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Note

Determination of glibenclamide, chlorpropamide and tolbutamide in plasma by high-performance liquid chromatography with ultraviolet detection

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A number of sulphonylurea derivatives are currently prescribed in various countries as oral hypoglycaemic agents for the treatment of non-insulin-dependent (type II) diabetes mellitus [1]. This group of drugs includes some older compounds, e.g. tolbutamide (TBA) and chlorpropamide (CPA), as well as the "second-generation" agents such as glibenclamide (GBA). Methods for monitoring therapeutic plasma levels are required, even for the incidence of side-effects. Since the pharmacology of sulphonylureas is still incompletely characterized, such methods are needed to support clinical studies.

The measurement of second-generation sulphonylureas, which are given to patients in low doses since they are generally much more potent than the older agents, requires very sensitive and specific methods [2—6].

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Various high-performance liquid chromatography (HPLC) techniques have been used for quantitative assays of sulphonylureas. The unchanged compounds are well separated and are detected by UV light. But the minimum detectable amount of TBA is 0.5 μg/ml of plasma and of CPA 0.7 μg/ml of plasma [7—12]. When smaller amounts of sulphonylureas have to be assayed, shorter wavelengths, between 200 and 230 nm, must be used, a range in which contaminants can easily interfere [5, 6], or suitable fluorophore derivatives must be made [4].

A sensitive and selective procedure for the analysis of GBA in blood samples from diabetic patients was developed and is described here. This method is based on the reaction of the sulphonylurea GBA with dinitrofluorobenzene (DNFB). The structure of the resulting derivative has been identified by gas chromatography—mass spectrometry (GC—MS), characterized by ultraviolet (UV) spectrophotometry, and utilized for quantitative purposes in the HPLC technique. The same procedure was also applied to the determination of CPA and TBA.

MATERIALS AND METHODS

The materials used were those commercially available; solvents and reagents of analytical grade were from BDH and Carlo Erba (Milan, Italy). Standard sulphonylureas were obtained from BBR (Milan, Italy), cyclohexylamine, *n*-butylamine and *n*-propylamine were purchased from Fluka (Buchs, Switzerland). Acetonitrile for HPLC was obtained from E. Merck (Darmstadt, F.R.G.).

Reagents

The deproteinizing reagent was 1 *M* hydrochloric acid. The derivatizing reagent was made weekly with 3 mg/ml DNFB (freshly recrystallized from diethyl ether) solution in *n*-butyl acetate and stored at 4° C in the dark.

Stock solutions of sulphonylureas (1 mg/ml) were prepared weekly in methanol and stored at 4° C. Working standard solutions (1 μg/ml) were made daily by dilution with water.

Instrumentation

Gas chromatography—mass spectrometry. A Finnigan Model 4000 quadrupole mass spectrometer was used in the electron-impact mode (ionizing energy 70 eV, multiplier energy 1500 V, trap current 100 μA, and ion source temperature 250° C). Data acquisition was performed by a Finnigan 6100 data system.

High-performance liquid chromatography. A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 2/2 high-performance liquid chromatograph equipped with a Perkin-Elmer LC-75 variable-wavelength UV detector and Autocontrol system was used. Samples were introduced by syringe into a Rheodyne 7105 (Berkeley, CA, U.S.A.) injection valve with 150-μl loop. A C_8 Perkin-Elmer column (5 μm particle size; 12.5 cm × 4.6 mm I.D.) was operated at room temperature. The mobile phase was acetonitrile—water (50:50) containing 0.15% phosphoric acid and the flow-rate was 1.5 ml/min. The column effluent was monitored at 350 nm as this wavelength provided the maximum signal-

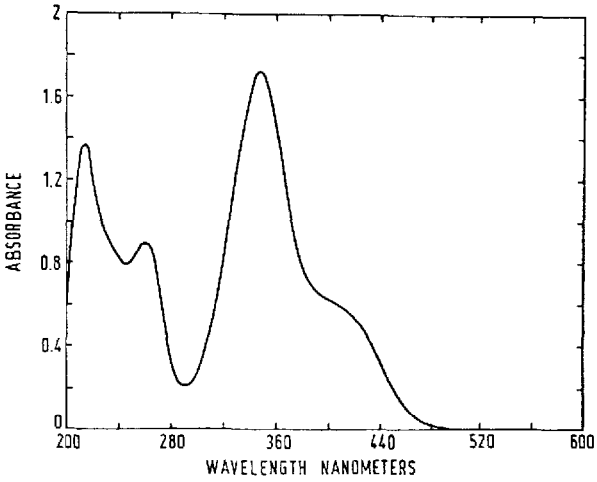

Fig. 1. UV spectrophotometric absorption of glibenclamide N-(2,4-dinitrophenyl)cyclohexylamine in mobile phase.

to-noise ratio in the UV spectrum recorded by the Autocontrol system (Fig. 1).

