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Note

Simultaneous determination of tolbutamide and its hydroxy and carboxy metabolites in plasma and urine by high-performance liquid chromatography

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Tolbutamide is an orally active sulphonylurea hypoglycaemic drug used for the treatment of maturity-onset diabetes mellitus. The dosage of, and hypoglycaemic response to, tolbutamide varies over a wide range in patients and this may be attributed, in part, to the large inter-individual variability in steady-state plasma tolbutamide concentrations [1]. Factors which have been found to contribute to this include age, disease, concurrent administration of other drugs and pharmacogenetic make-up [2].

Following oral administration of tolbutamide in humans, approx. 85% of the dose is excreted in urine as the carboxy metabolite (1-butyl-3-*p*-carboxyphenylsulphonylurea) and the hydroxymethyl metabolite (1-butyl-3-*p*-hydroxymethylphenylsulphonylurea) in the ratio 2:1 [3], and only the latter metabolite, usually referred to as the hydroxy metabolite, is pharmacologically active [4]. Because tolbutamide is metabolised primarily by hydroxylation, it has been used as a substrate probe to determine factors controlling a single hepatic metabolic reaction [5]. Analytical methods are essential to measure the drug and its two metabolites in biological fluids.

Although most of the recent high-performance liquid chromatographic (HPLC) methods have been able to measure the carboxy metabolite in plasma [6, 7] or the hydroxy metabolite in liver perfusate [8], rat plasma and urine [9], two studies have reported on the measurement of tolbutamide and its two metabolites in plasma [10] and in hepatocyte suspensions [11]. These methods have several drawbacks, including poor sensitivity and a long retention time for tolbutamide, and both methods did not provide details of assay variability. Two previous methods have also been reported for the determina-

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tion of tolbutamide and metabolites [12, 13]; these required a derivatisation procedure and gas chromatography with electron-capture or chemical-ionisation mass spectroscopy for detection.

We report here on a simple, rapid and sensitive HPLC method for the determination of tolbutamide and its two metabolites in plasma and urine.

EXPERIMENTAL

Reagents

All reagents were of analytical grade and included diethyl ether, hydrochloric acid and orthophosphoric acid (BDH, Port Fairy, Australia), methanol (Ajax, Sydney, Australia), distilled before use, and tetrabutylammonium hydrogen sulphate (Sigma, St. Louis, MO, U.S.A.). Acetonitrile (Mallinkrodt, South Oakleigh, Australia) was of HPLC grade. Tolbutamide, hydroxytolbutamide and carboxytolbutamide (Hoechst, Frankfurt, F.R.G.) and chlorpropamide (Pfizer, West Ryde, Australia) were all of analytical grade quality.

Apparatus

The liquid chromatograph consisted of an SP8770 isocratic pump (Spectra-Physics, San Jose, CA, U.S.A.), a Jasco Uvidec 100 V variable-wavelength UV detector (JASCO, Tokyo, Japan), a Waters WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe B-5000 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm × 4.6 mm I.D. stainless-steel column was home-packed with Spherisorb CN (cyano) 5-μm packing material batch 21/107 (Phase Separations, Queensferry, U.K.). The column was end-capped with an HPLC column end-capping agent (Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase was methanol–acetonitrile–0.1 *M* tetrabutylammonium hydrogen sulphate– distilled water (10:10:4.5:75.5) adjusted to pH 4.0 with phosphoric acid. The flow-rate through the column at ambient temperature was 1 ml/min, which produced a back-pressure of 13.8 MPa. The detector wavelength was set at 237 nm.

Stock solutions

Tolbutamide and its two metabolites were made up as 1 mg/ml stock solutions in methanol and were diluted to concentrations ranging from 0.1 to 10 μg/ml (hydroxy and carboxy tolbutamide) and from 0.1 to 100 μg/ml (tolbutamide) in blank plasma and from 0.5 to 200 μg/ml (all three compounds) in blank urine. Chlorpropamide stock solution (1 mg/ml in methanol) was diluted with distilled water to concentrations of 3 μg per 100 μl for plasma analysis and 30 μg per 100 μl for urine analysis.

