High-Pressure Liquid Chromatographic Analysis of Tolbutamide in Serum

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Abstract □ A high-pressure liquid chromatographic (HPLC) analysis of tolbutamide in serum is described. The assay requires only 1 ml of serum and is capable of measuring as little as 2 μg of tolbutamide. The metabolites of tolbutamide do not interfere in the assay. Human serum samples, taken after a 1-g oral dose of tolbutamide, were analyzed by the HPLC and an existing GLC procedure, and the results are compared.

Keyphrases □ Tolbutamide—high-pressure liquid chromatographic analysis, compared to GLC analysis, serum □ High-pressure liquid chromatography—analysis, tolbutamide in serum □ Antidiabetic agents—tolbutamide, high-pressure liquid chromatographic analysis in serum

Tolbutamide is utilized in the control of diabetes, and simple, sensitive analytical procedures for its determination in serum or plasma are needed. An analytical procedure capable of measuring small amounts of tolbutamide in 1 ml of serum with good precision and accuracy was needed and is the subject for this report.

A literature review (1) on tolbutamide includes various UV and colorimetric assays for tolbutamide in biological fluids. All of them, however, suffer from low sensitivity when compared to modern chromatographic procedures. Several GLC procedures (2–4) for tolbutamide in plasma use dimethyl sulfate as the derivatizing reagent. It has been reported (5) that more than one peak is produced using this reagent.

A reported (6) GLC pyrolysis procedure for tolbutamide requires frequent increases in temperature to remove column contamination and needs a 3.0-ml plasma sample for analysis. An electron-capture GLC procedure was reported (7, 8) for tolbutamide in biological fluids. However, the limited linear dynamic range of electron-capture detectors requires frequent sample dilutions or repeat analyses using smaller sample aliquots to bring the measurements into the observed[1] single-decade useful concentration range. The suggested, but inconvenient, alternative (7, 8) is to reserve the sensitive GLC procedure for samples containing less than 10 μg/ml and to use the colorimetric procedure for more concentrated samples.

A high-pressure liquid chromatographic (HPLC) procedure was reported (5) for the analysis of tolbutamide in compressed tablets. The procedure uses a citrate-buffered, aqueous methanol mobile phase and a hydrocarbon polymer stationary phase to achieve separation. A reported (9) HPLC procedure for the analysis of tolbutamide in serum is capable of measuring 25 μg of tolbutamide/ml of sample. The assay uses an extraction procedure previously reported (2).

This paper reports an HPLC procedure which avoids the GLC problems of on-column decomposition and nonlinear response and is capable of measuring as little as 2 μg of tolbutamide/ml of serum. The assay uses a simple extraction procedure and requires no derivative formation. The metabolites of tolbutamide (10) elute with the solvent front and do not interfere in the assay.

[1] D. G. Kaiser, The Upjohn Co., Kalamazoo, Mich., personal communication, 1974.

EXPERIMENTAL

Reagents and Solvents—Primary standard grade tolbutamide[2] was used as received. The internal standard, *N*-(*p*-methoxybenzenesulfonyl)-*N'*-cyclohexylurea[2], had been prepared from *p*-methoxybenzenesulfonamide and cyclohexyl isocyanate and was used as received, mp 184.2–186.9°; IR: λ_{max} (mineral oil mull) 3320, 3180, 1690, 1545, 1345, 1270, and 1160 cm^{-1}; UV λ_{max} (CH_3CN): 241 nm (ϵ = 17,250).

Anal.—Calc. for $C_{14}H_{20}N_2O_4S$: C, 53.82; H, 6.45; N, 8.96; S, 10.26. Found: C, 53.83; H, 6.50; N, 8.83; S, 10.66.

TLC of the internal standard gave a single spot in two different solvent systems. All solvents were distilled in glass grade[3], and only analytical grade reagents were used. The standard solutions of the internal standard and tolbutamide were prepared by accurately weighing approximately 25 mg of material into a 25-ml volumetric flask and dissolving in 25 ml of acetonitrile. Both the tolbutamide and the internal standard solutions are stable for at least 1 month at room temperature.

