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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GLIPIZIDE IN HUMAN PLASMA AND URINE

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SUMMARY

A sensitive and selective high-performance liquid chromatographic method for determination of intact glipizide in human plasma or urine has been developed. The plasma and urine samples were acid-buffered, before tolbutamide was added as internal standard. The samples were extracted with benzene, and the organic layer was evaporated to dryness. The residue was dissolved in equilibrated mobile phase (acetonitrile–0.01 *M* phosphate buffer pH 3.5, 35:65), and an aliquot of 20 μl was chromatographed on a Spherisorb ODS reversed-phase column. Quantitation was achieved by monitoring the ultraviolet absorbance at 275 nm. The response was linear (0–1000 ng/ml) and the detection limit was 5–10 ng/ml in plasma or urine. The within-assay variation was $\leq 10\%$. No interferences from metabolites or endogenous constituents were observed. The utility of the assay was demonstrated by determining glipizide in samples from a diabetic subject receiving a therapeutic dose of 5 mg of the drug.

INTRODUCTION

Glipizide (Fig. 1) is an effective oral hypoglycaemic agent, widely used in the treatment of non-insulin-dependent diabetes mellitus (type II diabetes) [1]. As a second-generation sulphonylurea, glipizide is given in low doses. In connection with pharmacokinetic and metabolic investigations of glipizide, a sensitive and selective method for the determination of plasma and urine levels of the drug is required.

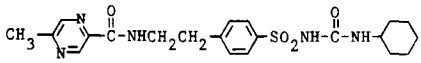

Fig. 1. Chemical structure of glipizide.

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A few bioassays for analysis of glipizide in plasma or serum have been reported [2–8]. The determination of glipizide in plasma has been performed with [^{14}C]glipizide [2, 3] or by radioimmunoassay techniques [4, 5]. However, the selectivity of these methods has not been verified. Possible metabolites or other sulphonylureas (e.g. glibenclamide) may cross-react and give rise to important disturbances in the assays. Gas chromatography (GC) has also been used for determination of glipizide in plasma or serum [6, 7]. However, GC requires a time-consuming derivatization step to give volatile and thermally stable derivatives. One of the GC techniques [7] also lacks selectivity, since structurally similar sulphonylureas (e.g. glibenclamide) may form identical derivatives. Wåhlin-Boll and Melander [8] described a sensitive high-performance liquid chromatographic (HPLC) technique for the measurement of glipizide concentrations in human serum. The method is limited by a relatively long elution time (25–30 min) for each sample. None of the previously published procedures [2–8] was tested for analysis of glipizide in urine samples.

A good method for monitoring plasma and urine levels is needed for defining accurate doses of glipizide and for analysis of samples in connection with hypoglycaemic crises and incidents with other kinds of severe side-effects. In order to find a sensitive, selective, simple and more rapid method for determination of intact glipizide in human plasma and urine samples, the HPLC assay described in this paper was developed. Plasma and urine samples from diabetic patients treated with glipizide were analysed to demonstrate the utility of the method.

EXPERIMENTAL

Equipment

A Milton Roy LDC Constametric I pump, equipped with a Milton Roy LDC Spectromonitor III variable-wavelength UV detector was used. Samples were introduced by syringe into a Rheodyne 7120 injector fitted with a 20-μl loop. A Spherisorb ODS (C_{18}) reversed-phase column (250 mm $\times$ 4.5 mm I.D.; 5 μm particle size; Jones Chromatography, Mid-Glamorgan, U.K.) was operated at room temperature. The chromatograms were recorded on a Philips PM 8251 chart recorder. For sample clean-up a vortex mixer, a BTL Multiwrist shaker (Baird & Tatlock, Chadwell Heath, U.K.), glass tubes with screw-caps with PTFE facing (ca. 12 ml capacity) and conical screw-capped tubes (ca. 3 ml capacity) were used. For centrifugation a Wifug Doctor centrifuge 6 was used. Glassware was cleaned with chromosulphuric acid, rinsed carefully with distilled water and dried before use.

Chemicals and drug standards

All solvents and reagents were of analytical grade. The 0.05 *M* hydrochloric acid was made by dilution of concentrated hydrochloric acid AR from E. Merck (Darmstadt, F.R.G.). Benzene AR, methanol AR and acetonitrile LiChrosolv® for HPLC were also obtained from Merck. The 0.01 *M* phosphate buffer solution (pH 3.5) was made from commercially available materials and redistilled water of high quality. The buffer solution was filtered through a Millipore® membrane filter (0.44 μm) before use.

Glipizide and 3-*cis*- and 4-*trans*-hydroxyglipizide were kindly supplied by Farmitalia Carlo Erba (Milan, Italy) and tolbutamide (internal standard) was kindly supplied by Hoechst (Frankfurt, F.R.G.).

