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ACKNOWLEDGMENTS AND ADDRESSES

Received June 23, 1975, from the *Biological Research and Development Laboratories, USV Pharmaceutical Corporation, Tuckahoe, NY 10707*

Accepted for publication December 1, 1975.

The authors thank Dr. Newton Birkhead, Medical Department, USV Pharmaceutical Corp., for monitoring the human oral dosing study and Dr. Micheal O'Hare for carrying out the GLC–mass spectrometry analysis.

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Assay of Sulfonylureas in Human Plasma by High-Performance Liquid Chromatography

S. SVED [x], I. J. McGILVERAY, and NICOLE BEAUDOIN

Abstract □ A sensitive and specific high-performance liquid chromatographic procedure for the determination of chlorpropamide or tolbutamide in plasma in the presence of their metabolites is described. The ether extract of acidified plasma is redissolved in the mobile phase, 17% acetonitrile in 0.05 *M* aqueous ammonium formate, and chromatographed on a reverse-phase column on a high-performance liquid chromatograph fitted with a UV absorbance detector. Quantitation of plasma samples containing less than 0.5 μg/ml of chlorpropamide and 5 μg/ml of tolbutamide is reported, using these drugs as mutual internal standards. The retention times of the metabolites are such that they do not interfere in the procedure. The assay method was tested in a human volunteer with both drugs and found suitable for single-dose pharmacokinetic studies.

Keyphrases □ Chlorpropamide—high-performance liquid chromatographic analysis in presence of metabolites, plasma □ Tolbutamide—high-performance liquid chromatographic analysis in presence of metabolites, plasma □ High-performance liquid chromatography—analysis, chlorpropamide or tolbutamide in presence of metabolites, plasma □ Sulfonylureas—chlorpropamide or tolbutamide, high-performance liquid chromatographic analyses in presence of metabolites, plasma □ Antidiabetic agents—chlorpropamide and tolbutamide, high-performance liquid chromatographic analysis in presence of metabolites, plasma

The two oral hypoglycemic drugs tolbutamide and chlorpropamide are widely used in the management of certain types of maturity-onset diabetes. Specific assay methods capable of measuring the unchanged drugs in plasma are desirable for monitoring clinical blood levels and for studies in pharmacokinetics (1) and drug interactions (2).

Early methods of estimation, based on UV absorbance, were neither specific nor sensitive. More recently, colorimetric procedures were introduced based on the method of Spingler (3) and modified (4, 5). They depend on the reaction of chlorpropamide or tolbutamide with 2,4-dinitrofluorobenzene, followed by measurement of the absorbance of the resultant *N*-propyl- or *N*-butyldinitroaniline at the appropriate wavelength. The sensitivity of these methods is limited, however, by the high and variable blanks from endogenous substances in the plasma. Furthermore, positive color is also obtained with some metabolites of the drugs. Attempts have been made to separate the metabolites by differential extraction prior to spectrophotometry (6, 7), but these methods are lengthy and the separation is incomplete[1].

A number of GC procedures were reported recently, involving methylation of the drugs with dimethyl sulfate (8) or diazomethane (9) or after reaction with 2,4-dinitrofluorobenzene (10). Most of these methods, however, suffer from the inability to distinguish between the parent drug and its metabolites (11), although this problem may have been overcome (12).

A TLC method employing a UV densitometer was described recently for the assay of chlorpropamide in serum (13).

High-performance liquid chromatography (HPLC) apparently has not been used for determining chlorpropamide and tolbutamide levels in the human plasma. Beyer (14) used a reverse-phase system for the determination of these drugs in pharmaceutical preparations, while Molins *et al.* (15) used an improved extraction method with a forward-phase HPLC system for the same purpose.

This work describes the use of a fast extraction procedure and reverse-phase HPLC for the estimation of chlorpropamide and tolbutamide in plasma at sufficiently high sensitivity and specificity to be used for single-dose bioavailability studies.

