

Determination of Metformin in Human Plasma and Urine by High-Performance Liquid Chromatography Using Small Sample Volume and Conventional Octadecyl Silane Column

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ABSTRACT – Purpose. To develop a selective and sensitive high-performance liquid chromatographic method for the determination of metformin in human plasma and urine, using a conventional reverse phase column and low specimen volume. **Methods.** Extraction of metformin and ranitidine (as internal standard) from plasma and urine samples (100 μ L) was performed with a 1-butanol-hexane (50:50, v/v) mixture under alkaline conditions followed by back-extraction into diluted acetic acid. Chromatography was carried out using a C18 column (250 mm \times 4.6 mm, 5 μ m). A mobile phase consisting of acetonitrile and KH_2PO_4 (34:66, v/v) and sodium dodecyl sulphate (3 mM) was pumped at an isocratic flow rate of 0.7 mL/min. **Results.** The calibration curves were linear (>0.995) in the concentration ranges of 10–5000 ng/mL and 2–2000 μ g/mL for metformin HCl equivalents in plasma and urine respectively. The mean absolute recoveries for 100 and 1000 ng/mL metformin HCl in plasma using the present extraction procedure were 93.7 and 88.5%, respectively. The intra- and inter-day coefficients of variation in plasma and urine were $<20\%$ at the lowest, and $<16\%$ at other concentrations. The percent error values were less than 2% in plasma while it reached $\sim 9\%$ in urine. The lower limits of quantification were 7.8 ng/mL and 1.6 μ g/mL of metformin base in plasma and urine respectively. **Conclusions.** The method showed high calibers of sensitivity and selectivity for monitoring therapeutic concentrations of metformin in both plasma and urine based on a 0.1 ml sample size.

INTRODUCTION

Metformin HCl (1,1-dimethylbiguanide HCl), first developed in 1957, is one of the most commonly used oral anti-hyperglycemic agents for the treatment of Type II diabetes mellitus. In 1995, the FDA approved metformin HCl for use in the United States, which led to a significant increase in clinical use (19). It is currently recommended as first-line therapy in overweight or obese patients with this condition (1).

Many methods are available for quantitation of metformin in biological samples. For its separation from endogenous components most of these methods rely on liquid or gas chromatographic steps (Table 1) (2-9,11-15,17-18,22-27). Others involving capillary electrophoresis have also been reported (21). Regarding sample preparation from biological fluids, extraction and clean-up of the sample is a critical first step in bioanalysis and requires high

selectively for the removal of interfering substances, while ensuring minimal analyte loss. The extraction of metformin from biological matrices is somewhat complicated by the highly polar nature of the molecule. This factor, in addition to the drugs widespread clinical use, may explain the relative abundance of different methods for analyzing metformin in biological specimens. Many of the current methods utilize varying techniques for sample cleanup, such as simple protein precipitation, solid phase extraction, ultrafiltration with column switching, chemical derivatization and liquid-liquid extraction.

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Table 1. Comparisons of some published methods for assaying metformin in human matrices

Volume of specimen (mL)	Validated plasma LLQ (ng/mL)	Type of human matrix	Sample preparation	Analytical column	Reference
0.5	30	Plasma	Protein ppt and dichloromethane wash	Phenyl	(17)
0.1 and 0.5	10	Plasma	Protein ppt and dichloromethane wash	Silica	(7)
1 serum NS urine	50 serum 2000 urine	Serum & urine	Ion pair SPE	C18 or F5HS	(23)
0.1	15.6	Plasma	Liquid-liquid	Silica	(3)
0.5	200	Plasma & urine	Protein ppt	CX	(5)
NS	10	NS	Protein ppt	Silica	(18)
0.3	100	Plasma	Ultrafiltration	CX	(24)
0.25	60	Plasma	Protein ppt	Cyano	(12)
0.1	250	Plasma	IPE	Silica	(21)
0.5	50	Plasma	SPE C8	Phenyl	(11)
1	10	Plasma & urine	IPE	C18	(13)
0.1	7.8*	Plasma & urine	Liquid-liquid	C18	Current method

Abbreviations: LLQ=lower limit of quantitation, SPE=solid phase extraction, protein ppt= protein precipitation, IPE= ion pair extraction, LLE= liquid-liquid extraction, F5HS= pentafluorohypersil, CX= cation exchange. NS denotes not specified or unclear. *Equivalent to 10 ng/mL of the HCl salt.

