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

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SUMMARY

A selective and sensitive high-performance liquid chromatographic method for determination of intact glibenclamide in human plasma or urine has been developed. With glibornuride as internal standard, acid-buffered plasma or urine was extracted with benzene. The organic layer was evaporated and the residue was dissolved in equilibrated mobile phase (acetonitrile—phosphate buffer 0.01 *M* pH 3.5, 50:50). An aliquot of 20 μl was chromatographed on a Spherisorb ODS reversed-phase column, and quantitation was achieved by monitoring the ultraviolet absorbance at 225 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 ⩽ 10%. No interferences from metabolites or endogenous constituents could be noted. The utility of the method was demonstrated by analysing glibenclamide in samples from diabetic subjects on therapeutic doses of the drug.

INTRODUCTION

Glibenclamide (Fig. 1) is one of the most widely used orally active sulphonylureas in the treatment of non-insulin-dependent (type II) diabetes mellitus [1]. Because of its low therapeutic doses (2.5—15 mg) UV spectrophotometric [2], fluorometric [2, 3] and colorimetric [2, 3] methods lack sufficient sensitivity and selectivity for determination of plasma levels of glibenclamide.

Gas chromatography (GC) has been used for the analysis of glibenclamide in biological material [4—6]. However, GC requires a time-consuming

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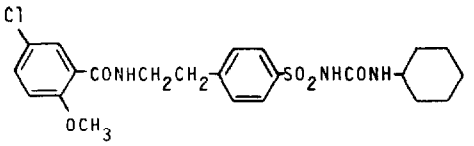

Fig. 1. Chemical structure of glibenclamide.

derivatization step to form volatile and thermally stable derivatives. Furthermore, the GC technique lacks selectivity, since the intact glibenclamide and certain of its metabolites, as well as the structure-like sulphonylurea glipizide, may form identical derivatives.

Sensitive radioimmunoassay techniques have been developed [7–9], although these may lack selectivity because of cross-reaction with glibenclamide metabolites or with glipizide.

More recently, high-performance liquid chromatographic (HPLC) methods [10–18] for determination of glibenclamide in serum or plasma have been reported. Some of these methods lack sufficient sensitivity for pharmacokinetic and/or metabolic studies of glibenclamide [10–12, 15]. In the most sensitive of the previously published methods [13, 17, 18], time-consuming derivatization or extraction steps were required. The detection limit (signal-to-noise ratio 2) for glibenclamide was reported to be ca. 5 ng/ml in the procedure by Uihlein and Sistovaris [16], but in our hands this method gave a detection limit of ca. 20 ng/ml, with a lot of disturbing peaks appearing. This finding resulted in an elution time of more than 30 min for each sample. In the assay described by Adams et al. [14], an unusual internal standard was used, which is not commercially available. None of the previously published procedures [10–18] was tested for analysis of glibenclamide in urine samples.

Methods for monitoring plasma and urine levels are required for adjustments of accurate doses of glibenclamide. More complete clinical studies have to be performed, owing to the fact that the pharmacology and pharmacokinetics of glibenclamide still are poorly characterized. A good assay is also needed for analysis of samples in connection with hypoglycaemic crises and incidents with other kinds of severe side-effects. With the aim of finding a sensitive, selective, simple and relatively rapid assay for determination of intact glibenclamide in human plasma and urine samples, the HPLC method described in this paper was developed. The utility of the method was demonstrated by analysing plasma and urine samples from diabetic patients treated with glibenclamide.

PHYSICOCHEMICAL PROPERTIES OF GLIBENCLAMIDE

The UV spectrum, solubility and partition of glibenclamide in buffer–diethyl ether, have been described in the literature [2, 19]. The partition in buffer–octanol in the pH range 5–12 was determined by Uihlein and Sistovaris [16] to be ca. 2300 for the free acid and 5 for the salt. The pK_a was determined by the same authors to 6.15–6.40. Glibenclamide is a very lipophilic compound as undissociated free acid (pH $\leqslant$ 4.5).

