

Determination of glibenclamide and its two major metabolites in human serum and urine by column liquid chromatography

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ABSTRACT

A simple reversed-phase liquid chromatographic method for the measurement of low concentrations of glibenclamide (glyburide) and its two major metabolites, 4-*trans*- and 3-*cis*-hydroxyglibenclamide, in human serum and urine has been developed. The compounds were extracted with *n*-hexane–dichloromethane (1:1). The UV detection wavelength was 203 nm. The minimum detectable serum level of glibenclamide was 1 ng/ml (2 nM), and the relative standard deviation was 8.9% ($n = 9$). When maximum sensitivity was desired the metabolites were chromatographed separately. Metabolites in urine were measured by the same method after five-fold sample dilution. The utility of the method was tested on a healthy volunteer who ingested 3.5 mg of glibenclamide. The parent drug was present in the serum for at least 18 h, and the metabolites in the urine for at least 24 h.

INTRODUCTION

Sulphonylurea drugs have been used for over 40 years in the treatment of non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). The most commonly employed agent is glibenclamide (glyburide), which is effective at very low concentrations [1–4]. So far, no analytical method has been sufficiently sensitive, specific and precise to appropriately describe the pharmacokinetics of glibenclamide and its metabolites in humans. UV spectrophotometric [5], colorimetric [5], fluorometric [5,6], gas chromatographic [7] and radioimmunoassay techniques [8] have all been reported. Liquid chromatographic (LC) methods have been described more recently and seem generally superior [9–16]. A method

for glipizide has been published [10], which also permits the determination of other sulphonylurea drugs including glibenclamide, but the sensitivity is not sufficient for pharmacokinetic studies on glibenclamide. A modification has been published that allows the measurement of low concentrations (5–10 ng/ml) of glibenclamide in human plasma and urine [15]. However, this is still not sufficient, and the assay employs benzene for extraction.

This paper describes a simple method with increased sensitivity and a less harmful extraction medium. It allows simultaneous extraction of glibenclamide and its metabolites, 4-*trans*- and 3-*cis*-hydroxyglibenclamide, from human serum. The metabolites can be detected using the same mobile phase as for glibenclamide, but re-analysis with a slower mobile phase may be necessary for quantification of the metabolites. The same technique can be used for the analysis of the metabolites in human urine. The utility of this method in pharmacokinetic studies was demonstrated.

EXPERIMENTAL

Apparatus

The chromatographic system comprised a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 A pump equipped with a U6K injector, a Chrompack reversed-phase guard column and a Chrompack Chromsep microsphere C₁₈ column (3 μ m, 100 mm \times 4.6 mm I.D.). A Waters Assoc. UV detector, Lambda-Max Model 481, was used. The detection wavelength was 203 nm and the sensitivity was kept at 0.008–0.064 a.u.f.s. Chromatograms were recorded with a chart speed of 5 or 10 mm/min using a Spectra-Physics Model 4290 integrator.

Chemicals and reagents

Glibenclamide and its metabolites, 4-*trans*- (M1) and 3-*cis*-hydroxyglibenclamide (M2) were supplied by Hoechst (Frankfurt, Germany) and the internal standard (I.S.) glibornuride was supplied by Hoffmann-La Roche (Basle, Switzerland) (Fig. 1). Acetonitrile was of specially pure HPLC S-grade (Rathburn Chemicals, Walkerburn, U.K.). Dichloromethane and *n*-hexane (LiChrosolv) of HPLC grade were bought from Merck, and methanol (pronalys AR) was obtained from AB Lab Kemi (Stockholm, Sweden). Tetrabutylammonium (TBA) hydrogen sulphate was supplied by Sigma (Stockholm, Sweden). All other buffer substances and reagents were of analytical grade. The buffers used were prepared from phosphate salts and distilled water.