Ion-pair liquid-liquid (13,21) or ion-pair solid-phase extraction (23) has been used to overcome clean up plasma samples, which is difficult to achieve owing to the high polarity of metformin. Use of other solid-phase extraction (11) and ultrafiltration with column switching (24) steps have also been incorporated into some methods, although they are not always yield optimal sensitivity, and may necessitate the use of certain devices which can add elements of expense and time into sample preparation. Chemical derivatization of metformin has been used in gas chromatography (4,15) and HPLC methods (22), but these can add complexity into the method with little advantage in terms of sensitivity. Recently, liquid chromatography-mass spectrometry (LC-MS) methods following plasma deproteination have been reported for metformin (6,9,25) and these techniques certainly enhance selectivity and sensitivity. They have a disadvantage however in that the instrumentation is quite expensive is not as

accessible as conventional HPLC in most laboratories, particularly in clinical laboratories.

Organic liquid-liquid extraction is a simple and effective method of affording sample cleanup for most analytes. Unfortunately, in the case of metformin, this approach is challenging because of the drug's polar characteristics. Herein, a method that successfully employs the liquid-liquid extraction of metformin and capable of measuring metformin in small volumes with high sensitivity is described. The extraction method was similar to that described by another group (3), although here a conventional reverse phase C18 column could be used for separation.

METHODS

Materials and reagents

Metformin HCl (purity of 97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ranitidine injection USP (Sandoz, Quebec, Canada) was used as a source of ranitidine HCl

for internal standard (IS) and purchased from the University of Alberta Hospitals (Edmonton, Alberta, Canada). Acetonitrile, hexane and water (all HPLC-grade) were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). Sodium dodecyl sulphate (SDS) was purchased from Anachemia (Mississauga, Ontario, Canada). Analytical grade 1-butanol and KH_2PO_4 were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada).

Instrumentation and chromatographic conditions

The chromatographic system consisted of a Waters (Milford, MA, USA) 600E multi-solvent delivery system pump, auto sampler with variable injection valve (Waters 717) and UV-visible tunable absorbance detector (Waters 486). The chromatograms were recorded using EZStart software (Scientific Software, Pleasanton, CA, USA) in a computer system for data collection and processing. Separation was performed on a 250 mm×4.6mm i.d., 5 μm particle size Alltima C18-column (Alltech, Deerfield II, USA). The mobile phase consisted of acetonitrile-potassium dihydrogen phosphate buffer pH 6.5 (34:66, v/v) and 3 mM SDS. It was prepared daily and degassed by filtering it under vacuum through a 0.045 μm nylon filter. The flow rate of the mobile phase through the analytical column was 0.7 ml/min, at room temperature. The detection wavelength was set at 236 nm.

Standard and stock solutions

A stock solution of 5 mg/mL metformin was prepared by dissolving 100 mg in 20 mL of water. Working solutions were prepared freshly on the day of experiment from the stock by successive dilutions with water. Calibration samples of plasma (0.1 mL) were prepared containing metformin HCl equivalent to 10, 30, 100, 500, 1000, 2000 and 5000 ng/mL. For urine, 0.1 mL samples containing metformin HCl equivalent to 2, 20, 100, 500, 1000, and 2000 $\mu\text{g}/\text{mL}$ were prepared. These samples were used for generation of standard curves.

An IS stock solution of 25 $\mu\text{g}/\text{mL}$ of metformin HCl was further diluted in water to prepare the working solution of 25 and 250

$\mu\text{g}/\text{mL}$ for plasma and urine analysis respectively. All stock solutions were kept at -5°C until use.

Extraction procedure

The extraction procedure was similar to that described by Amini et al. (2005), with some modification. To 100 μL matrix was added 20 μL of IS (25 $\mu\text{g}/\text{mL}$) solution in case of plasma, or 30 μL of IS (250 $\mu\text{g}/\text{mL}$) in case of urine. An 80 μL volume of 10 M NaOH was added to alkalize the media. Analytes were then extracted using a 3 mL mixture of 1-butanol:hexane (50:50 v/v). The tubes were vortex-mixed for 30 s and centrifuged at 3000 g for 3 min. The supernatant (organic layer) was transferred into clean glass tubes and acidified with 500 μL of 0.2% acetic acid, vortex-mixed for 60 s and centrifuged at 3000 g for 3 min. The supernatant was removed by aspiration using a pipette attached to a vacuum flask, and the remaining aqueous layer was evaporated to dryness *in vacuo*. The residues were reconstituted with 200 μL HPLC water. The injection volume into the chromatographic system ranged from 60-70 μL .

