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Determination of metformin in human plasma by high-performance liquid chromatography with spectrophotometric detection

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Abstract

A simple, selective, sensitive and precise high-performance liquid chromatographic plasma assay for the hypoglycemic agent metformin is described. Acidified samples of plasma were deproteinated with acetonitrile, washed with dichloromethane and the resulting supernatant injected. Chromatography was performed at 40°C by pumping a mobile phase of acetonitrile (250 ml) in pH 7, 0.03 *M* diammonium hydrogen phosphate buffer (750 ml) at a flow-rate of 1 ml/min through a silica column. Metformin and the internal standard (atenolol) were detected at 240 nm and were eluted 7.8 and 6.8 min, respectively, after injection. No endogenous substances were found to interfere. Calibration curves were linear ($r>0.999$) from 10 to 2000 ng/ml. The absolute recovery of both metformin and atenolol was greater than 76%. The detection limit and limit of quantitation were 2.5 and 10 ng/ml, respectively. The intra- and inter-day precision (C.V.) was 12%, or less, and the accuracy was within 6.2% of the nominal concentration. This method is suitable for clinical investigation and monitoring metformin concentration.

Keywords: Metformin

1. Introduction

Metformin (1,1-dimethylbiguanide) is an oral antihyperglycemic agent that has been used in the treatment of non-insulin-dependent diabetes mellitus (type II) since 1957. In 1995, metformin received FDA approval and renewal of interest in this drug has been observed in recent years [1]. In spite of the long-standing availability of metformin, there is relatively little information on its pharmacokinetics in man. There is also limiting data available regarding its disposition in different tissues or organs [1,2]. The most serious side effect associated with metformin is lactic acidosis, which may be related to high circulating concentrations of the drug [3]. In vitro exposure to high concentration of metformin has been shown to delay neural tube closure in early-somite mouse embryos [4]. More recently, metformin has been reported to induce acute hepatitis [5]. Although the risk of accumulation and lactic acidosis increases with the degree of impairment of renal function, the mechanism of metformin induced lactic acidosis is not yet sufficiently established [1,3]. And the role of therapeutic monitoring on metformin to improve the therapeutic efficacy and to minimize adverse side effects is still under investigation. To

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address all these issues, a suitable assay for quantitation of metformin in various biological fluids is deem necessary.

Several high-performance liquid chromatography (HPLC) methods, with [6–8] or without [9–18] chemical derivatization, were used for the determination of metformin in biological fluids. Previously described methods suffered from several disadvantages, such as lack of sensitivity [9,12,13,16,18], using complex extraction procedures which are tedious and time consuming [6–8], required the use of ultrafiltration and a column-switching system [18], and required relatively large sample volume [11,14]. The major investigations usually quoted were carried out using analytical methods that are indicated in the literature, but have not yet been published in detail nor have been fully validated for accuracy, precision and sensitivity [11,15].

For pharmacokinetic studies, a suitably sensitive method that allows an accurate measurement of low concentration of metformin in plasma is needed. For routine drug monitoring, assays that required a small sample volume are the most desirable. In this report, we present a simple, sensitive and specific HPLC method to determine metformin concentration in human plasma. The method has a lower detection limit than the previously published procedures and has been validated using both 0.1 and 0.5 ml of plasma. The applicability of this assay was demonstrated in animal and human pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Metformin hydrochloride (Lot 84H0451) and atenolol (Lot 106H0888) were purchased from Sigma (St. Louis, MO, USA). All chemicals were analytical-grade reagents and used as-received without further purification. HPLC-grade acetonitrile and dichloromethane were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Milli-Q Reagent Water (Millipore, Bedford, MA, USA) was used in the preparation of the mobile phase. Human plasma was from the Blood Bank of National Cheng Kung University Hospital (Tainan, Taiwan).

2.2. Instrumentation and chromatography

The HPLC system consisted of a Hitachi L-7100 pump, a Hitachi L-7200 autosampler, a Hitachi L-7400 UV detector (Tokyo, Japan) and an SISC Data Station (Scientific Information Service Corporation, Taipei, Taiwan). The analytical column, Hypersil HS Silica 5 μ, 25 cm×4.6 mm I.D., was protected by a precolumn (Hichrom Silica H5, 10×3.2 mm I.D.). The temperature of the column was maintained at 40°C using a Bio-Rad Column Oven (Hercules, CA, USA). The mobile phase, consisting of 25% acetonitrile and 75% pH 7, 0.03 *M* $(NH_4)_2HPO_4$, was pumped at a flow-rate of 1 ml/min. The prepared mobile phase was filtered through a 0.45-μm Millipore filter and degassed ultrasonically before used. The detector wavelength was set at 240 nm and peak areas were measured.

