GLC Determination of Plasma Levels of Intact Chlorpropamide or Tolbutamide

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Abstract □ A GLC procedure was developed for the quantitative estimation of intact chlorpropamide and tolbutamide concentrations in plasma; the drugs are used as mutual internal standards. After extraction of plasma containing the drug and internal standard with toluene, the dried residue is treated with ethereal diazomethane to form the methyl derivatives of tolbutamide and chlorpropamide. Aliquots of the ethereal solution are injected into a gas chromatograph equipped with a glass-lined injection port and glass column packed with a phenyl methyl silicone fluid (OV-25) on Chromosorb W, which facilitates the intact determination of the methyl derivatives of the drugs. The response to the flame-ionization detector was linear over a range of 0.20–25 μg/ml, with a 0.05-μg/ml limit of detectability for both drugs. The method compares favorably with a recently developed high-pressure liquid chromatographic procedure and is adequate for following blood level profiles of single doses of chlorpropamide (125 mg) and tolbutamide (250 mg). Mass spectral evidence showing that intact sulfonylureas are measured is presented.

Keyphrases □ Chlorpropamide—GLC analysis, plasma □ Tolbutamide—GLC analysis, plasma □ GLC—analysis, chlorpropamide and tolbutamide, plasma □ Antidiabetic agents—chlorpropamide and tolbutamide, GLC analysis, plasma

Chlorpropamide and tolbutamide, sulfonylurea derivatives, are widely used as oral hypoglycemic drugs in the treatment of diabetes. Most previously described procedures for the estimation of chlorpropamide and tolbutamide in biological fluids have been colorimetric or spectrophotometric methods (1–9). However, these methods suffer from a lack of specificity and/or sensitivity.

One GLC procedure for the estimation of plasma levels of chlorpropamide and tolbutamide involved methylation of the drugs with dimethyl sulfate prior to chromatography and detection with a flame-ionization detector (10). However, this procedure was found (11) to result in the degradation of the methylated derivatives of these drugs to methylated benzenesulfonamides in the injection port of the gas chromatograph. A similar GLC procedure involved methylation prior to chromatography (12).

The metabolism and pharmacokinetics of chlorpropamide were reviewed, and two assay methods were also described (13). One procedure utilized a GLC separation and quantitation with an electron-capture detector. The other was a high-speed liquid chromatographic (HSLC) procedure in which urinary metabolites of chlorpropamide were measured. Colorimetric and electron-capture methods were reported in which the nonspecific derivatives, *N*-propyl- and *N*-butyl-2,4-dinitroanilines, were formed by the reaction of 2,4-dinitrofluorobenzene with chlorpropamide and tolbutamide, respectively (14). These GLC procedures involved electron-capture detection or were nonspecific. Breakdown of the drugs to benzenesulfonamides in the injection port of the chromatograph appeared to be a problem in some cases (10–13).

The objective of the present study was to develop a simple and specific GLC procedure, employing flame-ionization detection for the determination of intact chlorpropamide and tolbutamide in plasma. This procedure was required for single-dose pharmacokinetic and bioavailability studies of these drugs.

EXPERIMENTAL

Reagents—Chlorpropamide[1], tolbutamide[2], toluene[3], and ether[4] were obtained commercially. Diazomethane was generated using a commercially available kit[5]. Plasma was obtained from the Red Cross Blood Bank. All other chemicals employed were analytical grade.

GLC—A gas chromatograph[6] equipped with a flame-ionization detector was employed. The column was coiled glass tubing, 1.83 m (6 ft) long by 3.0 mm i.d., packed with 5% phenyl methyl silicone fluid[7] (OV-25) on acid-washed, dimethyldichlorosilane-treated, high performance Chromosorb W[7] support (80–100 mesh).