Chromatographic technique

The mobile phase was acetonitrile–0.038 M phosphate buffer (pH 7.45–7.50) (27–28:73–72, v/v). This allowed the determination of glibenclamide and the detection of the metabolites. For quantification of the metabolites in serum or urine the mobile phase contained 22–24% acetonitrile. For quantification of very low

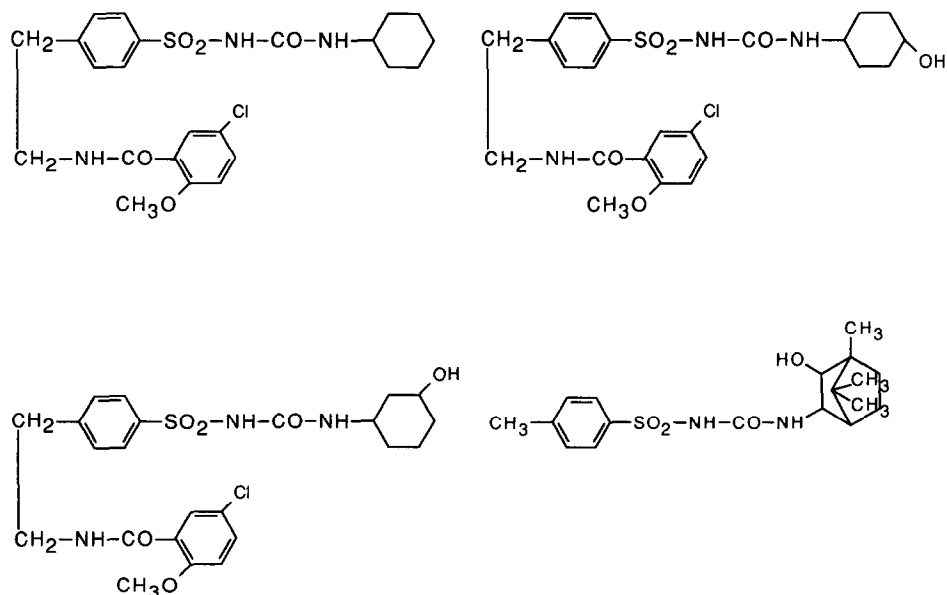


Fig. 1. Molecular structures of glibenclamide (upper left), 4-*trans*-hydroxyglibenclamide (M1) (upper right), 3-*cis*-hydroxyglibenclamide (M2) (bottom left) and glibornuride (I.S.) (bottom right).

(less than 10 ng/ml) concentrations of glibenclamide, 29% acetonitrile was used.

The mobile phase was degassed by filtration through a Millipore filter (HV 0.45 μm) or by sonication 15 min before use. The flow-rate was 0.7–1.0 ml/min, depending on the acetonitrile content. The mobile phase was not recirculated. The system was run at room temperature and pressures ranged from 12 to 17 MPa. Before any sample was introduced into the chromatograph, the system was equilibrated with the mobile phase for at least 1 h. A test solution containing glibenclamide, M1, M2 and the I.S. in methanol (4.0 $\mu\text{g}/\text{ml}$ of each substance) was injected to check the column efficiency and the retention times. The reduced plate height must not exceed 10 for any of the compounds, and after one more injection of the test solution the difference in peak-height ratios should not exceed 2%. The serum standard of 200 ng/ml (15 μl) was assayed twice, and the difference in peaks-height ratios should not exceed 3%. Then drug-free serum was injected and the calibration curve was obtained. The column was thoroughly cleaned overnight with acetonitrile–distilled water (30:70, v/v), pumped at 0.3 ml/min.

Stock solutions and standards

Methanol stock solutions of glibenclamide, M1, M2 (5.00 and 0.50 $\mu\text{g}/\text{ml}$ of each) and the I.S. (15.00 $\mu\text{g}/\text{ml}$) were stable for at least six months at 4–8°C. Known amounts of the solutions were added to 1.0 ml of drug-free serum or drug-free diluted urine to obtain calibration curves in the range 2.5–100, 10–200 or 50–500 ng/ml. Six standards were prepared for each curve.