2.3. Standards and controls

Master stock solutions of metformin hydrochloride (1 mg/ml) and the internal standard atenolol (1 mg/ml) were prepared in water monthly and kept tightly sealed at −80°C. The stock solution of metformin was diluted with drug-free plasma to give the calibration standards at concentrations of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml metformin hydrochloride. The quality controls were prepared independently at concentrations of 10, 100 and 1000 ng/ml prior to the start of sample collection and stored at −80°C until used. The working solutions of atenolol (0.1 mg/ml) were prepared from the stock solution. A complete calibration curve was generated with each analytical run.

2.4. Sample preparation

The samples to be analysed were removed from the freezer and thawed. Calibration standards, controls and unknown samples were processed in batches. To 0.5 ml of human plasma samples, in a 10-ml culture tube, 10 μl of atenolol (0.1 mg/ml), 50 μl of 1*M* HCl and 1.5 ml of acetonitrile were added. The contents of the tube were vortex-mixed for 30 s and centrifuged at 1763 *g* for 5 min. The transferred supernatant was washed with 1.5 ml of dichloromethane by vortex-mixing for 30 s. After

centrifugation (1763 *g*, 5 min), an aliquot of the aqueous layer was injected onto the column. When a smaller plasma volume of 0.1 ml was processed in 0.6-ml Eppendorf tubes, the volumes of all the reagents used were reduced proportionally except for the internal standard solution.

2.5. Quantitation

The model for the calibration curve of metformin used a natural logarithmic transformation (ln) of the peak area ratio of metformin to atenolol (PAR) and the metformin concentration (C), as given in the following equation: ln (PAR) = slope × ln (*C*) + (*y*-intercept). The slope and *y* intercept were determined by linear regression analysis (Excel 97 SR-1, Microsoft, Redmond, WA, USA) using nominal concentrations and measured PARs from calibration standards. Metformin concentrations were estimated from PARs using the formula:

$$C = e^{[\ln (PAR) - (y \text{ intercept})]/\text{slope}}.$$

2.6. Precision, accuracy and limit of quantitation

Intra-day precision was evaluated by analysing the spiked controls six times over 1 day in random order, while inter-day precision was evaluated from the analysis of each control once on each of six different days. Assay precision (coefficient of variation, C.V.) was assessed by expressing the standard deviation of the measurements as a percentage of the mean value. The accuracy was estimated for each spiked control by comparing the nominal concentration with the assayed concentration. The lower limit of quantitation was the lowest non-zero concentration level, which could be accurately (relative error $<$ 20%) and reproducibly (C.V. $<$ 20%) quantitated [19].

2.7. Recovery

Absolute recoveries of 10 and 2000 ng/ml concentrations of metformin in plasma were determined by assaying the samples as described above and comparing the peak areas of both metformin and atenolol with those obtained from direct injection of the compounds dissolved in aqueous supernatant of the processed blank plasma.

2.8. Stability

The freeze–thaw stability of metformin was determined in plasma samples comprising 10, 100 and 1000 ng/ml metformin for two freeze–thaw cycles. The stability of metformin (100 ng/ml) in processed samples left at ambient temperature (ca. 20°C) in autosampler vials was followed for 72 h. The long-term stability of metformin in samples stored at −80°C in screw cap vials was followed for 2 years.

2.9. Selectivity

Assay selectivity was examined in relation to interference from endogenous substances in the drug-free plasma. The retention times of a selection of drugs that may be potentially administered with metformin were measured following injection of solutions of these drugs in the mobile phase.

2.10. Clinical application

The assay was applied to an open-label, single dose (500 mg of metformin hydrochloride) pharmacokinetic study in young healthy volunteers. The study was approved by the Institutional Review Board of National Cheng Kung University Hospital. Subjects were screened by medical history, laboratory and physical examinations. Informed consent was obtained prior to the start of the study. After drug administration, blood samples for analytical determinations were collected at specific time intervals for 24 h. Plasma samples were stored at −80°C until analysis.