Sample preparation

Plasma. A 0.5-ml aliquot of plasma was pipetted into a 10-ml screw-capped glass centrifuge tube (Jobling Science Products, Sydney, Australia) to which were added 100 μl of the chlorpropamide standard plasma solution and 100 μl of 1 *M* hydrochloric acid. The mixture was briefly vortexed, 4 ml of diethyl ether were added and the glass tubes were placed on a rotary mixer for 15 min. The two phases were separated by centrifugation at 1600 *g* for 5 min. A

3.5-ml aliquot of the upper ether phase was transferred to a clean 10-ml tapered glass centrifuge tube (Stansen Scientific, Adelaide, Australia) and the ether evaporated to dryness under nitrogen at 40°C. The residue was redissolved in 100 μl of the mobile phase by vortexing for 1 min and 20 μl were injected on to the column via the automatic injector.

Urine. Urine was diluted 1:5 with distilled water and a 0.5-ml aliquot pipetted into the 10-ml screw-capped glass tube, to which were added 100 μl of 1 *M* hydrochloric acid and 100 μl of the chlorpropamide stock urine solution. The sample was then handled in exactly the same manner as that for plasma.

RESULTS

A chromatogram resulting from the injection of a solution of all four compounds (tolbutamide, hydroxytolbutamide, carboxytolbutamide and chlorpropamide) is shown in Fig. 1; Table I lists their retention times and capacity factors. Calibration curves in plasma and urine showed good linearity between peak-height ratios and concentration from 0.1 to 10 μg/ml (r^2 = 0.998) for the metabolites and between 0.1 and 100 μg/ml for tolbutamide (r^2 = 0.995) in plasma, and from 0.5 to 200 μg/ml for all three compounds (r^2 = 0.995) in

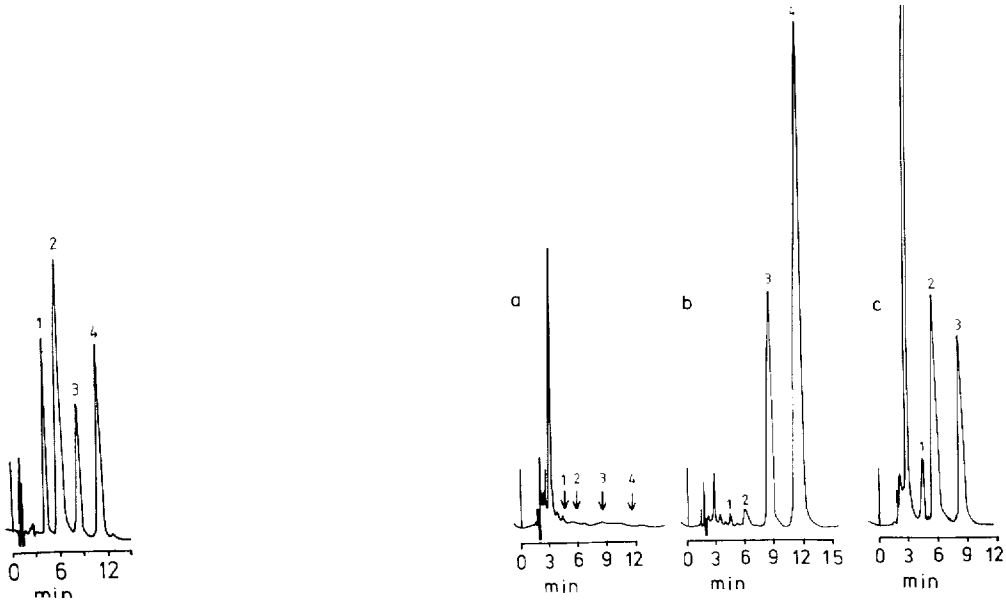

Fig. 1. Chromatogram of an injection of an aqueous solution containing hydroxytolbutamide (1), carboxytolbutamide (2), chlorpropamide (3) and tolbutamide (4).

