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§ Present address: Eli Lilly and Co., Indianapolis, IN 46206

ˣ To whom inquiries should be directed.

GLC Determination of Plasma Concentration of Phenylbutazone and Its Metabolite Oxyphenbutazone

K. K. MIDHAˣ, I. J. McGILVERAY, and C. CHARETTE

Abstract □ Sensitive specific methods are described for the determination of phenylbutazone and its metabolite oxyphenbutazone from the same plasma sample. The sample, to which an internal standard 5-(4-hydroxyphenyl)-5-phenylhydantoin (Standard II) is added, is first extracted with ether to remove interfering substances and then with *n*-heptane under acidic conditions to separate phenylbutazone, which is determined on a gas chromatograph by flash methylation (310°) with trimethylanilinium hydroxide using 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane as the external standard (Standard I). The aqueous acidic residue from which the phenylbutazone has been selectively removed is shaken again with ether to extract the oxyphenbutazone, which is analyzed with a different GLC system with flash methylation (310°) against Standard II. The methods are of sufficient sensitivity to determine plasma levels in humans after a 200-mg dose of phenylbutazone (phenylbutazone, 1 μg/ml; oxyphenbutazone, 0.5 μg/ml).

Keyphrases □ Phenylbutazone and its metabolite oxyphenbutazone—GLC analysis in plasma □ Oxyphenbutazone metabolite and phenylbutazone—GLC analysis in plasma □ GLC—analysis, phenylbutazone and oxyphenbutazone metabolite in plasma

Although several spectrophotometric methods (1–6) and a GLC method (7) have been reported for the estimation of phenylbutazone in biological fluids, fewer methods have been described for the estimation of oxyphenbutazone [1-phenyl-2-(*p*-hydroxyphenyl)-3,5-dioxo-4-*n*-butylpyrazolidine] and none for hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine], which are the two main metabolites of phenylbutazone.

The classical method of Burns *et al.* (1) is not of sufficient sensitivity to estimate phenylbutazone and oxyphenbutazone in biological fluids following single doses of phenylbutazone. Although the need for a sensitive specific method for estimating phenylbutazone in plasma has been met by the recently reported high-speed liquid chromatography (HSLC) and GLC procedures (8, 9), no suitable methods are available for estimating oxyphenbutazone and hydroxyphenylbutazone following single doses of phenylbutazone in humans.

For the current pharmacokinetic study in these laboratories (10), a GLC method that is specific and sensitive for measuring oxyphenbutazone in plasma following single doses of phenylbutazone has been developed. The liquid chromatographic method reported earlier (9) for phenylbutazone is adequate, but the present method measures the levels of oxyphenbutazone as well as phenylbutazone in the same plasma sample.

EXPERIMENTAL

Reagents—Ether[1] and *n*-heptane[2] were distilled in glass prior to use. Stock solutions of phenylbutazone[3] containing 100 μg/ml were prepared (9) and diluted with phosphate buffer (pH 7.4) to the concentrations required (2–64 μg/ml) before use. Stock solutions of oxyphenbutazone[3] containing 16 μg/ml in ether were freshly prepared daily and diluted to the required range (1–16 μg/ml). Stock solutions of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane[4] (Standard I), containing 50 μg/ml in *n*-heptane, and 5-(4-hydroxyphenyl)-5-phenylhydantoin[5] (Standard II), containing 22.2 μg/ml in 0.01 *N* NaOH, were prepared. A solution of Standard I was prepared daily and used as such. A solution of Standard II was prepared every week and diluted to 1.11 μg/ml with 0.01 *N* NaOH daily before use. A 0.2 *M* phosphate buffer (pH 11.2 ± 0.2) and 1 *N* and 2 *N* HCl were employed. A 0.2 *M* solution of trimethylanilinium hydroxide in methanol was synthesized according to the method of Barret (11).

