GLC Determination of Plasma Concentrations of γ-Oxo Metabolite of Phenylbutazone

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Abstract □ A quantitative method for the γ-oxo metabolite of phenylbutazone from plasma is described. The procedure involved an ethylene dichloride extraction of acidified plasma to which an internal standard, acenocoumarol, had been added. The extracted γ-oxo metabolite and the internal standard were methylated and analyzed by GLC. Determination of 0.25 μg of γ-oxo metabolite/ml with a relative standard deviation of 6.5% was accomplished.

Keyphrases □ Phenylbutazone γ-oxo metabolite—GLC analysis in plasma □ Metabolites—γ-oxo derivative of phenylbutazone, GLC analysis in plasma □ γ-Oxo metabolite of phenylbutazone—GLC analysis in plasma □ GLC—analysis, phenylbutazone γ-oxo metabolite in plasma □ Antirheumatic agents—phenylbutazone, γ-oxo metabolite, GLC analysis in plasma

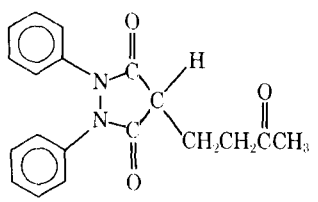
Phenylbutazone, an antirheumatic agent, undergoes biotransformation in humans to oxyphenbutazone; the γ-hydroxy, *p*,γ-dihydroxy, and γ-oxo derivatives; the C-4 glucuronides of phenylbutazone and the γ-hydroxy derivative; and, possibly, the γ-oxo derivative of oxyphenbutazone (1–5). Oxyphenbutazone and the γ-oxo metabolite (I) of phenylbutazone are both antirheumatic agents.

GLC (6–9) and high-speed liquid chromatography (10, 11) have been used to determine plasma levels of phenylbutazone, oxyphenbutazone, and the γ-hydroxy derivative of phenylbutazone, but methods for I have not been reported. A GLC procedure for the quantitative determination of plasma I concentrations is reported here; it is suitable for pharmacokinetic studies.

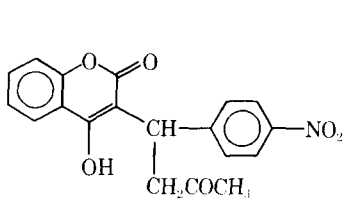
EXPERIMENTAL

Ethylene dichloride[1] and ether[2] were distilled in glass prior to use. Stock solutions containing 100 μg of I[3]/ml were prepared weekly in 0.1 *N* NaOH. Appropriate dilutions (0.25–20.0 μg/ml) were made immediately before use. Solutions containing 100 μg of the internal standard acenocoumarol[4] (II)/ml were prepared daily in 0.1 *N* NaOH (aqueous) and diluted to 8 μg/ml with distilled water. An ethereal solution of diazomethane was synthesized from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[5].

Plasma Level Study—Compound I (200 mg) was administered orally in solution, as described by McGilveray *et al.* (6) for phenylbutazone, to a healthy 85.6-kg male volunteer. Blood samples (10 ml) were withdrawn from the cubital vein, using heparinized evacuated tubes[6], before and at



I



II

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[3] United Pharmaceutical Works, Prague, Czechoslovakia.
[4] Geigy Pharmaceuticals, Dorval, Quebec, Canada.
[5] Diazald, Aldrich Chemical Co., Milwaukee, Wis.
[6] Vacutainers, Becton Dickinson Co., Mississauga, Ontario, Canada.