We determined a UV spectrum of glibenclamide in equilibrated mobile phase (see Experimental) and found the values λ_{max} = 225 nm and $\epsilon_{225\,nm}$ = $1.43 \cdot 10^4$.

Glibenclamide is decomposed under certain conditions. Hydrolysis takes place when an alkaline or an acidic aqueous solution of glibenclamide is heated. The stability of glibenclamide in methanol is described in the literature [19]. Glibenclamide may decompose slowly in methanol to form N-4-[2-(5-chloro-2-methoxybenzamide)ethyl] benzene sulphonylcarbamate [19]. However, we did not notice any decomposition of glibenclamide in the methanolic solutions used in this study.

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 × 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 Philipis 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 and rinsed carefully with distilled water before drying and using.

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.

Glibenclamide, 3-*cis*- and 4-*trans*-hydroxyglibenclamide were kindly supplied by Hoechst (Frankfurt, F.R.G.) and glibornuride was kindly supplied by Hoffman-La Roche (Basle, Switzerland).

Mobile phase

A mixture of acetonitrile—0.01 *M* phosphate buffer (pH 3.5) (50:50) was used for elution. The mobile phase was degassed by sonication for at least 15 min before use.

Standard solutions

Stock solutions of glibenclamide (50.0 μg/ml) and glibornuride (100.0 μg/ml) were prepared by dissolving accurately weighed samples in methanol. These solutions were found to be stable for at least three months when stored at 4°C. Working solutions of appropriate concentrations were made every week, by dilution of the stock solutions with methanol. The calibration curve standards contained 0—1000 ng/ml glibenclamide.

Plasma and urine samples

Drug-free human plasma or urine was used for making the calibration curves. Blood samples from diabetic patients were collected in Vacutainer® tubes for plasma, and the blood was centrifuged before transferring the plasma to plastic tubes fitted with plastic caps. The samples were stored frozen at −20° C until analysed. Urine samples from diabetic subjects were collected in polyethylene bottles and were stored at −20° C until analysed.

Extraction procedure (plasma and urine)

A 0.50-ml volume of plasma (or urine), 1.00 ml of 0.05 *M* hydrochloric acid and 25 μl of internal standard (conc. 10.0 μg/ml) were mixed with 3.00 ml of benzene in a 12-ml glass tube. The mixture was gently shaken 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 solvent flow-rate was 1.6 ml/min at 127 bar, with the UV detector set at 225 nm. The sensitivity of the detector was kept at 0.005—0.02 a.u.f.s. and the chromatograms were recorded with a chart speed of 150 mm/h.

Before any sample was injected into the chromatograph, the system was equilibrated with the mobile phase. In order to increase the lifetime of the column, it was thoroughly cleaned by pumping redistilled water and methanol through the column (15 + 15 min), after each day of chromatographing (ca. 25—30 samples).

Quantitation and calibration curves

Calibration curves were constructed by plotting the peak-height ratios of calibration curve standards versus their concentrations of glibenclamide, expressed in ng/ml of plasma or urine. The calibration curves were constructed on at least five different concentrations in the range 0—1000 ng/ml. A calibration curve was prepared once weekly, by adding known amounts of glibenclamide and internal standard to blank plasma or urine. The glibenclamide concentrations in patient samples were calculated from the calibration curve. At least two well corresponding samples were assayed in all unknown cases.

RESULTS AND PROCEDURES

Extraction procedure

In accordance with the physicochemical properties of glibenclamide, a pH of 3.0—3.5 seemed appropriate for the extraction of glibenclamide from plasma or urine samples. Various organic solvents were tried as extraction media, e.g. diethyl ether, dichloromethane, chloroform, benzene, toluene and *n*-pentanol—hexane (different compositions). Only benzene was found to be suitable, as the others either extracted glibenclamide poorly, or resulted in emulsions, or yielded interfering peaks in the HPLC chromatograms. This finding was in

agreement with the results reported by Wåhlin-Boll and Melander [11]. The average recovery of glibenclamide in the extraction step was ca. 80—85%, when benzene and acidified plasma or urine (pH ca. 3.5) were used.