3. Results and discussion

3.1. Chromatography

Chromatograms of drug-free plasma samples, spiked calibration samples and post-dose clinical study samples are shown in Figs. 1 and 2 for extracts of 0.5- and 0.1-ml plasma samples, respectively. A good separation of atenolol and metformin was achieved with the retention times of 6.8 and 7.8 min, respectively, without any interfering endogenous peaks. The specificity of the assay was further

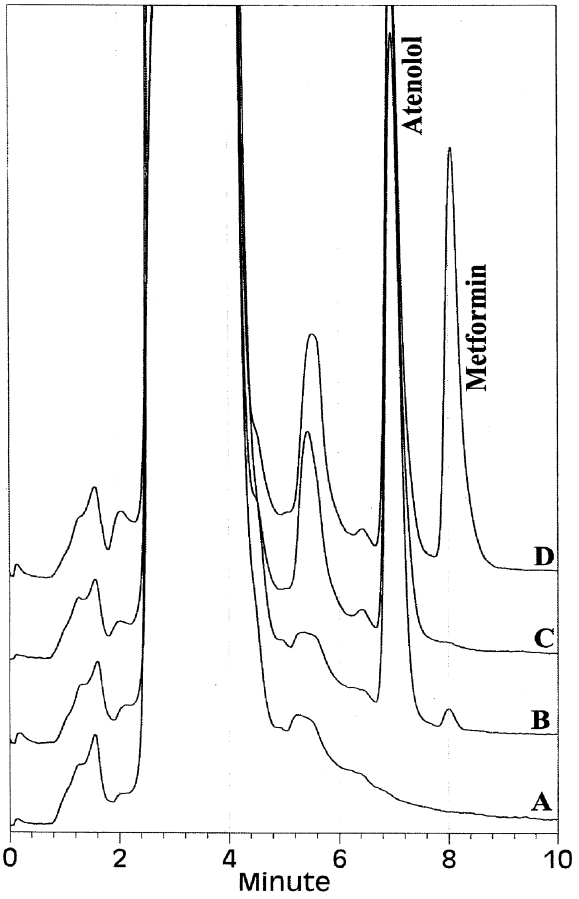

Fig. 1. Chromatograms of extracts of 0.5-ml human plasma: blank plasma (A), blank plasma spiked with 10 ng/ml metformin hydrochloride and the internal standard (B) and plasma samples obtained from a subject before (C) and 8 h (D) after administration of 500 mg of metformin hydrochloride. The response range was set at 5 mV.

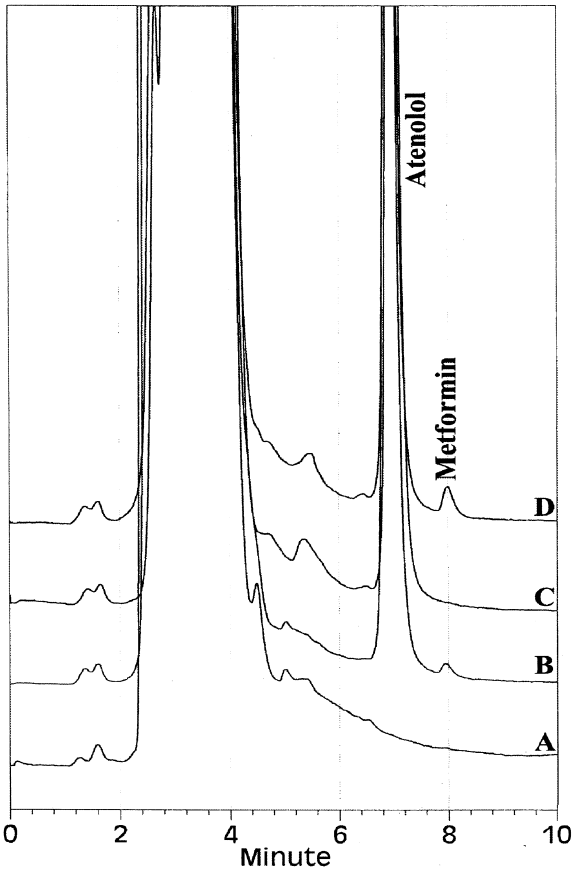

Fig. 2. Chromatograms of extracts of 0.1-ml human plasma: blank plasma (A), blank plasma spiked with 10 ng/ml metformin hydrochloride and the internal standard (B) and plasma samples obtained from a subject before (C) and 24 h (D) after administration of 500 mg of metformin hydrochloride. The response range was set at 5 mV.