Thin-layer chromatography and gas chromatography. Thin-layer chromatography (TLC) equipment was obtained from Chemetron (Milan). The TLC plates were prepared as described by Nair and Pinelli [13]. A Carlo Erba Fractovap gas chromatograph provided with a flame-ionization detection system was used. It was equipped with a column 2 × 0.4 cm I.D. and contained OV-17 3% as stationary phase on Gas-Chrom Q 80/100. The temperatures were 300°C for the injection block, 240°C for the column and 300°C for the detector. The flow-rate of the carrier gas was 50 ml/min.

Standard procedure

Aliquots (2 ml) of plasma, with 100 ng of TBA added, were placed in 12-ml tapered centrifuge tubes containing 0.5 ml of 1 *M* hydrochloric acid. Then 8 ml of chloroform were added to the test tubes which were shaken for 10 min on a reciprocal shaker. After centrifugation at 2000 *g* for 15 min, 7 ml of the organic lower phase were transferred into 12-ml tubes and evaporated to dryness under nitrogen at 45°C. The samples were redissolved in 100 µl of DNFB solution and heated at 120°C for 30 min in a dry block. The samples were evaporated to dryness under nitrogen at 60°C. The residues were redissolved in 100 µl of the mobile phase and 30—70 µl were injected into the chromatograph. In addition to the unknown samples, plasma calibration standards containing 25, 50, 100, 200 or 500 ng of GBA and 50 ng/ml of the internal standard TBA were prepared and aliquots injected. Calibration curves were drawn for GBA concentrations versus the ratio of the peak heights of GBA/TBA.

The percentage recovery from plasma was calculated when the data obtained for the standards added to plasma, extracted and derivatized were compared with values obtained when the standards were taken to dryness directly and derivatized.

The same procedure was applied to the analysis of TBA using CPA as

internal standard (30 μg/ml of plasma) and vice versa for analysis of CPA. Calibration curves for both compounds were prepared with 10, 25, 50, 100, and 150 μg/ml in plasma. For the last two compounds, plasma volumes of 100 μl can be processed.

Blood samples

Venous blood samples were obtained from diabetic patients administered orally with GBA (3.5 mg), TBA (500 mg) or CPA (500 mg) and introduced into tubes containing EDTA · 2K. After centrifugation, plasma was separated and stored at −40° C until analysis.

RESULTS AND DISCUSSION

Derivatization with DNFB

The reaction product of GBA with DNFB was studied by GC—MS. It has a molecular weight of 265, which corresponds to N-(2,4-dinitrophenyl)cyclohexylamine, a fragment of GBA (Fig. 2). To confirm this result pure cyclohexylamine was reacted with DNFB. The product showed the same mass spectrum as the compound mentioned above (Fig. 2).

GBA derivatized with DNFB and purified by TLC and the compound named N-(2,4-dinitrophenyl)cyclohexylamine had the same UV spectra.

Furthermore, the dinitrobenzene derivatives of GBA and cyclohexylamine had the same R_F value (0.42) when analysed by TLC with hexane—ethyl acetate—dioxane (65:25:10) as mobile phase and they showed the same retention time (8.7 min) when analysed by gas—liquid chromatography under the conditions described in Materials and methods.

It appears that, in butyl acetate at 120° C, GBA pyrolyses, liberating cyclohexylamine which is then alkylated by DNFB.

TBA and CPA were exposed to the same reaction conditions and behaved like GBA, forming dinitrobenzene butylamine and propylamine derivatives: these products were identified by mass spectra as described for GBA. The compounds after derivatization with DNFB are then suitable for HPLC analysis.

HPLC procedure

Fig. 3 shows chromatograms corresponding to (A) plasma blank and (B) standard plasma containing GBA (200 ng/ml), TBA (200 ng/ml) and CPA (200 ng/ml). The dinitrobenzene derivatives of GBA, TBA and CPA appear to be well separated and exhibited retention times of 3.4, 4.5 and 6.2 min, respectively, under the conditions described. Blank plasma extracts have no peaks interfering with the sulphonylureas (Fig. 3). Furthermore, there is a linear relationship between the peak height ratios of drug to internal standard versus drug concentration. Linearity for CPA and TBA was assessed in the range 0—150 μg/ml, for GBA 0—500 ng/ml, according to the therapeutic levels.