EXPERIMENTAL

Materials—Chlorpropamide[2], 2-hydroxychlorpropamide[2], 3-hydroxychlorpropamide[2], carboxytolbutamide[3], 4-hydroxytolbutamide[3], *p*-toluenesulfonamide[4], *p*-toluenesulfonylurea[4], *p*-chlorobenzenesulfonylurea[4], *p*-chlorobenzenesulfonamide[5], and tolbutamide[6] were used without further purification. Ether, anhydrous[7], was freshly redistilled before use. Acetonitrile[8] (UV grade)

[1] K. K. Midha and C. Charette of these Laboratories, personal communication.
[2] Supplied by Pfizer, Montreal, Quebec, Canada.
[3] Gift of Dr. J. Thiessen, University of Toronto, Toronto, Ontario, Canada.
[4] Synthesized in this laboratory.
[5] Baker, supplied by Canlab, Ottawa, Ontario, Canada.
[6] Strong-Cobb-Arner of Canada, Fort Erie, Ontario, Canada.
[7] Mallinckrodt Canada Ltd., Montreal, Quebec, Canada.
[8] Burdick & Jackson Laboratories Inc., Muskegon, Mich.

Table I—Retention Characteristics of Chlorpropamide, Tolbutamide, and Their Metabolites

Substance	k'[a,b]	t[c]
Solvent front (t_0)	0	1.1
Chlorpropamide	1.0	2.2
p-Chlorobenzenesulfonylurea	0.2	1.3
p-Chlorobenzenesulfonamide	1.4	2.6
2-Hydroxychlorpropamide	0.3	1.4
3-Hydroxychlorpropamide	0.4	1.5
Tolbutamide	3.0	4.4
Carboxytolbutamide	0.1	1.2
4-Hydroxytolbutamide	0.4	1.5
p-Toluenesulfonylurea	0.2	1.3
p-Toluenesulfonamide	1.7	3.0

[a] Chromatographic conditions were: column, silica gel–octadecylsilane; mobile phase, 17% acetonitrile in 0.05 *M* aqueous ammonium formate; flow rate, 2.5 ml/min; and temperature, 50°. [b] $k' = (t - t_0)/t_0$. [c] Retention time in minutes.

and ammonium formate[9] were used without further purification. All other chemicals were reagent grade. The chromatographic mobile phase was filtered by passing through a 0.2-μm pore size membrane filter[10]. Plasma was obtained from fresh heparinized blood of normal human volunteers. The plasma was pooled and stored at 4° in glass containers.

Apparatus—The chromatographic apparatus[11] was fitted with a UV absorbance detector at a fixed wavelength of 254 nm. The column[12] was a bonded octadecylsilane–silica gel type, 10-μm particle size, 4 × 300 mm internal dimensions. This column was used at room temperature or heated to 50° by means of a glass jacket through which water from a constant-temperature water bath was circulated.

Columns were also prepared in-house[13] by reacting silica gel with trichlorooctadecylsilane (16) and packing the product into stainless steel tubing, using a balanced density slurry method similar to that of Cassidy *et al.* (17).

Preparation of Internal Standard Solutions—Chlorpropamide, 4 mg, or tolbutamide, 20 mg, was dissolved in 1 ml of 0.1 *N* NaOH and diluted to 50 ml with water. These solutions were used without further dilution as internal standards by adding the chlorpropamide solution to the tolbutamide determinations and vice versa. Appropriate dilutions of these same standard solutions were made with blank plasma for the calibration curves.

Extraction of Plasma—*Method 1*—Plasma, 1 ml, in a capped culture tube[14] was mixed with 0.25 ml of the internal standard solution and acidified to pH 3 by the addition of 1 ml of 0.15 *M* phosphoric acid. The drugs were extracted[15] (30 min at 50 rpm) with 10 ml of ether. The mixture was centrifuged, and the ether layer was separated and evaporated to dryness in a conical centrifuge tube, using a dry bath[16] at 45–50° with a gentle stream of dry filtered nitrogen. The residue was redissolved in 0.2 ml of 0.1 *M* ammonium carbonate and 0.2 ml of ethylene dichloride by mixing[17]. Aliquots of 0.02 ml[18] of the aqueous phase were chromatographed.

Method 2—This method was identical to that described by Midha *et al.* (12). It consisted of extracting the acidified plasma with toluene, back-extracting the drugs into phosphate buffer (pH 11.2), acidifying the buffer, reextracting with toluene, and evaporating the toluene phase with nitrogen. The dried extract was redissolved in the chromatographic mobile phase and chromatographed as described.

