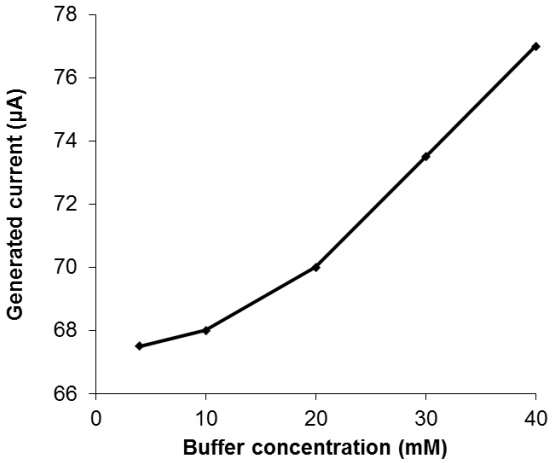


Fig. 5. Effect of borate buffer concentration on the generated current.

Effect of the applied voltage

The effect of the applied voltage was investigated between 15 and 25 kV, at 25 °C. As expected, migration time decreased and effective mobilities increased with increasing voltage; 23 kV was selected as a compromise between migration times and generated current.

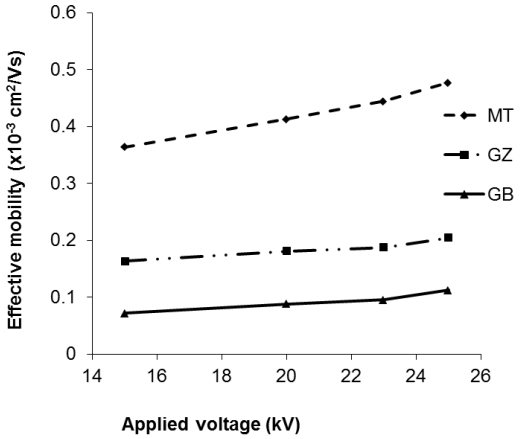


Fig. 6. Effect of applied voltage on effective mobility ($cm^2/V \cdot s$) of metformin, gliclazide and glibenclamide.

Effect of the temperature

The effect of the temperature was tested at 20, 25 and 30 °C (Fig. 7). Increasing temperature significantly decreased analysis time, most likely due to lowering the viscosity of the ME, 30 °C was selected for further studies.

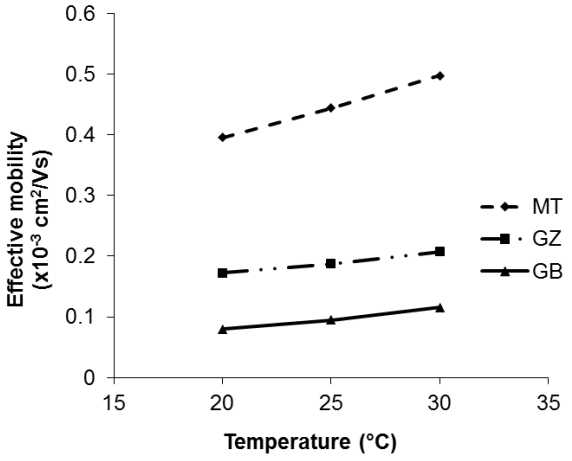


Fig. 7. Effect of temperature on effective mobility of metformin, gliclazide and glibenclamide.

Effect of amount of sample injected

The effect of the amount of sample injected was studied by varying the injection time from 3 to 10 s at 50 mbar, injection times of 7 s or higher produced deformed peaks, injection times under 5 s produced low peaks, an injection time of 5 sec was selected as optimum (Fig. 8).

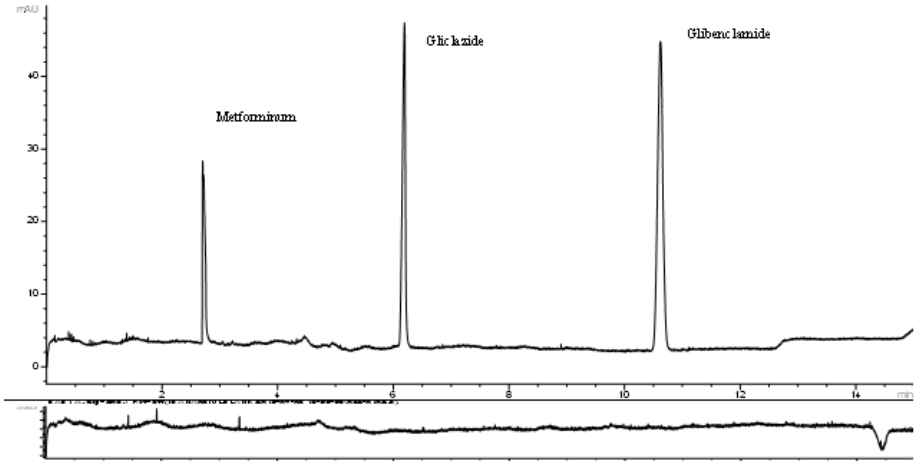


Fig. 8. Electrophoregram of a mixture of metformin, gliclazide and glibenclamide (250 µg/mL each), and electrophoregram of a blank injection, under final working conditions: temperature 30 °C; hydrodynamic injection 5 s 23 kV, at 50 mbar.

