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GLC Determination of Nylidrin in Human Urine Samples

HERSCHEL LI and PETER CERVONI [x]

Abstract □ A method for the detection of nanogram quantities of nylidrin in human urine is described. The method involves β-glucuronidase hydrolysis, extraction with chloroform, derivatization by silylation, and GLC determination. The suitability of the method was tested by analysis of urine samples of subjects after oral ingestion of nylidrin hydrochloride.

Keyphrases □ Nylidrin—GLC analysis, urine □ GLC—analysis, nylidrin in urine □ Vasodilators, peripheral—nylidrin, GLC analysis in urine

Nylidrin hydrochloride[1], 1-(*p*-hydroxyphenyl)-2-(1′-methyl-3′-phenylpropylamino)-1-propanol hydrochloride, is a sympathomimetic agent that acts predominantly by β-adrenergic receptor stimulation (1–3). It is a vasodilator used in the treatment of some peripheral vascular disorders (4, 5). The recommended human oral dose of nylidrin hydrochloride is 3–12 mg three or four times a day.

Two colorimetric analytical methods for measurement of nylidrin have been reported (6, 7), but they do not provide specificity or sufficient sensitivity for the determination of nylidrin in biological fluids.

Walle and Ehrsson (8, 9) developed a GC procedure for various alcoholic and amino compounds, using heptafluorobutyric anhydride and trimethylamine to prepare suitable derivatives for electron-capture detection. However, application of their method in this laboratory for the derivatization of nylidrin was only partially successful, since two peaks were observed in the chromatogram. Mass spectral analysis revealed that the major peak was the triheptafluorobutyryl derivative and the minor peak was the diheptafluorobutyryl derivative of nylidrin. Whether the diheptafluorobutyryl derivative was due to incomplete reaction of nylidrin with heptafluorobutyric anhydride as a result of steric hindrance at the secondary amino group of nylidrin or due to reverse hydrolysis of the triheptafluorobutyryl derivative is not clear at this time. An alternative approach was taken to provide a suitable method of determining urinary nylidrin in human subjects.

Preliminary studies performed on dogs in this laboratory showed that nylidrin is excreted in the urine as the free base and its glucuronide. The present method was designed to measure the total amount of nylidrin in urine after the urine was subjected to β-glucuronidase hydrolysis.

EXPERIMENTAL

Reagents—An aqueous standard stock solution was prepared by dissolving 5.00 mg of nylidrin hydrochloride[2] (equivalent to 89%

[1] Nylidrin hydrochloride is marketed by the USV Pharmaceutical Corp. under the trade name of Arlidin as 6- and 12-mg tablets. Subjects in this study received the 6-mg tablet orally.

[2] Nylidrin hydrochloride and nylidrin base were prepared in the Organic Chemistry Laboratories, USV Pharmaceutical Corp., Tuckahoe, NY 10707