Method 3—This method, used in an earlier series of experiments[19], was for the determination of tolbutamide only. Plasma, 0.2 ml, in a capped culture tube[14], was mixed with 0.05 ml of internal standard solution and with 0.2 ml of 0.05 *M* phosphate buffer (pH 9.8) and extracted[15] twice with 2 ml of ether. After centrifugation, the ether extract was discarded. The remaining plasma in the tube was acidified with 0.15 ml of 0.05 *M* phosphoric acid and extracted as before with 2 ml of ether.

The separated ether layer was evaporated to dryness in a dry bath[16] at 45–50° with a gentle stream of dry filtered nitrogen. The residue was redissolved in 0.025 ml of the mobile phase, and the entire solution was chromatographed as already described.

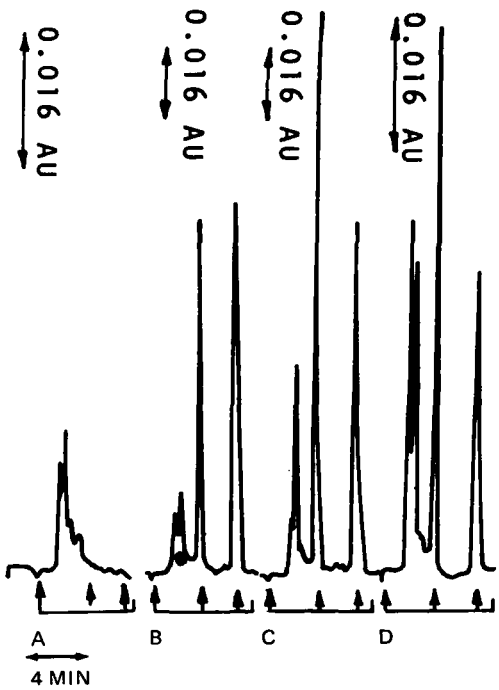

Figure 1—*Chromatograms of chlorpropamide and tolbutamide extracted from plasma by Method 1. Conditions for HPLC were as given in Table I. Key: A, blank plasma; B, blank plasma spiked with 20 μg of chlorpropamide/ml and 100 μg of tolbutamide/ml; C, plasma from an individual medicated with chlorpropamide (estimated value 31.1 μg/ml; internal standard, tolbutamide, 100 μg/ml); and D, plasma from an individual medicated with tolbutamide (estimated value 56.7 μg/ml; internal standard, chlorpropamide, 20 μg/ml).*

Chromatographic Conditions—HPLC was performed[11] on silica gel–octadecylsilane support, using 17% acetonitrile in 0.05 *M* aqueous ammonium formate as the mobile phase. At a flow rate of 2.5 ml/min, the back pressure for the column[12] at room temperature was approximately 2500 psi.

Recovery Experiments—The amounts of the drugs recovered after the extraction of the plasma by the different methods were estimated by comparing the peak heights obtained on chromatographing a known aliquot of the extract with the peak heights obtained from a known volume of a standard solution of the same drug.

Blood Profile Experiments—One volunteer (80 kg) was given orally, after fasting overnight, a 500-mg tolbutamide tablet[20] with 100 ml of water. Blood was withdrawn into heparinized vacuum tubes[21] at 0, 0.5, 1, 1.5, 2, 3, 4, 7, 24, and 28 hr. The samples were centrifuged immediately, and the plasma was kept at −20° until analysis by Method 1. After 1 week, the same subject received 250 mg of chlorpropamide[20]; the blood, sampled at 0, 1, 2, 3, 4, 5, 7, 24, 28, and 48 hr, was treated as described.