The sensitivity of this method was 5 ng/ml for GBA, 2 ng/ml for TBA and 2 ng/ml for CPA, with a signal-to-noise ratio of 2.

The application to the analysis of human plasma samples collected according to the above described conditions is shown in Fig. 4. The recovery was high even after single extraction with a medium polarity solvent such as chloroform

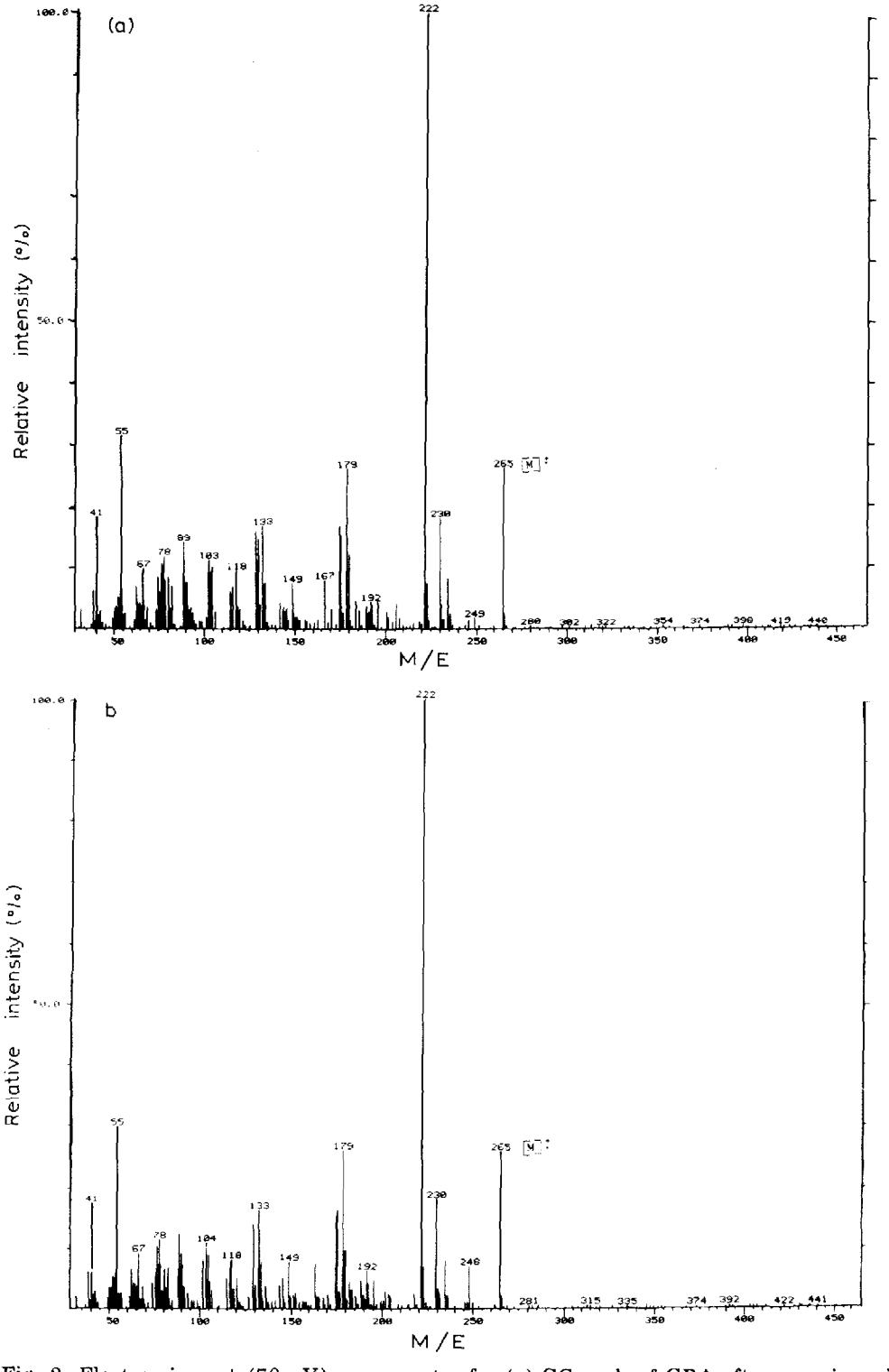

Fig. 2. Electron-impact (70 eV) mass spectra for (a) GC peak of GBA after warming with 1-fluoro-2,4-dinitrobenzene, and for (b) GC peak of N-(2,4-dinitrophenyl)cyclohexylamine.