Glassware—All glassware was silanized before use by soaking for 1 hr in 1% hexamethyldisilazane in ether and rinsing with ether, methanol, and finally water (distilled in glass) before oven drying. Evaporation tubes with narrow bore bases (12) were custom made from Teflon-lined screw-capped test tubes[5].

Plasma Level Study—Phenylbutazone was administered to two healthy male volunteers, in one case in a solution buffered at pH 7.2 [Subject 1, 30 years, 90.8 kg (200 lb)] and in the second case in 2 × 100-mg tablets[6] [Subject 2, 30 years, 95.3 kg (210 lb)]. Samples of blood (10 ml) were withdrawn from the cubital vein by means of heparinized containers[7] at 14 appropriate time intervals after dosing. The blood samples were centrifuged and the plasma was transferred before storing at −10°.

General Procedure—*Extraction of Phenylbutazone*—To 1-ml plasma samples (spiked or from dosed volunteers) in Teflon-lined screw-capped centrifuge tubes (20 ml) are added 1 ml of Standard

[1] Diethyl ether (anhydrous), Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[2] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[3] Ciba-Geigy, Canada.
[4] Aldrich Chemical Co., Milwaukee, WI 53233.
[5] Canadian Laboratory Supplies Ltd., Montreal, Quebec, Canada.
[6] Butazolidin, Geigy Pharmaceuticals, Montreal, Quebec, Canada.
[7] Vacutainers, Becton Dickinson & Co., Mississauga, Ontario, Canada.

