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The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma

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Abstract

This article describes the development of SPE and HPLC methods for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. Several extraction and HPLC methods have been described previously for the determination of each of these analytes in plasma separately. The simultaneous determination of these analytes is important for the routine monitoring of diabetic patients who take combination medications and for studying the pharmacokinetics of the combined dosage forms. In addition this developed method can serve as a standard method for the plasma determination of these analytes therefore saving time, effort and money. The recoveries of the developed methods were found to be between 76.3% and 101.9%. The limits of quantification were between 5 and 22.5 ng/ml. The intraday and interday precision (measured by coefficient of variation, CV%) was always less than 9%. The accuracy (measured by relative error %) was always less than 12%. Stability analysis showed that all analytes are stable for at least 3 months when stored at −70 °C.
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1. Introduction

Currently the most commonly prescribed medications for Type 2 diabetes are metformin and the second generation sulfonylureas which include glipizide, gliclazide, glibenclamide and glimperide. For many patients with Type 2 diabetes, monotherapy with an oral antidiabetic agent is not sufficient to reach target glycaemic goals and multiple drugs may be necessary to achieve adequate control [1]. In such cases a combination of metformin and one of the sulfonylureas (SU) is used [2]. This combination can be achieved by taking each of the drugs separately or alternatively fixed formulations have been developed. Combinations of metformin and glipizide or gliclazide or glibenclamide are available commercially as single tablets. A combination tablet formulation is beneficial in terms of its convenience and patient compliance. The measurement of the plasma concentrations of antidiabetic medications is important for studying the pharmacokinetics of these drugs, for adherence and drug monitoring in diabetic patients and for diagnostic purposes in factitious hypoglycaemia.

The choice of treatment for diabetic patients is mainly dependent on the doctors' choice which should be dependent on the patients' clinical characteristics and the pharmacological properties of the various agents available, thus, for certain diabetic populations we can find patients who are prescribed glipizide, gliclazide, glimperide, glibenclamide, metformin or a combination of metformin and one of the sulfonylureas.

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Therefore, therapeutic monitoring requires the availability of a single method that can be used for all these possibilities in order to save time, cost ad effort.

Several procedures have been developed to be used as standard methods for the analysis of sulfonylureas [3–6]. However, none of these methods were suitable for routine analysis. Some of them used solvent extraction in sample preparation [4,5] which is a time consuming process and loss of sample may frequently occur during extraction due to emulsion formation in addition to the reported low recovery. Even though Paroni et al. [3] used solid phase extraction (SPE) utilising OASIS® HLB cartridges, they used five washing steps during the extraction process which is not practical and is time consuming, in addition they used a gradient elution HPLC method. The SPE method developed by Strausbauch et al. [6] was only validated for urine samples. A more important limitation for implementing the previously mentioned procedures for routine analysis of antidiabetic medications is that they were all developed for estimation of glipizide, gliclazide and glibenclamide but not for glimperide or metformin.

Until now glimperide was only analysed in biological matrices by an HPLC method using derivatisation and solvent extraction which is tedious and time consuming [7]. Glimperide is relatively new and it was reported that the commonly used methods for sulfonylurea determination have low sensitivity for glimperide determination [7]. Niemi et al. [8] used an HPLC–mass spectrometry (MS) method for studying the effect of rifampicin on the pharmacokinetics of glimperide, however, the method was not validated and the use of MS has the disadvantage of that it is not available in many laboratories.

There is no single published method for the simultaneous determination of both metformin and any of the sulfonylureas in biological fluids. A method for determination of metformin and glipizide or gliclazide [9] and a method for the estimation of metformin and glibenclamide from their combined dosage forms [10] have been described previously for use in studying pharmaceutical preparations but not for analysis in biological fluids. When studying the pharmacokinetics of a new formulation containing a combination of metformin and glibenclamide Martha et al. [1] used two separate methods one for measuring the concentration of metformin and the other for glibenclamide.

In summary, in order to save time and money routine therapeutic monitoring requires the availability of a single method that can be used for the simultaneous determination of antidiabetic medications in plasma. Although, many methods have been reported in the literature for the estimation of metformin, gliclazide, glibenclamide and glipizide individually there is no single method reported for the simultaneous estimation of metformin and sulfonylureas. The reported general procedures for sulfonylurea determination did not include glimperide or metformin. In a previous study [11] the first ion pair solid phase extraction technique was developed for the specific HPLC determination of metformin. In this study that method was optimized for the simultaneous determination of a combination of metformin and sulfonylureas.

2. Experimental

2.1. Reagents and materials

Metformin (M), phenformin (P), glibenclamide (glyburide) (Gb), tolbutamide (T), glipizide (Gp), potassium dihydrogenphosphate (KH_2PO_4), sodium dodecyl sulphate (SDS, sodium lauryl sulphate), decane sulfonic acid sodium salt and heptanesulfonic acid sodium salt were purchased from Sigma (Poole, England). Glimperide (Gm) was provided by Aventis Pharma (Frankfurt, Germany). Gliclazide (Gc) was extracted (according to a British Pharmacopoeial procedure, 1998) from Gliclazide 80 mg tablets which were purchased from Generics (Herts, UK). Acetonitrile, methanol, and tetrahydrofuran (THF) were of HPLC grade and purchased from Romil (Cambridge, UK). Membrane filters F-4500.45 μm were obtained from Gelman Laboratory (Portsmouth, UK). Solid phase extraction cartridges [Waters Oasis® HLB and MCX cartridges (1 ml, 30 mg)] were purchased from AGB, Belfast. Extraction was carried on a Waters extraction manifold. Blank blood was donated from Northern Ireland Blood Transfusion Centre.

2.2. Internal standards

For the simultaneous determination of M and Gb or Gm (Method 1) the internal standard was P and for the simultaneous determination of M and Gp or Gc (Method 2) the internal standard was T. Stock solutions of the internal standards (P and T) were prepared by dissolving 0.016 g in 100 ml of water (initially dissolved in a few drops of methanol) and then further diluted with water (1:40). When preparing samples for extraction 50 μl of this stock was added to 1 ml of plasma to produce an internal standard concentration of 200 ng/ml plasma.