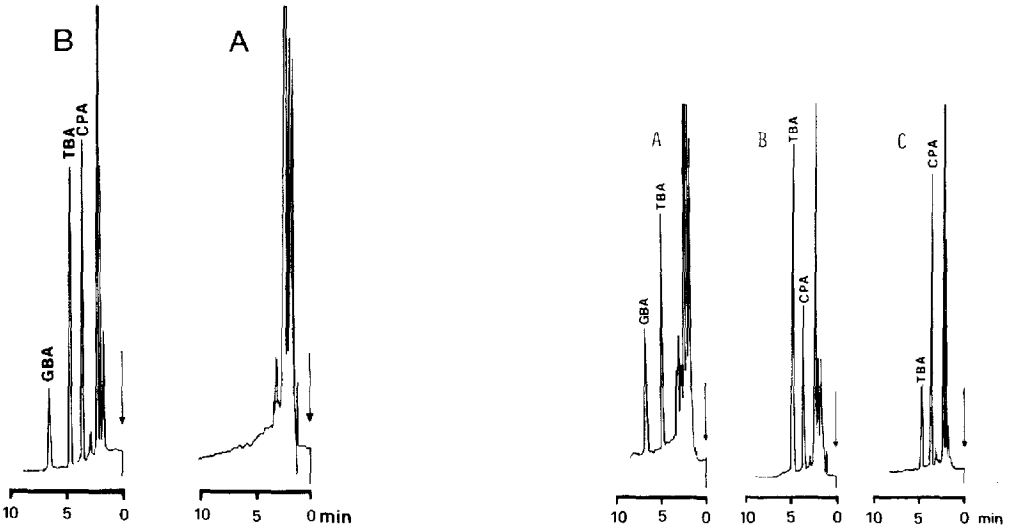

Fig. 3. Typical chromatograms of sulphonylureas, processed according to the described procedure. (A) Plasma blank, (B) plasma standard containing CPA 200 μg/ml, TBA 200 μg/ml and GBA 200 μg/ml.

Fig. 4. Chromatograms of plasma samples processed according to the described procedure, obtained from three diabetic patients. (A) After administration of GBA 3.5 mg (internal standard: TBA 150 ng/ml); (B) after 500 mg TBA (internal standard: CPA 5 μg/ml); (C) after 500 mg CPA (internal standard: TBA 5 μg/ml).

and was 92 ± 5% (n = 6) with an intra-assay coefficient of variation (C.V.) of 4.1% for GBA. The recovery for TBA was 91 ± 4% (n = 6) with a C.V. of 3.6% and 95 ± 4% (n = 6) with a C.V. of 3.2 for CPA.

Drug interference studies were carried out by analysis of plasma sulphonylureas in the presence of added acetaminophen, aspirin, diazepam, chlordiazepoxide, quinidine, diphenylhydantoin, theophylline and phenobarbital; none of these drugs interfered with sulphonylurea peaks.

CONCLUSIONS

The present assay allows the simultaneous analysis of three sulphonylureas. For TBA and CPA the sensitivity was higher than in all previously published HPLC methods [3, 7—12]. For GBA the detection limit (signal-to-noise ratio of 2) was lower than that obtained with the procedure by Uihlein and Sistovaris [5], which in our hands gave a detection limit of 15—20 ng/ml (signal-to-noise ratio of 2). In their method the column effluent is monitored at 200 nm and many peaks appear after that of GBA; thus an elution time of 20—30 min is needed for each sample. With our method, GBA, TBA and CPA are eluted in 6 min and one sample can be injected every 7 min. Furthermore, Uihlein and Sistovaris employ an unusual internal standard, which we were not able to obtain. The assay described by Besenfelder [4] has a comparable sensitivity of 5 ng/ml (signal-to-noise ratio of 3); however, he uses a fluorescence detector and a normal-phase column.

Selectivity also was very good due to the acid extraction, which eliminates all drug and endogenous compounds containing basic groups, and to the derivatization step. The column effluent was monitored at 350 nm, a wavelength which is absorbed by few molecules; thus interfering peaks do not appear in the chromatograms.

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