Serum and urine samples

Serum and urine samples were stored at -20°C in plastic polystyrene tubes (Eppendorf Reaktionsgefäße 3810) until assayed. Urine samples were diluted with distilled water five times before assay.

Extraction and analytical procedure

Determination of distribution and extraction constants of glibenclamide and glibornuride (I.S.). Equal phase volumes of *n*-hexane–dichloromethane (1:1, v/v) and phosphate buffers were shaken for 30 min at 24°C . UV spectrophotometry was used to measure the concentrations of glibenclamide and the I.S. in the aqueous phases. The extraction of glibenclamide and I.S. as ion-pairs with TBA was studied with a similar technique [17,18].

Method A. To a 1.0-ml volume of serum or five-fold diluted urine were added $25\ \mu\text{l}$ of I.S. solution and $100\ \mu\text{l}$ of 2 M hydrochloric acid. After the addition of 6.0 ml of *n*-hexane–dichloromethane (1:1, v/v) the glass tubes were rotated for 10 min. After centrifugation (700 g for 10 min), 5 ml of the organic phase were transferred to a conical tube and evaporated to dryness in a water-bath at 37°C under a gentle stream of air. The residue was redissolved in $50\ \mu\text{l}$ of equilibrated chromatographic mobile phase. Thereafter $10\text{--}25\ \mu\text{l}$ of the solution were injected onto the chromatograph. Standards and a blank of drug-free serum or urine were extracted in the same way. All samples were analysed in duplicate. Method A could be used in most cases.

Method B. When interfering peaks occasionally appeared in the chromatograms obtained with method A, a further step in the extraction process was used. The organic phases were washed, after extraction and separation, with 5.0 ml of 0.025 M TBA in 0.05 M phosphate buffer (pH 12.2) for 10 min by rotation. Standards and a blank of drug-free serum were treated in the same way. This method cannot be used for the determination of M1 and M2 as they are too hydrophilic.

Quantitation and evaluation

Capacity factors and retention times relative to the I.S. peak were used to identify relevant peaks. Peak-height ratios were calculated by dividing the glibenclamide, M1 or M2 peak heights by the I.S. peak height. Calibration curves were constructed by plotting the peak-height ratios of standards *versus* their concentrations.

Patient samples

One healthy male volunteer, 30 years old, height 1.75 m, weight 78 kg, fasted for 10 h prior to oral administration of 3.5 mg of glibenclamide (Daonil® tablet, Lot No. 257B246, Hoechst, Frankfurt, Germany), which was taken with 200 ml of tap water. After 30 min he had a breakfast of 1800 kJ. Blood was collected before tablet intake and at 23 intervals for 18 h after intake. Urine fractions were

collected during 24 h. The total urine volume of each voiding was measured and fractions were saved. The subject had lunch 5.5 h and supper 11 h after drug intake.

Method A has also been tested for interfering peaks in the chromatograms on serum samples from twelve patients with type 2 diabetes mellitus. Six of the patients were treated only with a controlled diet and six with diet and glibenclamide.

RESULTS AND DISCUSSION

Extraction

Glibenclamide is a weak acid with a pK_a of 6.15–6.40 [11] or 6.8 [19] and a partition coefficient of 65 (diethyl ether–buffer) [5] or 2300 (octanol–buffer) [11]. With *n*-hexane–dichloromethane (1:1, v/v) the partition coefficients were 206 (glibenclamide) and 12 (I.S.).

A phase-volume ratio of 6:1 was used for extraction. Recoveries from serum using method A were $100 \pm 2\%$ (glibenclamide and I.S.), $62 \pm 3\%$ (M1) and $80 \pm 3\%$ (M2).

The ion-pair extraction constants were 8800 (glibenclamide + TBA) and 260 (I.S. + TBA). Extraction recoveries as ion-pairs from serum were $100 \pm 2\%$ (glibenclamide) and $90 \pm 2\%$ (I.S.), and for M1 and M2 the recoveries were less than 15%, indicating that method B cannot be used for the metabolite assay. Despite the reported hydrolysis of sulphonylurea at high pH [7], no such degradation was observed.