2.3. Preparation of the mixed standards

For the determination of a combination of M and Gb or Gm (Method 1) the stock was prepared by dissolving 0.08 g of M and 0.04 g of Gb and Gm in 100 ml methanol. For the determination of a combination of M and Gp or Gc (Method 2) the stock was prepared by dissolving 0.04 g of Gp and 0.08 g of M and Gc in 100 ml methanol. From each of these two stock solutions 0.2 ml was taken and further diluted with water to 10 ml (this produced the first working standards with concentrations of 16 μg/ml for M and Gc and 8 μg/ml for G, Gb and Gp); these were further diluted with water to produce the remaining working standards. For Method 1; 50 μl of the aqueous solution of the internal standard (P) and 0.125 ml of the standards containing M, Gb and Gm were added to 1 ml plasma resulting in the following concentrations for M 50,

100, 250, 500, 1000, and 2000 ng/ml plasma and 25, 50, 125, 250, 500, 1000 ng/ml plasma for Gb and Gm. For Method 2; 50 μl of the aqueous solution of the internal standard (T) and 0.125 ml of the standards containing M, Gc and Gp were added to 1 ml plasma resulting in the following concentrations for M and Gc 50, 100, 250, 500, 1000, and 2000 ng/ml plasma and 25, 50, 125, 250, 500, 1000 ng/ml plasma for Gp. This procedure was used in order to use the lowest possible amount of methanol as the presence of methanol significantly affects the recovery of M. These concentrations were prepared as they were close to the actual plasma concentrations.

2.4. Sample preparation and extraction

Patients' blood samples were collected into glass tubes containing EDTA and centrifuged at 3000 rpm (1610 × *g*) for 15 min. The separated plasma was kept frozen at −70 °C until analysis. To 1 ml of patients' plasma samples or spiked plasma standards 50 μl of the internal standard solution and 0.125 ml of water were added. 0.1 ml of 0.75 M HCl for Method 1 and 0.1 ml of 0.4 M HCl for Method 2 was added to each of the standards and patients samples then vortex mixed for 30 s and centrifuged at 14,000 rpm (17,500 × *g*) for 5 min before extraction to prevent blockage of the cartridges.

The extraction procedure was an optimisation of one developed previously for SPE of M [ion pair solid phase extraction (IPSPE)] [11] the main difference was the addition of acid during the loading step.

The samples and standards were prepared as mentioned above and then extracted using the optimised ion pair solid phase extraction technique utilising Oasis® HLB cartridges (1 cc, 30 mg) (which was connected to Waters extraction vacuum Manifold) as following:

1. Condition1: 1 ml methanol followed by 1 ml of water;
2. Condition2: 1 ml aqueous solution of 2 mM sodium dodecyl sulphate (ion pair reagent);
3. Load: 1.275 ml spiked or patient plasma (as prepared above);
4. Wash: 1 ml 30% methanol;
5. Elute: 1 ml methanol;
6. Evaporate with nitrogen stream and reconstitute in 350 μl of the mobile phase and inject 150 μl onto the HPLC.

2.5. Chromatography

The chromatographic System consisted of Shimadzu LC-GA pump, Waters 712 WISP autosampler and UV absorbance detector (LDC 12 Milton Roy, Riviera Beach, USA). The separation was performed using a Discovery C_{18} Supelco analytical column (250 mm × 4.6 mm, 5 μ; Sigma, Poole, England). The guard column was a Supelco Discovery (20 mm × 4 mm, 5 μ; Sigma, Poole, England). Data recording was carried out using the Shimadzu Class VP system.

The mobile phase was an optimisation of one developed previously for M determination [11]. The main difference was the pH of the mobile phase for Method 2. The mobile phase was pumped at a flow rate of 1 ml /min and consisted of 2 mM sodium dodecyl sulphate, acetonitrile (37.5%) and potassium dihydrogenphosphate (62.5%) (from 0.02 M buffer to produce a final buffer concentration of 0.0125 M). For Method 1 the pH was adjusted to 7.3 using NaOH and for Method 2 it was adjusted to 5.3 using HCl for optimal separation. The prepared mobile phase was filtered through 0.45 μm Millipore filters and degassed ultrasonically before use. Based on the UV spectrum of the analytes the detector wavelength was set at 225 nm.

2.6. Assay characteristics for method validation

2.6.1. Specificity

To demonstrate the specificity of the method blank plasma from five different lots, spiked plasma samples and plasma samples of patients who had previously taken the drugs were analysed. Representative chromatograms were generated to show that other components that could be present in the sample matrix are resolved from the parent analyte [12].

2.6.2. Standard curve and linearity

The standard curve was determined on each day of the 5-day validation; the slope, intercept and the correlation coefficient were determined. For calculation of the standard curve plots of peak height ratios against concentration were used.

2.6.3. Accuracy and precision

Intraday precision, interday precision and the accuracy were calculated from data obtained during a 5-day validation. Three concentrations were chosen from the high medium and low range of the standard curve (50, 200, 750 ng/ml for Gp, Gb, and G and 100, 400, 1500 ng/ml for M and Gc). Plasma samples spiked at these three concentrations were analysed at each day of the 5-day validation ($n = 5$ at each concentration). During the 5 days validation the sample were left at room temperature. Precision was expressed as the coefficient of variation (CV%). Accuracy was expressed as the mean relative error (RE%). A precision (CV%) less than or equal to 15% and an accuracy (RE%) less than or equal to 15% are acceptable [13].

2.6.4. Limit of quantification (LOQ) and limit of detection (LOD)

Ten independent blank sample were measured once each. The LOD was expressed as the analyte concentration corresponding to the sample blank value plus three standard deviations. LOQ was expressed as the analyte concentration corresponding to the sample blank value plus five standard deviations [12].

2.6.5. Recovery

The recovery was determined at three concentrations (50, 200, 750 ng/ml for Gp, Gb, and G and 100, 400, 1500 for M and Gc) ($n = 6$ at each concentration), the recovery of the

internal standards was determined at the concentration used which was 200 ng/ml.

2.6.6. Stability

The stability of samples during storage was determined by analysing 12 spiked plasma samples twice [100 and 400 ng/ml for M and Gc and at 50 and 200 ng/ml for Gp, Gm and Gb ($n = 6$ at each concentration)] with a 3 months interval in between (after freezing at −70 °C) then the means (± SD) of the ratios between the two measurements were determined.

2.7. Calculations

Standard regression curve analysis was computed using class VP software without forcing through zero. Means and standard deviation were calculated using EXCEL® software (Microsoft Corporation, USA).