Mobile phase

A mixture of acetonitrile–0.01 *M* phosphate buffer pH 3.5 (35:65) was used for elution. The mobile phase was degassed by sonication before use.

Standard solutions

Stock solutions of glipizide (50.0 μg/ml) and the internal standard (tolbutamide, 100 μg/ml) were prepared by dissolving accurately weighed samples in methanol. These solutions were found to be stable for at least three to four months when stored at ca. 4 °C. Working solutions of appropriate concentrations were made every week, by dilution of the stock solutions with methanol. The calibration curve standards were prepared by adding known amounts of glipizide and internal standard to blank plasma or urine, and contained 0–1000 ng/ml glipizide.

Plasma and urine samples

Drug-free human plasma or urine was used to obtain the calibration curves. The venous blood samples from diabetic subjects on medication with glipizide were collected in Vacutainer® tubes for plasma. The blood was centrifuged, the plasma was transferred to plastic tubes fitted with plastic caps. Urine samples from the diabetic patients were collected in polyethylene bottles. The blood and urine samples were stored at −20 °C until assayed.

Extraction procedure (plasma and urine)

A 300-μl volume of internal standard (10.0 μg/ml tolbutamide) and 1.00 ml of 0.05 *M* hydrochloric acid were added to 0.50 ml of plasma (or urine), resulting in a pH of ca. 3. The mixture was extracted with 3.00 ml of benzene in a 12-ml glass tube, which was shaken gently for 15 min. After centrifugation for 5 min at 3250 *g*, the organic phase was transferred to a conical tube for evaporation to dryness under a stream of air (a well ventilated fume chamber was used because of the benzene vapour). The residue was redissolved in 50 μl of equilibrated mobile phase by vortexing. An aliquot of 20 μl was injected into the chromatograph.

Chromatographic conditions

The flow-rate of the solvent was 1.5 ml/min at 120 bar. The UV detector was set at 275 nm, and the sensitivity of the detector was kept at 0.005–0.02 a.u.f.s. The chromatograms were recorded with a chart speed of 150 mm/h.

Calibration curves and quantitation

The calibration curves were constructed by plotting the peak-height ratios of calibration curve standards versus their concentrations of glipizide, expressed in ng/ml of plasma or urine. The curves were constructed on at least five different concentrations in the range 0–1000 ng/ml. New calibration curves were prepared

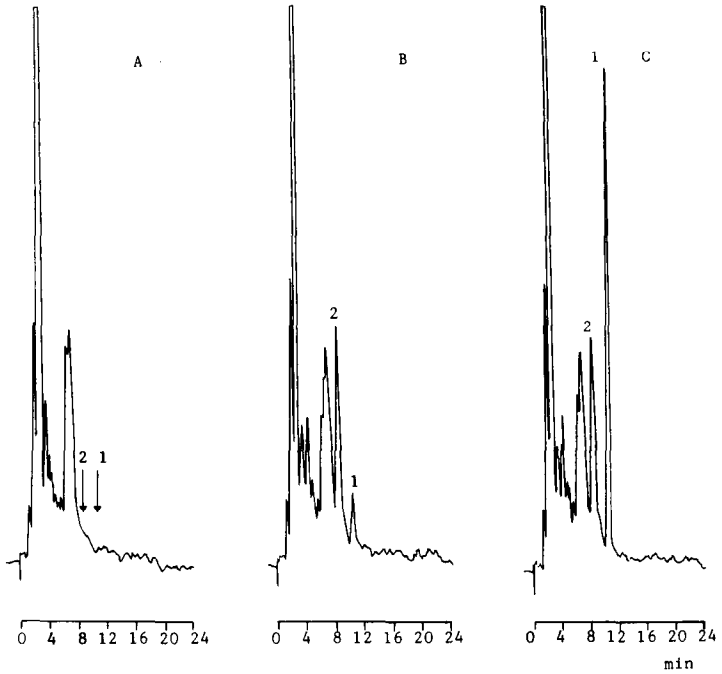

Fig. 2. Chromatograms of (A) plasma blank, (B) plasma blank spiked with glipizide (50 ng/ml) and tolbutamide, the internal standard (3300 ng/ml), and (C) a plasma sample 2 h after the administration of 5 mg of glipizide to a patient with diabetes. Peaks: 1 = glipizide; 2 = tolbutamide.

every week. Quantitation of the glipizide levels in samples from patients were calculated from the calibration curve.