The column was conditioned by maintaining the oven at 325° for 18 hr with low nitrogen flow. Operating conditions were: injection port temperature, 200°; column temperature, 220°; and detector temperature, 285°. The flow rate of nitrogen was 60 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Procedure—To 1-ml plasma samples in screw-capped[8] centrifuge tubes (20 ml) were added 1 ml of internal standard (7 μg of tolbutamide/ml for chlorpropamide or 4.25 μg of chlorpropamide/ml for tolbutamide) and 1.5 ml of 1 *N* HCl. The samples were extracted twice with 5-ml portions of toluene by shaking[9] for 10 min at 19 rpm, followed by centrifugation at 2500 rpm for 10 min. The 4- and 5-ml portions of the first and second toluene extracts, respectively, were transferred into a centrifuge tube (20 ml) containing 6 ml of 0.2 *M* phosphate buffer, pH 11.2

The tubes were mixed[9] for 10 min and then centrifuged for 10 min, after which the organic layer was discarded. The aqueous phosphate solution was acidified with 2.0 ml of 2 *N* HCl and extracted with 2 × 5-ml portions of toluene (mixed[9] 10 min, centrifuged 10 min). Four milliliters of the first extract and 5 ml from the second extract were transferred into an evaporating tube[10] (15). This combined toluene extract was evaporated to dryness at 85° under a stream of dry nitrogen.

An ethereal solution of diazomethane (0.5 ml) was added to the residue and left to react for 30 sec. The excess ethereal solution was evaporated at 45° under a stream of dry nitrogen to a volume of 25–50 μl, and aliquots (1–2 μl) were injected into a gas chromatograph equipped with a flame-ionization detector. The retention times of methylated chlorpropamide and tolbutamide were 4.50 and 5.90 min, respectively.

Standards and Calibration Curves—Chlorpropamide and tolbutamide standards were prepared by dissolving appropriate amounts of each in aqueous 0.01 *N* NaOH to obtain stock solutions of ≃50 μg/ml. Appropriate dilutions of these stock solutions were made to obtain concentrations over the 0.20–25.00-μg/ml range for chlorpropamide and tolbutamide.

[1] Chas. Pfizer & Co.
[2] Strong Cobb Arner of Canada, Fort Erie, Ontario, Canada.
[3] Caledon Laboratories, Georgetown, Ontario, Canada.
[4] Diethyl ether (anhydrous), Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[5] Catalog No. 710,025-0, Aldrich Chemical Co.
[6] Model 3920, Perkin-Elmer, Canada.
[7] Chromatographic Specialties, Brockville, Ontario, Canada.
[8] Lined with Teflon (duPont).
[9] Roto-Rack, Fisher Scientific Co., Montreal, Canada.
[10] Custom made by Canadian Laboratories Supplies Ltd., Montreal, Canada.

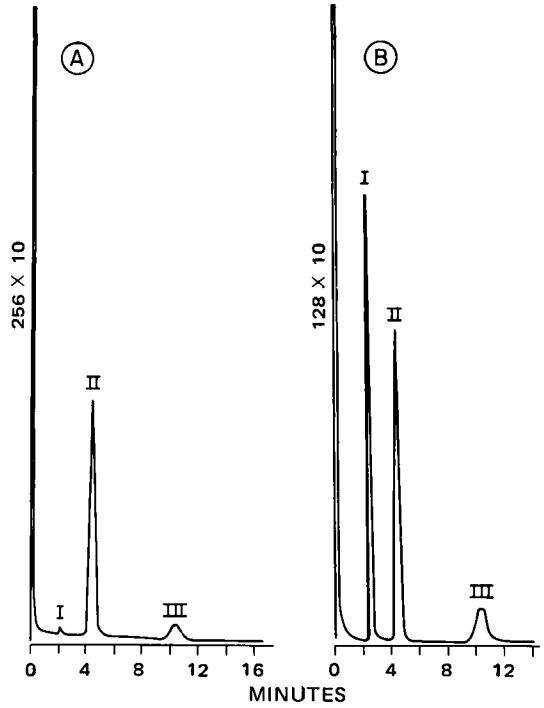

Figure 1—*Typical chromatograms of methylated chlorpropamide. Key: A, injection port temperature of 200°; and B, injection port temperature of 275°.*

Peak height ratios were calculated by dividing the height of the peak due to chlorpropamide or tolbutamide by the height of the other drug as the internal standard. Calibration curves were assembled from the results of spiked control plasma by plotting the peak height ratios against the concentrations of chlorpropamide or tolbutamide.