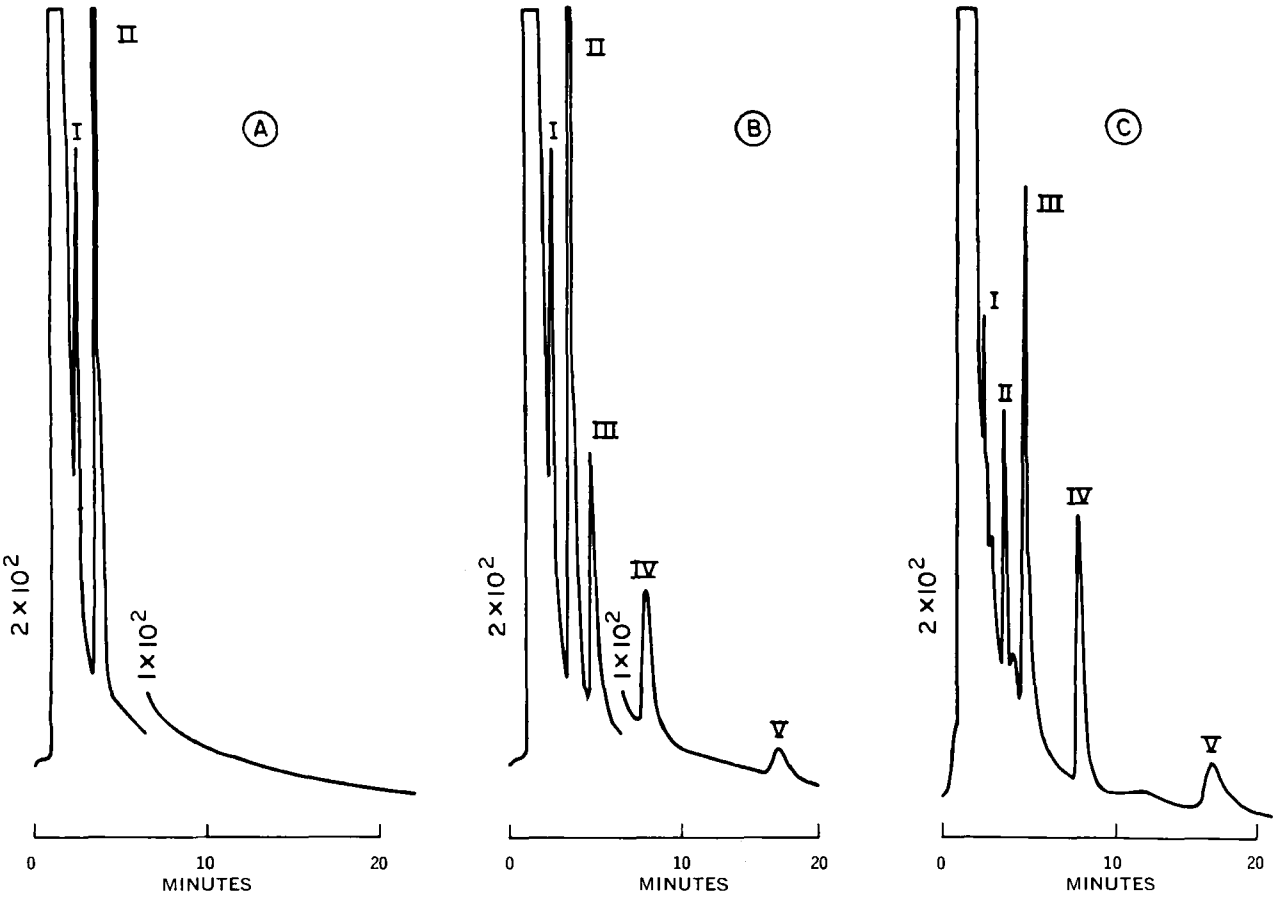


Figure 1—*GLC of human plasma. Key: A, control plasma; B, plasma containing 16 μg/ml of phenylbutazone and 50 μg/ml of Standard I; C, plasma from a human volunteer who had been given 400 mg of phenylbutazone; peaks I and II, endogenous material from plasma; peak III, Standard I; and peaks IV and V, major and minor peaks of flash-methylated phenylbutazone.*

II (1.11 μg/ml), 2 ml of 1 *N* HCl, and 5 ml of ether. The samples are extracted by mixing[8] for 10 min at 19 rpm followed by centrifugation at 2500 rpm for 10 min. Four milliliters of the ethereal layer is transferred into another Teflon-lined screw-capped centrifuge tube (20 ml) containing 5 ml phosphate buffer (pH 11.2). After repeating the extraction with 7 ml of ether, 7 ml of the organic layer is added to the previous extract. The combined extracts are mixed[8] with the phosphate buffer (10 min) and centrifuged (10 min) and the ethereal layer is discarded. The aqueous phosphate solution is acidified with 2 ml of 2 *N* HCl and extracted with 2 × 5-ml portions of *n*-heptane (mix[8] 10 min and centrifuge 10 min). Four milliliters of the *n*-heptane layer is transferred from the first extract and 5 ml from the second into an evaporating tube containing 1 ml of Standard I (50 μg/ml in *n*-heptane). The *n*-heptane extract is evaporated to dryness at 60° under a stream of dry nitrogen, and 50 μl of a methanolic solution of trimethylanilinium hydroxide (0.2 *M*) is added. The contents are dissolved by swirling on a mixer[9] before injecting 1–2 μl into gas chromatograph I.

Extraction of Oxyphenbutazone—The tubes containing the acidified phosphate buffer and remaining *n*-heptane are carried over for the determination of oxyphenbutazone. The remaining *n*-heptane is removed with a Pasteur pipet, a further 2-ml portion of *n*-heptane is added, the tube is swirled gently by hand, and *n*-heptane is removed with a Pasteur pipet. The aqueous acid is then extracted with 2 × 5-ml portions of freshly distilled ether by mixing[8] (15 min each time) followed by centrifugation (10 min each time) and removal of 4 and 5 ml of the ether extract successively into an evaporating tube. The ether extract is then evaporated in a dry bath at 40° under a stream of dry nitrogen; 20 μl of trimethylanilinium hydroxide (0.2 *M*) in methanol is added with gentle rotation followed by mixing[8]. One to two microliters is injected into gas chromatograph II.

