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High-Performance Liquid Chromatographic Assay of Tolbutamide and Carboxytolbutamide in Human Plasma

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Received October, 10, 1980, from the *Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmaceutics, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.* Accepted for publication March 4, 1981.

Abstract □ A high-performance liquid chromatographic method was developed for the simultaneous measurement of tolbutamide and its major metabolite, carboxytolbutamide, in plasma. The assay involves the ether extraction of 1 ml of plasma, using chlorpropamide as an internal standard. The extract is dried, the residue is taken up in acetonitrile, and 5 μl is injected into a reversed-phase column. The mobile phase consisted of 35% acetonitrile and 65% 0.05 *M* phosphoric acid buffer (pH 3.9). A fixed-wavelength detector was set at 254 nm. The sensitivity limits for the tolbutamide and carboxytolbutamide assay were 2 and 0.1 μg/ml, respectively. The ratio of carboxytolbutamide to tolbutamide in plasma obtained from a subject given a 500-mg tolbutamide tablet was ~1:20.

Keyphrases □ Tolbutamide—simultaneous high-performance liquid chromatographic assay with carboxytolbutamide, human plasma □ Carboxytolbutamide—simultaneous high-performance liquid chromatographic assay with tolbutamide, human plasma □ High-performance liquid chromatography—simultaneous assay of tolbutamide and carboxytolbutamide

Tolbutamide is an oral hypoglycemic agent used for the chronic treatment of diabetes mellitus of the maturity-onset type. After oral administration to humans, ~85% of the dose is excreted in the urine as the carboxy metabolite (1-butyl-3-*p*-carboxyphenylsulfonylurea) and the hydroxymethyl metabolite (1-butyl-3-*p*-hydroxymethylphenylsulfonylurea) (1), both inactive.

BACKGROUND

The pharmacokinetics of tolbutamide in patients is highly variable, with apparent half-lives ranging from ~2 to 25 hr (2, 3). Because of this variability, it was suggested that plasma tolbutamide concentrations should be determined in patients receiving this drug (2, 4). The bioavailability of tolbutamide after oral administration was shown to be influenced by the salt form (5), the surface area (6), and the excipients present in the formulation (7). These observations are particularly significant since changes in the serum glucose concentrations show a good relationship to the time course of tolbutamide in plasma (5, 7). Therefore, an assay method for tolbutamide and its metabolites would be useful in pharmacogenetic studies, therapeutic drug monitoring, and bioavailability studies.

Colorimetric (8–12) and UV (13) methods lack sensitivity and specificity. Methods involving GLC (14–17) require derivatization of the drug. More recently, high-performance liquid chromatographic (HPLC) methods were developed (18, 19). One method (18) is suitable for the determination of tolbutamide and its major metabolite, carboxytolbutamide, but does not use an internal standard and is not sensitive enough to quantitate the low metabolite levels reported previously (20). Another method (19) uses chlorpropamide as an internal standard but does not measure carboxytolbutamide simultaneously.

EXPERIMENTAL

Reagents and Chemicals—Tolbutamide[1], carboxytolbutamide[1], chlorpropamide[2], ether[3], acetonitrile[3], hydrochloric acid[4], and phosphoric acid[5] were used.

HPLC—Deionized water (1 ml) and 1 ml of chlorpropamide internal standard (30 μg/ml) were added to 1 ml of plasma in a 20-ml centrifuge tube with a polytef-lined screw cap. After mixing, 0.1 ml of 1.3 *N* HCl was added; the mixture was extracted with 10 ml of ether for 15 min by gentle shaking on a platform shaker. After centrifugation at −10° for 15 min at 3000 rpm, the ether layer was transferred to a 15-ml conical tube and evaporated under nitrogen at 40°. The residue was taken up in 100 μl of acetonitrile and vortexed for 30 sec, and 5 μl was injected into the HPLC system.

The HPLC system consisted of a sample injector[6], a mobile phase pump[7] operated at 2.5 ml/min (2000 psi), a reversed-phase column[8], a fixed-wavelength UV absorbance detector[9] at 254 nm (aufs 0.01), and a recorder[10] operated at 0.5 cm/min. The mobile phase consisted of 35% acetonitrile and 65% 0.05 *M* phosphoric acid buffer (pH 3.9). Column temperature was maintained at 28°. After each use, the column was washed with water followed by methanol to avoid damage by the buffer in the mobile phase.