RESULTS AND DISCUSSION

To achieve good separation of chlorpropamide and tolbutamide from each other and from their metabolites, alternative chromatographic conditions were tried. The conditions finally chosen utilized a microparticulate bonded reverse-phase packing with a mobile phase consisting of 17% acetonitrile and 83% 0.05 *M* aqueous ammonium

[9] Catalog No. 15 6264, Aldrich Chemical Co. (Canada) Ltd., Montreal, Quebec, Canada.
[10] Millipore Teflon membrane filter, Catalog No. FGLP02500, Millipore Ltd., Mississauga, Ontario, Canada.
[11] Model ALC 202, consisting of a model 6000 pump, model U6K injector, and UV detector, Waters Associates Inc., Milford, Mass.
[12] MicroBondapak-C_{18}, Waters Associates Inc., Milford, Mass.
[13] By A. Butterfield and L. Wilson of this laboratory.
[14] Catalog No. T1356-1, Canlab, Ottawa, Ontario, Canada.
[15] Roto-Rack, Fisher Scientific, Ottawa, Ontario, Canada.
[16] Model SC/48, Brinkmann Instruments (Canada) Ltd., Rexdale, Ontario, Canada.
[17] Vortex Genie, Scientific Instruments, Springfield, Mass.
[18] Catalog No. 702N microliter syringe, Hamilton Co., Reno, Nev.
[19] Presented by S. Sved, N. Beaudoin, and I. J. McGilveray at the Twenty-Second Canadian Conference on Pharmaceutical Research, Montreal, Canada, 1975.
[20] Obtained commercially.
[21] Vacutainer, Becton, Dickinson & Co., Canada, Ltd., Mississauga, Ontario, Canada.

Table II—Recoveries and Statistical Parameters of the Calibration Curves for the Assay of Chlorpropamide and Tolbutamide

Drug	Correlation Coefficient (r^2)	Coefficient of Variation		Regression		Recovery[a], %
		Minimum	Maximum	Intercept	Slope	
Chlorpropamide[b,c]	0.998	3.23	6.08	−0.0032	0.0476	95.7 ± 2.9
Tolbutamide[b,c]	0.989	3.46	8.80	+0.0031	0.0095	93.3 ± 4.8
Tolbutamide[c,d]	0.980	3.78	5.95	−0.0049	0.0140	50.3 ± 2.9

[a] Mean ± *SD*. [b] Extraction by Method 1. [c] Conditions for HPLC as in Table I. [d] Extraction by Method 3.

formate. The retention times and capacity factors, k', for chlorpropamide, tolbutamide, and some of their metabolites are shown in Table I. All metabolites tested have retention times different from chlorpropamide and tolbutamide and thus do not interfere.

The column routinely used was commercially prepared, but similar results were obtained using other columns, including two that were bonded and packed in this laboratory. (The exact proportion of acetonitrile in the mobile phase had to be adjusted to the retentivity of each column.) An increase in temperature from ambient (~25°) to 50° made little difference in the resolution but markedly decreased the back pressure as well as the retention time. These reduced retention times (~4 min for the most retained drug, tolbutamide) permitted up to 12 analyses/hr.

Chromatograms of fresh human plasma extracts are shown in Fig. 1. In these experiments, control blank plasma was either extracted as such (trace A) or spiked with 20 μg of chlorpropamide/ml together with 100 μg of tolbutamide/ml and then extracted (trace B). The last two traces were obtained from a volunteer after the administration of chlorpropamide (Fig. 1C) or tolbutamide (Fig. 1D) and using the other drug as the internal standard. All extractions were done by Method 1.

No interfering peaks appeared in the blank plasma with a retention time similar to that of chlorpropamide, and only a very small peak appeared with a retention time similar to that of tolbutamide. However, dated plasma from the blood bank[22] contained substantial amounts of interfering materials having long retention times (23–28 min, $k' \simeq 20$–25), possibly leached out from the plastic bags used for storage (18).

Figure 2 shows the calibration curves for chlorpropamide and tolbutamide using blank plasma spiked with the appropriate amounts of the drugs, extracted, and chromatographed according to Method 1. The concentration range for chlorpropamide was 0.5–40 μg/ml with 100 μg of tolbutamide/ml as the internal standard. The concentration range for tolbutamide was 5–100 μg/ml with 20 μg of chlorpropamide/ml as the internal standard. The ratios of peak heights of the drug to internal standard plotted against drug concentration fell on a straight line. All points were within 1 *SE* of the means of five determinations from the curves. The statistical parameters of the calibration curves, as well as recoveries, are summarized in the first two lines of Table II. The overall extraction efficiencies were between 93 and 97%; the correlation coefficients, r^2, were 0.99 or better; and the intercepts were negligible.