Instrumentation—Samples were extracted using a two-speed reciprocating shaker[4]. Derivatization reactions were mixed using a vibromixer[5]. The GLC data were collected[6] using a 1.8-m × 3-mm i.d. glass column packed with 3% OV-17 on 60–80-mesh Gas Chrom Q. The column, injector, and ^{63}Ni-electron-capture detector temperatures were 233, 263, and 270°, respectively. Nitrogen and purge gas flow rates were 40 and 30 ml/min, respectively. Injections of 1–2 μl were made.

The mobile phase used in the liquid chromatography[7] was based on work previously reported (5) and was composed of 30% methanol and 70% water and was 0.01 *M* in monobasic sodium citrate. The flow rate at 500 psig was 0.40 ml/min. All measurements were at 26°. Detection was by UV absorption at 254 nm using sensitivity settings of 0.01–0.08 absorbance unit full scale (aufs). Quantification was by peak height ratio. Three-microliter samples were injected using a 10-μl syringe[8].

Assay Procedure—*Preparation of Standard Curve Samples*—Using microliter pipets[9] or calibrated microliter syringes[8], put 125, 100, 50, and 25 μl of a 1.0-mg/ml tolbutamide standard solution into separate 35-ml screw-capped[10] centrifuge tubes. Put 50 μl of the 0.10-mg/ml tolbutamide standard into a separate 35-ml centrifuge tube. Add 25 μl of the 1.0-mg/ml internal standard solution to each tube and also to an empty tube to be used as a blank. Blow all tubes dry with a gentle nitrogen stream. Add 1 ml of blank serum and 2 ml of pH 4.4, 0.1 *M* citrate buffer and mix well on a vibromixer. It is also possible to use 0.1 *M*, pH 4.5 phosphate buffer.

Preparation of Test Serum Samples—Put 25 μl of the 1.0-mg/ml internal standard solution into 35-ml centrifuge tubes and gently blow dry with nitrogen. Add 1 ml of test serum and 2 ml of pH 4.4 citrate buffer to each tube and mix well on a vibromixer.

[2] The Upjohn Co., Kalamazoo, Mich.
[3] Burdick & Jackson Laboratories, Muskegon, Mich. It is important to use tetrahydrofuran containing 250 ppm of butylated hydroxytoluene to avoid oxidizing the internal standard.
[4] Eberbach & Sons, Ann Arbor, Mich.
[5] Scientific Products, McGaw Park, Ill.
[6] Model MT-220, Tracor Inc., Austin, Tex.
[7] Model 820 equipped with a 1-m ETH Permaphase column, DuPont Instrument Products, Wilmington, Del.
[8] Precision Sampling Corp., Baton Rouge, La.
[9] Lang-Levy lambda pipets, Bio-Rad Labs., Richmond, Calif.
[10] Lined with Teflon (du Pont).

Table I—Extraction Efficiencies of Tolbutamide from Buffered Serum[a]

Solvent	Extraction Efficiency, %
5:95 Tetrahydrofuran–hexane	24.8
5:95 Isopentyl acetate–hexane	14.7
5:95 Butyl chloride–hexane	2.7
5:95 Methylene chloride–hexane	8.1
5:95 Isopentyl alcohol–hexane	73.1
8:92 Isopentyl alcohol–hexane	81.6

[a] Tritium-labeled tolbutamide in 4 ml of serum plus 8 ml of 0.1 *M*, pH 4.5 citrate buffer was extracted with 2 × 10 ml of organic solvent.

Treatment of Standard and Test Samples—Extract all tubes with 2 × 10 ml of 5% tetrahydrofuran in hexane for 10 min. Transfer the upper hexane layer to a 15-ml screw-capped[10] centrifuge tube and reduce the volume to about 5 ml using a warm water bath and a nitrogen stream. If emulsions form, centrifuge (3000 rpm for 10 min) and/or freeze the samples to break the emulsion. Use a pasteur pipet to transfer the hexane.

Extract the 5 ml of hexane with 10 ml of 0.1 *M* Na_2CO_3 for 10 min, centrifuge, and remove the upper hexane layer with suction.