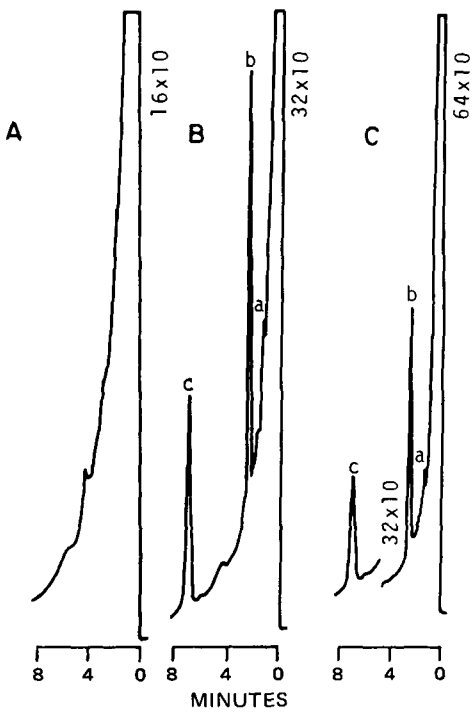


Figure 1—*Gas chromatograms of human plasma. Key: A, control plasma; B, spiked plasma containing 3 μg of I/ml and 8 μg of II/ml; and C, plasma from a dosed volunteer (55 hr) containing 7.75 μg of I/ml and 8 μg of II/ml. Peaks a and b are due to methylated I, and peak c is due to methylated acenocoumarol.*

18 appropriate time intervals over 223 hr following the dose. The blood samples were centrifuged, and the separated plasma was stored at −15° before analysis.

Extraction of I—To a 1-ml plasma sample ("spiked" or from dosed volunteers) in a glass-stoppered centrifuge tube[7] (50 ml) were added 1 ml of II (8 μg/ml), 3 ml of 3 *N* HCl, and 20 ml of ethylene dichloride. The samples were extracted and derivatized with diazomethane as previously described (12), except that the organic extract was not dried.

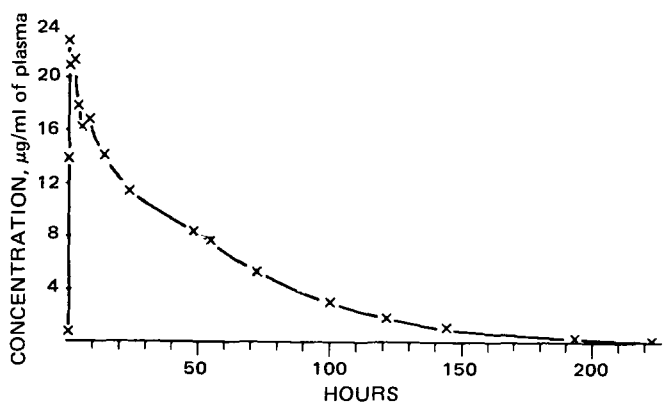


Figure 2—*Plasma profile of an 85.6-kg volunteer who received a 200-mg solution of I orally.*

[7] Fisher Scientific Co., Montreal, Quebec, Canada.

