Journal of Chromatography, 490 (1989) 355–363
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4686

SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TOLBUTAMIDE AND ITS METABOLITES IN HUMAN PLASMA AND URINE USING PHOTODIODE-ARRAY DETECTION

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(First received June 30th, 1988; revised manuscript received January 10th, 1989)

SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of tolbutamide and its metabolites in human plasma and urine. The compounds examined were extracted with diethyl ether from the acidified biological fluid. Chlorpropamide was used as internal standard, and 235 nm was chosen as the wavelength for diode-array detection. A study of the relationship between the capacity factor and the mobile phase composition and pH showed that acetonitrile–2-propanol–0.1% orthophosphoric acid (17.17:66, v/v) was the best eluent on a C_8 reversed-phase column. The method is precise, sensitive and suitable for pharmacological investigations.

INTRODUCTION

The sulphonylurea drug tolbutamide (1-butyl-3-*p*-tolylsulphonylurea; TBA) is an oral hypoglycaemic agent used for the treatment of non-insulin-dependent diabetes mellitus; it is particularly important in clinical pharmacological research. TBA is predominantly metabolized by the hepatic cytochrome P-450-dependent mixed-function oxidase system. The initial step in its metabolism is hydroxylation of the methyl group to hydroxytolbutamide (1-butyl-3-*p*-hydroxymethylphenylsulphonylurea; HTBA). HTBA is further oxidized to carboxytolbutamide (1-butyl-3-*p*-carboxyphenylsulphonylurea; CTBA).

The metabolism of TBA in humans has been extensively studied, as it is one

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of the probe drugs for examination of genetic polymorphism in drug oxidation [1], and it is a probe drug for assessing the degree of enzyme inhibition or induction that may affect the capacity of an individual to oxidize drugs [2–4].

The clinical pharmacological studies required the measurement of the concentration of TBA and both its metabolites in biological fluids.

First attempts at their determination involved spectrophotometry [5], but the separation of metabolites was tedious and incomplete. A number of gas chromatographic methods have been reported [6–8], but they involved some derivatization procedure or mass spectrometry for detection [9]. Previous high-performance liquid chromatographic (HPLC) assays could determine only TBA, because of the low capacity factor (k') of metabolites [10], or TBA and CTBA [11]. Peart et al. [3] did not report any chromatogram or any data on the resolution of endogenous peaks and HTBA; the latter has a very short retention time ($t_R = 2.4$ min). Keal et al. [12] published the only HPLC method able to measure TBA and its two metabolites in one run with appropriate sensitivity and speed, but they applied the less commonly used cyano column together with ion-pair chromatography.

We offer here a simple HPLC assay for simultaneous measurement of TBA, HTBA and CTBA in human plasma and urine on a C_8 reversed-phase column with photodiode-array detection.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile, diethyl ether and 2-propanol were purchased from Merck (Darmstadt, F.R.G.). Disodium phosphate, sodium hydroxide, monopotassium phosphate and orthophosphoric acid (Reanal, Budapest, Hungary) were of analytical grade. TBA was obtained from Chinoin (Budapest, Hungary). HTBA and CTBA were kindly supplied by Hoechst (Frankfurt, F.R.G.) and chlorpropamide was a gift from Pfizer (Brussels, Belgium).

Preparation of stock solutions

TBA, CTBA or HTBA (10 mg) were dissolved in 1 ml of 0.1 *M* sodium hydroxide and diluted to 10 ml with distilled water. These solutions were further diluted to concentrations ranging from 0.05 to 100 μg/ml. A 1 mg/ml solution of chlorpropamide (CPA) was prepared similarly and further diluted four- and twenty-fold. A 100-μl volume of these diluted solutions was added to urine and plasma samples, respectively.

Sample handling

Plasma samples for assay were obtained at 0, 3, 6 and 12 h, and urine samples were collected for the periods 0–6, 6–12 and 12–24 h. The plasma samples and 5-ml aliquots of urine samples were stored at −18°C until analysed.

Calibration

Calibration curves were prepared from drug-free plasma or urine by spiking with the standard solutions and carrying the samples through the analytical procedure. The linear calibration curves were obtained by the least-squares method and tested for correlation coefficients.

Calculation of k'

Values for k' were calculated from expression $k' = (t_R - t_0)/t_0$, where t_R was the retention time of the sample and t_0 was that of a non-retained material. The value of t_0 was measured by injecting methanol onto the column.