examined by the absence of co-eluting peaks when the following drugs were injected: baclofen, caffeine, diphenidol, gliclazide, metoclopramide, paroxetin, quinine, salicylic acid, terfenadine, theophylline, tramadol, probenecid, triamterene; anti-arrhythmics — procainamide, quinidine; antibiotics — cefaclor, cefadroxil, cefamandole, cefixime, ceftriaxone, cefuroxime, cephalexin; antipsychotics — chlorpromazine, haloperidol; beta-blockers — alprenolol, propranolol; and H_2-receptor antagonists — cimetidine, famotidine, ranitidine.

3.2. Calibration and validation

The calibration curves were linear from 10 to 2000 ng/ml. The mean±SD regression equation for 12 replicated calibration curves constructed using 0.5-ml plasma samples on different days was: ln (PAR) = (1.02±0.02) × ln C + (−5.90±0.14), r^2 = 0.9993±0.0005. And the equation for six replicated calibration curves constructed using 0.1-ml plasma samples was: ln (PAR) = (1.04±0.02) × ln C + (−7.66±0.10), r^2 = 0.999±0.001. The slopes were essentially identical, whereas the difference in inter-

Table 1
Intra-day and inter-day accuracy and precision for the determination of metformin in human plasma using 0.1 or 0.5 ml of plasma ($N=6$)

$C_{nominal}$ (ng/ml)	Intra-day			Inter-day		
	$C_{est.}$ (ng/ml)	C.V. (%)	Error (%)	$C_{est.}$ (ng/ml)	C.V. (%)	Error (%)
0.1 ml of plasma						
10	9.5	11.3	−5.5	9.7	5.6	−3.0
100	103.0	4.2	3.0	100.0	5.2	0.2
1000	996.0	4.2	−0.4	999.0	4.8	−0.1
0.5 ml of plasma						
10	9.5	4.9	−4.6	9.4	12.0	−5.6
100	106.0	0.7	6.2	103.0	3.5	2.6
1000	1009.0	0.4	0.9	1029.0	5.5	2.9

$C_{nominal}$ = Nominal concentration; $C_{est.}$ = estimated concentration; C.V. = coefficient of variation.

cepts corresponded to the different concentration of atenolol in the processed samples. Virtually the same assay results were obtained for clinical samples irrespective of the volume of plasma samples processed.

Precision and accuracy (10–1000 ng/ml) were investigated by replicated analyses of spiked controls (Table 1), and in all cases the intra-day and inter-day precision was acceptable at a C.V.-value of 12% or less. In addition, accuracy was within 6.2% when compared with nominal concentrations across this range. For this validation, the limit of quantitation (LOQ) was set at 10 ng/ml. The limit of detection was 2.5 ng/ml as determined using a signal-to-noise ratio of 3.

Satisfactory assay sensitivity was assisted by relatively high and reproducible recoveries for metformin and atenolol as shown in Table 2. The suitability of atenolol as the internal standard is demonstrated as the relative recovery of metformin, determined by comparing the peak area ratios (metformin/atenolol) of the processed samples with that of unprocessed control samples was closed to 100%.

3.3. Stability

The mean concentration for the quality control samples (9.8, 102.9 and 1004 ng/ml) was within 2.9% of nominals for metformin following the second freeze–thaw cycle. Metformin was stable in human plasma at ambient temperature for up to 48 h [17,18]. Vallee et al. also reported that metformin was stable for 72 h in autosampler vials following sample preparation via solid-phase extraction [17]. However, significant decomposition of metformin in the final plasma extract stored at 4°C has been shown to occur using the solid-phase extraction method [11]. In this study, metformin (100 ng/ml) was stable in processed samples left at ambient temperature for up to 72 h with the estimated concentrations (mean±SD, $N=3$) at 24, 48 and 72 h of 100.1±8.8, 96.3±3.5 and 101.1±4.1 ng/ml, respectively. Long-

Table 2
Recovery of metformin in spiked human plasma in the presence of internal standard (mean±SD, $N=5$)

Concentration (ng/ml)	Absolute recovery (%)		Relative recovery (%)
	Metformin	Internal standard	
10	76.9±7.6	77.2±7.6	99.8±10.6
2000	76.7±0.7	75.9±1.3	101.1±1.0

term stability of metformin in plasma stored frozen at −10, −20 and −70°C has been investigated previously for up to 5, 8 and 8 months, respectively [14,18]. In this study, satisfactory stability was found during storage of up to 2 years at −80°C (Fig. 3), which permits stockpiling of clinical samples during collection periods for subsequent batch analysis.

