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Simultaneous determination of the sulphonylurea glimepiride and its metabolites in human serum and urine by high-performance liquid chromatography after pre-column derivatization^a

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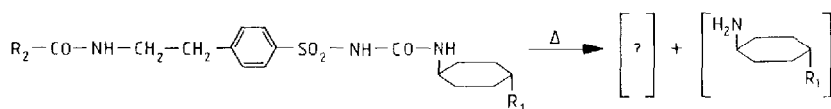
SUMMARY



A sensitive and selective high-performance liquid chromatographic method has been developed for a new sulphonylurea, glimepiride, and its metabolites. The assay involves extraction with diethyl ether, thermolysis of the sulphonylureas at 100°C and trapping of the resulting amines with 2,4-dinitrofluorobenzene. The derivatives were quantitated on a reversed-phase column by absorbance at 350 nm using a step gradient for the three compounds in serum and an isocratic run for the metabolites in urine. Analogous compounds were used as internal standards. The detection limit was 5 ng/ml for glimepiride and metabolite II and 10 ng/ml for metabolite I using 1 ml of serum. The method has been applied to the analysis of serum and urine samples from pharmacokinetic studies in humans.

INTRODUCTION

Glimepiride (HOE 490) is a new sulphonylurea that reduces blood glucose with high efficacy [1]. Thus, the therapeutic dosage in humans is very low (1–3 mg per day). The measurement of such sulphonylureas in serum and urine samples requires very sensitive and specific methods. Various high-performance liquid chromatographic (HPLC) techniques used to determine the related sulphonylurea glibenclamide [2–6] failed with respect to sensitivity and specificity for the determination of glimepiride, especially in the ng/ml range.

^aDedicated to Prof. Dr rer. nat. Hansgeorg Gareis on the occasion of his sixtieth birthday.



Compound	R ₁	R ₂
GLIMEPIRIDE	-CH ₃	
I	-CH ₂ OH	"
II	-CO ₂ H	"
III	-C ₂ H ₅	"
IV	-OH	

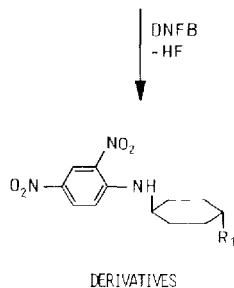


Fig. 1. Structures of glimepiride, I, II and both internal standards (III, IV) and the corresponding derivatives.

Therefore, a method was applied based on thermal cleavage of the sulphonylurea and trapping of the resulting amine in situ with 2,4-dinitrofluorobenzene (DNFB) [7,8]. The 2,4-dinitrophenylamine derivatives (Fig. 1) produced by this method are stable and can be detected at 350 nm, a wavelength that only a few molecules absorb.

The results of metabolism studies in humans [9] supported the application of this method of determination. The metabolites I and II (Fig. 1) were formed, although the major portion in serum is still glimepiride. In contrast, no glimepiride was detected in urine. The metabolites I and II account for more than 80% of the renally excreted dose. This means that both the relevant metabolites show structural changes only in the cyclohexylamine part (Fig. 1) of the sulphonylurea structure. Thus, this derivatization procedure could be extended to the metabolites.

This paper describes the simultaneous determination of glimepiride and its metabolites I and II in serum and the determination of the metabolites I and II in urine.

EXPERIMENTAL

Reagents

The following solvents and reagents were used without specific purification: acetonitrile (HPLC grade S, Rathburn, Walkerburn, U.K.), acetic acid *n*-butyl ester A.R., diethyl ether A.R., citric acid 1-hydrate A.R. and ready-for-use hydrochloric acid-potassium chloride buffer pH 1 (0.05 M) (Riedel de Haen,

Seelze-Hannover, F.R.G.), 1-fluoro-2,4-dinitrobenzene A.R. and perchloric acid 70–72% A.R. (Merck, Darmstadt, F.R.G.) and dimethylchlorosilane (DMCS) (Macherey-Nagel, Düren, F.R.G.).

Standards

Glimepiride and metabolites I and II were supplied by Hoechst (Frankfurt, F.R.G.). Stock solutions (100 $\mu\text{g}/\text{ml}$) of glimepiride and internal standard III (Fig. 1) were prepared by dissolving 10 mg of each compound in 100 ml of ethanol. The stock solutions (100 $\mu\text{g}/\text{ml}$) of I, II and IV (Fig. 1) were prepared by dissolving 1 mg in 10 ml of methanol. All stock solutions were protected from light and kept between 20 and 30°C to prevent precipitation. They were stable for at least one month. Serum calibration samples were prepared by dilution of 50 μl of each stock solution of glimepiride and I and II to 25 ml with drug-free serum to give final concentrations of 200 ng/ml for glimepiride, I and II.