The resulting chromatograms of a serum blank, and of serum samples spiked with 1.25 and 10 ng/ml drug and treated according to method B are shown in Fig. 2.

Serum gave fewer interfering peaks than plasma. Air was chosen for evaporation as no sign of sulphonylurea decomposition was observed.

Detection wavelength, retention and mobile phase composition

The maximum absorption wavelengths were 202 nm (glibenclamide and I.S.) and 204 nm (M1 and M2) in the mobile phase containing 28% acetonitrile. The corresponding molar absorptivities were 50 000 (glibenclamide), 51 000 (M1), 46 000 (M2) and 30 000 for the I.S. For glibenclamide, this is an improvement of more than three-fold on the previously reported value [15]. The background absorbance was below 0.2, giving acceptably low baseline noise [20], without affecting the detection limit.

Fig. 3 demonstrates the influence of different concentrations of acetonitrile on the capacity factors of M1, M2, the I.S. and glibenclamide. The column efficiency was excellent; reduced plate heights of less than 5.0 were reached at $k' = 1$ –10 when a test solution was injected into the chromatograph. The three columns were used for four, five and seven months, respectively, and *ca.* 1350, 400 and 800

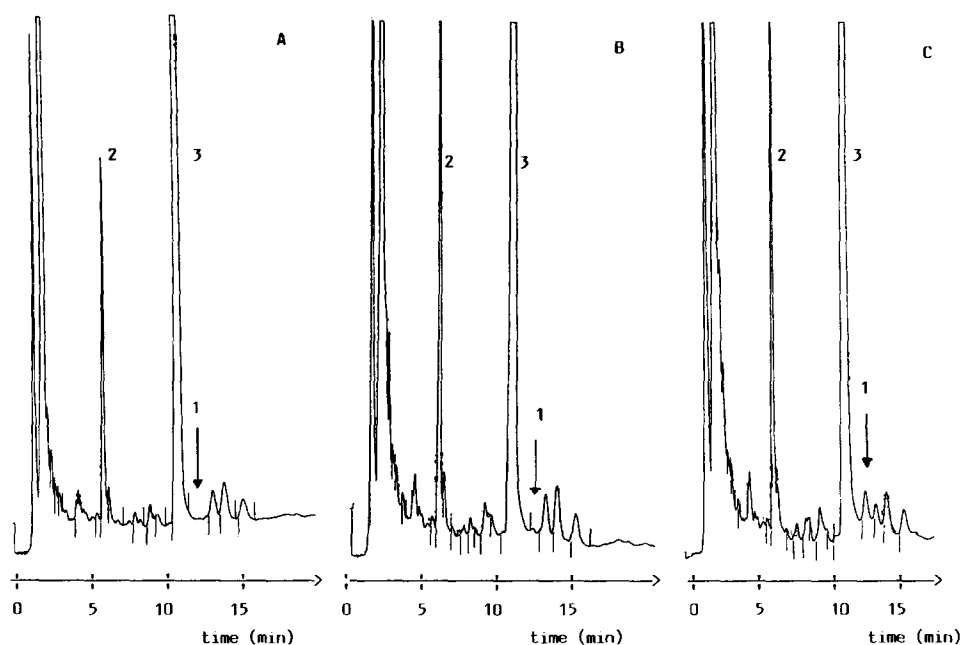


Fig. 2. Chromatograms of (A) a serum blank containing the I.S., (B) a serum blank spiked with glibenclamide (1.25 ng/ml) and the I.S. and (C) a serum blank spiked with glibenclamide (10 ng/ml) and the I.S. Extraction was according to method B. Injection volumes: (A) 15 μ l; (B) and (C) 20 μ l. Mobile phase, acetonitrile-phosphate buffer pH 7.49 (28:72); flow-rate, 0.7 ml/min; detection wavelength, 203 nm; response, 0.016 a.u.f.s.; chart speed, 10 mm/min. Peaks: 1 = glibenclamide (12.7 min); 2 = I.S. (6.0 min); 3 = endogenous compound.