RESULTS AND DISCUSSION

Extraction procedure

Glipizide is a weak acid with a pK_a of 5.94 [9], and a relatively lipophilic compound as undissociated free acid ($pH \leq 4.5$). In accordance with these properties, a pH of 3.0–3.5 seemed to be appropriate for the extraction from plasma or urine samples. Different organic solvents have previously been studied as extraction media by Wåhlin-Boll and Melander [8]. Only benzene was found to be suitable for the extraction of glipizide from plasma. The average extraction recovery of glipizide was ca. 85% when benzene and acidified plasma or urine (pH ca. 3.5) were used. The internal standard (tolbutamide) was also extracted to a high extent (ca. 80%) under the same conditions.

Chromatographic procedure

Various compositions of acetonitrile and phosphate buffer were tried as mobile phase, and different ionic strengths of the buffer component were evaluated. A 35:65 mixture of acetonitrile–phosphate buffer (0.01 *M*, pH 3.5) was found to be optimal.

The detection wavelength was set to 275 nm, at which the absorption coeffi-

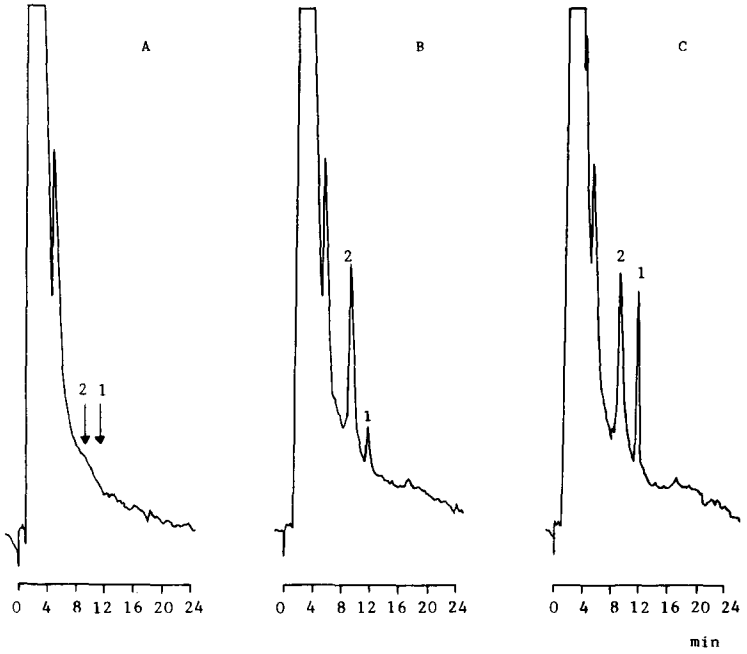

Fig. 3. Chromatograms of (A) urine blank, (B) urine blank spiked with glipizide (50 ng/ml) and tolbutamide, the internal standard (3300 ng/ml), and (C) a urine sample (fraction 2–4 h after the dose) after the administration of 5 mg of glipizide to the same patient as in Fig. 2. Peaks: 1 = glipizide; 2 = tolbutamide.

cient is sufficiently high for both glipizide ($1.01 \cdot 10^4$) and tolbutamide ($0.07 \cdot 10^4$). Wåhlin-Boll and Melander [8] chose a wavelength of 225 nm for detection of glipizide and their internal standard, glibornuride, at which wavelength the absorption coefficients are $2.40 \cdot 10^4$ and $1.60 \cdot 10^4$, respectively. However, when we used a wavelength of 225 nm a number of interfering peaks appeared. Thus we selected a wavelength of 275 nm, which, in spite of the decreased absolute sensitivity, resulted in cleaner chromatograms.

The separation was performed at room temperature on the reversed-phase column at a flow-rate of 1.50 ml/min. Under these conditions, the capacity factors (k') for glipizide and tolbutamide were determined as 5.9 and 4.7, respectively. The corresponding retention times were 10.5 and 8.2 min, respectively. The total chromatographic time was ca. 15 min per sample (both for plasma and urine). This time was somewhat shorter than the corresponding time found by Wåhlin-Boll and Melander [8].

Typical chromatograms are shown in Fig. 2 for plasma and Fig. 3 for urine.

Linearity and sensitivity

The calibration curves for plasma and urine were linear over the range 0–1000 ng/ml, a range that is adequate for therapeutic levels of glipizide. Calibration curve intercepts were not significantly different from zero.