Recovery

The recoveries were determined with metformin HCl concentrations of 100 and 400 ng/mL of plasma, and 100, 2000 $\mu\text{g}/\text{mL}$ of urine using four replicates for each concentration. The extraction efficiency was determined by comparing the extracted peak heights of analyte in samples to the peak heights of the same amounts of analyte directly injected to the instrument, without extraction.

Calibration, accuracy and validation

Full inter and intraday validation assessment was undertaken in human heparinised plasma samples. After validation was established in plasma, for urine a partial (one day) validation was undertaken. Samples were processed by adding IS and known amounts of metformin HCl to 100 μL human matrix providing a metformin HCl concentration range of 10-5000 ng/mL in case of plasma and 2-2000 $\mu\text{g}/\text{mL}$ in case of urine. The ratios of metformin base to IS peak height were calculated and plotted vs nominal metformin concentrations to construct

calibration curves. Data for calibration curves were weighted by a factor of 1/concentration due to the wide range of concentration used.

Intraday, accuracy and precision of the assay were determined using a range of concentrations of metformin HCl in both matrices. The concentrations were selected at 10, 30, 100, 500 and 1000 ng/mL and at 2, 20, 100, 500 and 2000 µg/mL for human plasma and urine respectively. The urinary concentrations were selected to be higher because the drug is excreted extensively in urine, and concentrations were expected to be high as a result (16). Each concentration had a replicate of five samples. Regarding human plasma samples, to permit the assessment of interday accuracy and precision, the assay was repeated on three separate days. For each daily run, a set of calibration samples separate from the validation samples were prepared to permit quantification of the peak height ratios of metformin to IS. Precision was assessed by percentage coefficient of variation (CV%) while accuracy was represented by determining mean intra- or inter-day percentage error.

Application

The method was used to determine metformin concentration in plasma and urine samples from two volunteers, after oral administration of 1000 mg as 2 tablets containing 500 mg of metformin per tablet. Tablets were administered with a glass of water following an overnight fast. Subjects provided written consent, and the study was approved by the University of Alberta Health Research Ethics Board. After dosing, serial blood samples were taken for up to 24 h. The samples were centrifuged for 10 min after sampling and plasma separated and frozen at -20°C until assayed. The terminal phase half-lives were determined by applying regression analysis to the log linear terminal portion of the concentration vs. time curve. The linear trapezoidal rule was used to calculate the area under the plasma concentration vs. time curve from the time of dosing to the last measured concentration. The maximal concentration (C_{max}) and the time to achieve it (t_{max}) were determined by visual examination of the data.

RESULTS

The chromatographic retention times were 9.5 min for metformin and 13.5 min for IS. The method provided specificity, with baseline resolution of IS and metformin with a lack of interfering peaks from endogenous components in plasma or urine (Fig. 1). The peaks were symmetrical. The average recoveries were 93.7 and 88.5% with 100 and 1000 ng/mL metformin HCl in plasma, respectively. In urine, recovery was 83.0% and 89.4 for 100 and 2000 µg/mL, respectively. The average extraction recoveries for IS were 83.3 and 82.7% in plasma and urine respectively. There were excellent linear relationships ($r^2 > 0.995$) noted between the peak height ratios and concentrations over metformin HCl ranges of 10–5000 ng/mL plasma, and 2–2000 µg/mL of urine.

Based on regression analysis of the concentration vs. peak height ratios of metformin to IS, in plasma and urine average slopes of 0.00041 and 0.0274 were observed, respectively. Corresponding intercepts were 0.00594 and 0.6385 respectively. The mean r^2 for plasma and urine standard curves were 0.999 and 0.9972 respectively. The CV of intra- and inter-day assessments for both matrices were less than 19% (Table 2 and 3). Mean inter-day error in human plasma was less than 2% (Table 2). In urine, mean percentage error was up to 9% (Table 3). Based on the inter- and intra-day CV% and mean error, it could be determined that the lower limits of quantitation (LLQ) of metformin base were 7.8 ng/mL and 1.6 µg/mL based on 0.1 mL of human plasma and urine respectively (Tables 2 and 3).