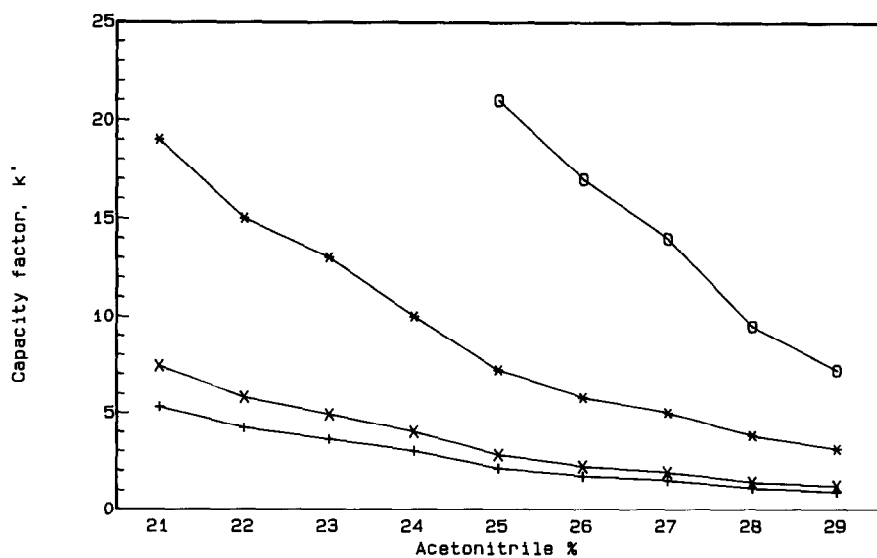


Fig. 3. Influence of acetonitrile concentration in the mobile phase on the capacity factors of glibenclamide (○), I.S. (*), M1 (+) and M2 (×). Stationary phase, Chromsep microspher C_{18} column (3 μ m, 100 mm \times 4.6 mm I.D.); mobile phase, acetonitrile (21–29%)–0.038 M phosphate buffer pH 7.45–7.50 (79–71%).