GLC—GLC was effected on two gas chromatographs[10], both equipped with flame-ionization detectors. Stainless steel columns [2.4 m × 0.3 cm (8 ft × 0.125 in.) o.d. for phenylbutazone, column I; 1.2 m × 0.3 cm (4 ft × 0.125 in.) o.d. for oxyphenbutazone, column II] packed, respectively, with 3.0 and 5.0% OV-7 coated on acid-washed, DMCS treated, 80–100-mesh, high performance Chromosorb W[11] were used throughout the study. Both columns were conditioned by injecting a silylating mixture[12] and maintaining the columns at 300° for 18 hr with low nitrogen flow. For both columns the injection port and detector temperatures were 310 and 300°, respectively, and the column oven temperature was 260°. Nitrogen, as a carrier gas, was maintained at 40 ml/min for column I and 27 ml/min for column II. Hydrogen and compressed air flow rates were adjusted to give maximum response with each gas chromatograph.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to phenylbutazone or oxyphenbutazone by the height of the respective standard. Calibration curves were constructed from the results of spiked control plasma samples by plotting the concentration of phenylbutazone or oxyphenbutazone (micrograms per milliliter plasma) against the respective peak height ratios.

RESULTS AND DISCUSSION

Flash-heater methylation of phenylbutazone with trimethylanilinium hydroxide gave two peaks with retention times of 7.1 and 14.9 min (IV and V, Fig. 1B). The heights of the respective peaks were in the ratio 5:1. This ratio was independent of oven temper-

[8] Roto-Rack, Fisher Scientific Co.
[9] Vortex Genie, Fisher Scientific Co.
[10] Model F/11, Perkin-Elmer, Canada.
[11] Chromatographic Specialties, Brockville, Ontario, Canada.
[12] Silyl 8, Pierce Chemical Co., Rockford, Ill.