3. Results and discussion

3.1. Optimisation of the Mobile phase

3.1.1. Preliminary experiments [11]

In a previous study [11] we described the optimisation of the mobile phase for HPLC M plasma determination. Our preliminary experiments indicated that using different concentrations of acetonitrile and even different pHs of the buffers in acetonitrile did not produce a suitable retention of M. Hence, a reversed phase ion-pair chromatographic (IPC) method was developed for M determination and the ion pair reagent which was most suitable for M was SDS (heptane sulfonic acid and decane sulfonic acid were not able to produce optimal retention of M and P). The developed mobile phase was 2 mM sodium dodecyl sulphate in acetonitrile (37.5%) and potassium dihydrogenphosphate (62.5%) (from 0.02 M buffer to produce a final buffer concentration of 0.0125 M). The pH was adjusted at 7.3 using NaOH.

In this study a series of experiments were conducted in order optimise this mobile phase for the simultaneous determination of M and Gb, Gm, Gp or Gc.

3.1.2. Two mobile phases differing only in pH

Because of their polarity and pK_a values Gm ($pK_a = 6.2$) and Gb ($pK_a = 6$) tend to have different retention pattern than Gp ($pK_a = 5.9$) and Gc ($pK_a = 5.8$). Using the previously developed mobile phase (AbuRuz et al. [11]) the sulfonylurea peaks overlapped with each other and Gp, Gc and T had short retention times. When decreasing the pH to less than 6.8 (at mobile phase organic content of 35%–40%) T and Gp have good retention times but Gm and Gb are not detected. Changing the organic phase content did not solve the problem, therefore it was decided to use two mobile phases that only differed in the pH; one for the determination of M and Gm or Gb using P as the internal standard (Method 1) and the other for the determination of M and Gp or Gc using T as the internal standard (Method 2).

For Method 1 the same conditions that were developed for M determination was used and the retention times were: for M was 4.7 min, P 12.5 min, Gm 8.4 min and Gb 6.2 min. For Method 2 decreasing the pH to 6.4 M retention time was 5 min, Gp was 8.1 min, Gc was 15.6 min and T was 10.1 min. When these mobile phases applied to the plasma the pH of the mobile phase for Method 2 was reduced to 5.3 in order to avoid interferences.

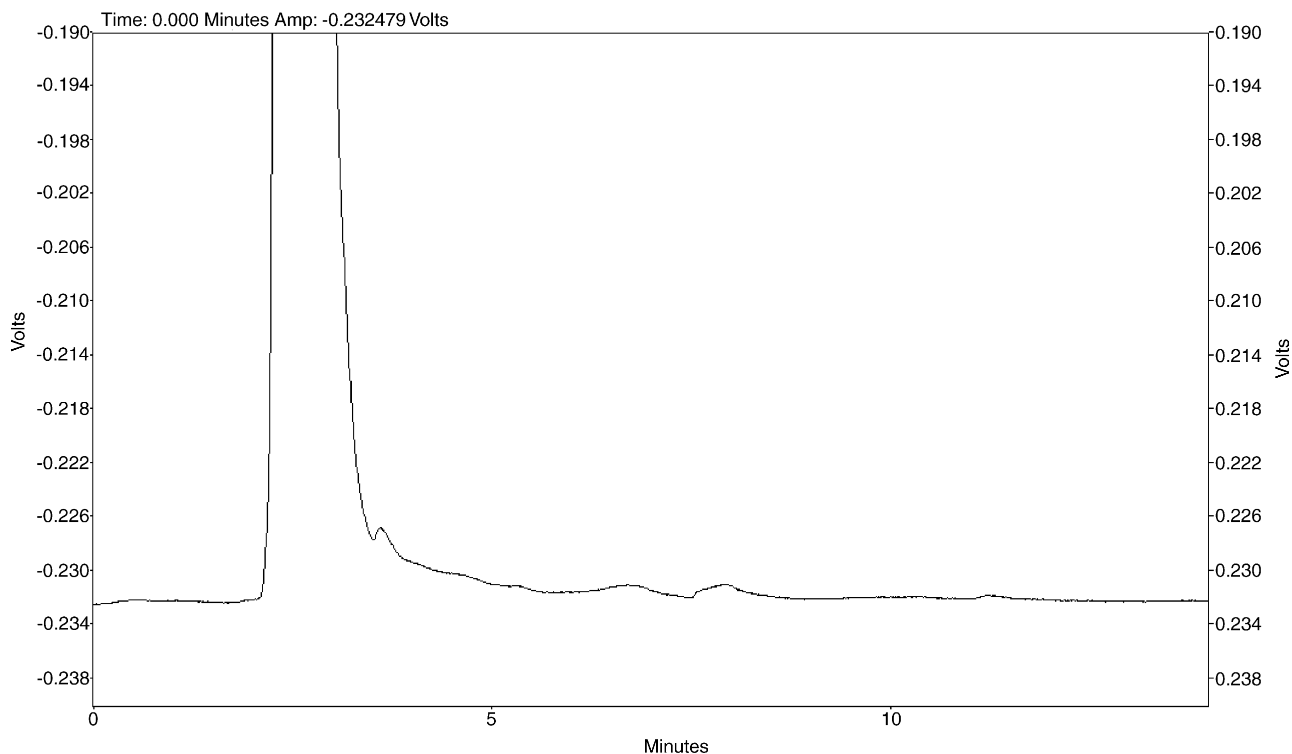
3.2. Optimisation of the extraction techniques