With an injection volume of 20 μl the detection limit (signal-to-noise ratio 2)

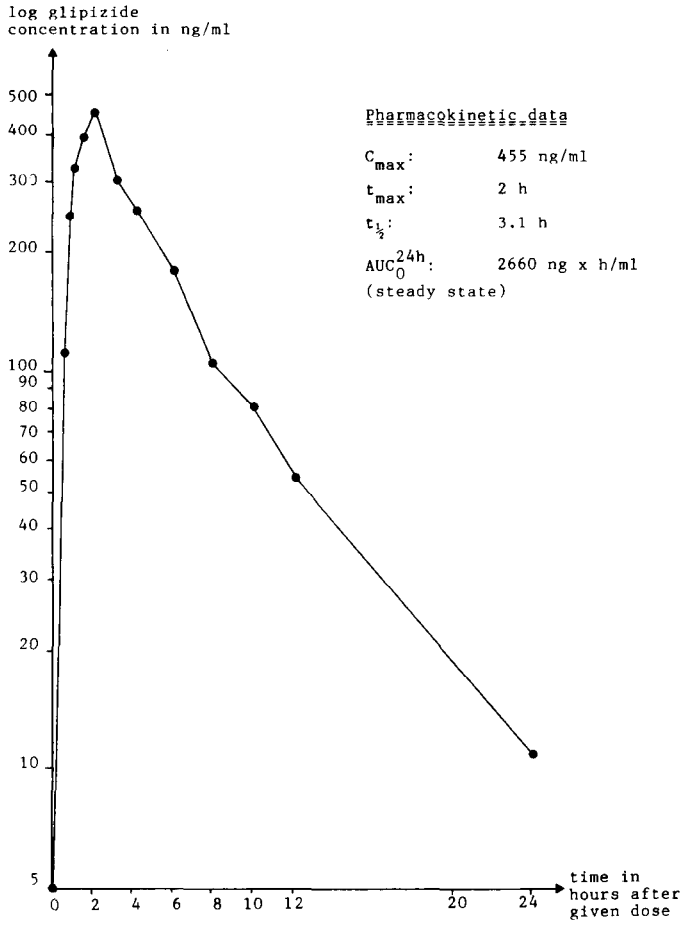

Fig. 4. Plasma concentrations and pharmacokinetic parameter values of glipizide during a 24-h period, obtained for a diabetic subject (the same as in Figs. 2 and 3) after oral administration of 5 mg of the drug (steady-state conditions).

for glipizide was ca. 5 ng/ml. The sensitivity was kept at 0.005 a.u.f.s. and the relative standard deviation (R.S.D.) at 5 ng/ml was ca. 14%.

Precision and selectivity

The precision of the method was determined by calculating the R.S.D. at three different concentrations of glipizide in plasma and urine, respectively. At least ten samples at each level were analysed. Within-assay studies gave the following results. Plasma: $\leq 10\%$ at 10 ng/ml; 7.2% at 30 ng/ml; 2.9% at 250 ng/ml. Urine: 9.1% at 10 ng/ml; 4.7% at 50 ng/ml; 1.4% at 250 ng/ml.

Between-assay R.S.D. values were not determined. Instead, new calibration curve solutions were made and new calibration curves constructed every week.

Both glipizide and tolbutamide were determined with great selectivity by the described assay. The two main metabolites of glipizide (3-*cis*- and 4-*trans*-hydroxyglipizide) were eluted with the solvent front and did not interfere in the

chromatograms. No interferences from normal endogenous plasma or urine constituents were observed (see Figs. 2 and 3).

Applicability of the method

The utility of the assay was demonstrated by analysing glipizide in plasma and urine samples from a diabetic patient on an oral dose of 5 mg. Fig. 4 shows a representative plot of log (concentration) versus time for plasma from this patient. Certain pharmacokinetic data calculated from the curve are listed in Fig. 4. These values accord well with previously reported values in both diabetic subjects and volunteers [2, 3, 7, 10, 11].

Glipizide is almost completely metabolized by the liver, to at least five metabolites, mainly 4-*trans*- and 3-*cis*-hydroxyglipizide [1, 11]. The metabolites are rapidly excreted by the kidneys. The excretion of unchanged drug in the urine is normally low (3–10% within 24 h after dosing) [11]. We also found low levels of intact glipizide in the urine samples from the diabetic subject (a total excretion of ca. 3.5% of the given dose during 0–24 h after administration). Normally it is of little value to analyse unchanged glipizide in urine. However, it may be valuable to determine glipizide in urine samples from subjects with impaired liver function or low metabolizing capacity. It could also be interesting to perform urine analyses in drug interaction studies, when other drugs may increase or decrease the metabolic transformation of glipizide.

CONCLUSION

The HPLC assay described in this paper is suitable for monitoring glipizide in clinical therapy and in pharmacokinetic and/or metabolic studies, and may also be suitable in cases of hypoglycaemic crises. The assay is sufficiently selective, sensitive, rapid and simple to allow accurate and precise measurements of both plasma and urine levels of glipizide under therapeutic conditions.

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