Chromatographic procedure

Various compositions of acetonitrile and phosphate buffer pH 3.5 were tried as mobile phase. Different ionic strengths of the buffer component were also evaluated, and an ionic strength of ca. 0.01 appeared to be the best. A 50:50 mixture of acetonitrile—phosphate buffer (0.01 *M*, pH 3.5) was found to be the optimal composition of the mobile phase.

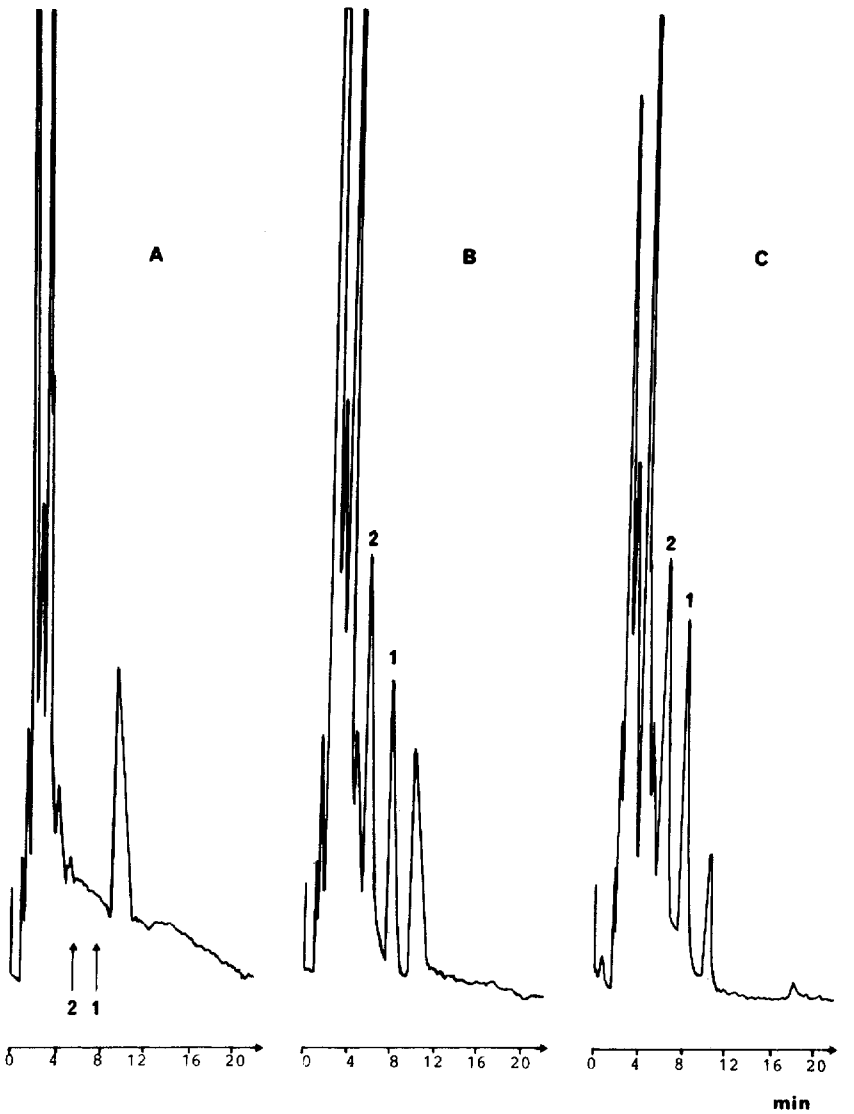

Fig. 2. Chromatograms of (A) plasma blank, (B) plasma blank spiked with glibenclamide (300 ng/ml) and glibornuride, the internal standard (500 ng/ml) and (C) a plasma sample 6 h after the administration of 10 mg of glibenclamide to a patient with diabetes. Peaks: 1 = glibenclamide; 2 = glibornuride.