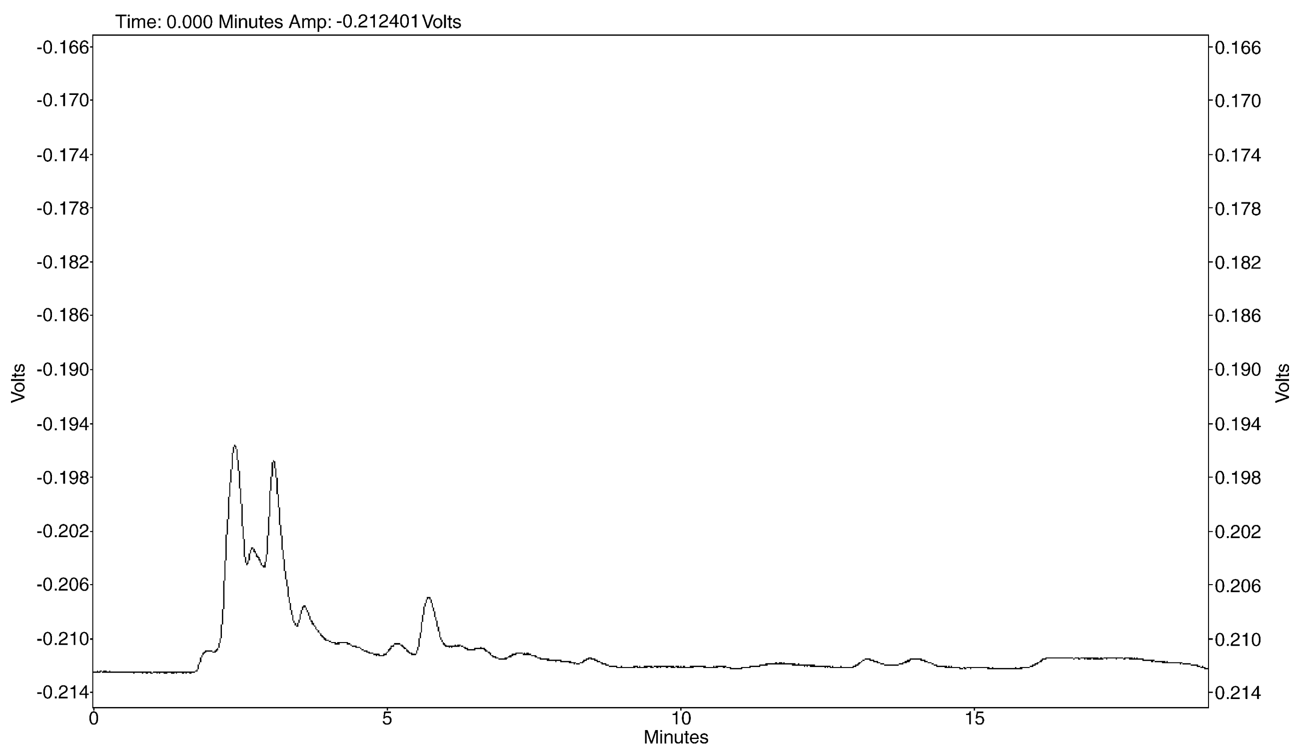
M has a very high polarity; therefore it was not possible to extract it from biological fluids using organic solvents or conventional SPE techniques. In order to overcome this problem other authors have used different methods and strategies which did not result in an optimum extraction of M (low recovery, low sensitivity, tedious solvent extraction ...). Therefore, our previous study on M resulted in the development of the first ion pair solid phase extraction for M extraction [11]. The principle of this procedure is equilibrating the SPE cartridges with ion pair reagent (by passing 1 ml of IPR solution (SDS) through the cartridge) the IPR will be retained in the column by a hydrophobic interaction. Upon loading the sample, M will form a complex with the retained IPR which then can be easily eluted using methanol.

When this procedure was applied to SU (from plasma) low recoveries resulted 37.9% for Gp and 20.3% for Gc and less than 10% for Gb and Gm. This is because the procedure did not involve addition of acid at the loading step. SU (unlike M) are strongly bound to plasma protein, therefore, acid addition is important to disrupt drug-protein binding and improving the recovery. The acid was not used in the case of M only analysis as it was noticed that by increasing the acid loading the recovery of M decreases (probably by affecting

Table 1
Summary of the extraction method development

	Recovery of M from solution	Recovery of SUs from solution	Recovery of M from plasma	Recovery of SUs from plasma
Oasis® HLB cartridges	<10.0%	>90%	Negligible	>90% (Depending on the acidity at the loading step)
Oasis® MCX cartridges	100% (only by breaking the resin using 1 M HCl)	>90%	Negligible	>90% (Depending on the acidity at the loading step)
IPSPE (Oasis® HLB cartridges)	>90% (no acid during loading)	>90%	>75% (with 100 μL 0.3 M HCl)	>80% with acid loading





Figs. 1 and 2. Chromatograms showing the result of extraction of blank plasma samples for Method 1 (up) and Method 2 (down).

the ionisation of the IPR and therefore affecting its ability to bind to M). By adding acid at the loading step the recovery of sulfonylureas increased (>80%). Table 1 summarises the experiments that led to the development of the IPSPE technique for M and SU.

The best acid loading (the one with the optimum recoveries for all the analytes) was determined by testing different acid concentrations at the loading step and it was found that the best acid loading for Method 1 is 100 μl 0.75 M HCl and for Method 2 is 100 μl 0.4 M HCl. It was noticed that the acid

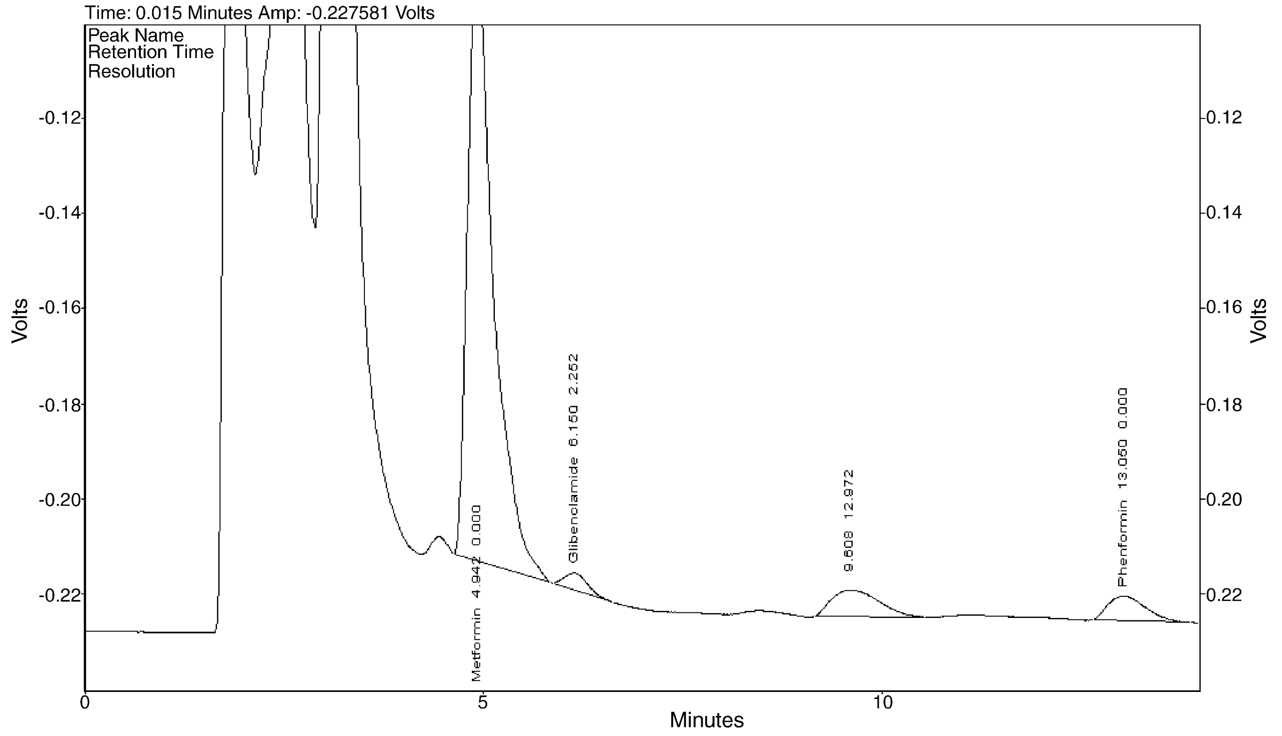


Fig. 3. A chromatogram showing the result of extraction of a plasma sample taken from a patient who was on M (850 mg three times daily) and 2.5 mg Gb twice daily using Method 1 and showing M 3600 ng/ml, Gb 250 ng/ml and P 200 ng/ml.