Chromatographic conditions

A Hewlett-Packard HP 1090A liquid chromatograph was used, equipped with a diode-array detector, an HP 85B system master, an HP 9121 dual disc drive and an HP 3390A recording integrator. The separations were performed on a BST C_8 or a BST C_{18} (7 μm particle diameter, 250 mm × 4.0 mm I.D.) column (BST, Budapest, Hungary) at room temperature.

The isocratic mobile phase was acetonitrile–2-propanol–0.1% orthophosphoric acid (17:17:66, v/v). The UV absorption was detected at 235 nm. The flow-rate was 1.2 ml/min.

Extraction procedure

The extraction was almost identical with those described in the literature [3,11,12]. To 0.5 ml of heparinized plasma (or 0.2 ml of urine) 5 (or 25) μg of CPA internal standard in 100-μl solution, 100 μl of 1 *M* hydrochloric acid and 4 ml of diethyl ether were added to a tapered glass centrifuge tube. After extraction for 10 min on a shaker, the sample was centrifuged at 2000 *g* for 2–3 min at room temperature. Then the upper layer was transferred to another test-tube and evaporated under a stream of nitrogen at 40°C. The dry residue was dissolved in 100 μl of acetonitrile–distilled water (1:1, v/v), 5 μl of which were injected into the HPLC column.

RESULTS AND DISCUSSION

Composition of the mobile phase

Most of the previous studies [3,10,11] dealing with the HPLC determination of TBA and its metabolites used a C_{18} column with acetonitrile in a weakly acidic medium as mobile phase. The problem with these systems was that the metabolites could be separated only with difficulty from endogenous substances, and in such cases TBA had a very long retention time.

We have compared how acetonitrile, 2-propanol and a 50% (v/v) mixture of acetonitrile and 2-propanol influenced the retention. As the endogenous substances interfering in the measurement of metabolites needed 4–4.2 min to

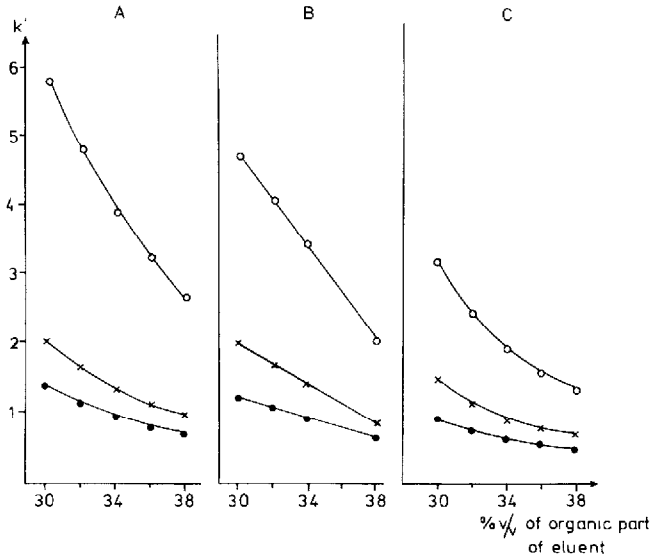

Fig. 1. Relationship of the k' values of HTBA (●), CTBA (×) and TBA (○) to the percentage of the organic part of the eluent. (A) Acetonitrile; (B) acetonitrile–2-propanol (1:1, v/v); (C) 2-propanol.

elute from the column and we decided to have a run no longer than 10–11 min (t_0 was ca. 2.1–2.4 min), we looked for an eluent that not only separated the compounds of interest, but also kept their retention times between 4.5 and 11 min, i.e. the k' values were between 0.9 and 3.5.

Fig. 1 shows the relationship between k' and the percentage of organic solvent in the eluent, the aqueous part being distilled water adjusted to pH 2.0 with orthophosphoric acid.

Our trials with acetonitrile on a C_8 column (Fig. 1A) yielded similar results to the former reports on C_{18} columns [3,10,11]. We could not find a composition where the k' values of the examined peaks were within the desired limits. 2-Propanol (Fig. 1C) decreased the k' value of TBA to a greater extent than those of the metabolites, but it increased the pressure of the system to an undesirable degree and HTBA had such a short retention time that it could not be well resolved from endogenous substances. The best conditions were obtained by using 34% acetonitrile–2-propanol (1:1, v/v) (Fig. 1B) as the organic part of the mobile phase. Even HTBA could be well separated, TBA had an acceptable retention time, and the pressure was moderate (130 bar).

Effect of pH

In the earlier methods, either the authors used an acidic pH of 3.9 [11,12] or no numerical value was reported [3,10].