Urine calibration samples were prepared by dilution of 0.5 ml of each stock solution of I and II to 25 ml with drug-free urine to give final concentrations of 2 $\mu\text{g}/\text{ml}$ for I and II. The serum and urine calibration samples were aliquoted and stored frozen at -20°C. They were stable for at least one month. The working solution of both internal standards for serum samples was produced by dilution of 1 ml of each stock solution of III and IV to 10 ml with methanol.

The working solution of internal standard for urine samples was produced by dilution of 1 ml of stock solution of IV to 10 ml with methanol.

HPLC conditions

Serum. Chromatographic separation of the derivatives was achieved on a 125 mm \times 4.6 mm I.D. column filled with Spherisorb ODS, particle size 5 μm (Phase Separations, Queensferry, U.K.). A step gradient [10] was applied: the first mobile phase (A) was 0.05 M perchloric acid-acetonitrile (60:40, v/v); after 6 min, the valve was switched to mobile phase B, which was 0.05 M perchloric acid-acetonitrile (42:58, v/v); after a further 8 min the valve was switched to mobile phase A; 2 min later the next sample was injected.

The injection volume was 100 μl . To prevent phase separation during large series of analyses the prepared samples in the vials of the autoinjector were mixed again shortly before injection by repeatedly aspirating and dispensing the sample in the vials or by bubbling a small volume of air through the sample. This was easily carried out by the programmable Model 231 auto-injector (Gilson).

HPLC analysis was performed at room temperature (ca. 25°C), and the flow-rate was 2 ml/min. Under these conditions the retention times were 3.4 min for the derivative of internal standard IV, 4.3 and 5.2 min for the derivatives of the metabolites II and I, respectively, 11.3 min for the derivative of glimepiride and 13.4 min for the derivative of internal standard III.

Urine. The same chromatographic column was used to separate the derivatives of I, II and IV. The mobile phase was 0.05 M perchloric acid-acetonitrile (60:40, v/v). The flow-rate was 2 ml/min. The mobile phase and the column were at room temperature (ca. 25°C). The injection volume was 100 µl. Under these conditions the retention times were 3.5 min for the derivative of the internal standard IV and 4.8 and 5.6 min for the derivatives of the metabolites II and I, respectively.

Sample preparation and derivatization

Serum. A 40-µl volume of the internal standard solution (containing III and IV) and 1 ml of buffer (pH 1) were added to 1 ml of serum and shaken with 5 ml of diethyl ether for 20 min. After centrifugation at 2500 g for 5 min, 4 ml of the organic phase were transferred to a conical centrifuge tube, which had been silanized with dimethylchlorosilane (DMCS), and evaporated at 30°C under nitrogen. A 100-µl volume of DNFB solution (30 µl of DNFB in 10 ml of acetic acid *n*-butyl ester) were added to the residue and kept for exactly 20 min at 100°C in a heating block. The acetic acid *n*-butyl ester was then evaporated at 60°C under nitrogen, and the residue was taken up in 200 µl of mobile phase A.

Urine. A 50-µl volume of the internal standard solution (containing only IV) and 1 ml of citrate buffer (pH 3, 0.4 M) were added to 1 ml of urine and shaken with 5 ml of diethyl ether for 20 min. All further steps were identical with those in the serum method described above.

Instrumentation

Derivatization of samples was carried out in a TCS heating block (Labor Technik Barkey, Bielefeld, F.R.G.). The chromatographic equipment was a 300 B solvent pump (Gynkotek, Germering, F.R.G.), an electric-driven TMV-6 switching valve (Latek, Heidelberg, F.R.G.), a Model 231 autoinjector (Gilson, Villiers-le-Bel, France), a BT 3030 UV detektor (Biotronik, Maintal, F.R.G.) set at 350 nm, and an SP 4270 integrator (Spectra-Physics, Darmstadt, F.R.G.) giving the peak heights.

Calculations

Quantification was based on the peak-height ratios of the substance of interest and the corresponding internal standard. For glimepiride the internal standard was the ethyl analogue III, for the metabolites I and II the internal standard was IV.