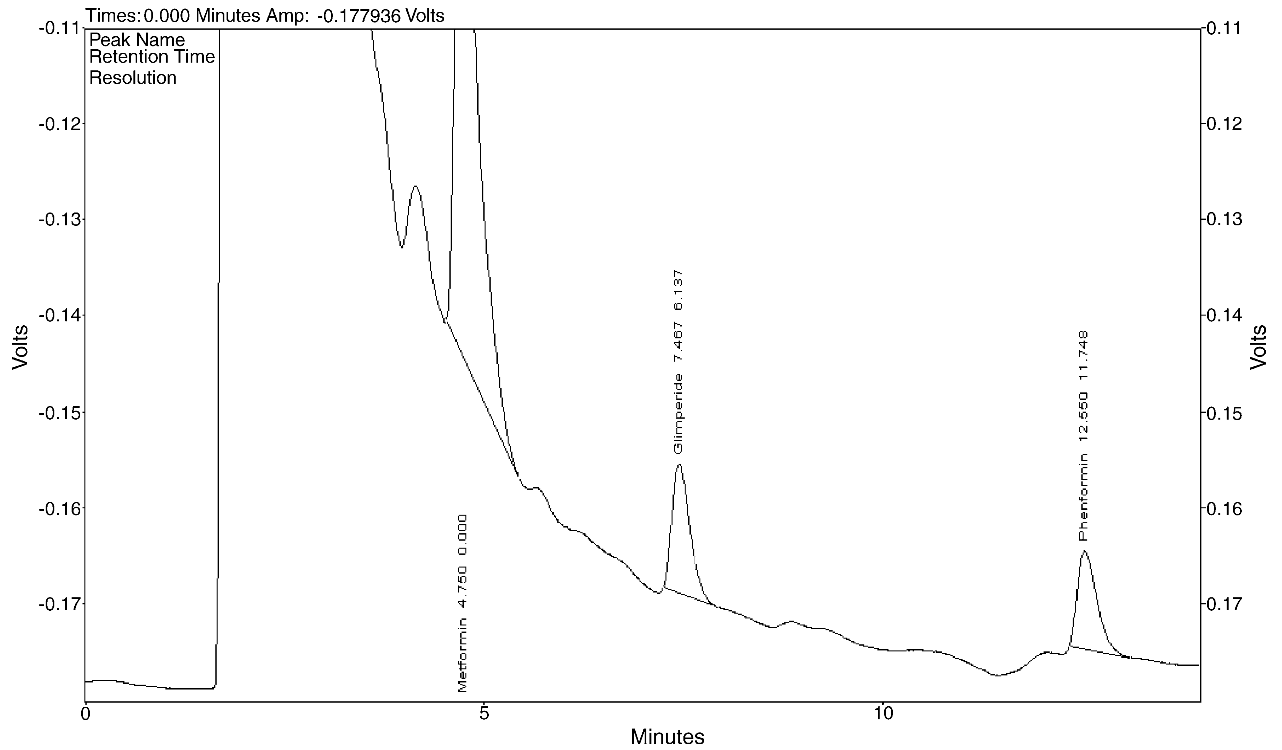


Fig. 4. A chromatogram showing the result of extraction of a plasma sample taken from a patient who was on 850 mg M once daily and 3 mg Gm once daily using Method 1 and showing M 1980 ng/ml, Gm 550 ng/ml and P 200 ng/ml.