Take an 8-ml aliquot of the sodium carbonate layer and transfer to a 35-ml screw-capped centrifuge tube. Add 2.00 ml of 1.0 *M* HCl and mix to expel gas. Extract with 10 ml of methylene chloride for 10 min, centrifuge, and remove bottom methylene chloride layer with a 5-ml gastight syringe[11] equipped with a 17.8-cm (7-in.), 20-gauge, square-cut tipped needle. Transfer the methylene chloride to 5-ml pointed, glass-stoppered centrifuge tubes and blow dry with a gentle nitrogen stream. Rinse the residue into the tip of the tube with several washes of methylene chloride and blow dry.

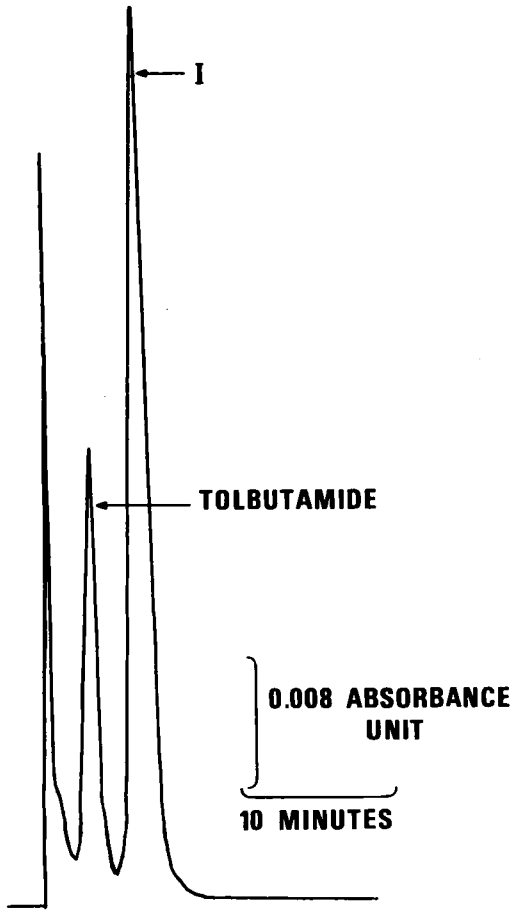
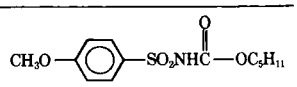
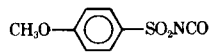
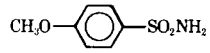
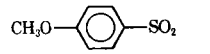
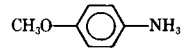
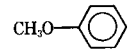


Figure 1—*Typical HPLC chromatogram of tolbutamide and the internal standard (I) from a serum sample. Sensitivity is 0.08 aufs, and the retention times of tolbutamide and the internal standard (I) are 6.0 and 9.0 min, respectively.*

Table II—Principal Peaks in the Mass Spectra of the Reaction Product of Isopentyl Alcohol with Internal Standard

m/e	Presumed Structure
301 (molecular ion)	
213	
187	
171	
124 (base peak)	
107	
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Redissolve the residue in 40 μl of acetonitrile and inject 3-μl aliquots into the liquid chromatograph. Calculate the peak height ratios and plot the standard curve to be used to determine the tolbutamide content of the test samples.

Blood Level Study in Humans—Informed written consent was given by a normal human male with a fasting blood sugar not exceeding 110 mg/100 ml. The drug was given as two 0.5-g tablets of tolbutamide[12] after an 8-hr fast. Food was allowed 3 hr after drug administration. Blood samples (18 ml) were taken at the indicated time intervals and allowed to clot; the serum, harvested by centrifugation, was immediately frozen to await analysis.

GLC Assay Procedure—The procedure of Matin and Rowland (7) was used for the GLC analysis of the serum samples. The assays were run as part of a much larger study, so considerable expertise has been generated in using the procedure. Due to the nonlinearity of the calibration curve, it was necessary to use 0.1-ml serum samples instead of the 0.5-ml samples suggested by Matin and Rowland (7).