RESULTS AND DISCUSSION

Assay validation

For assay validation, glimepiride and metabolites I and II were mixed over the concentration ranges 6–1000 ng/ml with drug-free human serum and 50–

TABLE I

STATISTICAL VALIDATION OF THE ASSAY FOR GLIMEPIRIDE, I AND II IN SERUM ($n=8$)

Compound	Concentration added (ng/ml)	Concentration found (mean \pm S.D.) (ng/ml)	Coefficient of variation (%)
Glimepiride	6	7.1 \pm 1.9	26.4
	23	26.1 \pm 1.1	4.3
	226	233.1 \pm 6.6	2.8
	1130	1117.8 \pm 36.2	3.2
I	16	20.3 \pm 2.6	12.9
	63	67.0 \pm 3.0	4.5
	157	155.3 \pm 4.0	2.6
	244	245.3 \pm 4.7	1.9
II	7	7.5 \pm 0.5	7.1
	35	34.8 \pm 1.4	4.0
	178	175.3 \pm 3.6	2.1
	355	354.4 \pm 9.8	2.8

TABLE II

STATISTICAL VALIDATION OF THE ASSAY FOR I AND II IN URINE ($n=6$)

Compound	Concentration added (ng/ml)	Concentration found (mean \pm S.D.) (ng/ml)	Coefficient of variation (%)
I	53	60.3 \pm 5.9	9.7
	204	207.2 \pm 11.2	5.4
	1051	1020.7 \pm 34.9	3.4
	5021	5018.3 \pm 198.5	4.0
II	55	55.8 \pm 4.5	8.0
	210	215.7 \pm 11.9	5.5
	1079	1091.2 \pm 35.8	3.3
	5277	5225.0 \pm 160.4	3.1

5000 ng/ml with human drug-free urine. Each mixture was divided into several portions. Determination of serum (urine) samples were carried out in four (three) series on four (three) separate days. These data provided us with a measure of accuracy, precision and linearity of the assay (Tables I and II).

Extraction yield

The extraction yields of glimepiride and metabolites I and II from serum was 85, 50 and 63%, respectively, determined at 1 μ g/ml by comparing the peak heights obtained by direct injection of standard aqueous solutions with those

obtained after diethyl ether extraction. This assay was carried out using a modification of the method described in ref. 2. The extraction yields of metabolites I and II from urine were 75 and 80%, respectively, determined by comparing the peak heights obtained by derivatization of the pure compounds with those obtained after diethyl ether extraction of urine samples spiked with 2 $\mu\text{g}/\text{ml}$ I and II.

Detection limit

In serum samples the detection limits (signal-to-noise ratio of 3) were 5 ng/ml for glimepiride, 10 ng/ml for I and 5 ng/ml for II. In urine samples, variable 'blank' values in the range of 0–30 ng/ml were sometimes observed. Therefore, 50 ng/ml was taken as a preliminary limit of determination for both compounds.

Stability

The compounds were found to be stable in serum and urine at room temperature (20–26°C) and at 37°C for 24 h and also after four freeze–thaw cycles. According to the defined criteria [11] no relevant decrease in concentration was observed. The long-term stability at –20°C is still under investigation. Until now (four months for serum, two months for urine), there have been no indications of instability of the compounds in serum and urine.

Selectivity

Serum (Fig. 2). For glimepiride, no significant interferences were detected in 'blank' human serum samples. For I and II, blank values of ca. 20 and 10 ng/ml, respectively, were sometimes observed.