RESULTS AND DISCUSSION

Methylation of chlorpropamide with diazomethane gave three peaks (peaks I–III, Fig. 1A) with retention times of 2.2, 4.5, and 10.5 min, respectively, under the described GLC conditions. GLC–mass spectrometric[11] analysis of compounds giving rise to peaks I–III indicated that peak I was due to *N*-methyl-*p*-chlorobenzenesulfonamide (major ions at *m/e* 205–207, 175–177, and 111–113), peak II was due to *N*-methylchlorpropamide (diagnostic ions at *m/e* 261–263, 232–234, 226–228, 175–177, and 111–113), and peak III was due to a methyl enol ether of chlorpropamide, most likely the result of attack of diazomethane on carbonyl oxygen and loss of the more acidic sulfonamide proton (diagnostic ions at *m/e* 290–292, 258–260, 175–177, and 111–113).

Figure 1B shows a typical chromatogram of methylated chlorpropamide obtained with a complete glass system (*i.e.*, glass-lined injection port and glass column) with the injection port held at 275°. Figure 1A shows a typical chromatogram of methylated chlorpropamide obtained when the injection port temperature was dropped to 200°. From Fig. 1, it is apparent that the amount of *N*-methyl-*p*-chlorobenzenesulfonamide (peak I) increased at the expense of *N*-methylchlorpropamide (peak II) as the injection port temperature was raised. The amount of the methyl enol ether of chlorpropamide (peak III) did not seem to be influenced by the temperature changes in the injection port.

This thermal degradation of methylated chlorpropamide to *N*-methyl-*p*-chlorobenzenesulfonamide was tested by varying the injection port temperatures from 175 to 275°; 200° was best. At 175°, no decrease in the amount of *N*-methyl-*p*-chlorobenzenesulfonamide, as monitored by peak I, was observed. With injection port temperatures above 200°, the breakdown increased (Fig. 1B).

Similarly, methylation of tolbutamide with diazomethane and subsequent GLC analysis under similar conditions gave three peaks (peaks I–III, Fig. 2). GLC–mass spectral evidence indicated that peak I (retention time of 2.0 min) was due to *N*-methyl-*p*-toluenesulfonamide (major ions at *m/e* 185, 155, 141, 121, and 91), peak II (retention time of 5.9 min) was due to *N*-methyltolbutamide (diagnostic ions at *m/e* 241, 220, 212, 185, 155, 121, and 91), and peak III (retention time of 13.9 min) was due to a methyl enol ether of tolbutamide (diagnostic ions at *m/e* 284, 269, 255, 241, 184, 155, 121, and 91).