EVALUATION OF CERTAIN VALIDATION PARAMETERS

We evaluated certain validation parameters (linearity, limits of detection and quantification, repeatability, intermediate precision) following ICH Q2(R1) guidelines [9].

Published CE methods (including MEKC and MEEKC) commonly use peak areas for quantitative analysis, although according to Ph.E and USPE in MEKC peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response, and also to compensate for the different responses of sample constituents with different migration times [24]. Other sources advise the same for CE coupled with UV detection, because detection occurs when separation is still taking place, as such the time analytes spent in the detector is affected by their different velocities, and this affects the respective peak areas [3, 17].

We calculated results both from peak areas and from the ratio of peak areas *versus* migration time respectively, for the developed method.

Linearity, limit of detection (LOD) and quantification (LOQ)

Linearity was performed using seven different concentrations of MT, GZ and GB respectively; each concentration was analyzed in 5 consecutive runs. Both average peak areas and the average ratios of peak areas *versus* migration times were plotted against the corresponding concentrations to obtain the calibration curves. The response factor of the UV detector was found to be linear in the studied range of concentrations.

The drug concentrations calculated based on the regression equations were plotted against the known amounts, the correlation coefficients being above 0.999 (Table 2). The method was found to be linear between 30 and 300 µg/mL for each drug.

Limits of detection (LOD) and quantitation (LOQ) were calculated based on the standard deviation of the response (standard deviation of the intercepts of regression lines) and the slope of the calibration curve. Linearity data along with LOD and LOQ are summarized in Table 2.

Table 2

Regression equations and calculated limits of detection and quantification for linearity data

		Regression parameters	Correlation coefficient	LOD (µg/mL)	LOQ (µg/mL)
Metformin	Area (mAU·s)	y = 0.3329 x – 0.2254	0.9995	5.47	16.58
	Area/Time (mAU·s/min)	y = 0.1209 x – 0.1378	0.9998	5.01	15.18
Gliclazide	Area (mAU·s)	y = 0.5808 x – 0.2211	0.9999	7.34	22.25
	Area/Time (mAU·s/min)	y = 0.0927 x – 0.051	0.9999	8.85	26.81
Glibenclamide	Area (mAU·s)	y = 1.0704 x + 0.3872	0.9998	9.51	28.82
	Area/Time (mAU·s/min)	y = 0.0997 x – 0.03801	0.9997	9.01	27.32

Accuracy and precision

For all the substances investigated the correlation between the known and calculated amounts of substance used for the regression line is very good (Table 3).

Table 3

Correlation between known amounts and calculated concentrations for linearity data

	Slope of the regression line of known concentrations *vs*. calculated concentrations using regression curves from Table 2	
	area regression equation	area/time regression equation
Metformin	1	1
Gliclazide	1.000545	1
Glibenclamide	1.00139	1

Repeatability was determined in ten replicates at the concentration of 90 µg/mL for each drug (mixture of 92.40 µg/mL MT, 90.24 µg/mL GZ and 90.48 µg/mL GB, all within the confidence interval of 95%), and was expressed as percent relative standard deviations (%RSD) of migration times, peak areas, and ratios of peak areas *vs* time (Table 4).

Table 4

Repeatability

	Migration time (min)	RSD %	Area (mAU·s)	RSD %	Area/Time (mAU·s/min)	RSD %
MT	2.83 ± 0.02	1.24	30.98 ± 0.31	1.60	10.96 ± 0.07	1.07
GZ	6.63 ± 0.06	1.52	57.68 ± 2.14	5.99	8.70 ± 0.27	4.92
GB	11.72 ± 0.15	2.09	120.89 ± 2.45	3.28	10.31 ± 0.15	2.41

Intermediate precision was studied at six different concentrations (75, 175, 275, 160, 200, 240 µg/mL for each drug) on three different days for each concentration (in 3 or more consecutive injections, n = 72 injections, within the confidence interval of 95%), and was represented as percent relative standard deviation (%RSDs) of percent recoveries (Table 5).

Table 5

Intermediate precision

	Recovery % (Area)	RSD%	Recovery % (Area/Time)	RSD%
MT	101.69 ± 1.64	6.99	98.64 ± 1.26	5.52
GZ	107.33 ± 2.16	8.78	99.02 ± 1.73	7.56
GB	113.07 ± 2.03	7.72	99.57 ± 1.51	6.53

CONCLUSION

A MEEKC method was developed for the simultaneous determination of metformin, gliclazide and glibenclamide, three frequently used oral antidiabetics. The method was found to be linear. We compared results calculated according to peak areas with results calculated according to peak areas *vs* time ratios. For all three studied drugs, the variation coefficients of calculated concentrations were slightly lower when using area *versus* time ratio regression equation, than when using area regression equation. More significantly, in the case of lipophilic substances, strongly interacting with the pseudostationary phase, expressing quantitative results as peak area *versus* time ratio provides better recovery than in respect to peak areas. For gliclazide recovery improved from 107.33% to 99.02%, whereas for glibenclamide, which is completely retained in the ME droplet due to

its highly lipophilic nature, recovery improved from 113.07% to 99.57%. In the case of metformin which is highly hydrophilic, thus not retained in the ME droplet, using area *versus* time ratio did not bring a significant improvement in recovery.

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