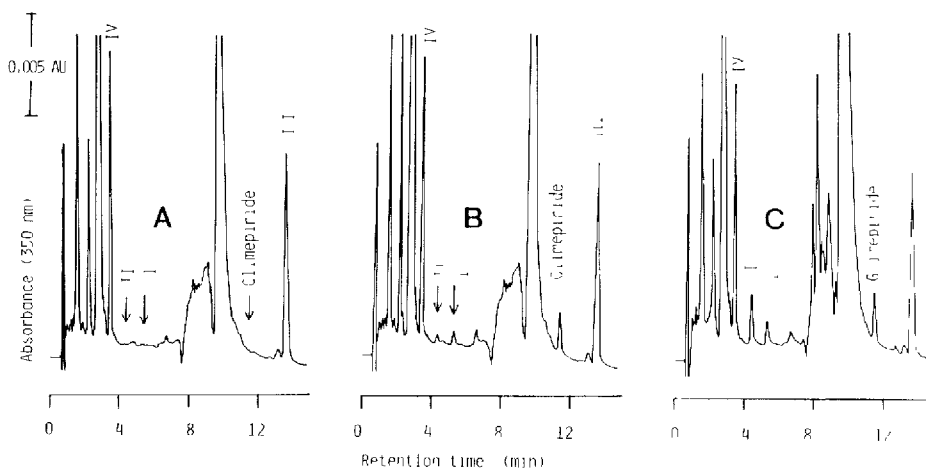


Fig. 2. Chromatograms of derivatized serum samples from a volunteer before (A) and 2.5 h after (B) oral administration of 1 mg of glimepiride. Sample B contained 7 ng/ml II, 25 ng/ml I and 43 ng/ml glimepiride. Sample C was a spiked sample with 51 ng/ml II, 47 ng/ml I and 56 ng/ml glimepiride.

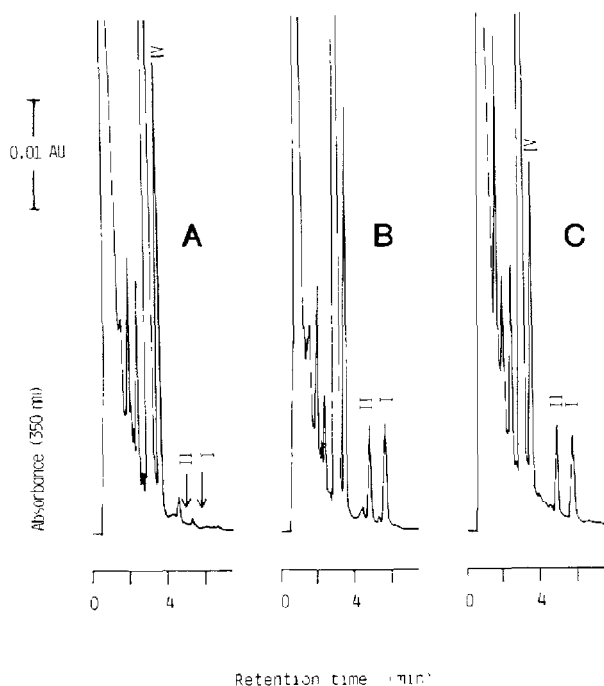


Fig. 3. Chromatograms of derivatized urine samples from a volunteer before (A) and 4–8 h after (B) oral administration of 0.5 mg of glimepiride. Sample B contained 461 ng/ml I and 379 ng/ml II. Sample C was a spiked sample with 475 ng/ml I and 435 ng/ml II.

Serum samples containing glibenclamide and glibornuride produced a peak at 9.5 min after derivatization, and tolbutamide produced a peak at 8.6 min. All the other commercially available sulphonylurea compounds also differ structurally at the cleavable amine part from the derivatives. However, with the overlapping application of glibenclamide, its metabolite IV cannot be used as internal standard and, therefore, III must be used in its place as the internal standard for all compounds.

Urine (Fig. 3). In drug-free urine samples endogenous interferences (below 30 ng/ml) for I and II were sometimes detected. Comedication with other sulphonylureas should not interfere with the peaks of I and II since they all differ structurally at the cleavable amine part. However, with the interference of glibenclamide, its metabolite IV cannot be used as internal standard and, therefore, an external standard method must be used, with some reduction in accuracy.

A crucial point of the specificity of both assays for serum and urine is the fact that during sample preparation the molecule is cleaved, thus only a fragment of the molecule is available for detection. This means that metabolic changes in the residual part of the molecule will not be detected. Metabolism

studies in humans [9], however, showed that alongside glimepiride in serum, only the metabolites I and II, with the altered cyclohexylamine moieties, are present in serum and urine in appreciable amounts.

Use of two internal standards in serum

The use of an appropriate internal standard is indicated for extraction, derivatization, reconstitution in mobile phase and chromatography. Since there are large differences in polarity between glimepiride and its metabolites, the homologue III was used as an internal standard for glimepiride and IV was used as internal standard for the closely related metabolites. A further advantage is that each derivative is also eluted with the corresponding mobile phase of the step gradient.