Preparation of Standard Curves—A standard solution was prepared to contain 100 μg of tolbutamide/ml and 5 μg of carboxytolbutamide/ml in 0.1 *N* NaOH. Aliquots of this solution were diluted with deionized water to yield standards containing 2, 5, 10, 20, 40, 60, and 80 μg of tolbutamide/ml and 0.1, 0.25, 0.5, 1, 2, 3, and 4 μg of carboxytolbutamide/ml. The internal standard solution contained 30 μg of chlorpropamide/ml in 0.0005 *N* NaOH. One milliliter of the tolbutamide–carboxytolbutamide solution and 1 ml of the chlorpropamide solution were added to 1 ml of pooled human plasma, and the mixture was assayed. Standard curves were prepared using duplicate samples at each concentration by plotting peak height ratio (tolbutamide to internal standard or carboxytolbutamide to internal standard) *versus* drug or metabolite concentration.

Stability Studies—Three 20-ml samples of pooled plasma were spiked with 20 ml of water, 20 ml of an aqueous standard containing 10 μg of tolbutamide/ml and 0.5 μg of carboxytolbutamide/ml, or 20 ml of an aqueous standard containing 80 μg of tolbutamide/ml and 4 μg of carboxytolbutamide/ml. Each portion of spiked plasma was separated into individual 8-ml portions and stored frozen at −10° for subsequent assay after 1, 2, and 4 weeks. A sample of each also was assayed immediately after preparation.

[1] Provided by The Upjohn Co., Kalamazoo, Mich.
[2] Provided by Pfizer, Brooklyn, N.Y.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Mallinckrodt, Paris, Ky.
[6] Model U6K, Waters Associates, Milford Mass.
[7] Model M6000, Waters Associates, Milford, Mass.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Model 440, Waters Associates, Milford, Mass.
[10] Recordall Series 5000, Fisher Scientific Co., St. Louis, Mo.

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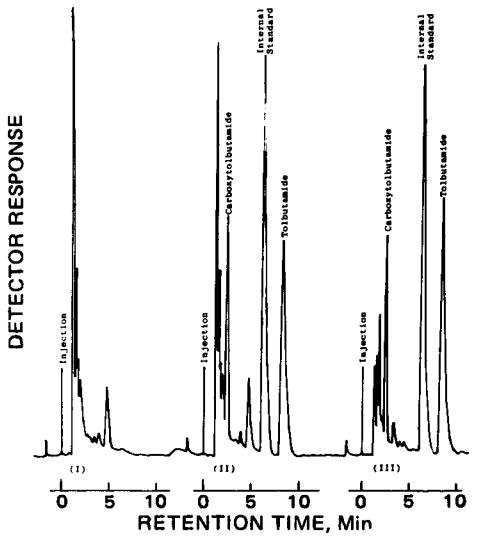

Figure 1—*Chromatograms for plasma extracts. Key: I, blank pooled plasma; II, pooled plasma spiked with 40 μg of tolbutamide/ml, 2 μg of carboxytolbutamide/ml, and 30 μg of chlorpropamide/ml as the internal standard; and III, plasma sample obtained from human subject 4 hr after a 500-mg oral dose of tolbutamide, with chlorpropamide added as internal standard.*

Human Study—A healthy male volunteer received a single commercially available 500-mg tolbutamide tablet. Blood samples (10 ml) were obtained just before and at 0.5, 1, 2, 3, 4, 6, and 24 hr after drug administration. These samples were assayed for tolbutamide and carboxytolbutamide levels using the described procedures.

RESULTS AND DISCUSSION

Tolbutamide, carboxytolbutamide, and chlorpropamide each exhibited a 235-nm wavelength of maximum absorbance when present in the mobile phase. A wavelength of 254 nm was selected to permit utilization of a fixed-wavelength detector. The absorptivities of tolbutamide, carboxytolbutamide, and chlorpropamide in the mobile phase were 3.2, 27.9, and 9.3 liters/g cm, respectively, at 254 nm.

Typical chromatograms for extracted plasma are shown in Fig. 1 for a pooled plasma sample containing no drug, a pooled plasma sample containing 40 μg of tolbutamide/ml and 2 μg of carboxy metabolite/ml, and a plasma sample obtained from a subject 4 hr after a 500-mg oral dose of tolbutamide. Chlorpropamide (30 μg/ml) was added to the latter two samples. The metabolite, internal standard, and drug peaks were well resolved with retention times of 1.8, 5.6, and 7.8 min, respectively. A peak with a retention time of 4.0 min appeared in extracts of pooled plasma but did not interfere in the assay, provided the mobile phase was carefully maintained at pH 3.9. This peak did not appear in the plasma samples freshly obtained from a human subject. The recoveries of tolbutamide, the carboxy metabolite, and chlorpropamide were ~99, 80, and 60%, respectively, relative to a direct HPLC assay of aqueous standards. Thus, the greater sensitivity of the assay for carboxytolbutamide was due to absorptivity differences rather than extraction efficiency.