RESULTS AND DISCUSSION

Extraction Efficiency of Tolbutamide and Internal Standard from Serum Samples—Various organic solvents (tetrahydrofuran, isopentyl acetate, butyl chloride, methylene chloride, and isopentyl alcohol) in hexane were tested, and only 5% tetrahydrofuran in hexane was satisfactory. This solvent system, under the conditions of the procedure, gave an extraction efficiency of 78.7% for tolbutamide and 62% for the internal standard. Addition of 0.5 g of anhydrous sodium sulfate to the aqueous phase increased the extraction efficiency only about 3%. Tetrahydrofuran must be preserved with 250 ppm of

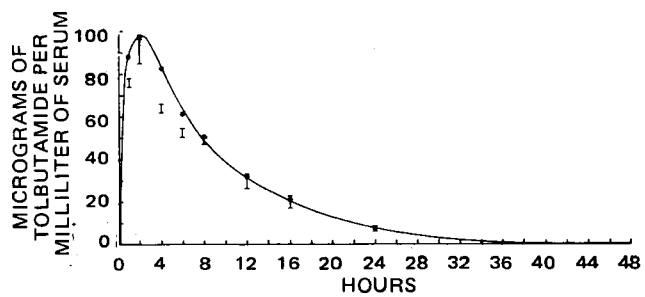

Figure 2—*Serum tolbutamide levels in a human subject after a 1-g oral dose. Key: ●, HPLC data; and* I, *GLC data. The GLC data represent the range of duplicate determinations.*

[11] Hamilton Co., Reno, Nev.

[12] Orinase, The Upjohn Co., Kalamazoo, Mich.

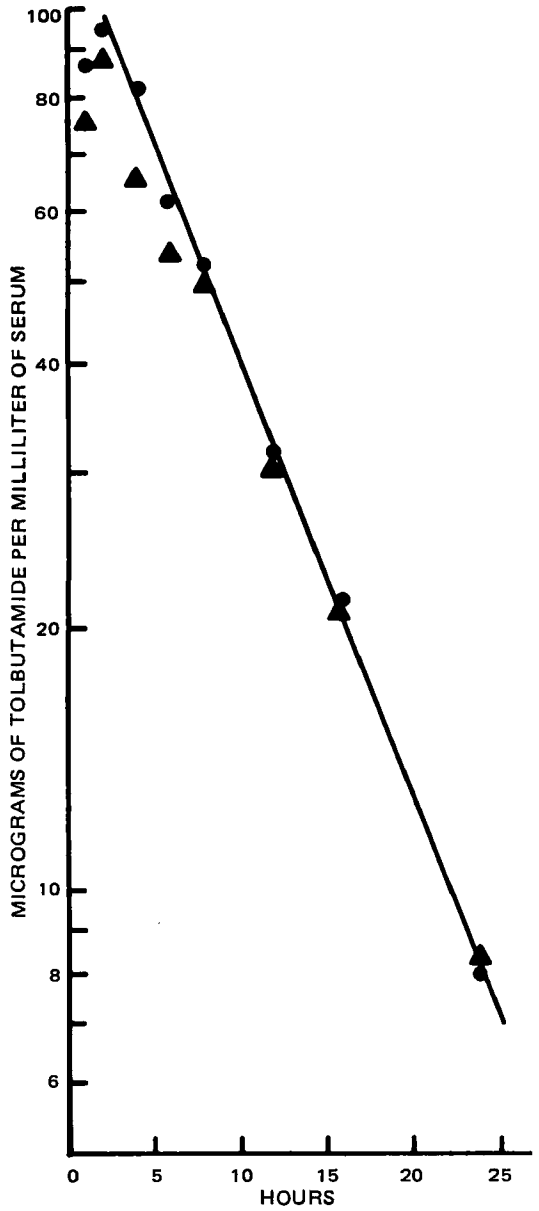

Figure 3—*First-order plot of serum tolbutamide levels after a 1-g oral dose. The* $t_{1/2}$ *is 6.03 hr. Key:* ●, *HPLC data; and* ▲, *GLC data.*

butylated hydroxytoluene to avoid oxidation of the internal standard. A comparison of extraction efficiencies of tritium-labeled tolbutamide from buffered serum using various organic solvents is shown in Table I.