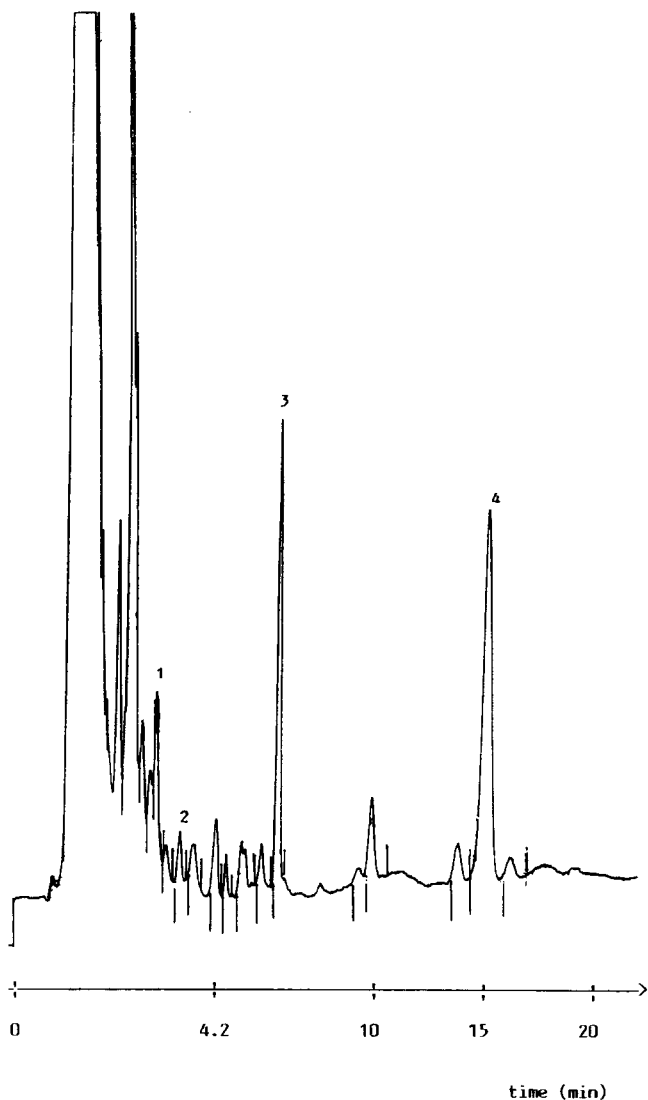


Fig. 4. Chromatogram of a serum sample 105 min after the administration of 3.5 mg of glibenclamide to an 87-year-old woman with type 2 diabetes mellitus. Extraction was according to method A. Injection volume, 10 μ l; mobile phase, acetonitrile-phosphate buffer pH 7.50 (27:73); flow-rate, 0.8 ml/min; detection wavelength, 203 nm; response, 0.016 a.u.f.s.; chart speed, initially 10 mm/min then after 4.2 min 5 mm/min. Peaks: 1 = M1, 104 ng/ml (2.92 min); 2 = M2, 42 ng/ml (3.41 min); 3 = I.S. (6.61 min); 4 = glibenclamide, 406 ng/ml (15.2 min).

samples, respectively, were injected before column degradation was observed.

In serum, M1 and M2 could be detected simultaneously with glibenclamide quantification if the acetonitrile content was 27–28%. However, for quantification of the metabolites in serum or urine the acetonitrile concentration should be

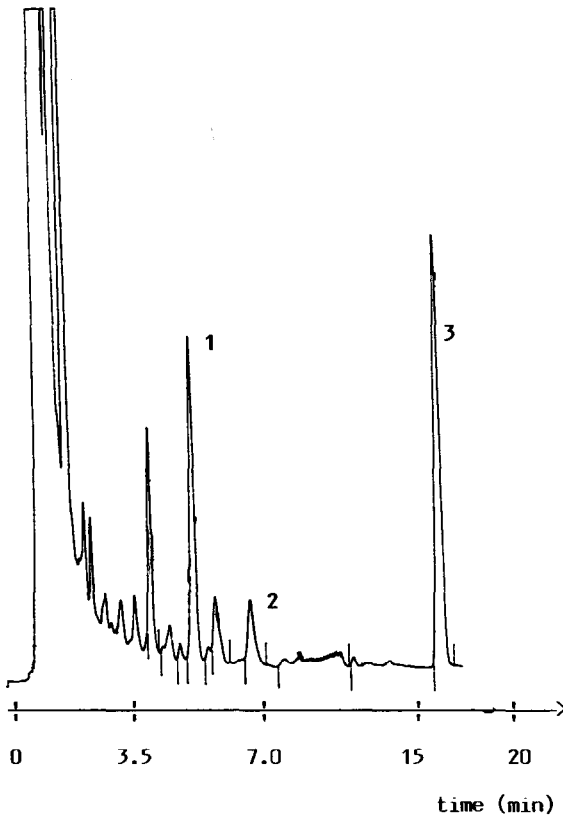


Fig. 5. Chromatogram of a five-fold diluted urine sample fraction collected 1–2 h after the administration of 3.5 mg of glibenclamide to a healthy volunteer. Extraction was according to method A. Injection volume, 20 μ l; mobile phase, acetonitrile–phosphate buffer pH 7.45 (23:77); flow-rate, 0.8 ml/min; detection wavelength, 203 nm; response, 0.032 a.u.f.s.; chart speed, initially 10 mm/min then after 7.0 min 5 mm/min. Peaks: 1 = M1, 107 ng/ml (5.3 min); 2 = M2, 25 ng/ml (6.8 min); 3 = I.S. (15.7 min).

lower (22–24%). Fig. 4 shows a chromatogram of a serum sample from a patient with type 2 diabetes mellitus containing M1, M2 and glibenclamide, and Fig. 5 shows a chromatogram of urine containing M1 and M2.