3.4. Application

The assay was applied to a preliminary pharmacokinetic experiment. A single dose of 500 mg of metformin hydrochloride was administered to healthy volunteers after an overnight fast. Blood samples were collected at scheduled intervals. The plasma concentration–time profile is illustrated in Fig. 4. The results show that this simple and rapid method is sufficiently sensitive to accurately follow blood level for at least three half-lives after peak time. The present assay has also been applied successfully to study the hepatic disposition of metformin in isolated perfused rat liver [2].

3.5. Method development

Several methods for the measurement of plasma metformin have been developed, most of which have been based on HPLC with spectrophotometric detection in the range of 230–240 nm (Table 3).

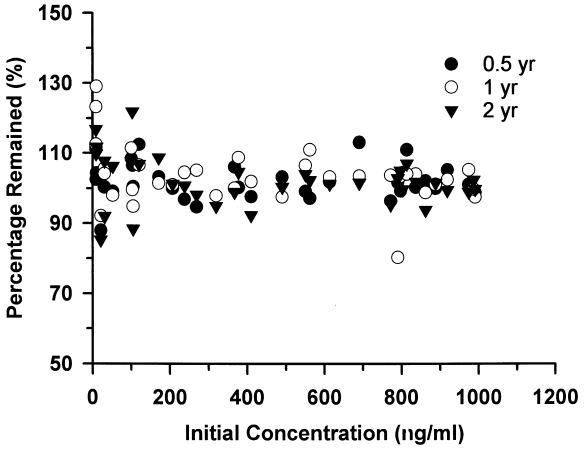

Fig. 3. The long-term stability of metformin in frozen human plasma stored at −80°C.

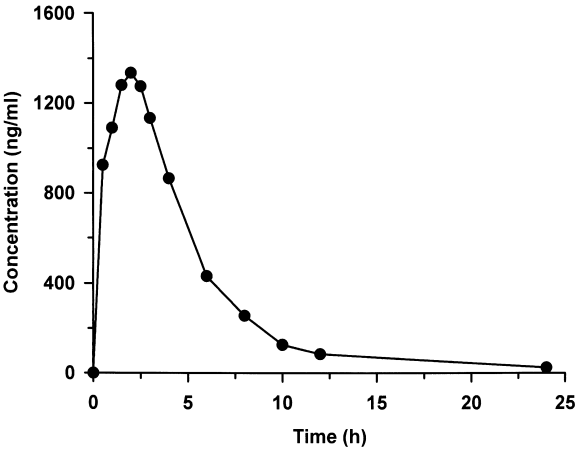

Fig. 4. Plasma concentration–time profile after oral administration of a 500-mg metformin hydrochloride tablet to a healthy subject.

Metformin can be eluted using silica [15,20], cyano [11,16], C_{18} [9,14], phenyl [13] or cation-exchange columns [10,12,18] with mobile phases consist of a mixture of phosphate buffer (pH 2.4–7.5) and acetonitrile. Some reports used organic amines as mobile phase additives [7,8]. In the present method, both metformin and atenolol show acceptable peak symmetry without the addition of organic amines to improve peak shape.

Due to its high polarity, metformin is particularly difficult to extract. Early attempt using bromothymol blue as the pair ion, extracted by ether–dichloromethane followed by back-extraction with tetrabutylammonium hydroxide resulted in a low absolute recovery (27%) even after double extraction (43%) [14]. A higher relative recovery for metformin (59–80%) was obtained using bromothymol blue as the pair ion followed by double extraction with chloroform, however, the recovery was concentration-dependent and associated with poor reproducibility [20]. Protein precipitation that uses dilution with acetonitrile [9] and methanol–$ZnSO_4$–ethylene glycol solution [10] reduces sensitivity, whereas the use of trichloroacetic acid [12] or perchloric acid [16] results in an acidic supernatant, which is generally not suitable for direct injection onto the column and further dilution may be necessary. Using ultrafiltration and an online extraction column, an absolute recovery of 98.5% was obtained [18]. In the present

Table 3
Analytical characteristics of reported HPLC methods for the determination of metformin in human plasma