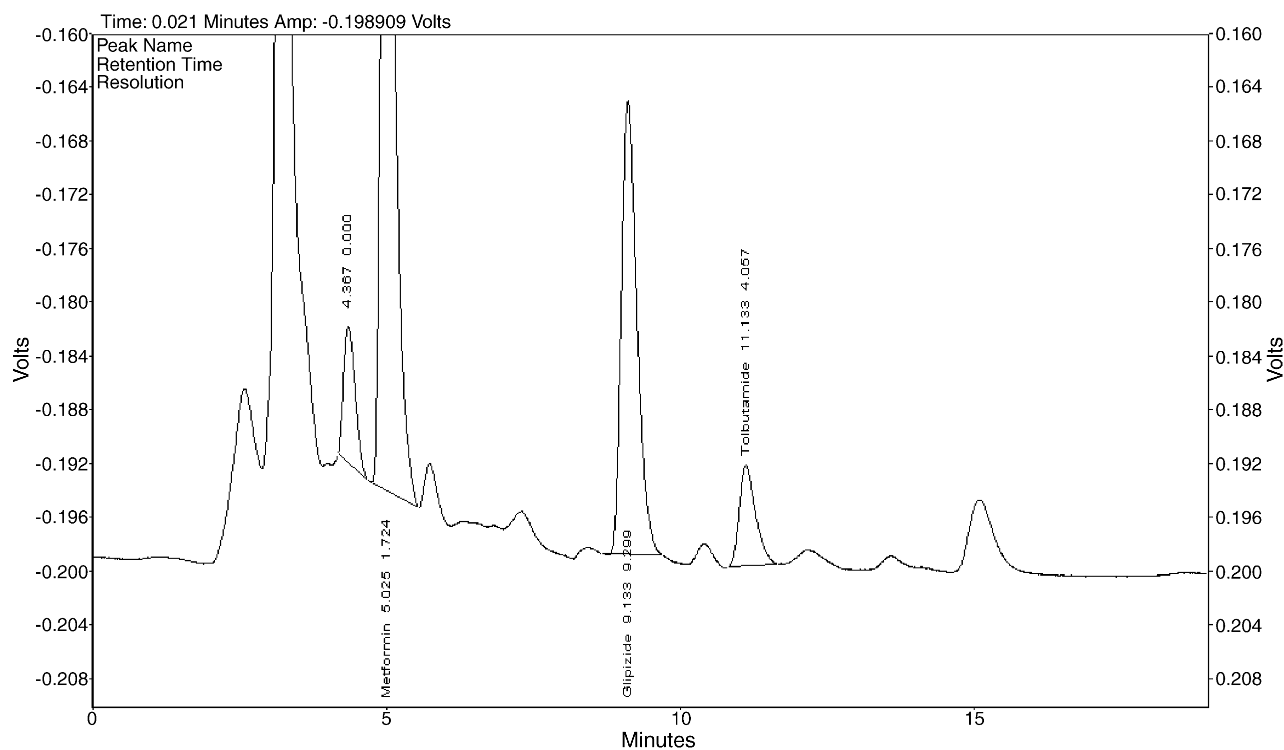


Fig. 5. A Chromatogram showing the result of extraction of a plasma sample taken from a patient who was on 500 mg M once daily and 15 mg Gp once daily using Method 2 and showing M 960 ng/ml, Gp 730 ng/ml and T 200 ng/ml.

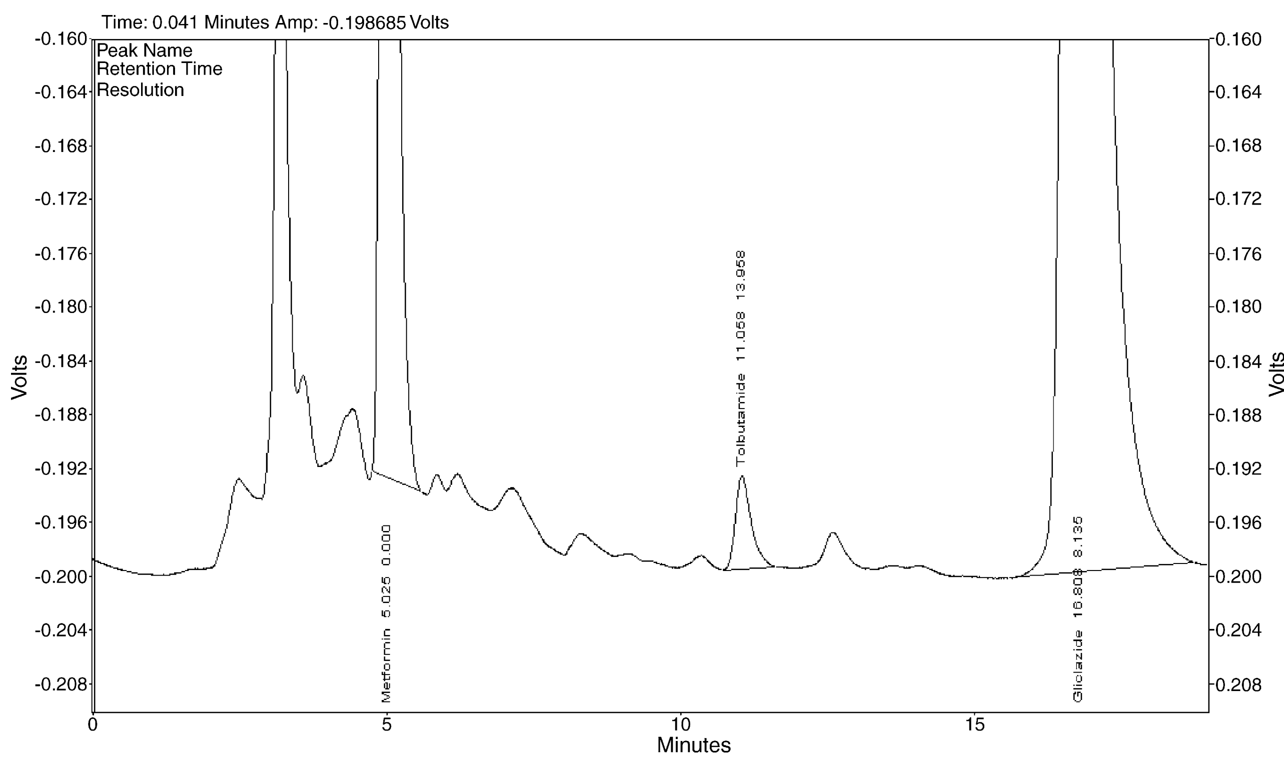


Fig. 6. A Chromatogram showing the result of extraction of a plasma sample taken from a patient who was on 500 mg M three times daily and 80 Gc twice daily using Method 2 and showing M 2700 ng/ml, Gc 14,000 and T 200 ng/ml.

used should be as little as possible as M recovery decreases with increasing acidity on the loading step.

Plasma samples should be centrifuged before SPE to prevent them from blocking the cartridges. The washing step should be one step only and followed by the elution step (i.e. not two washing steps) as it was noticed that the ion pair reagent is eluted when using two washing steps.

3.3. Validation

3.3.1. Specificity

Figs. 1 and 2 are chromatograms of blank plasma samples for Methods 1 and 2. Figs. 3–6 are chromatograms of plasma samples from patients who were taking a combination of M and Gb, a combination of M and Gm, a combination of M and Gp and a combination of M and Gc, respectively. There were no endogenous substances in the plasma that interfered with the peaks of interest as the blank analysis gave no responses at the retention times of the peaks of interest and the resolutions between all the peaks is more than 2.

3.3.2. Standard curve and linearity

The standard curve was determined on each day of the 5-day validation, the slope, the intercept and the correlation coefficient was determined. Table 2 shows the mean ± SD for slope and intercept for 5 calibration curves for Methods 1 and 2. By examining the calibration curves and the table it was concluded that the relationship between height ratio (or area ratio) and concentration was linear within the studied concentration range.

3.3.3. Accuracy and precision

The values obtained during the 5-day validation for plasma intraday and interday precision and accuracy are summarised in Tables 3 and 4. All values of accuracy and precision where within recommended limits. Intraday precision ranged between 0.6% and 7.8% where as the interday precision was between 1.5% and 13.1%. The intraday mean error was between 0% and 12.8% whereas the interday mean error was between 0% and −11.1%.

Table 2
Calibration curves summary statistics for the studied drugs

	Slope (mean ± SD)	Intercept (mean ± SD)	Correlation coefficient (r)
Method 1			
Metformin	1.2655 ± 0.0381	0.0242 ± 0.0603	≥0.997
Glibenclamide	1.4116 ± 0.0672	0.1537 ± 0.0696	≥0.998
Glimperide	1.3436 ± 0.0696	0.1130 ± 0.0330	≥0.998
Method 2			
Metformin	0.4782 ± 0.0329	−0.0177 ± 0.0871	≥0.999
Glipizide	0.8643 ± 0.0924	0.0246 ± 0.0275	≥0.998
Gliclazide	2.5338 ± 0.1634	0.0039 ± 0.0876	≥0.999

Slopes and intercepts where determined automatically by the Class Vp software program.