We studied the effect of pH in a system consisting of 17% (v/v) of acetoni-

trile, 17% (v/v) of 2-propanol and 66% (v/v) of 0.06 *M* phosphate buffer (pH 7.0 or 5.5) or distilled water, the pH of which was adjusted to 4.0, 3.0 and 2.0 with orthophosphoric acid. Fig. 2 shows the relationship of k' and pH. It can be seen that at pH 2 the resolution was at its best and the k' values were in the range 0.9–3.5.

However, pH 7.0 was further investigated as means of keeping the retention time of TBA short. By decreasing the proportion of the organic part of the mobile phase, we tried to increase the k' values between the optimal values. Although the retention of TBA increased quickly, that of the metabolites was hardly altered (Fig. 3). Therefore, the possibility of using a basic eluent was rejected.

To avoid the difficulty of daily adjustment to pH 2.0 in the aqueous part of the mobile phase, application of 0.1% (v/v) of orthophosphoric acid in distilled water provided the same result. The final composition of eluent was ace-

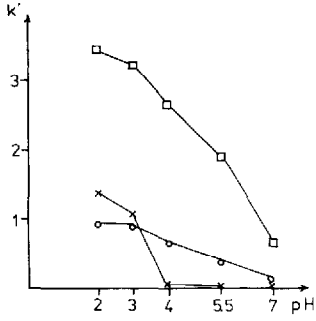

Fig. 2. Relationship of k' values of HTBA (○), CTBA (×) and TBA (□) to pH. The eluent was 17% (v/v) acetonitrile–2-propanol–aqueous (17.17:66, v/v) of varying pH. The pH in the aqueous part of the mobile phase was fixed with 0.06 *M* phosphate buffer (pH 7.0 and 5.5) or with 0.1% (v/v) orthophosphoric acid in distilled water (pH 4.0, 3.0 and 2.0).

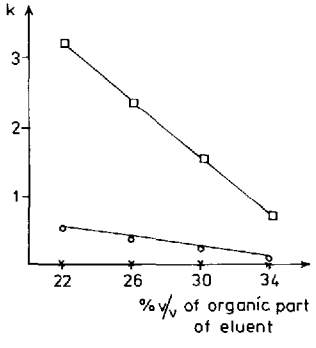

Fig. 3. k' values of HTBA (○), CTBA (×) and TBA (□) as a function of the percentage of acetonitrile–2-propanol (1:1, v/v) in the mobile phase. (The pH of the aqueous part was 7.0.)

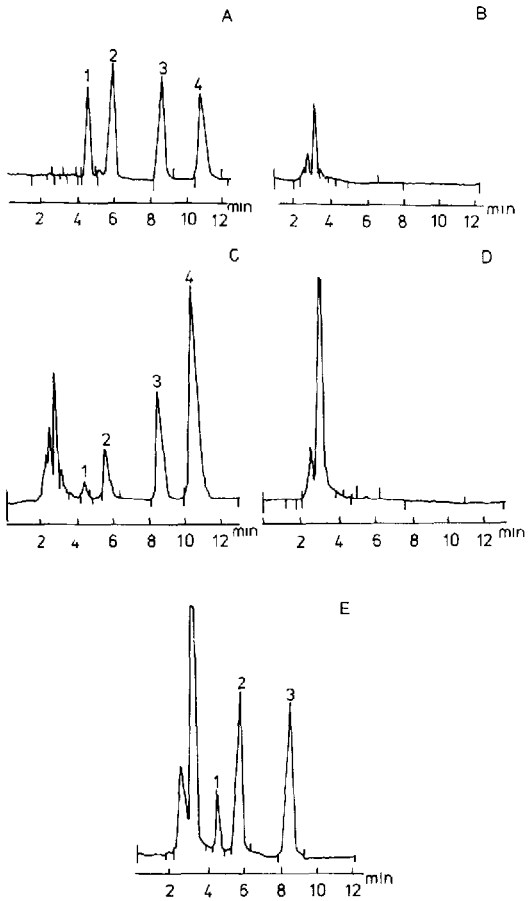

Fig. 4. (A) Representative chromatogram of HTBA, CTBA, CPA and TBA in aqueous standard solution. (B) Chromatogram of extract from drug-free plasma. (C) Chromatogram of extract from plasma of a patient, 3 h after oral administration of 500 mg of TBA (plasma levels 0.3 μg/ml HTBA, 8.1 μg/ml CTBA and 34.8 μg/ml TBA). (D) Chromatogram of extract from drug-free urine. (E) Chromatogram of extract from a 6-h urine sample of a patient, administered orally with 500 mg of TBA (the urine sample contained 43.8 μg/ml HTBA and 207.4 μg/ml CTBA). Peaks: 1 = HTBA; 2 = CTBA; 3 = CPA; 4 = TBA.

tonitrile–2-propanol–0.1% orthophosphoric acid (17:17:66, v/v); this gave the chromatograms shown in Fig. 4.