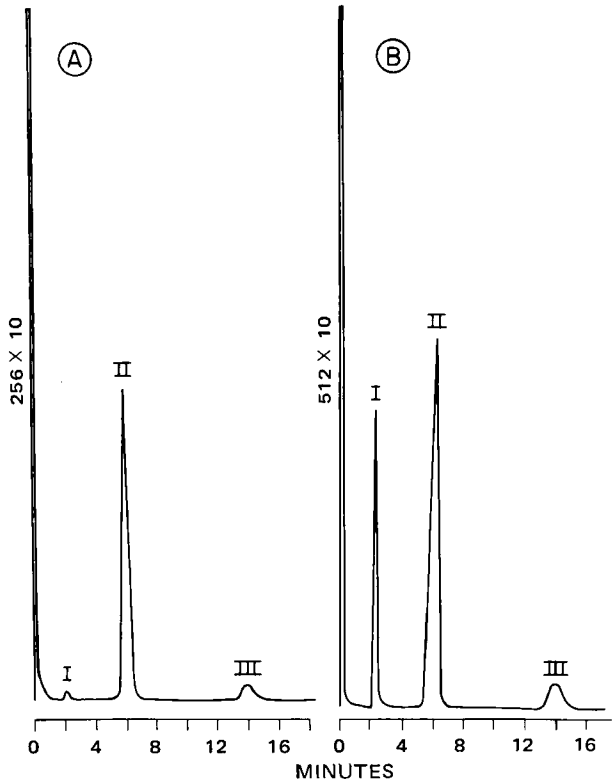

Figure 2—*Typical chromatograms of methylated tolbutamide. Key: A, injection port temperature of 200°; and B, injection port temperature of 275°.*

As the injection port temperature was dropped from 275 to 200° (Fig. 2), peak I due to *N*-methyl-*p*-toluenesulfonamide decreased and peak II due to *N*-methyltolbutamide increased. Thermal breakdown of methylated tolbutamide to *N*-methyl-*p*-toluenesulfonamide was reported previously (11), and it was observed (16) that methylation of tolbutamide also gave a methyl enol ether of tolbutamide as a minor product separate from the principal product *N*-methyltolbutamide.

The findings reported herein indicate that methylation of tolbutamide or chlorpropamide with diazomethane forms the desired *N*-methylated derivatives of these compounds together with minor amounts of methyl enol ethers. However, when the *N*-methylated derivatives were injected into the gas chromatograph, breakdown occurred to the respective *N*-methylbenzenesulfonamides. Careful choice of the injection port temperature minimized the breakdown of *N*-methyl derivatives of chlorpropamide and tolbutamide. Thus, the major peaks given by *N*-methylchlorpropamide (peak II, Fig. 1A) and *N*-methyltolbutamide (peak II, Fig. 2A) were used for the quantitative estimation of the respective drugs. Since these peaks were well resolved from each other (retention times of 4.5 and 5.9 min, respectively) under the study conditions and thermal breakdown was minimal when the injection port temperature was 200°, *N*-methyltolbutamide was used as an internal standard for *N*-methylchlorpropamide and vice versa.

Several other derivatization procedures, such as flash-heater methylation and methoxime formation with methoxyamine, were investigated in an attempt to form suitable derivatives of chlorpropamide and tolbutamide for GLC analysis. Flash-heater methylation of tolbutamide and chlorpropamide with trimethylanilinium hydroxide gave single peaks for each drug when a metal injection port was used. GLC–mass spectral analysis of these peaks indicated that *N,N*-dimethyl derivatives of benzenesulfonamides were

[11] Hitachi Perkin-Elmer model RMSU mass spectrometer coupled to a Perkin-Elmer model 990 gas chromatograph through a two-stage jet separator.