Table 3
Intraday precision and accuracy data for assays of metformin and sulfonylureas in plasma ($n = 5$)

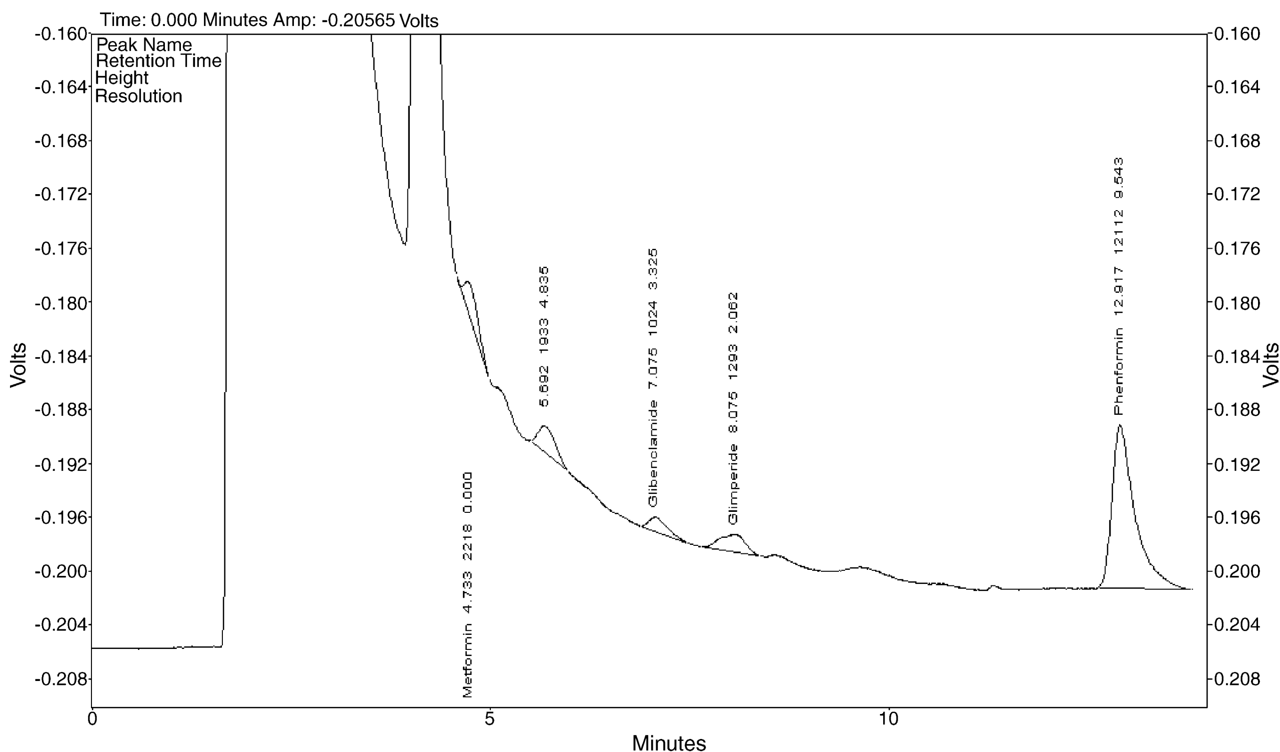
Nominal concentration (ng/ml)	Precision		Accuracy mean relative errors (%)
	M ± SD	CV%	
Method 1			
Metformin 100	102.8 ± 3.43	3	2.8
400	405.8 ± 17.8	4.4	1.5
1500	1500.0 ± 38.9	2.6	0.0
Glibenclamide 50	51.8 ± 1.4	2.8	3.6
200	194.1 ± 2.2	1.1	−2.9
750	738.8 ± 22.4	3.0	−1.5
Glimperide 50	54.3 ± 3.0	5.6	8.6
200	203.7 ± 14.8	7.3	1.9
750	762.7 ± 26.2	3.4	1.7
Method 2			
Metformin 100	112.8 ± 6.3	5.6	12.8
400	400.8 ± 31.2	7.8	0.2
1500	1466.7 ± 53.2	3.6	−2.2
Glipizide 50	52.9 ± 2.4	4.5	5.8
200	208.0 ± 14.7	7.0	4.0
750	711.3 ± 32.6	4.6	−5.16
Gliclazide 100	94.4 ± 1.0	1.9	−5.6
400	406.5 ± 2.4	0.6	1.63
1500	1503.9 ± 11.5	0.77	0.26

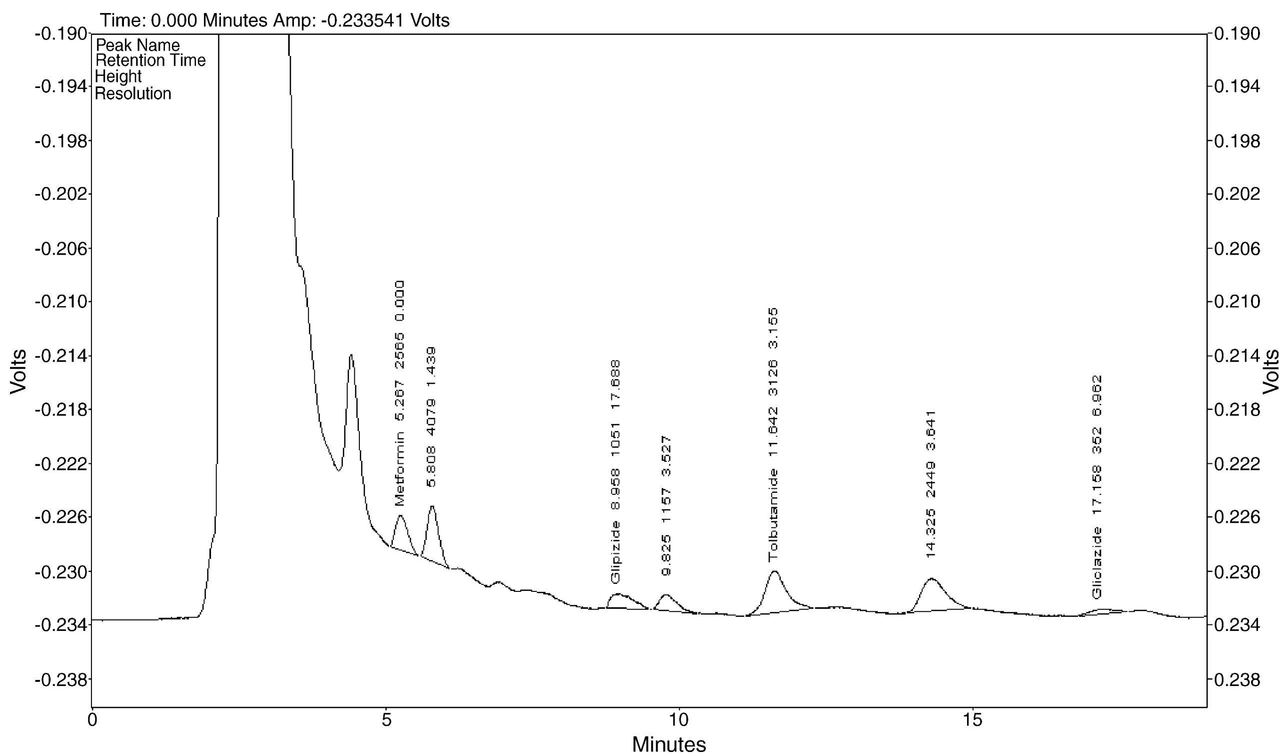
3.3.4. Limit of detection and limit of quantification