The separation was performed at room temperature on a Spherisorb ODS reversed-phase column at a flow-rate of 1.60 ml/min. The detection wavelength was chosen to 225 nm, where the ϵ values are high for both glibenclamide ($1.43 \cdot 10^4$) and internal standard ($1.12 \cdot 10^4$).

The capacity factors (k') for glibenclamide and internal standard were 4.3 and 2.9, respectively. The corresponding retention times were 8.0 and 5.8 min, respectively. The total chromatographic time was ca. 20 min per sample (both for plasma and urine).

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

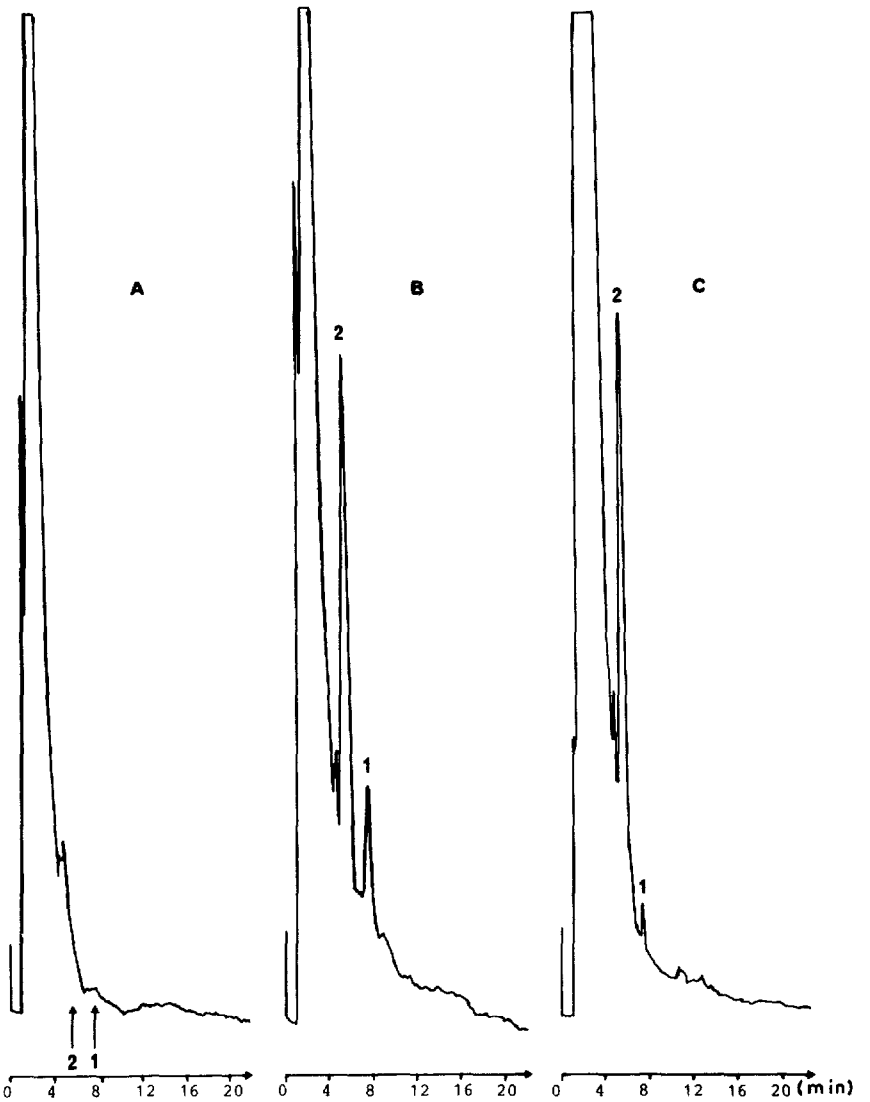

Fig. 3. Chromatograms of (A) urine blank, (B) urine blank spiked with glibenclamide (100 ng/ml) and glibornuride, the internal standard (500 ng/ml) and (C) a urine sample (fraction 12—24 h after the dose) after the administration of 10 mg of glibenclamide to the same patient as in Fig. 2. Peaks: 1 = glibenclamide; 2 = glibornuride.

urine. The peaks are sharp, symmetrical and well defined with respect to the baseline.