In the test of assay applicability, plasma concentrations could be followed for up to 24 h following administration of the metformin dose. The observed C_{max} were 2.47 and 2.37 mg/L, occurring at corresponding t_{max} of 2.1 and 1.6 h respectively, for each of the two volunteers. The plasma elimination half-life was 3.7 h for both volunteers. The areas under the plasma concentration time curve from 0 h extrapolated to infinity (AUC_{0-∞}) were 11.9 and 10.7 mg·h/L. As expected, the measured concentrations of metformin in the 24 h cumulative urine samples were higher, being 149 and 168 µg/mL in the two volunteers.

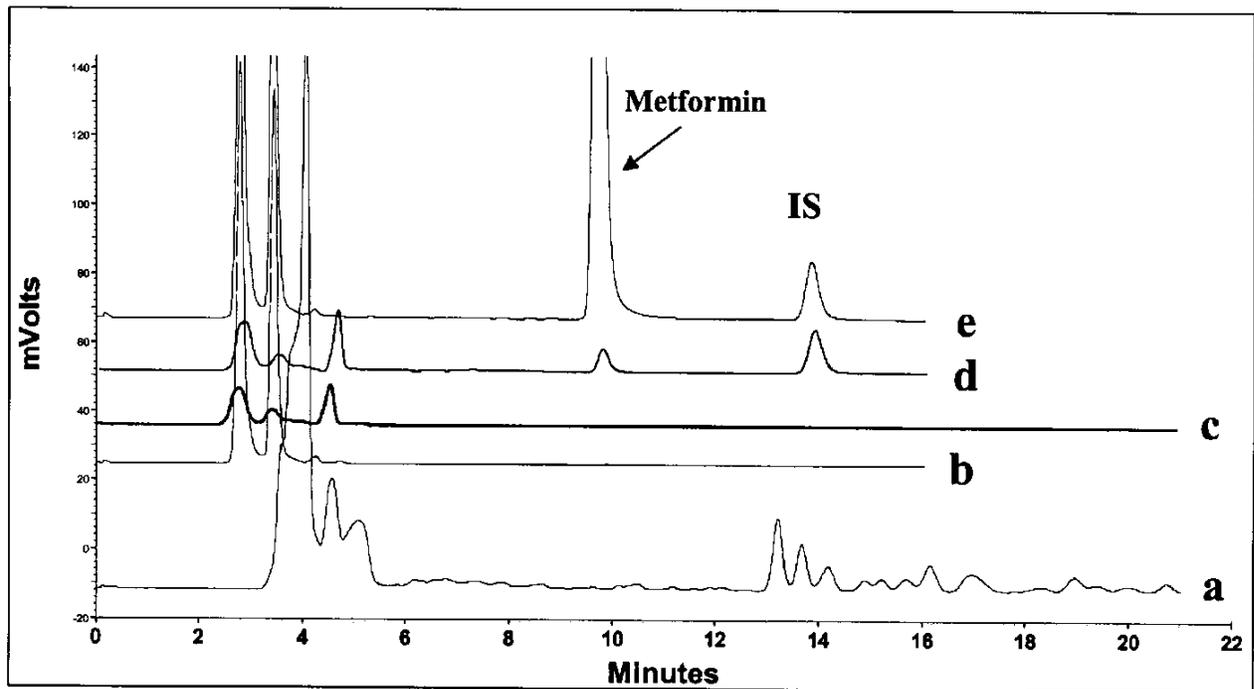


Figure 1. HPLC-UV chromatograms obtained for blank human plasma extracted by protein precipitation method (a), blank human urine (b), blank human plasma (c), human plasma sample after metformin administration (d) and human urine sample collected during 24 after metformin administration (e). In contrast to chromatogram a, chromatograms b through e were extracted using the described liquid-liquid extraction technique.

Table 2. Validation data for the assay of metformin in human plasma, n=5

Nominal concentration of HCl salt*, ng/mL	Intraday			Interday		
	Mean±SD ng/mL* (CV%)	Mean±SD ng/mL*	CV% Error %	Mean±SD, ng/mL*	CV% Error %	
10	7.46±0.51 (6.81)	10.4±1.11 (10.7)	10.6±1.78 (16.8)	9.50±1.77	18.6	-5.01
30	29.3±4.12 (14.1)	27.3±1.30 (4.76)	31.2±0.62 (1.98)	29.3±1.95	6.64	-2.37
100	97.2±5.06 (5.21)	95.5±10.4 (10.9)	97.9±1.86 (1.90)	96.9±1.26	1.31	-3.14
500	492±3.44 (0.70)	493±6.67 (1.35)	522±34.4 (6.58)	502±17.4	3.46	0.46
1000	975±13.10 (1.34)	982±40.3 (4.10)	1096±39.7 (3.63)	1017±67.8	6.66	1.79

*To convert to base, multiply by 0.78.