Isopentyl alcohol (5%) in hexane also gave a satisfactory extraction efficiency for tolbutamide and the internal standard, but significant amounts of the isopentyl carbamate of the internal standard and tolbutamide were formed after 20 min at room temperature. The thermal dissociation of alkylsulfonylureas to give first the corresponding sulfonyl isocyanate and then, on reaction with alcohols, the alkyl carbamate is well known (11, 12). However, the reaction is usually run at 80° or higher. Therefore, the ease with which the reaction occurs, especially with the internal standard, is surprising. The reaction with the internal standard, based on peak heights, varied from 9 to 19% while tolbutamide reacted from 2 to 7%.

The identity of the reaction products was confirmed by isolating the HPLC peaks and comparing their mass spectra with those of authentic samples. The principal peaks found in the mass spectra of the reaction product of isopentyl alcohol and the internal standard are given in Table II.

Accuracy and Precision—A typical chromatogram of tolbutamide and the internal standard in a serum sample is shown in Fig. 1.

Table III—Accuracy and Precision of HPLC Assay for Tolbutamide in Human Serum

Tolbutamide Taken, μg		Amount Found, μg
128		129.9
		129.5
		133.0
		130.7
	Mean	130.7
	95% *CL*	±2.2 μg
	RSD	1.02%
64		65.5
		64.2
		65.5
		65.5
		67.7
	Mean	65.7
	95% *CL*	±1.4 μg
	RSD	1.7%
6.4		6.00
		6.31
		5.82
		5.88
	Mean	6.00
	95% *CL*	±0.30 μg
	RSD	3.1%

There was baseline separation between the drug and internal standard peaks. The calibration curve was linear from 2 to 160 μg of tolbutamide/ml of serum. The slope and associated 95% confidence interval was 0.0399 ± 0.0008, and the relative standard deviation for the slope was 1.8%. The curve passed through the origin, indicating no interference when assaying a blank serum sample.

Replicate calibration curves on different days (n = 3) gave a mean slope of 0.0381 and a mean relative standard deviation of 3.7%. The data in Table III show the accuracy and precision of the assay. The internal standard peak was measured at 0.08 aufs while the tolbutamide peak was measured at 0.01–0.08 aufs and then normalized to 0.01 aufs. It may be possible, assuming no interference, to increase the sensitivity from 2 to 0.1 μg/ml by using a variable wavelength detector at the absorption maximum (228 nm) of tolbutamide.

Comparison of GLC and HPLC Analyses—The results for the GLC and HPLC analyses of human serum samples obtained after the ingestion of 1 g of tolbutamide are given in Fig. 2. The points corresponding to the HPLC data form a smooth curve. There is a noticeable difference between the GLC and HPLC data at 4 and 6 hr, but it is not very likely that the serum tolbutamide concentration actually goes through a sharp change in slope as seen in the GLC data between 6 and 8 hr. The data from the logarithmic phase of Fig. 2 are plotted in Fig. 3 and show excellent linearity, as expected for simple first-order elimination.

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Synthesis and Properties of Mesoionic Pyrimido[1,2-*b*]pyridazine-2,4-diones and Mesoionic Pyridazino[2,3-*a*]-*s*-triazine-2,4-diones: Mesoionic Analogs Structurally Related to Fervenulin

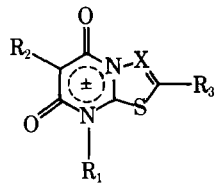
R. A. COBURN ˣ **and R. A. CARAPELLOTTI**

Abstract □ Derivatives of two new and unusual classes of heterocycles, possessing structural similarities to the broad spectrum antibiotic fervenulin, were synthesized and examined for *in vitro* antimicrobial activity. Only three of 17 mesoionic pyrimido[1,2-*b*]pyridazine-2,4-diones exhibited evidence of antimicrobial activity while seven of eight mesoionic pyridazino[2,3-*a*]-*s*-triazine-2,4-diones were active against one or more microorganisms. Susceptibility toward attack by nucleophiles of both mesoionic pyridazino[2,3-*a*]-*s*-triazine-2,4-diones and fervenulin was observed.