Pharmacokinetics of glimepiride in humans

This method was used in the analysis of serum and urine samples from volunteers dosed with glimepiride. Fig. 4 shows the serum concentration-time curves of glimepiride and its metabolites after oral administration of a single dose of 3 mg of glimepiride^a. The serum concentration of glimepiride reached

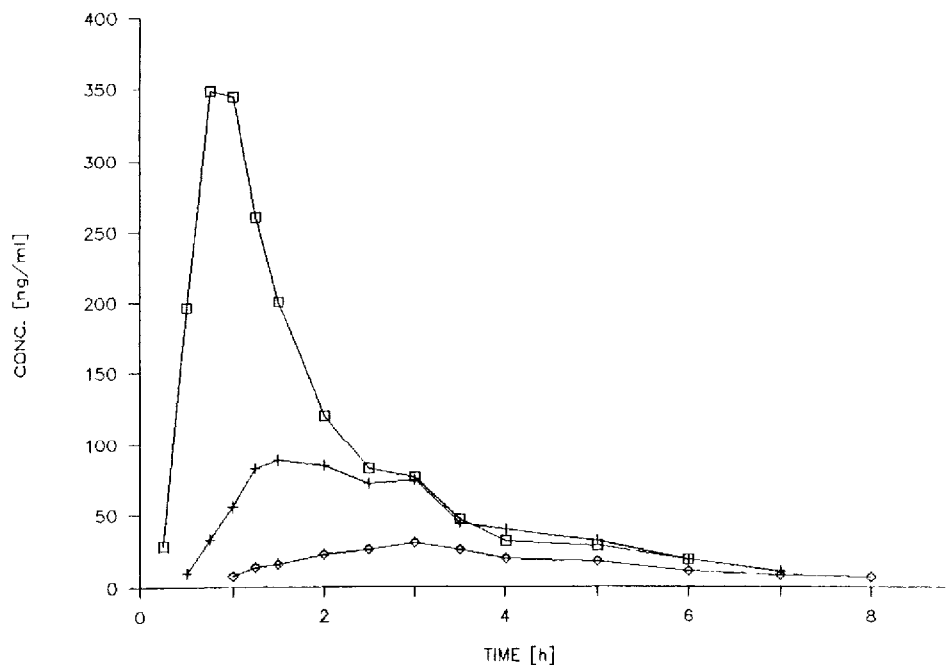


Fig. 4. Serum concentration-time curves of glimepiride (□), I (+) and II (◇) after a single oral dose of 3 mg of glimepiride in humans

^aWe thank Prof. Gries and Dr. Koschinsky (Düsseldorf, F R G.) for performing this study.

a maximum (348 ng/ml) at 45 min after dosing, whereas the metabolites I and II reached maxima (88 and 31 ng/ml, respectively) at 90 and 180 min, respectively. After 12 h all concentrations were below the detection limit.

For the same patient 37.9% of the administered dose was recovered as metabolites I and II in urine within 24 h of dosing. The relative proportion of I was 67.1% and of II it was 32.9%.

CONCLUSION

A sensitive and selective method has been developed for the simultaneous determination of glimepiride and its metabolites I and II. This method is especially suited to human serum and urine samples, where the metabolism is well known and, owing to the low dose, the levels are in the ng/ml range.

REFERENCES

- 1 K. Geisen, *Arzneim.-Forsch.*, 38 (1988) 1120.
- 2 M. Uihlein and N. Sistovaris, *J. Chromatogr.*, 227 (1982) 93.
- 3 H. Pötter and M. Hülm, *J. Chromatogr.*, 273 (1983) 217.
- 4 H. Möller, S.L. Ah and U. Gundert-Remy, *Med. Welt*, 34 (1983) 949.
- 5 M. Nieder, C. Dilger and H. Jaeger, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 102.
- 6 H. Emilsson, S. Sjöberg, M. Svedner and I. Christenson, *J. Chromatogr.*, 383 (1986) 93.
- 7 D. Castoldi and O. Tofanetti, *Chim. Chim. Acta*, 93 (1979) 195.
- 8 L. Zecca, S. Trivulzio, A. Pinelli, R. Colombo and O. Tofanetti, *J. Chromatogr.*, 339 (1985) 203.
- 9 I. Hornke and H. Jantz, Internal Report, Hoechst, Frankfurt/Main, 1990.
- 10 F. Erni, R.W. Frei and W. Lindner, *J. Chromatogr.*, 125 (1976) 265.
- 11 U. Timm, M. Wall and D. Dell, *J. Pharm. Sci.*, 74 (1985) 972.