Linearity

The calibration curves for plasma and urine were linear over the range 0—1000 ng/ml (correlation coefficient, r = 0.98), a range that is adequate for the therapeutic levels of glibenclamide in both plasma and urine. Calibration curve intercepts were not significantly different from zero.

Sensitivity

The detection limit (signal-to-noise ratio 2) for glibenclamide was 5—10 ng/ml, with an injection volume of 20 μl. The sensitivity was kept at 0.005 a.u.f.s. The relative standard deviation (R.S.D.) at 10 ng/ml was $\leqslant$ 10%.

Precision

The precision of the assay was studied by calculating the R.S.D. at three different concentrations of glibenclamide in plasma, and at least ten samples of each of these concentrations were analysed. Within-assay studies gave $\leqslant$ 10% at 10 ng/ml, 8.1% at 30 ng/ml and 7.9% at 250 ng/ml. Between-assay R.S.D. values were not determined. Instead, we performed new calibration curve solutions and calibration curves every week. Within-assay R.S.D. values for different concentrations of glibenclamide in urine were lower than the corresponding values in the plasma assay.

Selectivity

Glibenclamide and glibornuride were determined with great selectivity by the described method. Neither of the two main glibenclamide metabolites found in human plasma or urine (3-*cis*- and 4-*trans*-hydroxyglibenclamide) interfered in the chromatogram. Both metabolites were eluted with the solvent front. Furthermore, no interference was noted from normal endogenous plasma or urine constituents (see Figs. 2 and 3).

Drug interference studies were carried out by analysis of glibenclamide in the presence of glipizide, chlorpropamide, tolbutamide, trimetoprim, sulphamethoxazole, naproxene, thioridazine, furosemide, theophylline and flunitrazepam. None of these drugs interfered with glibenclamide or glibornuride. However, it should be pointed out that this statement cannot be definitive, because of the possible appearance of metabolites of the drugs mentioned above. Interference studies must be done whenever other drugs are used in the medication.

Method applicability

Plasma and urine samples from diabetic subjects, treated with glibenclamide, were analysed by the described HPLC procedure. Fig. 4 shows a representative log(concentration) versus time curve for plasma from a diabetic patient. The plot indicates that the method permits analysis of samples during at least a 24-h period after an oral dose of 5 mg. Certain pharmacokinetic parameter values are calculated from the curve and are listed in Fig. 4. These values are in accordance with the values previously reported by Rupp et al. [20] and by