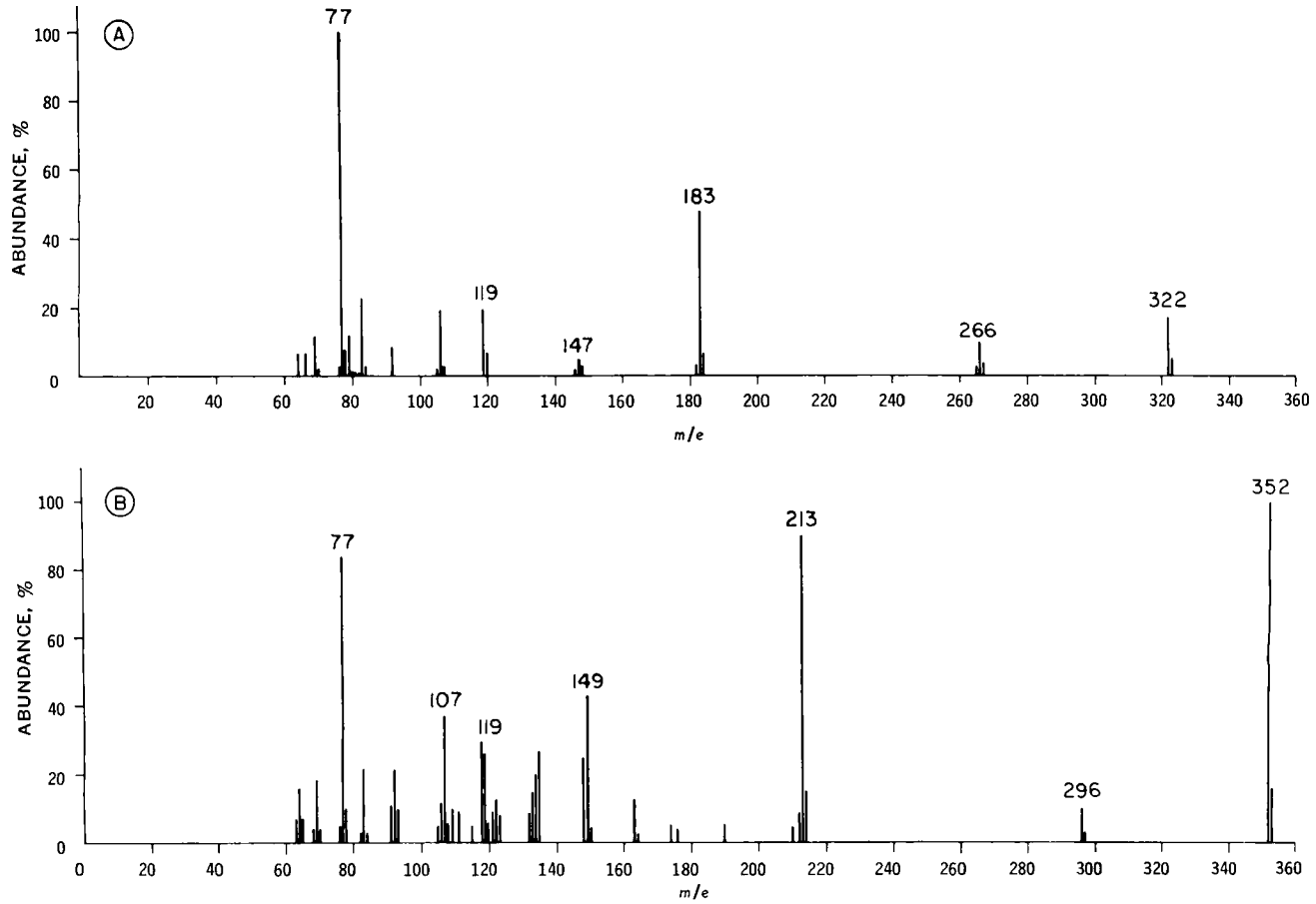


Figure 2—*GLC–mass spectra of major peaks of flash-methylated phenylbutazone (A) and oxyphenbutazone (B).*

ature, instrument, and sample size; therefore, the major peak that elutes first was used for quantitation. Standard I gave a single peak at 4.0 min upon flash methylation (III, Fig. 1B). To establish the structures of the compounds giving rise to these two peaks from trimethylanilinium hydroxide treatment of phenylbutazone, eluates from the GLC column were fed directly into the mass spectrometer[13] and the mass spectra of IV and V were recorded. The mass spectrum of each eluate showed a molecular ion peak at *m/e* 322. Abundant ions of the major component retention time (7.1 min, IV, Fig. 1B) were located at *m/e* 77, 119, 147, 183, and 266 (Fig. 2A). Structures I–V have been postulated for these ions (Scheme IA). These fragmentations suggest that the major derivative of flash-methylated phenylbutazone has the structure 1,2-diphenyl-3-methoxy-4-*n*-butyl-5-oxopyrazoline.

Flash-heater methylation of oxyphenbutazone with trimethylanilinium hydroxide gave two peaks with retention times of 9.3 min for the major peak and 18.4 min for the minor peak (VI and IX, Fig. 3C). As with phenylbutazone, both were formed in the same height ratio and this ratio was also independent of oven temperature, instrument, and sample size. GLC–mass spectroscopy of these two derivatives of oxyphenbutazone was employed. The mass spectrum of the derivative giving rise to the major peak (retention time 9.3 min) showed the molecular ion at *m/e* 352 (VII, Scheme IB) and that giving rise to the minor peak retention time (18.4 min) showed the molecular ion at *m/e* 338. The mass spectrum of the major product (Fig. 2B) had abundant ions at *m/e* 77, 107, 119, 149, 213, and 296, for which Structures I–VI (Scheme IB) have been postulated. The major product of flash-methylated oxyphenbutazone was tentatively assigned the structure 1-phenyl-2-(*p*-methoxyphenyl)-3-methoxy-4-*n*-butyl-5-oxopyrazoline and was used for quantitation.