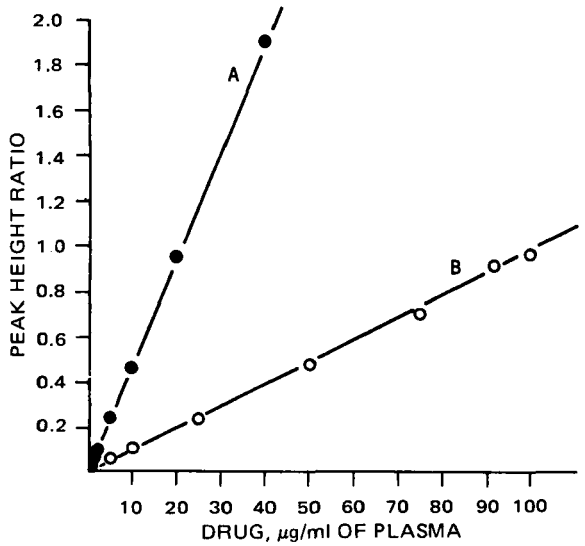

Figure 2—*Calibration curves for chlorpropamide and tolbutamide in plasma. Key: A (●), 0.5–40 μg of chlorpropamide/ml plus 100 μg of internal standard/ml (tolbutamide); and B (○), 5–100 μg of tolbutamide/ml plus 20 μg of internal standard/ml (chlorpropamide). Extraction was by Method 1. Conditions for HPLC were as given in Table I.*

[22] Canadian Red Cross, Ottawa, Ontario, Canada.

These results show that a single extraction with ether at pH ~3 quantitatively separated the drugs from most substances that could interfere in this study. After evaporation of the extract, the drug could be redissolved in aqueous ammonium carbonate buffer. However, it was desirable to add a small amount of ethylene dichloride at this stage to ensure dissolution of any drug that might be occluded inside some neutral lipids.

Use of this method allowed the preparation of all extracts for 1 day's analysis in less than 2 hr; dried extracts could be kept indefinitely in the deep freeze. Figure 3 shows the application of Method 1 to the plasma concentration of chlorpropamide (Fig. 3A) over 48 hr and tolbutamide (Fig. 3B) over 28 hr in a human volunteer following single doses of each drug at different time intervals.

In an earlier study[19], HPLC was compared with GLC in the analysis of samples obtained from a human volunteer treated successively with 125 mg of chlorpropamide or 500 mg of tolbutamide. In these experiments, the chlorpropamide profile was obtained using the extraction procedure of Midha *et al.* (12) (Method 2) while the tolbutamide profile was obtained using Method 3. In both cases, HPLC was performed as described. The parameters for the calibration curve using Method 3 are given in the last line of Table II.

The results of these experiments, published recently (12), show that the profiles are essentially the same whether assayed by GLC or high-speed liquid chromatography. The close similarity between the two methods is demonstrated in Table III. Differences between the drug levels found by GLC and HPLC amount to 4–7% average (means of absolute differences). None of the differences between the individual values exceeded 8 or 14% for chlorpropamide or tolbutamide, respectively, and the areas under the curve agreed within 2%. While the extraction procedures used in this series of experiments are not strictly equivalent to Method 1, the latter, with its improved recovery, should give similar results.

In conclusion, the HPLC procedure using extraction Method 1 is

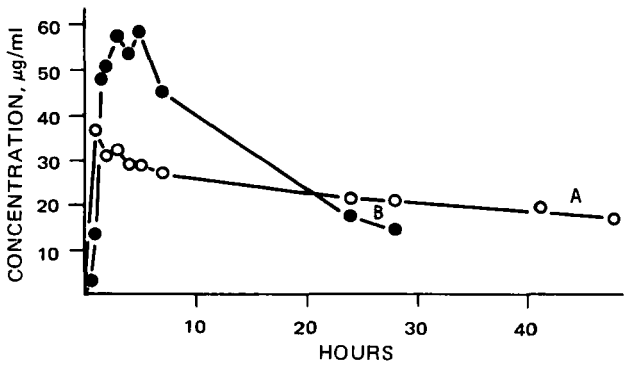

Figure 3—*Plasma profile of absorption and elimination of chlorpropamide and tolbutamide. The drugs were administered orally to a normal human volunteer after overnight fasting. Analysis was done by Method 1. Key: A (○), 250 mg of chlorpropamide; and B (●), 500 mg of tolbutamide. Conditions for HPLC were as given in Table I.*