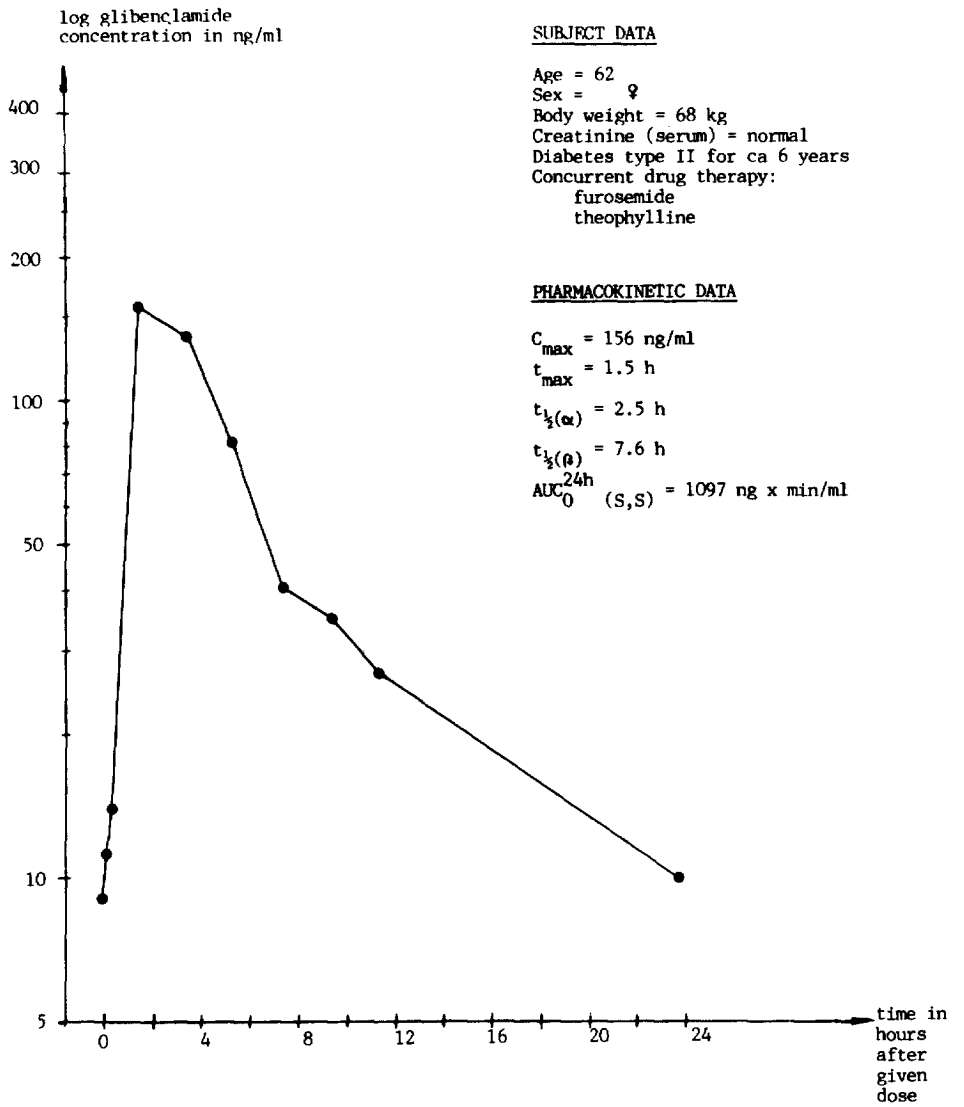

Fig. 4. Plasma concentrations and pharmacokinetic parameter values of glibenclamide during a 24-h period, obtained for a diabetic subject after oral administration of 5 mg of the drug (steady-state conditions).

Fucella et al. [21]. Their values were obtained from studies with [^{14}C]glibenclamide (5 mg orally to volunteer subjects).

Glibenclamide is extensively metabolized by the liver, and ca. 50% of the metabolites are eliminated in the faeces and the rest in the urine. The main metabolites are 3-*cis*- and 4-*trans*-hydroxyglibenclamide. The excretion of intact glibenclamide in the urine is normally very low [22]. We have also found very low amounts of glibenclamide in urine samples from diabetic subjects, with normal kidney and liver function after doses in the range 2.5—15 mg. The mean cumulative excretion of glibenclamide during 0—24 h was $\leqslant$ 1% of the given dose. It seems therefore to be of small value to analyse intact

glibenclamide in urine. However, it can be valuable to determine glibenclamide in urine samples from patients with impaired liver function or low metabolizing capacity, e.g. the hydroxylating capacity. It can also be interesting to perform urine analysis in drug interaction studies. Different drugs may increase or decrease the metabolic transformation of another drug, through enzymatic induction or inhibition. The excretion pattern can be changed, and one or more of the pharmacokinetic parameters can be affected. Enzyme inhibition has been observed when sulphaphenazole, for example, was administered with the antidiabetic agent tolbutamide [22]. The elimination half-life for tolbutamide was prolonged for this reason. A similar inhibition cannot be excluded when glibenclamide is given together with certain other drugs.

CONCLUSIONS

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

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