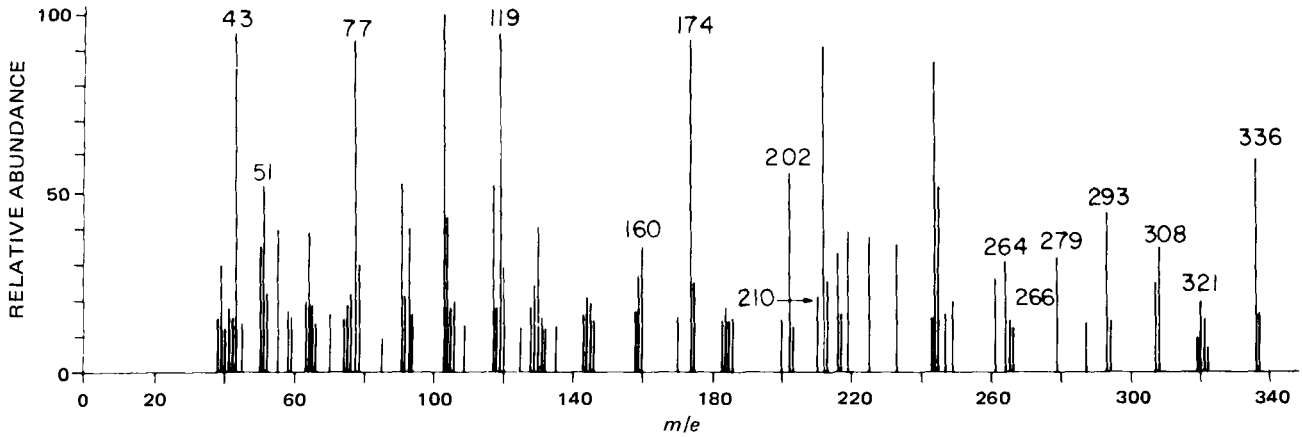


Figure 3—*GLC–mass spectrum (normalized) of the compound giving rise to the peak for methylated I from pooled plasma extracts from the dosed human volunteer.*

GLC—A gas chromatograph[8] equipped with a flame-ionization detector was employed. The column was a spiral glass tube, 180 × 0.25-cm i.d., packed with 3% phenyl methyl dimethyl silicone[9] coated on acid-washed, dimethylchlorosilane-treated, 80–100-mesh, high-performance flux-calcined diatomite support[10].

The column was conditioned by maintaining it at 340° for 18 hr with a low nitrogen flow. For the analyses, the injection port, detector, and column oven temperatures were maintained at 300, 310, and 285°, respectively. The nitrogen carrier gas flow rate was maintained at 70 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to methylated I (retention time of 2.6 min) by that from methylated II (7.1 min). Calibration curves were constructed from the results of spiked control plasma samples by plotting the peak height ratios against the concentration of I (micrograms per milliliter of plasma).

RESULTS AND DISCUSSION

Methylation of I with diazomethane gave two peaks with retention times of 1.5 and 2.6 min (peaks a and b, Fig. 1B) on GLC analysis. The heights of the respective peaks were in the ratio 1:6.5. This ratio was independent of oven temperature, instrument, and sample size. Therefore, the major peak, with a retention time of 2.6 min, was used for quantitation. GLC–mass spectrometry[11] of peaks a and b gave a molecular ion at *m/e* 336 and other diagnostic ions at *m/e* 321, 308, 293, 279, 264, 202, 177, 160, 119, 77, 51, and 43.

It was demonstrated[12] by preparative TLC and subsequent PMR characterization that the major product of diazomethane-methylated phenylbutazone is 1,2-diphenyl-4-methyl-4-*n*-butyl-3,5-pyrazolidinedione and the minor product is 1,2-diphenyl-3-methoxy-4-*n*-butyl-3,5-pyrazolinedione. On this basis, the compound giving rise to peak b (retention time of 2.6 min) was tentatively assigned the structure 1,2-diphenyl-4-methyl-4-(2-oxo-*n*-butyl)-3,5-pyrazolidinedione; the compound giving rise to peak a was tentatively assigned the structure 1,2-diphenyl-3-methoxy-4-(2-oxo-*n*-butyl)-5-pyrazolinone.

Methylation of II with diazomethane gave one peak on GLC analysis with a retention time of 7.1 min (peak c, Fig. 1B). The methylated derivative of II has the structure 3-(α-acetonyl-*p*-nitrobenzyl)-4-methoxycoumarin (12).

Attempts to prepare derivatives of I by the safer flash-heater methylation technique using trimethylanilinium hydroxide gave irreproducible results. Several other liquid phases such as saturated hydrocarbon lubricant[13], methyl silicone gum[14], phenyl methyl silicone fluid[15], and phenyl methyl dimethyl silicone fluid[16] were tested, but the major peak for I exhibited either tailing or interference from plasma constituents.

Carbon disulfide, employed as the injection solvent, rendered a very small solvent peak. Plasma extracts reacted with diazomethane but not dissolved in carbon disulfide were stable for up to 12 hr at room temperature; however, in the unreacted plasma extracts, I was much less stable.