Table III—Observed Differences between GLC and HPLC Assay of Drug Blood Profiles

Drug	Percent Difference: Maximum Observed	Percent Difference: Average[a]	AUC[b], % Difference
Chlorpropamide[c,d]	8.60	4.25	1.13
Tolbutamide[d,e]	14.10	7.41	1.90

[a] Mean of absolute differences. [b] Area under the plasma concentration–time curve (chlorpropamide, 0–168 hr; and tolbutamide, 0–28 hr). [c] Chlorpropamide was extracted by Method 2 for both HPLC and GLC. [d] Conditions for HPLC as in Table I. [e] Tolbutamide was extracted by Method 2 for GLC and by Method 3 for HPLC.

fast, sensitive, and specific for the determination of chlorpropamide and tolbutamide. Application of the method to plasma profiles in human volunteers showed that it can be used in single- and multiple-dose pharmacokinetic studies.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1975, from the *Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario K1A OL2, Canada.*

Accepted for publication December 2, 1975.

The authors thank Dr. K. K. Midha and Mr. A. Butterfield for invaluable discussions and Mrs. F. Matsui and Mr. C. Charette for providing some sulfonylurea derivatives.

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Effects of Intravenous Dantrolene Sodium on Respiratory and Cardiovascular Functions

K. O. ELLIS [x], F. L. WESSELS, and J. F. CARPENTER

Abstract □ Dantrolene sodium, a peripherally acting skeletal muscle relaxant, at doses up to 30 mg/kg iv had no effect on respiratory volume, respiratory rate, blood pressure, or heart rate in anesthetized dogs. The ED_{50} for inhibition of skeletal muscle contractions was 4.5 mg/kg in anesthetized dogs. In anesthetized sheep, the ED_{50} for skeletal muscle relaxation was 3.2 mg/kg under methoxyflurane anesthesia and 1.7 mg/kg under pentobarbital anesthesia. Unanesthetized sheep administered doses up to 30 mg/kg iv evidenced no dose-related cardiovascular effects. Respiratory volume decreased and respiratory rate increased, with the net result that the respiratory minute volume was not affected by dantrolene sodium. The results indicate that dantrolene sodium has no effect on the cardiovascular or respiratory systems that would preclude its use intravenously in acute conditions where direct relaxation of skeletal muscle is required, as in the management of malignant hyperthermia.

Keyphrases □ Dantrolene sodium—effects on respiratory and cardiovascular functions, dogs and sheep □ Respiratory functions—effects of dantrolene sodium, dogs and sheep □ Cardiovascular functions—effects of dantrolene sodium, dogs and sheep □ Relaxants, skeletal muscle—dantrolene sodium, effects on respiratory and cardiovascular functions, dogs and sheep

Dantrolene sodium[1], a new skeletal muscle relaxant, has therapeutic utility in chronic spasticity (1–4). The primary site of action of dantrolene sodium is outside the central nervous system (CNS) (5–8). It does not alter neuromuscular transmission or affect the electrical excitability of the muscle membrane (9, 10) but acts by uncoupling excitation–contraction mechanisms (11, 12). The hypothesized mechanism of dantrolene sodium's action is a decrease in release of Ca^{+2} from the sarcoplasmic reticulum (9, 13, 14).

The unique pharmacological action of dantrolene sodium suggests that it might be useful in the treatment of a condition characterized by muscle rigidity and elevated myoplasmic calcium (*i.e.*, malignant hyperthermia) (15). Recently, in the established syndrome of malignant hyperthermia in susceptible (MHS) swine, dantrolene sodium caused a rapid loss of muscle rigor commencing within 5 min, an immediate cessation of the increase in deep muscle temperature followed by a

[1] Dantrium, Eaton Laboratories, a subsidiary of Norwich Pharmacal Co., Division of Morton-Norwich Products, Norwich, N.Y.