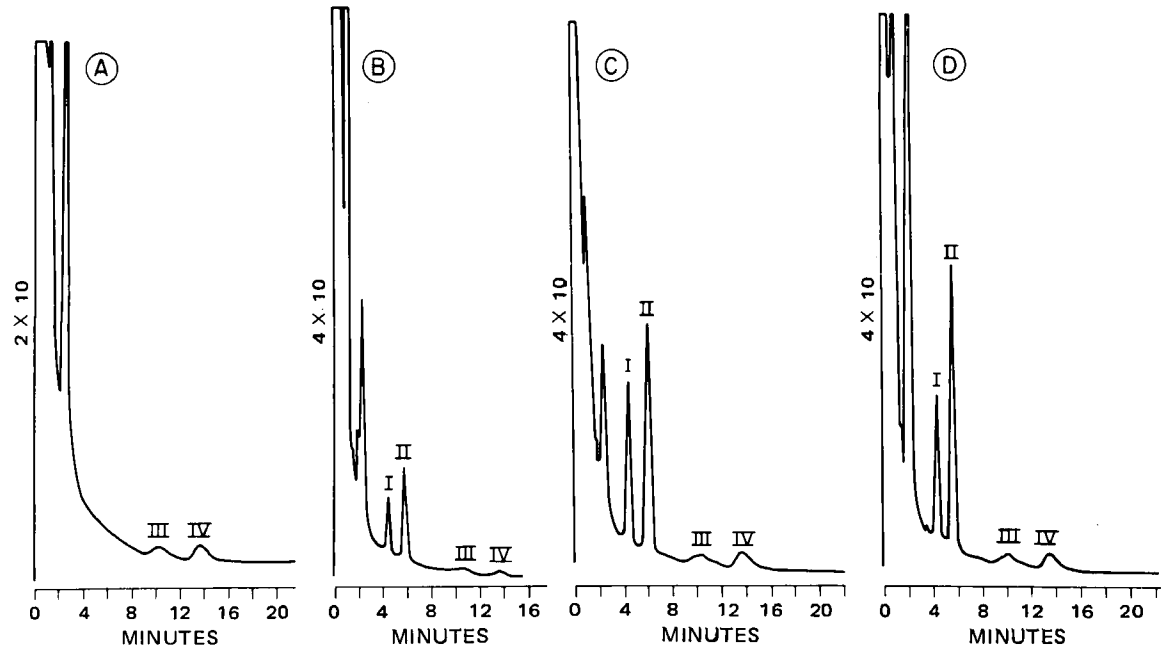

Figure 3—*Typical chromatograms of human plasma. Key: A, control plasma; B, plasma containing 4.25 μg/ml of chlorpropamide and 6.65 μg/ml of tolbutamide; C, plasma from a human volunteer who had been given 125 mg of chlorpropamide (amount is 4.75 μg); D, plasma from a human volunteer who had been given 500 mg of tolbutamide (amount is 15.10 μg); and peaks I and II, due to methylated chlorpropamide and tolbutamide, respectively.*

being formed. This observation is in agreement with previous results (17). This mode of derivatization was abandoned, since *p*-chlorobenzenesulfonamide, a metabolite of chlorpropamide[12], and *p*-toluenesulfonamide, a metabolite of tolbutamide[12], are extracted in the extraction procedure described and also undergo flash-heater methylation with trimethylanilinium hydroxide to *N,N*-dimethyl derivatives, which interfere in the analysis of these drugs.

Formation of methoxime derivatives of chlorpropamide and tolbutamide with methoxyamine hydrochloride in pyridine and subsequent GLC analysis gave a single peak for each drug on a column packed with a mixture of 1% dimethyl silicone fluid (OV-101) and 1% polyamide (Poly A-103). GLC–mass spectral analysis of these peaks indicated that the peaks were due to the respective benzenesulfonamides. Since these benzenesulfonamides are metabolites of these drugs[12], they would interfere.

Figure 3A shows a typical chromatogram obtained with control blank plasma processed as described under *Experimental* but with the internal standard, tolbutamide or chlorpropamide, omitted. Extraneous peaks III and IV at retention times 10.0 and 13.4 min, respectively, did not interfere with chlorpropamide and tolbutamide. Peaks appearing in the first 3 min after injection did not interfere with peak I (Fig. 3B) due to *N*-methylchlorpropamide or peak II (Fig. 3B) due to *N*-methyltolbutamide.

A chromatogram obtained when the method was applied to spiked plasma containing 4.25 μg of chlorpropamide and 6.65 μg of tolbutamide is shown in Fig. 3B. It is clear that the extraneous peaks do not interfere with the peaks due to chlorpropamide (peak I, 4.5 min) and tolbutamide (peak II, 5.9 min). Figure 3C shows a chromatogram obtained from a 72-hr plasma sample (1 ml) of a volunteer (73 kg) who received a commercial tablet of chlorpropamide (125 mg). Figure 3D shows a chromatogram obtained from a 24-hr plasma sample (1 ml) of a volunteer (73 kg) who received a commercial tablet of tolbutamide (500 mg).