Figure 1A shows a typical chromatogram obtained by processing fresh blank plasma from humans as described under *Experimental.* No extraneous peaks were observed in any chromatograms from human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 3.0 μg of I/ml and 8.0 μg of II/ml is shown in Fig. 1B. Figure 1C shows a chromatogram obtained from a 55-hr sample (1 ml) from the human volunteer who ingested a 200-mg dose of I as described under *Experimental.*

The overall recoveries of I (47.42 ± 1.64%) and II (99.9 ± 1.25%) from plasma were identical to those reported earlier for the analysis of II using I as the internal standard (12). The accuracy and precision of the GLC assay are demonstrated in Table I. Results were based on at least five determinations at each I concentration, ranging from 0.25 to 20.0 μg/ml. The overall relative standard deviation was 3.55%.

The calibration curve was linear ($y = mx$) over the concentration range of 0.25–20.0 μg of I/ml of plasma. A mean slope value of 0.617 ± 0.005 ($p = 0.05$; $r^2 = 0.999$) was obtained. During the analysis, the peak height ratio of the drug and the internal standard was used as the index of detector performance and overall efficiency.

Application of the method to plasma level determinations in a human male is demonstrated in Fig. 2. Plasma profiles observed over 223 hr indicate that the half-life of this drug (~32 hr) was approximately half that of phenylbutazone (~70 hr) in the same subject (6). The specificity of the analytical procedure was determined by pooling the remaining plasma from the dosed volunteer, extracting without the addition of II, and derivatizing as described under *Experimental.* A GLC–mass spectrum (Fig. 3) of the compound giving rise to the resultant peak for I was obtained and compared with that of authentic methylated I. No ions other than those for methylated I were observed in the mass spectrum of the peak from pooled plasma samples, indicating virtually no interference.

The described GLC procedure is sensitive and specific for the determination of I following single doses of I.

Table I—Estimation of I Added to Plasma by GLC[a]

Added I, μg	*n*	Mean Peak Height Ratio, I/II	*SD*	*CV*
0.25	5	0.170	0.011	6.50
1.0	5	0.684	0.016	5.35
2.0	6	1.440	0.053	3.65
3.0	7	2.026	0.092	4.56
5.0	5	3.052	0.043	1.42
10.0	5	6.076	0.106	1.74
20.0	5	12.328	0.207	1.68
			Mean *CV* =	3.56

[a] The equation is $y = mx$, where $m = 0.617 \pm 0.005$ ($r^2 = 0.999$).

[8] *Model 3920,* Perkin-Elmer, Montreal, Quebec, Canada.
[9] OV-11, Chromatographic Specialties, Brockville, Ontario, Canada.
[10] Chromosorb W, Chromatographic Specialties, Brockville, Ontario, Canada.
[11] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi Perkin-Elmer model RMSU mass spectrometer through a jet separator.
[12] Dr. D. V. C. Awang, Drug Research Laboratories, Health Protection Branch, National Health & Welfare, Ottawa, Ontario, Canada K1A 0L2, personal communication.
[13] Apiezon-L, Chromatographic Specialties, Brockville, Ontario, Canada.
[14] SE-30, Chromatographic Specialties, Brockville, Ontario, Canada.
[15] OV-17, Chromatographic Specialties, Brockville, Ontario Canada.
[16] OV-7, Chromatographic Specialties, Brockville, Ontario, Canada.

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Synthesis and Antibacterial Evaluation of 2-(Substituted Phenylureido)-4-thiocyanatobenzothiazoles

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Received March 14, 1977, from the *Chemistry Division and Pharmacometrics Division, Norwich-Eaton Pharmaceuticals, Division of Morton-Norwich Products, Inc., Norwich, NY 13815.* Accepted for publication May 2, 1977.

Abstract □ The synthesis and antibacterial evaluation of a number of 2-(substituted phenylureido)-4-thiocyanatobenzothiazoles are described. The more active compounds against the test organisms *in vitro* generally were those substituted with halogens on the phenyl and benzothiazole rings.