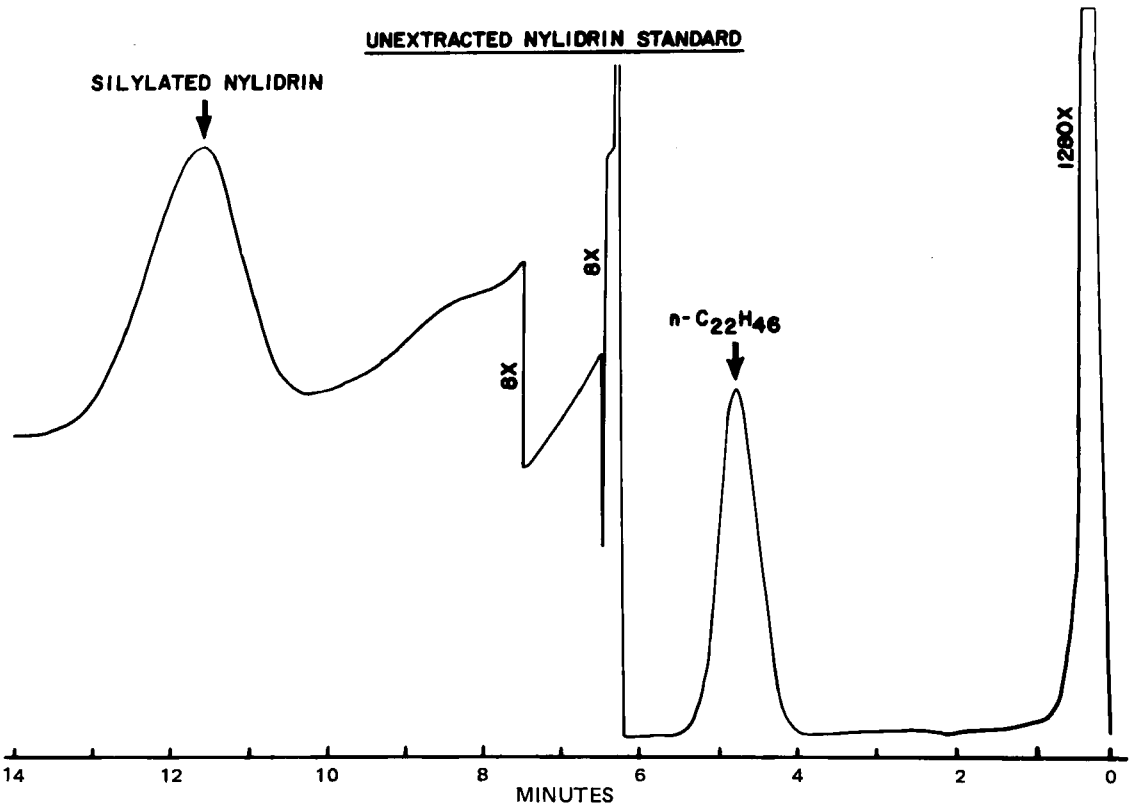

Figure 1—*Gas chromatogram of unextracted nylidrin standard after silylation* (*see* Experimental).

nylidrin free base moiety) in 1000 ml of distilled water. This solution was used in the preparation of a standard curve for nylidrin in urine from which nylidrin in the unknown urine samples was calculated.

Another standard stock solution containing 55.7 ng/μl was prepared by dissolving nylidrin as the free base[2] in spectral grade chloroform[3]. This solution was used in recovery experiments and for establishing the reference retention time for GLC analysis. A 0.2% solution of normal docosane[4] in spectral grade chloroform was used as the internal standard.

Urine Sample Collections—Normal male subjects were fasted overnight, and urine voided and collected just before oral administration of nylidrin hydrochloride tablets[1] served as the 0-hr control urine samples. Thereafter, the urine was voided *ad libitum* and the total volume was collected and stored in a freezer. Sample collection continued for as long as 30 hr after ingestion of nylidrin hydrochloride tablets.

β-Glucuronidase Hydrolysis—After adjustment of the urine to pH 5 with acetic acid[3], the volume was measured. A 5.0-ml aliquot was then pipetted into a 100-ml glass-stoppered bottle, and 5 ml of 0.1 *M* acetate buffer solution (pH 5.0) containing 5000 units of β-glucuronidase[5] was added. The samples were then kept in a Dubnoff metabolic shaking incubator[6] at 37° for 18 hr in the presence of air.

The hydrolyzed urine mixture was saturated with sodium carbonate and extracted three times with 25 ml of spectral grade chloroform. The chloroform extracts were pooled and dried over anhydrous sodium sulfate and condensed to a small volume. The drug was then back-extracted two times with 2.5 ml of 0.1 *N* HCl.

A 4.0-ml aliquot of the pooled acid extract was transferred to a glass-stoppered conical tube, and the acid was evaporated to dryness in a freeze-drying unit[7]. The side of the tube was washed with a small volume of methanol, and the methanol solution was evaporated to complete dryness under a stream of dry nitrogen for derivatization.

Preparation of Derivative—The trimethylsilyl derivative of nylidrin was prepared by adding 30 μl of bis(trimethylsilyl)trifluoroacetamide[8] and 20 μl of a 0.2% chloroform solution of docosane into conical tubes containing the dried acid extracts. The reactants were then thoroughly mixed using a vortex agitator. Silylation was carried out in an oil bath at 55–60° for 1 hr. Five microliters of the reaction mixture was injected directly into the chromatograph for analysis.