The response of the flame-ionization detector to chlorpropamide and tolbutamide was linear with concentrations over the 0.20–25.00-μg/ml range. The ratio of the peak heights of chlorpropamide or tolbutamide and their respective internal standard (tolbutamide or chlorpropamide) plotted against concentration in the 0.20–25.00-μg/ml range gave straight lines passing through the origin (r = 1.0). Mean slope values of 0.146 ± 0.002 and 0.290 ± 0.003 were obtained for chlorpropamide and tolbutamide, respectively. The overall coefficients of variation were 3.4% (Table I) and 3.1% (Table II), respectively. The overall recoveries of chlorpropamide and tolbutamide from plasma were of the order of 72.1 ± 3.3 and 83.9 ± 1.5%, respectively (Table III). The described GLC procedure for tolbutamide and chlorpropamide appears specific since the known metabolites of both drugs do not interfere in the assay.

Table I—GLC Estimation of Chlorpropamide Added to 1 ml of Plasma

Chlorpropamide Added, μg	n	Mean Peak Height Ratio	Coefficient of Variation, CV, %[a]
0.212	4	0.049	6.05
0.425	4	0.085	0.91
0.920	3	0.143	4.03
1.840	3	0.303	3.81
3.680	3	0.553	2.09
7.360	4	1.137	2.72
13.800	3	2.093	4.12
23.000	3	3.350	3.44

[a] Mean CV = 3.40%, $y = mx$, where m = 0.146; and r = 1.0.

Table II—GLC Estimation of Tolbutamide Added to 1 ml of Plasma

Tolbutamide Added, μg	n	Mean Peak Height Ratio	Coefficient of Variation, CV, %[a]
0.332	4	0.095	4.19
0.665	4	0.191	1.62
1.330	4	0.466	3.52
3.325	8	0.993	3.21
6.650	6	1.964	3.73
13.300	5	3.860	3.05
26.200	4	7.744	2.47

[a] Mean CV = 3.11%, $y = mx$, where m = 0.290; and r = 1.0.

[12] There is some doubt about the status of these sulfonamides. While they occur in urine of patients treated with the drugs (13), the trace amounts suggest that they are degradation products rather than metabolites. They also occur as impurities in dosage forms and would interfere, as stated, in certain assays.

Table III—Recovery of Chlorpropamide and Tolbutamide from Plasma Determined by GLC Assay

Micrograms Added to 1 ml of Plasma	n	Mean Recovery, μg	Mean Recovery, %	*SD* of Percent Recovery
		Chlorpropamide		
3.68	4	2.71	73.63	4.77
7.36	4	5.19	70.59	1.76
Mean 72.11	3.27			
		Tolbutamide		
7.00	5	5.87	83.92	1.49

The major metabolites of tolbutamide are *p*-toluenesulfonamide[12], *p*-toluenesulfonylurea, hydroxytolbutamide, and carboxytolbutamide. When treated with diazomethane and chromatographed under the conditions described under *Experimental*, the metabolites gave retention times different from those of *N*-methyltolbutamide and *N*-methylchlorpropamide. *N*-Methyl-*p*-toluenesulfonamide had a retention time of 2.0 min. Methylated *p*-toluenesulfonylurea gave two peaks with retention times of 2.0 and 2.45 min. Methylated hydroxytolbutamide gave peaks with retention times of 9.7 and 23.6 min. Chromatography of carboxytolbutamide gave rise to two peaks with retention times of 10.8 and 12.9 min.

The major metabolites of chlorpropamide are *p*-chlorobenzenesulfonamide[12], *p*-chlorobenzenesulfonylurea, 2-hydroxychlorpropamide, and 3-hydroxychlorpropamide. None of these metabolites interfered with the assay; on methylation with diazomethane followed by chromatography under the described conditions, they gave retention times different from those of the methylated derivatives of the drug and the internal standard. Methylated *p*-chlorobenzenesulfonylurea gave two peaks with retention times of 2.2 and 2.7 min.