Keyphrases □ Benzothiazoles, various substituted—synthesized, evaluated for antibacterial activity □ Antibacterial activity—evaluated for various substituted benzothiazoles □ Structure–activity relationships—various substituted benzothiazoles evaluated for antibacterial activity

Previously, the synthesis and antiparasitic screening of a series of thiocyanatobenzothiazoles were reported (1). These compounds exhibited significant anthelmintic and antifungal activities. As an extension of this work, the synthesis of some 2-(substituted phenylureido)-4-thiocyanatobenzothiazoles is reported here. These compounds possess a high degree of *in vitro* activity against the Gram-positive bacteria *Staphylococcus aureus* and *Corynebacterium liquefaciens*.

DISCUSSION

The method of preparation of the 2-(substituted phenylureido)-4-thiocyanatobenzothiazoles (V–XII) (Table I) involved the reaction of the appropriate 2-amino-4-thiocyanatobenzothiazole (I–IV) with a phenyl isocyanate. The synthesis of I, II, and IV was reported previously (1, 2); the synthesis of III is reported here. The general procedure for the preparation of V–XII is shown in Scheme I. The assignment of structures for other compounds of the V–XII type was discussed previously (1).

The compounds were tested for antibacterial activity against *S. aureus* and *C. liquefaciens* using *in vitro* serial dilution techniques (3). Compounds V–XII exhibited activity against the test organisms at levels of from 0.048 to 6.25 μg/ml of test media. The test results for V–XII and the reference standard, nifuradene[1] (XIII) (4), are shown in Table I.

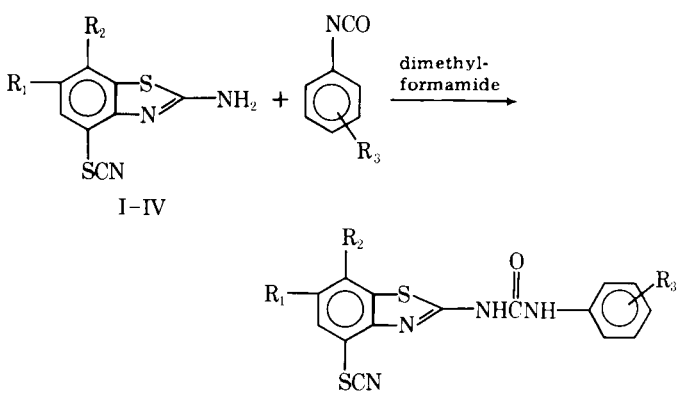
[1] U.S. Adopted Name for 1-[(5-nitrofurfurylidene)amino]-2-imidazolidinone.

Table I—Antibacterial Evaluation of Phenylureidothiocyanatobenzothiazoles

Compound	R_1	R_2	R_3	Minimal Inhibitory Concentration, μg/ml	
				S. aureus[a]	*C. liquefaciens*[b]
V	CH_3	H	4-NO_2	0.75	0.75
VI	Cl	H	H	0.38	3.1
VII	Cl	H	4-Cl	0.19	0.75
VIII	*n*-Bu	H	4-Br	6.25	6.25
IX	Cl	Cl	4-Cl	0.19	1.5
X	Cl	Cl	4-Br	0.38	0.048
XI	Cl	Cl	3,4-$(Cl)_2$	3.1	6.25
XII	Cl	Cl	4-F	0.38	1.5
XIII	Nifuradene			3.10	12.5

[a] Strain No. Mi-12, Norwich Pharmacal Co. [b] Strain No. Co-11, ATCC 11828.

In general, the compounds possessing the highest degree of antibacterial activity against the test organisms were substituted with halogen atoms on both the phenyl and benzothiazole rings (VII and IX–XII). However, no other structure–activity trends were apparent among the eight compounds tested.



Scheme I