Standard curves prepared for both tolbutamide and the carboxy metabolite exhibited excellent linearity over the concentration ranges employed for each material. The least-squares slope, intercept, and correlation coefficient for the tolbutamide calibration curve were 0.0140, 0.0052, and 0.9999, respectively. The same values for the carboxy metabolite were 0.303, −0.013, and 0.9999, respectively. The precision of the assay for tolbutamide and carboxytolbutamide was determined by the analysis of six replicate samples at each of three concentrations. Plasma samples containing 10, 40, and 80 μg of tolbutamide/ml exhibited relative standard deviations of 1.8, 0.5, and 0.4%, respectively. Plasma samples containing 0.5, 2.0, and 4.0 μg of carboxytolbutamide/ml exhibited relative standard deviations of 7.9, 2.2, and 1.4%, respectively. The lower limits of sensitivity were 2 μg/ml for tolbutamide and 0.1 μg/ml for the carboxy metabolite. Analysis of the spiked pooled plasma samples that had been stored frozen for 1, 2, and 4 weeks indicated no significant loss of tolbutamide or the carboxy metabolite over that period.

Figure 2 summarizes the tolbutamide and carboxy metabolite concentrations determined in a subject who received a 500-mg of tolbutamide

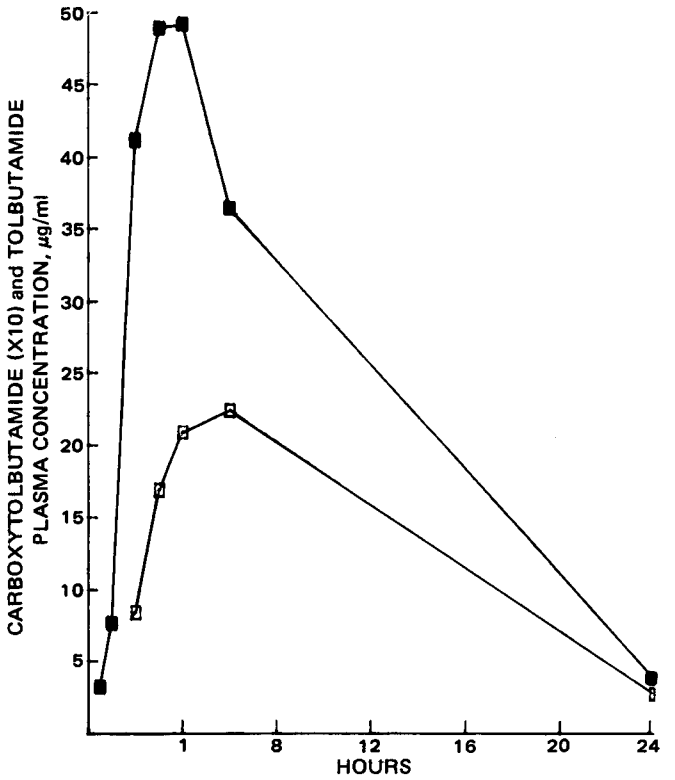

Figure 2—*Plasma concentrations of tolbutamide (■) and carboxytolbutamide (□) following administration of a 500-mg oral dose of tolbutamide to a human subject.*

tablet. The peak plasma tolbutamide concentration of 49 μg/ml was in the range of peak levels reported in other studies with human subjects who received 500-mg oral doses of the drug (17, 21). The peak plasma carboxy metabolite concentration was 2.2 μg/ml, which was ~1/20 the tolbutamide concentration. Matin and Knight (20) found a ratio of ~50:1 for tolbutamide to the carboxy metabolite after administration of a 1-g iv dose to a diabetic patient. They also reported the presence of a hydroxymethyl metabolite in this patient. The concentration of the metabolite paralleled that of the carboxy metabolite but was ~50% that of the major metabolite. Since an authentic sample of the hydroxymethyl metabolite was unavailable, it was not possible to determine the potential for this compound to interfere in the present assay or to be quantitated by the assay. However, chromatograms obtained with the assay did not reveal any evidence of an additional peak that could be attributed to this metabolite.

Based on these data, the reported method exhibits adequate sensitivity and specificity for single-dose bioavailability studies as well as for the monitoring of plasma tolbutamide concentrations in patients. In addition, the method is applicable to pharmacokinetic studies of the drug and its primary metabolite. It also should be applicable to the determination of plasma chlorpropamide concentrations with tolbutamide as the internal standard.