Flash-heater methylation of Standard I with trimethylanilinium hydroxide consistently gave a sharp peak on column I under the described experimental conditions. Flash methylation of 5-(4-hydroxyphenyl)-5-phenylhydantoin (Standard II) was described previously (15). The combined GLC–mass spectral evidence obtained in the present study was consistent with a trimethylated structure for Standard II (the molecular ion being at *m/e* 310). The use of trimethylanilinium hydroxide as a methylating reagent offers the advantage that purified plasma extracts at high temperatures do not need to be completely moisture free—a factor that saves time. The reagent is stable over a long period and is easily prepared. The methylation reaction is instantaneous in the injection port at a temperature of 300° (14). One important precaution is that care must be taken to avoid transfer of trace amounts of acid during the extraction carried out at acidic pH since methylation with trimethylanilinium hydroxide does not occur under acidic conditions.

Figure 1A shows a typical chromatogram obtained by processing fresh blank plasma as described in the *Experimental* section for phenylbutazone but omitting the external standard (Standard I). The extraneous peaks (I and II) at retention times 1.6 and 2.8 min were observed in chromatograms of all human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 16 μg/ml of phenylbutazone is shown in Fig. 1B, where it is clear that the extraneous peaks I and II do not interfere with the peaks from Standard I (peak III, retention time 4.0 min) or phenylbutazone (peak IV, retention time 7.1 min). Figure 1C shows a chromatogram from a 1-ml plasma sample from a volunteer who received 400 mg of phenylbutazone. The time required for one analysis is 18 min. Oxyphenbutazone and hydroxyphenylbutazone do not interfere with the assay because they are extracted with *n*-heptane only to the extent of 7% and have different retention times. The major and minor peaks of oxyphenbutazone on column I have retention times of 15.2 and 31.0 min, respectively. Hydroxyphenylbutazone[14], under similar condi-

[13] Hitachi Perkin-Elmer model RMS4 mass spectrometer with Perkin-Elmer gas chromatograph model 990.

[14] Reference specimen was provided by Dr. A. Sallman, Ciba-Geigy, Switzerland.