GLC—GLC separations were performed on a gas chromatograph[9] equipped with a flame-ionization detector. The 1.8-m × 0.63-cm (6-ft × 0.25-in.) o.d. glass column was packed with 0.2% OV-1 on 80–100-mesh Corning GLC-110 dimethylchlorosilane-treated glass beads[4]. The chromatographic conditions used were: injector and manifold temperature, 230°; column temperature, initially 170° and programmed at the rate of 0.5°/min; helium flow rate, 50 ml/min; hydrogen pressure, 27 psi; and air pressure, 30 psi. The recorder chart speed was 152.4 cm (60 in.)/hr.

RESULTS AND DISCUSSION

Derivatization—The derivative of nylidrin, when chromatographed under various column temperature conditions both with isothermal and temperature-programmed modes, showed only one derivative peak as compared to the reagent blank sample. The reaction was rapid and complete. Comparison of relative areas using a constant amount of nylidrin and internal standard at reaction times of 5, 20, 30, 45, 60, 90, and 120 min showed no further increase after 30 min.

The derivative was stable at room temperature in a tightly sealed reaction tube. However, upon exposure to moisture, reversible hydrolysis of the trimethylsilyl groups apparently took place. The following observation supports this postulate. If a reaction tube is opened for sampling on the 1st day and the same sample is reinjected into the chromatograph on a subsequent day, an occasional decrease in the relative peak area is observed. This loss in peak area can be restored upon resilylation with a fresh portion of bis(trimethylsilyl)trifluoroacetamide[8].

To assure a quantitative yield in derivative preparation and to prevent hydrolysis of the derivative, the following measures were

[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Applied Science Laboratories, State College, Pa.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] Precision Scientific Co., Chicago, Ill.
[7] Thermovac model FDC-2-MH-CT-Mod., Thermovac Industries Corp., Copiague, N.Y.
[8] Regisil, Regis Chemical Co., Chicago, Ill.
[9] Model 900, Perkin-Elmer Corp., Norwalk, Conn.