Assay validation

Two systems were validated: (1) glibenclamide in serum (mobile phase 29% acetonitrile); (2) metabolites in urine (mobile phase 23% acetonitrile). Method A was used for extraction in both systems.

Linearity. Six standards in the range 10–200 ng/ml (system 1) or 50–500 ng/ml (system 2) were assayed. The calibration curves were found to be linear and could be described by the equations

$$y = 0.00393x + 0.0122, r = 0.9994 \text{ (glibenclamide in serum)}$$

$$y = 0.00860x + 0.0424, r = 0.9984 \text{ (M1 in urine)}$$

$$y = 0.00761x - 0.0465, r = 0.9986 \text{ (M2 in urine)}$$

Sensitivity. The minimum detectable concentration (MDC) of glibenclamide in serum was 1 ng/ml (signal-to-noise ratio 5:1). The relative standard deviation at this level was 8.9% ($n = 9$) (see *Precision*). The MDC for the metabolites was 5 ng/ml in serum or diluted urine.

Precision. The reproducibilities of systems 1 and 2 were determined by analysing standards at different levels in the normal concentration range, and for system 1 also separately in the low concentration range under optimal conditions, *i.e.* only 75 μg of the I.S. were added to each standard instead of the usual 375 μg in order to receive higher peak-height ratios. For measurements in the low concentration range, the sensitivity of the detector was kept at 0.008 a.u.f.s. and 40 μl of sample were injected (Table I).

Selectivity. Retention times relative to the I.S. or capacity factors were used to identify glibenclamide and its metabolites, and it was apparent that all three substances were assayed with great selectivity by the described methods. Blanks were used to localize peaks of endogenous compounds. No interference was seen from other tested sulphonylureas: glipizide, tolbutamide and chlorpropamide.

Recovery. See *Extraction*.

Patient samples

The described methods were used to determine glibenclamide and the metabolites in serum (Fig. 6), and the metabolites in urine (Fig. 7). After 3.5 mg of glibenclamide was ingested on an empty stomach, the drug was present in the serum for at least 18 h, and the metabolites were detected in the urine for at least 24 h. The absorption half-life of glibenclamide was 0.26 h ($k = 2.67$ l/h, $r = 0.9708$) and maximum serum concentration of 237 ng/ml was observed after 1.0 h. The elimination half-life was 1.8 h ($k = 0.388$ l/h, $r = 0.9986$) between 2 and 10

TABLE I
PRECISION AND REPRODUCIBILITY DATA

Concentration (ng/ml)	Relative standard deviation (%)			
	System 1		System 2	
	Normal concentration range ($n = 6$)	low concentration range ($n = 9$)	M1 ($n = 6$)	M2 ($n = 6$)
1		8.9		
5		5.3		
10	10.1	3.0		
50		3.3	6.7	3.5
75	4.7			
200	3.9		1.9	2.3
500			1.6	1.8