Ref.	Column[a]	Mobile phase	Internal standard	Flow (ml/min)	Run time (min)	UV (nm)	Volume (ml)	Pretreatment/ extraction[a]	LOQ (ng/ml)
Cheng and Chou	Si	30 m*M* $(NH_4)_2HPO_4$ (pH 7)–CH_3CN =75:25	Atenolol	1	10	240	0.1,0.5	CH_3CN–CH_2Cl_2	10
Bonfigi et al. [10]	CX	50 m*M* KH_2PO_4–CH_3CN=76:24 (pH 5.3)	Buformin	1	21	236	0.2	CH_3OH–$ZnSO_4$–EG	20
Vesterqvist et al. [18]	CX	0.4 *M* $(NH_4)_2HPO_4$	No	2	12	232	0.3	Ultrafiltration	100
Yuen and Peh [16]	Cyano	10 m*M* KH_2PO_4 (pH 3.5)–CH_3CN=6:4	No	1	8	234	0.25	PCA	60
Song et al. [20][b]	Si	0.1 *M* Phosphate buffer (pH 2.5)	Phenformin	?	13	195	0.1	IPE	250
Vallee et al. [17]	?	?	?	?	?	?	?	SPE (?)	20
Sambol et al. [15]	Si	10 m*M* $(NH_4)_2HPO_4$–CH_3CN=8:2 (pH 7.5)	Propylbiguanide	?	?	235	?	CH_3CN–CH_2Cl_2	10
Caille et al. [11]	Cyano	5 m*M* Phosphate (pH 3)–CH_3CN =6:4 (+dibutylamine)	Phenformin	1.4	8?	?	1	SPE (CN)	?
Huupponen et al. [13]	Phenyl	10 m*M* KH_2PO_4(pH 7)–CH_3CN=6:4 (+diethylamine)	Phenformin	1.35	6	236	0.5	SPE (C_8)	50
Keal and Somogyi [14]	C_{18}	3 m*M* Heptanesulfonic acid (pH 4)+ 50 m*M* KH_2PO_4–CH_3CN=92:8	Propylbiguanide	1	9	234	1	IPE	10
Benzi et al. [9]	C_{18}	10 m*M* Phosphate–CH_3CN=5:5	No	?	10	254	0.3	CH_3CN	75
Charles et al. [12]	CX	30 m*M* $(NH_4)_2HPO_4$ (pH 2.4)	Propylbiguanide	3	12	230	0.5	TCA	200

[a] CX=Cation-exchange, EG=ethylene glycol, IPE=ion-pair extraction, PCA=perchloric acid, SPE=solid-phase extraction, TCA=trichloroacetic acid.
[b] Capillary electrophoresis.

study, dichloromethane was used to wash the aqueous phase and to separate it from the organic phase of the processed sample mixture. The volume of the final aqueous supernatant was slightly less than the initial volume of plasma; therefore, the dilution caused by acetonitrile was minimized.

Several structure-related biguanides have been used as the internal standard in various assays of metformin (Table 3). As propylbiguanide is not commercially available, assays that used it as the internal standard cannot be readily applied [12,14,15]. In a recent report, the retention time of the internal standard buformin (19.5 min) was more than twice of that for metformin (9.5 min), which inevitably prolongs the total run time for assay [10]. Several groups used phenformin as the internal standard [7,8,14]. The absolute recovery of phenformin (75%) was less than that of metformin (85%) using solid-phase extraction [11]. Phenformin is less hydrophilic than metformin and it binds to plasma protein. Although adequate retention for phenformin can be achieved in our LC system, it was found that the extraction recoveries of phenformin varied among different batches of plasma (data not shown), whereas those of metformin remain unchanged and were greater than that for phenformin. The exact causes were not known, although the variations in pH and the protein/lipid content of plasma were speculated. Atenolol was employed as the internal standard in the present method, even though structurally it is less similar to metformin as compared with the other biguanides. Both atenolol and metformin are weak bases and predominantly ionized at acidic pH. The recovery of atenolol was the same as that of metformin and did not vary with plasma sources.

4. Conclusion

This new method has proved to be simple, useful and appropriate, not only for clinical research but also animal studies. It offers the sensitivity and selectivity for monitoring therapeutic concentrations of metformin. The method is flexible and requires only 0.1 ml of plasma, making it suitable for the

study of pharmacokinetics of metformin in man and rat.

Acknowledgements

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