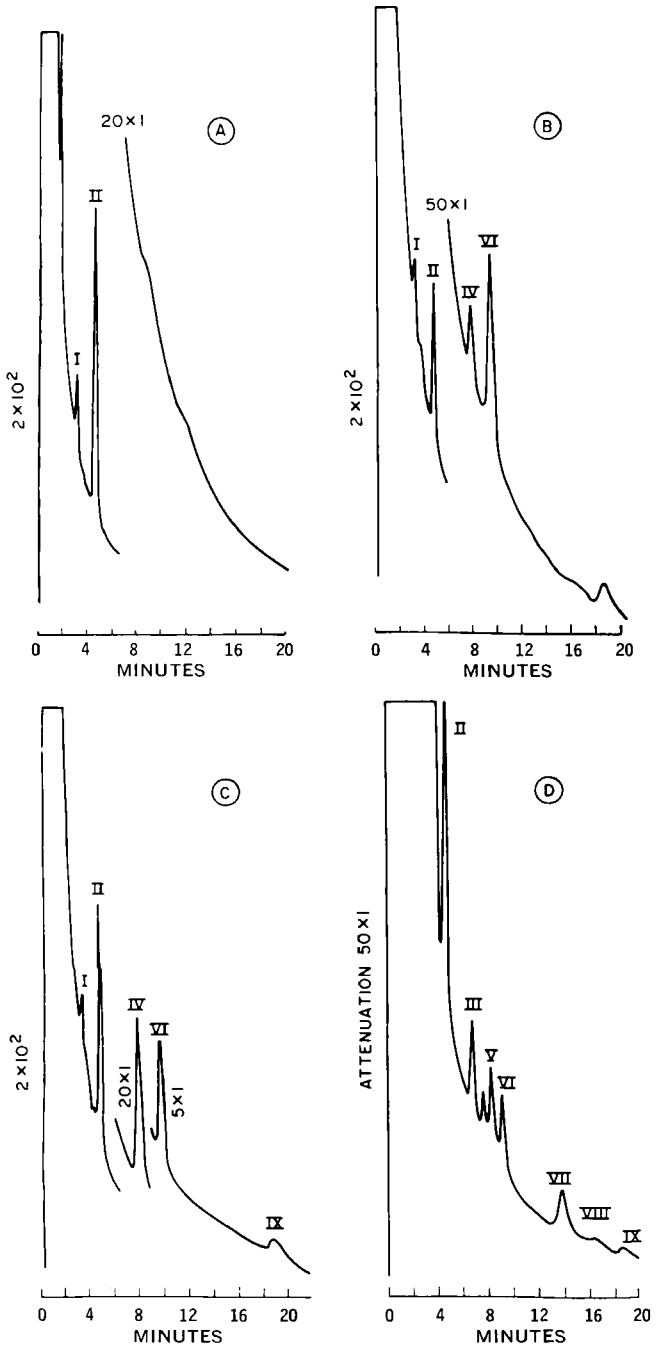
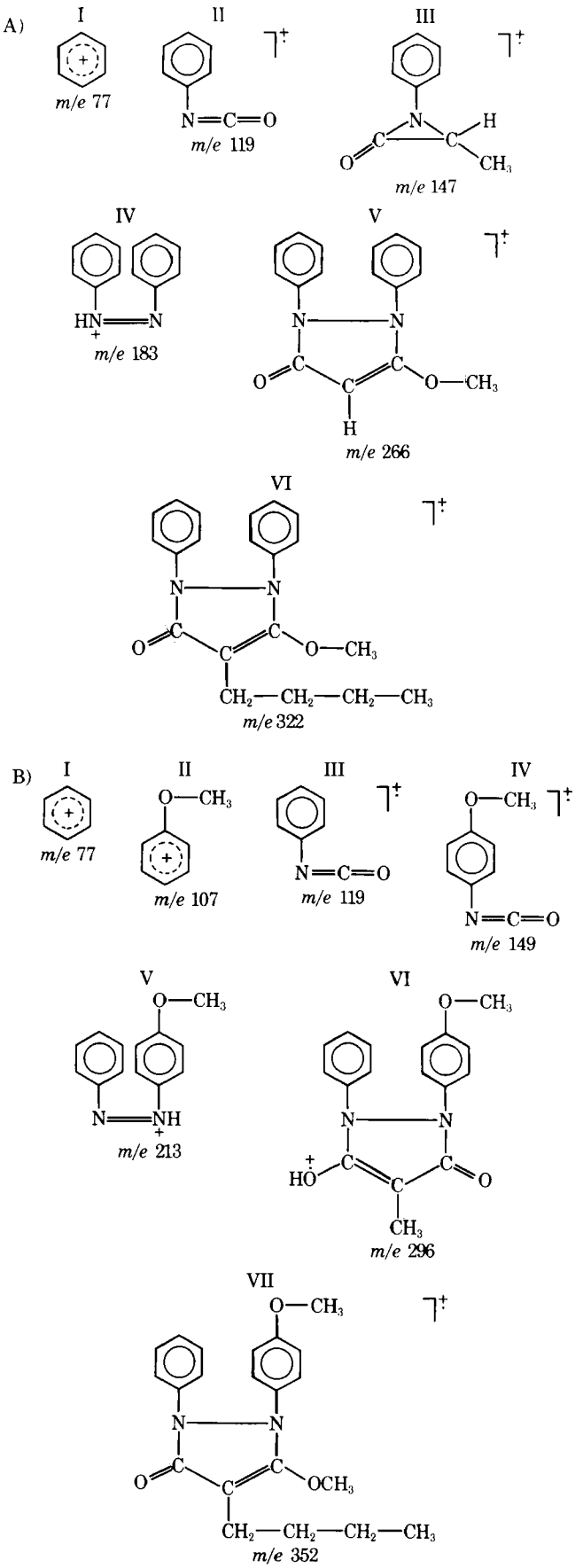