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Human Blood Preservation: Effect on *In Vitro* Protein Binding

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Abstract □ *In vitro* plasma protein binding for phenytoin, meperidine, and bretylium tosylate was affected by the type of preserved human blood used for its estimation. Fresh heparinized plasma and serum gave equivalent fractions bound at the concentrations studied for all three drugs. However, the *in vitro* plasma binding of phenytoin and meperidine decreased ~9–50% when estimated in fresh citrated plasma or commercially available lyophylized human serum at the concentration levels investigated. The fraction of bretylium tosylate bound to plasma protein decreased ~30–40% when estimated in fresh citrated plasma but was unchanged when estimated in the lyophylized human serum.

Keyphrases □ Binding, plasma protein—effect of blood preservation, humans □ Blood—preservation, effect on *in vitro* plasma protein binding □ Pharmacokinetics—effect of human blood preservation on *in vitro* protein binding

The influence of plasma protein binding on the distribution and elimination of drugs was recently reviewed (1). Accurate evaluation of the fraction of drug bound is essential for a thorough understanding of a drug's pharmacokinetics. Plasma/serum protein binding is normally estimated using several *in vitro* techniques such as equilibrium dialysis, ultrafiltration, gel filtration, differential spectrophotometry, and electrophoresis (2). Several investigators determined plasma protein binding in fresh plasma from subjects participating in pharmacokinetic studies (3, 4), and other sources of blood were utilized in other studies. A frequently reported source is fresh blood bank plasma (5, 6). Blood bank plasma is normally preserved with a standard anticoagulant/preservative, citrate phosphate dextrose USP. However, the presence of preservative has been implicated in lowering the protein binding of quinidine (5). Therefore, it is important that the effect of blood constituents and preservatives be evaluated.

The purpose of this investigation was to determine if a standard anticoagulant/preservative would interfere with the binding of other drugs and the suitability of a commercially available lyophilized serum[1] as a substitute for blood bank plasma when large volumes of plasma/serum are required. The drugs studied, phenytoin, meperidine, and bretylium tosylate, are chemically different and have been reported[2] to exhibit a binding of 90, 60, and 10%[2], respectively (3, 7).

EXPERIMENTAL

Blood was obtained from one healthy male volunteer, 27 years of age, who was not using any medication. Blood was collected in a clean glass flask containing either 400 IU of heparin/20 ml of blood or 63 ml of citrate phosphate dextrose/450 ml of blood. The blood to be used as serum was collected in clean glass test tubes and allowed to clot. Lyophilized serum[1], reconstituted according to the manufacturer's suggestion, was used as the commercial source of lyophilized human serum.

Table I—Effect of Blood Preservation Procedure on Fraction of Drug Bound [a]

Drug	Concentration, μg/ml	Fresh Plasma Heparin	Fresh Plasma Citrate	Fresh Serum	Lyophilized Serum
Phenytoin	15.0	0.89 (0.90, 0.88)	0.80 (0.76, 0.84)	0.89 (0.89, 0.89)	0.80 (0.79, 0.81)
	5.0	0.90 (0.89, 0.90)	0.81 (0.79, 0.83)	0.90 (0.91, 0.89)	0.80 (0.80, 0.80)
Meperidine	0.98	0.58 (0.56, 0.60)	0.25 (0.23, 0.27)	0.54 (0.52, 0.56)	0.26 (0.22, 0.30)
	0.12	0.60 (0.58, 0.62)	0.27 (0.22, 0.28)	0.58 (0.52, 0.62)	0.23 (0.18, 0.28)
Bretylium tosylate	1.2	0.13 (0.14, 0.12)	0.04 (0.04, 0.04)	0.10 (0.12, 0.09)	0.12 (0.12, 0.12)
	0.3	0.11 (0.12, 0.11)	0.06 (0.06, 0.05)	0.11 (0.10, 0.12)	0.10 (0.08, 0.12)

[a] Mean of two determinations; individual values are given in parentheses.

A standard solution of [^{14}C]meperidine, [^{14}C]bretylium tosylate, or [^{14}C]phenytoin was added to plasma at two concentrations within the respective therapeutic ranges. Protein binding was then determined by equilibrium dialysis. The samples were dialyzed across a cellophane membrane against phosphate buffer (0.067 *M*, pH 7.4) at 37° using a dialysis cell. Equilibrium was achieved for each drug by 24 hr. A control

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