Fig. 2. Chromatograms of an injection from an extract of: (a) blank plasma; the arrows indicate the position of hydroxytolbutamide (1), carboxytolbutamide (2), chlorpropamide (3) and tolbutamide (4); (b) a plasma sample taken 3 h following ingestion of 250 mg tolbutamide in a human subject; peaks: 1 = hydroxytolbutamide (0.50 μg/ml), 2 = carboxytolbutamide (0.75 μg/ml), 3 = chlorpropamide, internal standard (30 μg/ml) and 4 = tolbutamide (28.3 μg/ml); (c) a 12-h urine sample taken from a human subject following ingestion of 250 mg tolbutamide; peaks: 1 = hydroxytolbutamide (39.6 μg/ml), 2 = carboxytolbutamide (192.8 μg/ml), 3 = chlorpropamide, internal standard (300 μg/ml); tolbutamide was undetectable.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS FOR TOLBUTAMIDE, ITS METABOLITES AND CHLORPROPAMIDE

The dead time was calculated as the time for elution of the solvent front.

Compound	Retention time (min)	Capacity factor (k')
Hydroxytolbutamide	4.2	0.83
Carboxytolbutamide	5.9	1.57
Chlorpropamide	8.4	2.65
Tolbutamide	10.9	3.74

TABLE II

ASSAY REPRODUCIBILITY OF TOLBUTAMIDE AND METABOLITES IN PLASMA AND URINE

Compound	Concentration (μg/ml)	Coefficient of variation (%)	n
Plasma			
Hydroxytolbutamide	2.5	4.4	5
	0.2	7.5	5
Carboxytolbutamide	2.5	5.6	5
	0.2	6.9	5
Tolbutamide	25	2.3	5
	2	6.1	5
Urine			
Hydroxytolbutamide	100	7.5	8
	10	4.8	8
Carboxytolbutamide	100	5.5	8
	10	3.8	8

urine. The accuracy of the plasma calibration curves was 5.7% for tolbutamide, 8.4% for hydroxytolbutamide and 8.0% for carboxytolbutamide (in all cases, $n = 6$). In urine, it was 5.6% for hydroxytolbutamide and 6.6% for carboxytolbutamide (in both cases, $n = 5$). Fig. 2 shows chromatograms of a blank plasma sample, a sample from a subject 3 h following ingestion of 250 mg tolbutamide and a urine sample from the same subject during a dosing interval. Human plasma samples occasionally showed an endogenous peak substance which interfered with the determination of carboxytolbutamide; this was resolved by the addition of more (1%) ion-pairing agent (tetrabutylammonium hydrogen sulphate) to the mobile phase.

The detection limit for tolbutamide and metabolites was 0.1 μg/ml, defined as four times the baseline noise level. The precision of the assay for the determination of tolbutamide and metabolites in plasma and urine at the different concentrations is given in Table II. Tolbutamide concentrations in the urine of subjects administered tolbutamide are less than the assay detection limit and hence its variability in that biological medium was not deemed necessary.

DISCUSSION

The presently described assay for the determination of tolbutamide and metabolites in plasma and urine utilises information derived from previous HPLC methods. The use of chlorpropamide as internal standard allowed for the adequate separation of the two metabolites from endogenous substances without prolonged retention times for tolbutamide and chlorpropamide. Similarly, the use of diethyl ether as extracting solvent resulted in clean chromatograms which aided in the detection of low concentrations of the compounds of interest. Various C_{18} columns were tried, but all were found not to adequately resolve the metabolites from each other and from endogenous plasma components, irrespective of the mobile phase composition. The described resolution was achieved with the 5-μm cyano column and the mobile phase reported here. The assay is simple and rapid, and in a normal working day 48 samples can be easily prepared and injected automatically overnight. The use of this assay has provided the opportunity of investigating the hydroxylation clearance of tolbutamide and its perturbation by inhibitors of drug metabolism such as cimetidine [14]. The method provides adequate sensitivity and selectivity to make it applicable to pharmacokinetic studies of tolbutamide at a much reduced cost, that is HPLC versus gas chromatography—mass spectrometry.

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