Table 3. One day validation data for the assay of metformin in human urine, n=5.

Nominal concentration of HCl salt* $\mu\text{g/mL}$	Mean \pm SD*, $\mu\text{g/mL}$	CV%	%Error
2	2.09 \pm 0.04	1.95	4.56
20	20.1 \pm 0.19	0.93	0.34
100	105 \pm 2.09	2.00	4.72
500	543 \pm 5.12	0.94	8.66
2000	2047 \pm 89.3	4.36	2.34

*To convert to base, multiply by 0.78.

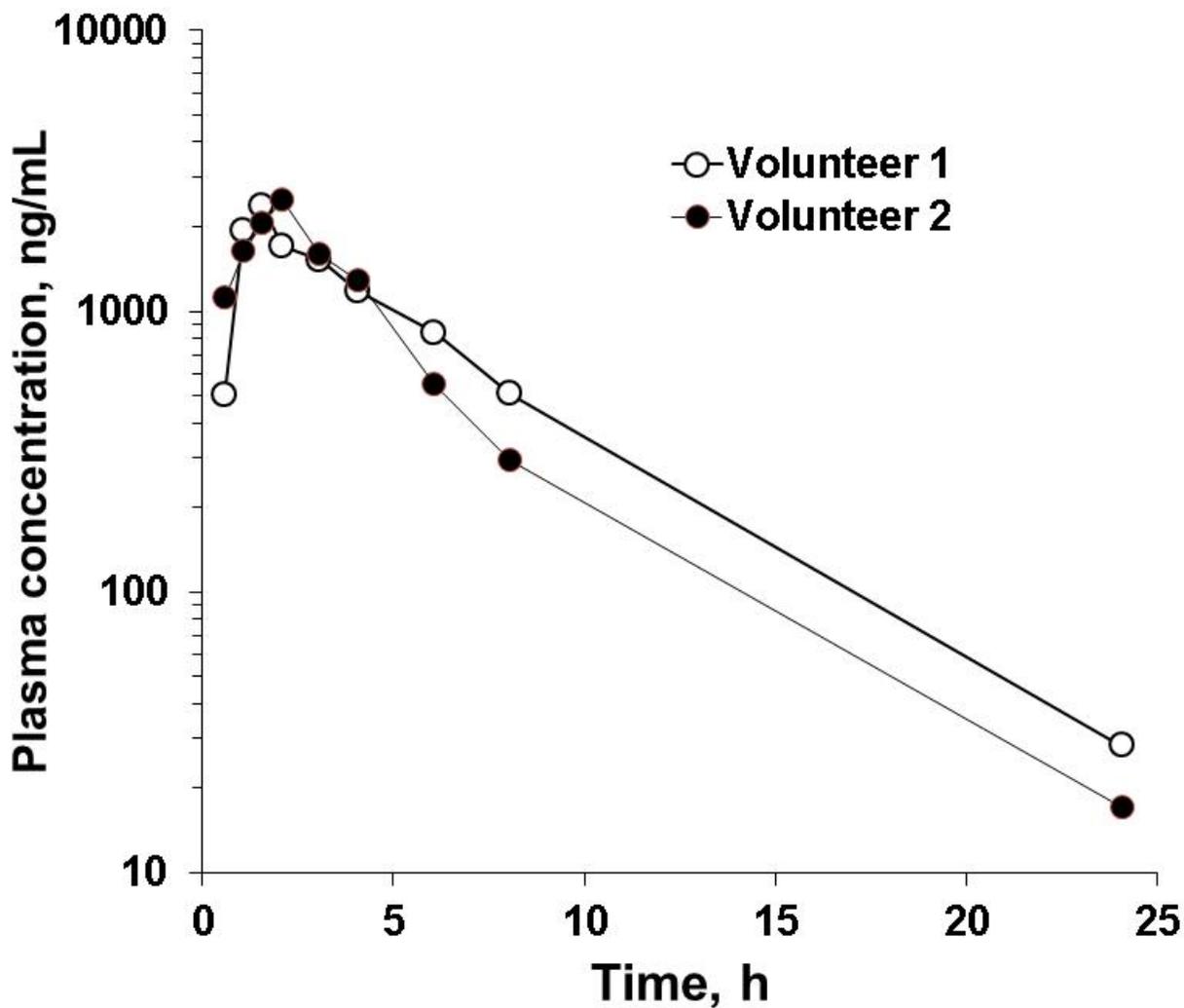


Figure 2. Plasma metformin concentration vs. time profiles after oral administration of 1000 mg metformin HCl to two healthy individuals.