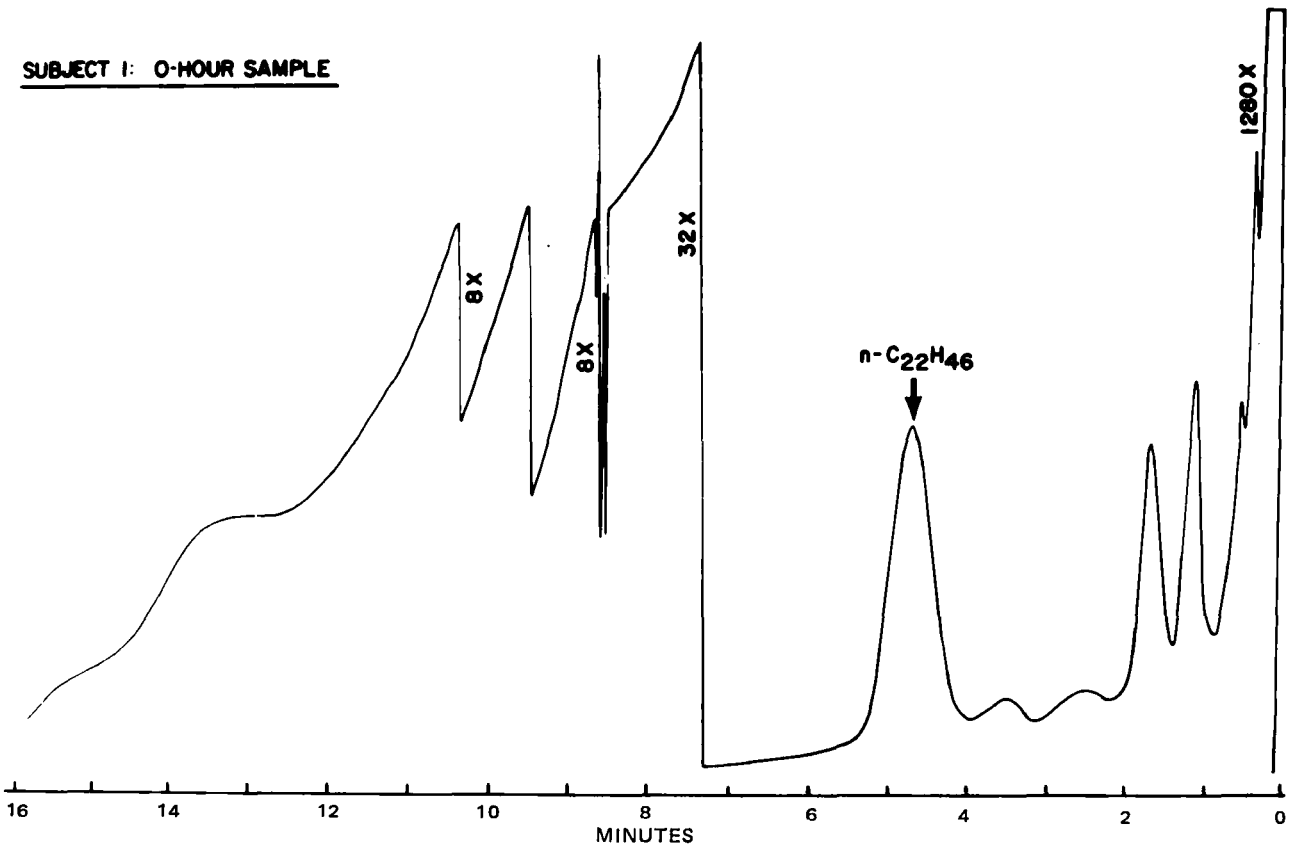

Figure 2—*Gas chromatogram obtained from the extract of a 5-ml 0-hr urine sample of Subject 1 after silylation.*

employed: (*a*) excess reaction time; (*b*) excess silylating reagent, which increases the protective capacity against moisture; (*c*) analysis of samples on the same day as the samples are derivatized; and (*d*) running of all samples in duplicate.

GLC–Mass Spectrometry—The derivative of nylidrin employed in this method was subsequently studied by GLC–mass spectrometry[10]. The electron-impact mass spectrum showed a strong peak at *m/e* 176. The molecular ion peak was not observed. However, the chemical ionization mass spectrum, using isobutane as the reactant gas, showed a strong quasimolecular ion at *m/e* 444 and a base peak at *m/e* 176.

The peak at *m/e* 444 was postulated to be the M + 1 ion of di(trimethylsilyl)nylidrin, the molecular weight of which is 443. The peak at *m/e* 176 probably was obtained after the cleavage between the α- and β-carbons. This result indicates that silylation took place at the phenolic and β-hydroxyl groups and that the sterically hindered nitrogen was not derivatized.

Chromatography—Under the described chromatographic conditions, the retention times of trimethylsilylnylidrin and docosane were approximately 11.7 and 4.7 min, respectively. The relative retention time of trimethylsilylnylidrin was 2.46–2.49.

Figure 1 shows the chromatogram obtained from a standard solution of nylidrin in chloroform. Figure 2 represents the chromatogram from an extract of a 5.0-ml 0-hr urine sample of Subject 1. No interfering peaks were observed in the region of the trimethylsilylnylidrin peak. Figure 3 shows the chromatogram obtained from the extract of a 5.0-ml 0-hr urine sample from Subject 1 with 1 μg of nylidrin hydrochloride standard added. The trimethylsilylnylidrin peak appears in the same region as the trimethylsilylnylidrin peak for the unextracted nylidrin standard shown in Fig. 1.

Standard Curve and Sensitivity—The areas of the trimethylsilylnylidrin and docosane peaks were measured by the method of triangulation. The relative area was determined by the ratio of the area of the trimethylsilylnylidrin peak to that of the docosane peak.

A standard curve was prepared for each experiment by adding aqueous stock solutions of nylidrin hydrochloride to 5.0 ml of the 0-hr urines, and these samples were carried through the same procedure as the unknown urine samples. Both the urine standards and unknown urine samples were extracted and derivatized by the same procedure and injected into the chromatograph under the same conditions.