Wavelength

Variation of the wavelength in the narrow range from 220 to 240 nm, where all four compounds of interest have an appropriate absorption, made very little difference to the absorption of endogenous compounds, so was of no help in the separation and detection.

As the absorption maxima of TBA, HTBA, CTBA and CPA are between 225 and 240 nm, 235 nm was chosen as the wavelength of detection.

Columns

We tested the developed mobile phase on a C_{18} column and found that chromatographic parameters were very similar to those on C_8.

Recovery and reproducibility

The extraction efficiency was assessed by adding 2 and 20 μg of each compound or 0.2 μg of metabolites to drug-free plasma or urine aliquots ($n=6$). The areas under the peaks of the extracted samples were compared with those of unextracted samples in distilled water. The recovery values are given in Table I.

A linear concentration versus area-under-peak relationship, with correlation coefficients better than 0.994, was obtained in the range 0.1–100 μg/ml for the compounds of interest in plasma and urine. The equation of the line is $y=0.0228x+0.0317$ for TBA, $y=0.0309x-0.0302$ for HTBA and $y=0.0472x+0.0027$ for CTBA ($n=5$). The accuracy was determined by comparing the added amounts of materials with the measured ones. It was defined as a percentage of the target value and was between 95.0 and 106.5%. The detailed results can be seen in Table I.

The detection limits were 40, 20 and 50 ng/ml for HTBA, CTBA and TBA, respectively.

The coefficient of variation (C.V.) for six injections can be seen in Table I.

Since no TBA was found in any urine samples obtained from TBA-administered individuals, its recovery, accuracy and C.V. were not measured in urine.

TABLE I

PRECISION OF ASSAY ($n=6$)

Compound	Medium	Amount added (μg)	Amount measured (μg)	Accuracy (%)	Recovery (%)	Coefficient of variation (%)
HTBA	Plasma	0.2	0.19	95.0	84	7.9
	Plasma	2 0	1.94	96.5	83	4.7
	Urine	20.0	18.98	97.8	80	5.6
CTBA	Plasma	0 2	0.21	105.0	77	4.2
	Plasma	2.0	2.05	102.5	77	4.7
	Urine	20.0	19.27	96.4	82	3.9
TBA	Plasma	2.0	2.13	106.5	79	6.6
	Plasma	20.0	20.53	102.7	75	5.2

Human study

As the assay described here will be used in future clinical pharmacological studies, the plasma concentration of TBA and its metabolites and the concentration of metabolites in urine of a volunteer receiving 500 mg of TBA orally were measured.

The healthy non-smoking male volunteer, 42 years old, 85, kg, had not taken any medication for four weeks before the study and gave informed consent.

Former quantitative determinations [2,3] showed that the hydroxylation procedure, predominantly forming CTBA, accounted for 38.4–72.5% of the dose in urine. HTBA is equivalent to 4.6–23% of the dose of TBA. We found values of 48.3% for CTBA and 8.2% for HTBA. Plasma concentrations were 34.8, 89.2 and 17.2 μg/ml for TBA, 0.3, 9.0 and 0.6 μg/ml for HTBA and 8.1, 21.2 and 5.9 μg/ml for CTBA at 3, 6 and 12 h after administration, respectively (Fig. 4).

CONCLUSION

Our aim was to develop an easy-to-use method using reversed-phase HPLC for the measurement of TBA and its metabolites in biological fluids. We tried to eliminate the drawbacks of former methods that used C_{18} columns, but we have kept many parts of the procedures: e.g. the extraction procedure in acidic medium and CPA as internal standard.

The application of pH 2 in the aqueous part of the mobile phase and of acetonitrile–2-propanol (1:1, v/v) instead of acetonitrile as the organic part of the mobile phase made possible the adequate separation of TBA, CTBA, HTBA, CPA and the endogenous compounds, while the time of the assay did not increase. The new method is accurate and reproducible and ensures the sensitive measurement of all compounds of interest for routine pharmacokinetic studies. It is thus an alternative to ion-pair chromatography on cyano-bonded columns [12].

ACKNOWLEDGEMENTS

The authors thank Hoechst AG for supplying reference standards if HTBA and CTBA, Dr. C.F. Cheuvart, Pfizer Ltd. (Brussels, Belgium) for the CPA standard and Mrs. S. Orosz for technical assistance.

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