The peak of retention time 2.2 min was due to *N*-methyl-*p*-chlorobenzenesulfonamide, the breakdown product and perhaps a metabolite[12]. Methylated 2-hydroxychlorpropamide and methylated 3-hydroxychlorpropamide broke down mainly under the GLC conditions to give *N*-methyl-*p*-chlorobenzenesulfonamide. Minor peaks for intact methylated derivatives of alcohols, with retention times of 7.84 and 10.41 min, were well separated from those of methylated drug and the internal standard.

Application of the method to plasma level determination of chlorpropamide by GLC is shown in Fig. 4 where it is compared with a recently developed high-pressure liquid chromatographic (HPLC) procedure (18). A 125-mg dose of chlorpropamide (one-half of a tablet) was given to a healthy male volunteer (73 kg); blood was withdrawn at intervals over 168 hr, and aliquots of the plasma were assayed for chlorpropamide separately by the GLC and HPLC procedures. The methods compared favorably with an overall difference of 5.7%. When areas under the curves to 168 hr

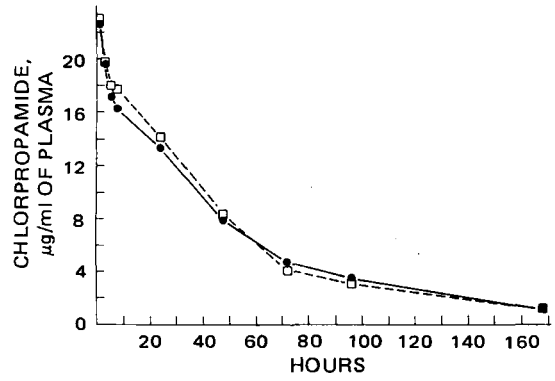

Figure 4—*Comparison of plasma chlorpropamide levels by GLC method with that of an HPLC method following a single oral dose of a 125-mg tablet of chlorpropamide to a human volunteer (73 kg). Key: ●, GLC method; and □, HPLC method.*

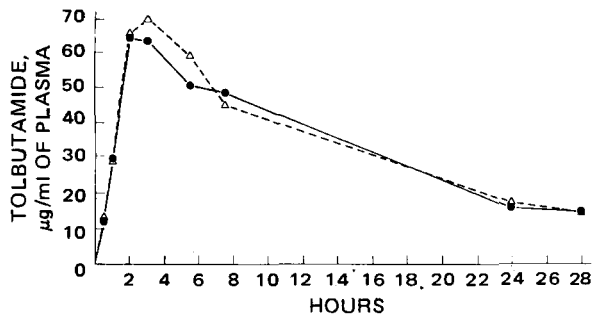

Figure 5—*Comparison of plasma tolbutamide levels by GLC method with that of an HPLC method following a single oral dose of a 500-mg tablet of tolbutamide to a human volunteer (73 kg). Key: ●, GLC method; and △, HPLC method.*

were compared, a difference of 1.1% was obtained. Application of the method to plasma level determinations of tolbutamide is demonstrated in Fig. 5, and comparison with a recently developed HPLC method (18) is also shown.

A 500-mg tablet of tolbutamide was given to a healthy male volunteer (73 kg); blood was withdrawn at intervals over 28 hr and analyzed separately by the GLC and HPLC procedures. The methods compared with an overall difference of 7.0%. When areas under the curves were compared to 28 hr, a difference of 1.9% was obtained.

The GLC method is simple, sensitive, and specific, because intact methylated chlorpropamide and tolbutamide are measured and the drugs are distinguished from their respective metabolites. The method can be employed for single- as well as multiple-dose pharmacokinetic studies. The procedure also gives promise of applicability to other sulfonylurea drugs.

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