The standard curve was obtained by plotting the relative area of the trimethylsilylnylidrin peaks against the nylidrin concentration, expressed as the free base equivalent. The slope and intercept of the standard curve were determined by the method of least squares. The total urinary nylidrin excreted during each time interval was calculated:

$$\text{total urinary nylidrin} = \text{ng/injection} \times \frac{\text{adjusted total urine volume (ml)}}{\text{5-ml aliquot}} \times \text{dilution factor} \quad \text{(Eq. 1)}$$

To examine the variabilities in the eight experiments when the unknown samples were being assayed, the values for the relative areas obtained from the same nylidrin concentrations used in determining the urine standard curves in these experiments were grouped together as replicates. These values were then applied to regression analysis. The equation for the regression line thus obtained was (n = 49, r = 0.82):

$$Y = -0.00354 + 0.00013X \quad \text{(Eq. 2)}$$

when $Y = 0$, and $X_0 = 68$ ng/ml of urine. The X intercept is interpreted as the average lower limit of detection of urinary nylidrin in

Table I—Recovery of Nylidrin from Urine[a]

Nylidrin in Urine, ng/ml		
Theoretical	Experimental	Recovery, %
178	106.75 ± 14.25	60 ± 8
356	206.75 ± 8.75	58 ± 2
534	358.0 ± 24.0	67 ± 4
712	477.75 ± 44.0	67 ± 6
890	639.0 ± 9.5	72 ± 1
1068	850.0 ± 27.0	80 ± 2

[a] Mean of three runs ± *SE* of the mean.

[10] Dupont 491 gas chromatograph–mass spectrometer, Dupont Instruments, Wilmington, Del.