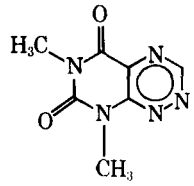
Keyphrases □ Pyrimido[1,2-*b*]pyridazine-2,4-diones—synthesized, *in vitro* antimicrobial activity screened □ Pyridazino[2,3-*a*]-*s*-triazine-2,4-diones—synthesized, *in vitro* antimicrobial activity screened □ Heterocycles—substituted pyrimido[1,2-*b*]pyridazines and pyridazino[2,3-*a*]-*s*-triazines synthesized, screened for antimicrobial activity □ Structure–activity relationships—substituted pyrimido[1,2-*b*]pyridazines and pyridazino[2,3-*a*]-*s*-triazines synthesized, antimicrobial activity screened □ Antimicrobial activity—substituted pyrimido[1,2-*b*]pyridazines and pyridazino[2,3-*a*]-*s*-triazines □ Mesoionic compounds—substituted pyrimido[1,2-*b*]-pyridazines and pyridazino[2,3-*a*]-*s*-triazines series synthesized, antimicrobial activity screened

The discovery of *in vitro* antibacterial activity of mesoionic thiazolo[3,2-*a*]pyrimidine-5,7-diones (I*a*, X = CH) and mesionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidine-5,7-diones (I*b*, X = N) was reported recently (1). In particular, the most active I*b* compounds possess obvious structural similarities to the broad spectrum antibiotic fervenulin (II) (2, 3) (replacement of N=N by a sulfur atom). These findings prompted the examination of two other ring systems structurally similar to fervenulin, mesoionic pyrimido[1,2-*b*]pyridazine-2,4-diones[1] (III) and mesoionic pyridazino[2,3-*a*]-*s*-triazine-2,4-diones[2] (IV).

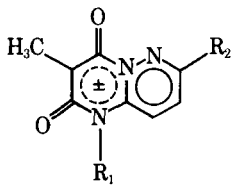
Reported here are the syntheses of a number of derivatives of mesoionic Structures III and IV and an examination of their chemical properties compared with those of fervenulin (II). These compounds were screened for *in vitro* antibacterial and antifungal activities and for *in vivo* antimalarial activity as part of an initial pharmacological investigation.



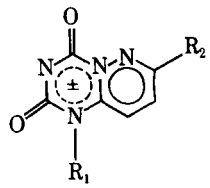
I*a*: X = CH
I*b*: X = N



II



III

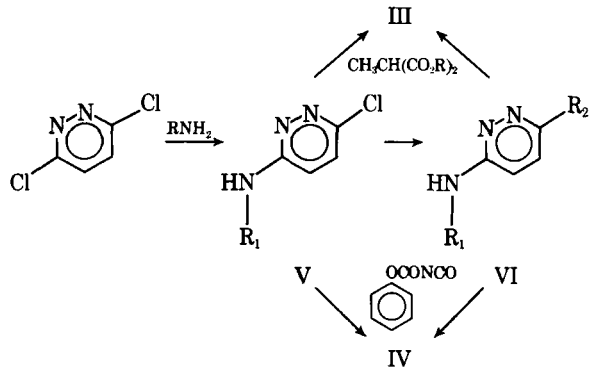


IV

CHEMISTRY

Compounds III*a*–III*q* (Table I) were prepared by the condensation of 3-aminopyridazines (secondary amines V and VI) with bis(2,4,6-trichlorophenyl) methylmalonate as previously described (1, 4), and IV*a*–IV*h* (Table II) were prepared by reaction of V and VI with phenoxycarbonyl isocyanate (Scheme I). The 3-(*N*-substituted amino)pyridazines (V) were prepared by the displacement of one chloro group of 3,6-dichloropyridazine with a primary alkyl-, aryl-, or aralkylamine. The chloro group of V could then be displaced to give VI with 6-substituents such as methoxy, morpholino, hydrogen, anilino, and *N*-methylpiperazyl (Table III).

In contrast to mesoionic thiazolopyrimidinediones (I*a*), which readily acylate benzylamine (4), mesoionic pyrimidopyridazinediones



Scheme I

[1] *anhydro*-1-Substituted 2-hydroxypyrimido[1,2-*b*]pyridazinium-4-one hydroxide.

[2] *anhydro*-1-Substituted 2-hydroxypyridazino[2,3-*b*]-*s*-triazinium-4-one hydroxide.