Table 5 details the LOD and the LOQ for each of the investigated analytes. Concentrations down to the LOQ were detected with acceptable accuracy and precision using this method (CV% and RE% <15%) (Figs. 7 and 8). The mean background was also calculated and found to be 6.5% (glipizide), 14% (gliclazide), 6% (glibenclamide), 5.5%

Table 4
Interday precision and accuracy data for assays of metformin and sulfonylureas in plasma ($n = 5$)

Nominal concentration (ng/ml)	Precision		Accuracy mean relative errors (%)
	M ± SD	CV%	
Method 1			
Metformin 100	97.8 ± 5.1	5.2	−2.2
400	385.6 ± 27.9	7.2	−3.6
1500	1573 ± 58.3	3.7	4.9
Glibenclamide 50	49.9 ± 2.8	5.5	0.2
200	177.9 ± 7.2	4.0	−11.1
750	737.1 ± 15.2	2.0	−1.7
Glimperide 50	51.8 ± 1.3	2.4	3.6
200	202.3 ± 9.3	4.6	1.2
750	750.4 ± 16.7	2.2	0.1
Method 2			
Metformin 100	101.0 ± 5.4	5.3	1.0
400	419.5 ± 27.3	6.5	4.9
1500	1499.6 ± 133.3	8.9	0.0
Glipizide 50	46.6 ± 6.1	13.1	6.8
200	208.2 ± 3.1	1.5	4.1
750	766.7 ± 26.1	3.4	2.2
Gliclazide 100	100.4 ± 8.5	8.5	0.4
400	406.6 ± 7.9	1.9	1.7
1500	1566.9 ± 35.2	2.2	4.5





Figs. 7 and 8. Chromatogram showing the result of extraction of spiked plasma showing M, Gm, Gb, Gp, and Gc at concentrations close to the limit of quantification.

(glimperide), 5% (metformin Method 1) and 10.5% (metformin Method 2) of the response at the Limit of Quantification. The absence of significant background interference can be observed through examining Figs. 1 and 2.

3.3.5. Recovery

Table 6 shows data for extraction recovery ($n = 6$). The highest recovery of M was using Method 2 (97.7%) and the lowest recovery was using Method 1 (76.3%). This was to be

Table 5
LOD and LOQ data for assays of metformin and sulfonylureas in plasma (ng/ml)

	LOD	LOQ
Metformin/Method 1	3.0	5.0
Metformin/ Method 2	9.9	16.5
Glibenclamide	4.0	7.0
Glimperide	4.5	7.5
Glipizide	4.5	7.5
Gliclazide	13.5	22.5

Table 6
The recovery data for assays of metformin and sulfonylureas in plasma ($n = 6$)

Nominal concentration (ng/ml)	M ± SD	CV%
Method 1		
Metformin 100	76.3 ± 7.7	10.1
400	89.7 ± 4.5	5.0
1500	89.2 ± 2.4	2.7
Glibenclamide 50	81.8 ± 6.8	8.3
200	86.0 ± 5.0	5.7
750	87.3 ± 2.9	3.3
Glimperide 50	94.7 ± 6.7	7.1
200	101.5 ± 10.1	10.0
750	101.9 ± 1.8	1.7
Phenformin 200	93.3 ± 4.6	4.9
Method 2		
Metformin 100	97.1 ± 1.8	1.8
400	95.9 ± 3.8	3.9
1500	87.8 ± 2.5	2.8
Glipizide 50	90.4 ± 4.9	5.4
200	79.7 ± 5.8	7.3
750	80.0 ± 2.0	2.6
Gliclazide 100	97.7 ± 1.2	1.2
400	94.4 ± 1.7	1.8
1500	87.6 ± 2.5	2.8
Tolbutamide 200	98.7 ± 6.6	6.7

expected as the acid loading used in Method 2 was less than the one in Method 1 where a high acidity was used in the loading step to increase the recovery of SU. The recoveries of SU ranged between 79.7% and 101.9%

3.3.6. Stability

The means and standard deviations of the ratios between the two repeat measurements that were carried out 3 months apart (plasma stored at −70 °C) for M ($n = 12$), Gb ($n = 12$) and Gm ($n = 12$) were: 1.04 ± 0.04; 0.99 ± 0.05 and 1.0 ± 0.05 respectively. For Method 2 the same procedure was used and the means and standard deviations for the ratios between the two measurements for M ($n = 12$), Gp ($n = 12$) and Gc ($n = 12$) were 1.07 ± 0.06, 1.0 ± 0.05 and 0.96 ± 0.06, respectively. These data indicate that all analytes are stable for at least three months when stored at −70 °C.

4. Conclusion

The developed method is a suitable and valid method for the determination of a combination of M and SU. The use of this method can save time and effort when monitoring a population of diabetic patients who take several diabetes medications; there is no need to have more than one HPLC system or to change the HPLC column to measure plasma from patients on different medication regimes. The validity, LOQ and the linearity range of the method makes it an acceptable method for clinical studies in diabetes patients taking these medications. The method was successfully used in the analysis of more than 100 samples obtained from patients on a combination of M and a SU.

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