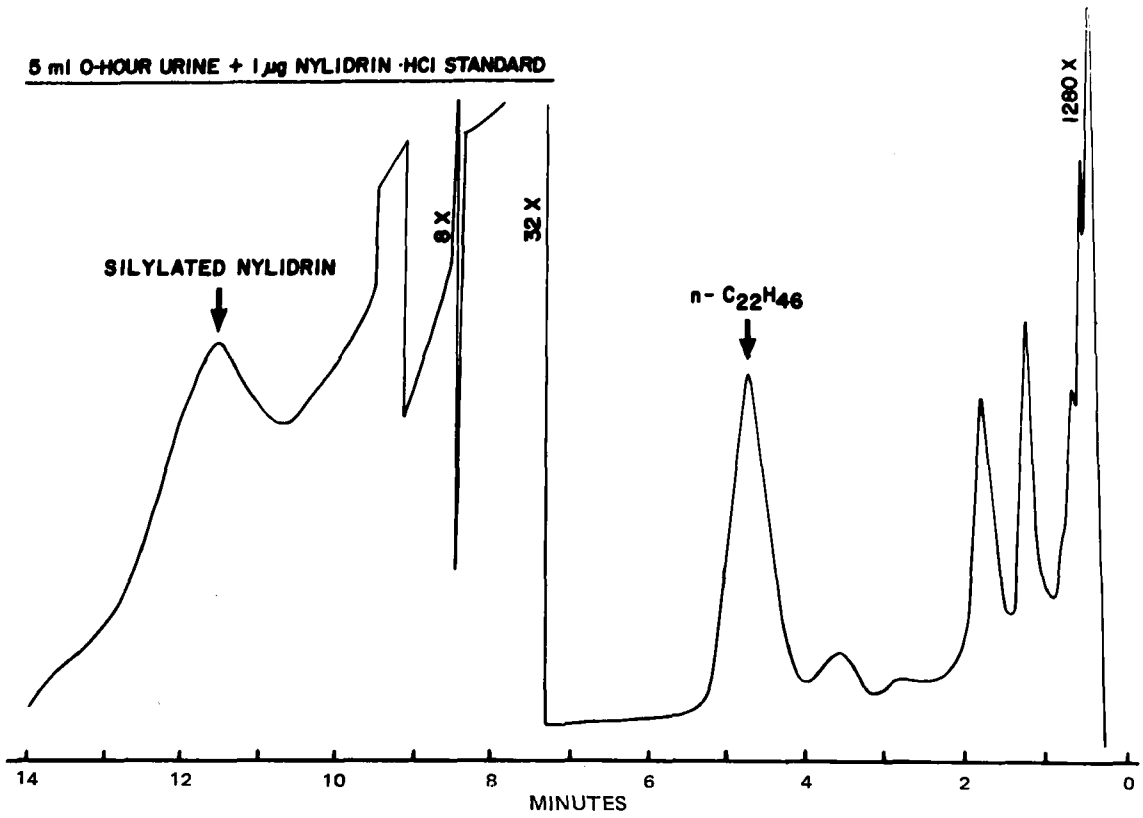

Figure 3—*Gas chromatogram obtained from the silylated extract of a 5-ml 0-hr urine sample of Subject 1 with 1 μg of nylidrin hydrochloride standard added.*

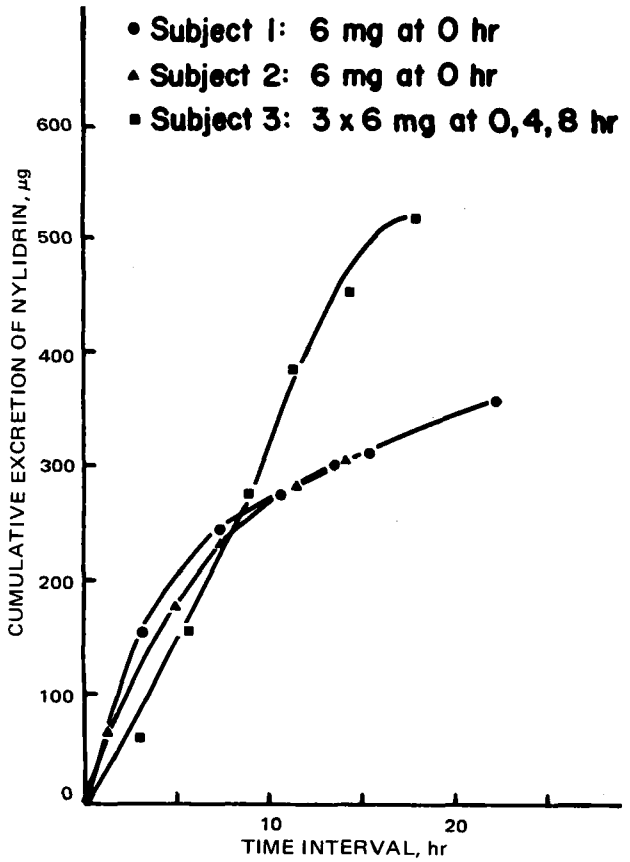

Figure 4—*Cumulative urinary excretion curves of nylidrin in three subjects after different oral dosage regimens of nylidrin hydrochloride tablets* (see Results and Discussion).

eight experiments.

Extraction Efficiency—To assure that nylidrin was adequately extracted from urine, a recovery experiment was conducted. The amount of nylidrin recovered from the urine after β-glucuronidase treatment was calculated on the basis of the values obtained from the unextracted standard samples.

Table I shows the recovery with different nylidrin concentrations. It is apparent that greater loss (lower recovery) was experienced in urine samples with low nylidrin concentrations. Therefore, unknown urine samples were calculated from the urine standard curve and not the unextracted standard curve to account for the differences in the extraction efficiency over the range of concentrations studied.

Application—The described assay method was applied to the analysis of urine samples of human subjects who received different oral dosage regimens of nylidrin hydrochloride tablets. Subjects 1 and 2 received one 6-mg tablet at 0 hr; Subject 3 received one 6-mg tablet at 0, 4, and 8 hr. Figure 4 shows the nylidrin cumulative excretion patterns in these subjects. The nylidrin excretion curves for Subjects 1 and 2 were identical. Excretion was rapid up to 8 hr and slowed thereafter throughout the remainder of the study. The excretion curve for Subject 3 was almost linear throughout the entire study.

Summary—The described GLC method, using flame-ionization detection, is capable of determining nylidrin in urine of human subjects given a single therapeutic dose. As reported for two subjects, varying oral doses of nylidrin hydrochloride tablets can be shown to result in a varied excretion pattern (Fig. 4). This method will be used in studies concerning the pharmacodynamic aspects of nylidrin.

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

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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.