DISCUSSION

Several of the pre-existing methods cited for assay of metformin are presented in Table 1. To the best of our knowledge, there has been no HPLC method developed to determine metformin concentration in both human plasma and urine utilizing volume sizes as small as 0.1 mL, using a liquid-liquid extraction procedure with a column specifically designed for reverse phase chromatography (e.g. C18 column).

The retention of metformin was examined on C18, C8 and phenyl columns with mobile phases that consisted of a mixture of phosphate solutions and acetonitrile. Metformin retention was very close to the column dead volume and no separation could be achieved from endogenous components using such columns and mobile phase composition. This is likely due to the polar nature of metformin inhibiting its ability to interact with the hydrophobic chains of C8, C18 and phenyl moiety. To overcome this problem, we tried manipulating the mobile phase composition by incorporating different concentrations of SDS. SDS was first used in metformin separation within tablet formulations by Kolte et al. (14) in a concentration of 5 mM. It is a well-known anionic surfactant used as a pseudo-stationary phase. At concentrations above the critical micelle concentration (CMC), SDS forms micelles which provide a pseudo-stationary phase that can effectively separate compounds based on partitioning between the aqueous mobile phase and the hydrophobic interior of the SDS micelles (10). Accordingly, it decreases the polarity of metformin, permitting its partitioning with the highly hydrophobic long C18 chain. As the amount of SDS was added, generally the longer would be the retention time of metformin on C18 column. A concentration of 3 mM was found to be appropriate for the separation and both metformin and IS, and showed acceptable peak symmetry and suitable retention times (Fig. 1d,e).

Regarding extraction of metformin from biological matrices, protein precipitation followed by an organic solvent wash has used for plasma sample preparation for metformin (Table 1). As others have found, protein

precipitation by itself was ineffective in removing all of the endogenous substances (Fig 1a). A disadvantage of protein precipitation is a lowering of sensitivity due to the sample dilution that it entails. Alkaline conditions have been used to extract metformin into a suitable organic solvent (3). For the present method workup, the extractability of metformin from plasma was tested in recovery experiments using dichloromethane, hexane, TBME and 1-butanol/n-hexane (50:50 % v/v) mixture. Both the TBME and 1-butanol/hexane mixtures were found to be most effective in terms of removing endogenous substances, but in terms of recovery, 1-butanol and hexane provided the optimal recovery. Both evaporation of extracting solvent and back-extraction into 0.2% acetic acid were tested. Back extraction of metformin into an acidic aqueous medium yielded a satisfactory outcome in terms of both selectivity and removal of interferences.

Use of the structurally related biguanide compound phenformin, which had been used as internal standard in other assays (2,11), proved to be unsuitable for the chromatographic conditions employed, due to its delayed elution time. In those methods phenformin retention times were two to three-fold longer than that for metformin, thereby prolonging the total run time for the assay. In method workup, atenolol (7) and propranolol (17) were tried but were not suitable using the chromatographic conditions used. Ranitidine was used as reported by Amini et al. (3) and with our analytical conditions found it to offer high and reproducible recovery and reasonable retention time.

The assay was successfully used to monitor plasma concentrations of metformin for up to 24 h after dosing of 1000 mg to two volunteers (Fig. 2). Each of the pharmacokinetic parameters measured in the subjects were in line with previous dose-normalized observations in volunteers (20).

CONCLUSION

The method displayed high calibers of sensitivity and selectivity for monitoring concentrations of metformin in both plasma and

urine, based on a small (0.1 mL) sample volume. It was simple in terms of sample preparation and involved liquid-liquid extraction, which was rapid and accessible by the virtue of using a single C18 column. The method was also shown to be applicable for research involving both human plasma and urine specimens as part of a pharmacokinetic experiment.

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