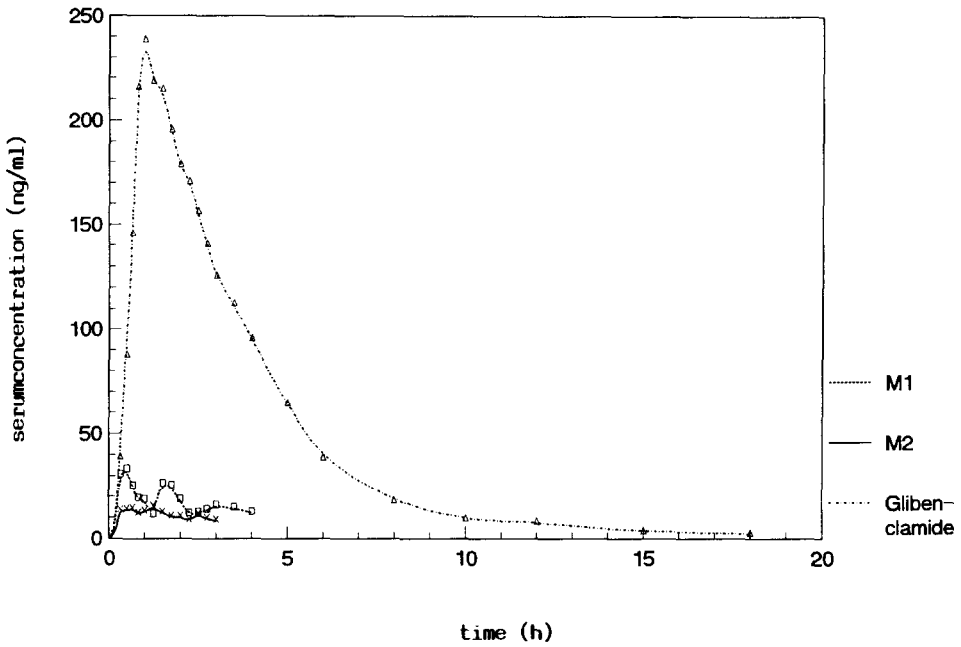


Fig. 6. Serum concentrations of glibenclamide and its two major metabolites after an oral dose of 3.5 mg of glibenclamide in a healthy volunteer.

h, and longer, 3.4 h ($k = 0.205$ l/h, $r = 0.9829$), between 10 and 18 h. The elimination half-lives in urine of M1 and M2 were similar, 3.4 h ($k = 0.203$ l/h, $r = 0.9934$ and $k = 0.205$ l/h, $r = 0.9988$, respectively). The excreted amount of M1 was four to five times that of M2. After 24 h the cumulative amount of M1 excreted in the urine was 1.11 mg and that of M2 was 0.25 mg.

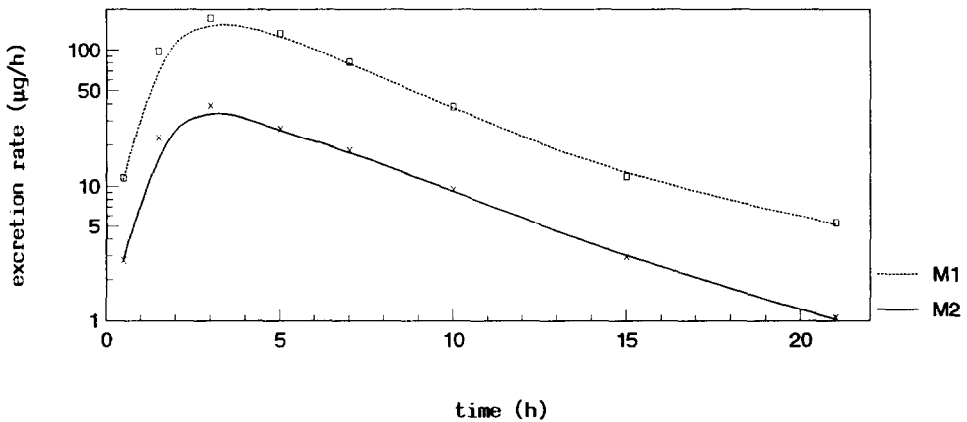


Fig. 7. Semilogarithmic plot of the rate of excretion of M1 and M2 against the midpoint time of urine collection, after an oral dose of 3.5 mg of glibenclamide in a healthy volunteer.

The analysis of serum samples from the patients showed that the method works well with material from a type 2 diabetic population. The peak for glibenclamide was interfered with by other peaks in one of the twelve patients. For M1 and M2 the results were 3/12 and 1/12, respectively.

CONCLUSION

The described method was shown to be suitable for pharmacokinetic studies of glibenclamide and its two major metabolites.

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