Figure 3—*GLC of human plasma. Key: A, control plasma; B, plasma containing 14.75 μg/ml of oxyphenbutazone and 1.11 μg/ml of Standard II; C, plasma from a human volunteer who had been given 200 mg (2 × 100-mg tablets) of phenylbutazone; D, plasma containing 9.44 μg/ml of oxyphenbutazone, 15.56 μg/ml of hydroxyphenylbutazone, and 1.11 μg/ml of Standard II; peaks I and II, endogenous material; peaks III, V, VII, and VIII, peaks due to flash-methylated hydroxyphenylbutazone; peak IV, Standard II; and peaks VI and IX, peaks due to flash-methylated oxyphenbutazone.*

tions, gives peaks with retention times of 10.5 and 14.2 min and thus does not interfere.

The response of the flame-ionization detector to phenylbutazone was linear with concentration in the 1.0–64.0-μg/ml range. The peak height ratio of the drug and external standard was used as the index of detector performance and overall efficiency of the analytical procedure. The overall recovery of phenylbutazone from plasma was more than 98%, a figure comparable to that reported earlier (8, 9). Figure 4 represents the calibration curve ob-



Scheme I—Postulated fragmentation of phenylbutazone (A) and oxyphenbutazone (B)

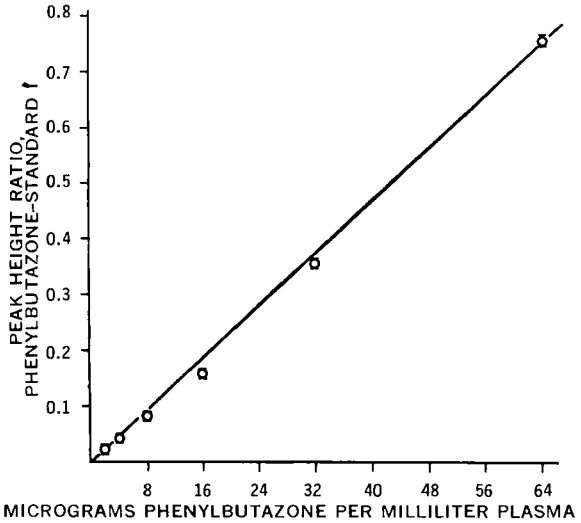


Figure 4—*Calibration curve of phenylbutazone extracted from human plasma. Each point is a mean of at least four determinations, except for 2 μg where a mean of eight determinations was taken.*

tained by plotting the peak height ratio of phenylbutazone/Standard I *versus* the concentration of phenylbutazone. The plot is a straight line ($y = mx$) over the range of 2.0–64.0 μg phenylbutazone/ml plasma. A mean slope value of 0.0117 ± 0.0003 ($r^2 = 1$) was obtained. The overall coefficient of variation was 4.65%.

A typical chromatogram obtained by processing fresh blank plasma as described in the *Experimental* section for oxyphenbutazone but omitting the internal standard (Standard II) is shown in Fig. 3A. The extraneous peaks (I and II) at retention times 3.2 and 4.6 min were observed in chromatograms of plasma from all human subjects (total of eight) including the pooled plasma from the Red Cross blood bank. The extraneous peaks (I and II) do not interfere with peaks due to Standard II (IV, retention time 7.7 min) and oxyphenbutazone (VI, retention time 9.3 min), as shown in the chromatogram for spiked plasma (Fig. 3B) containing 14.75 μg/ml of oxyphenbutazone. The time required for one analysis is 20 min. Phenylbutazone does not interfere because it is removed by partitioning into *n*-heptane. This partition of phenylbutazone into *n*-heptane was checked by preparing plasma samples containing various concentrations of phenylbutazone (up to 60 μg/ml) and treating each sample as described for the oxyphenbutazone extraction. No phenylbutazone was detected in the final ether extract.

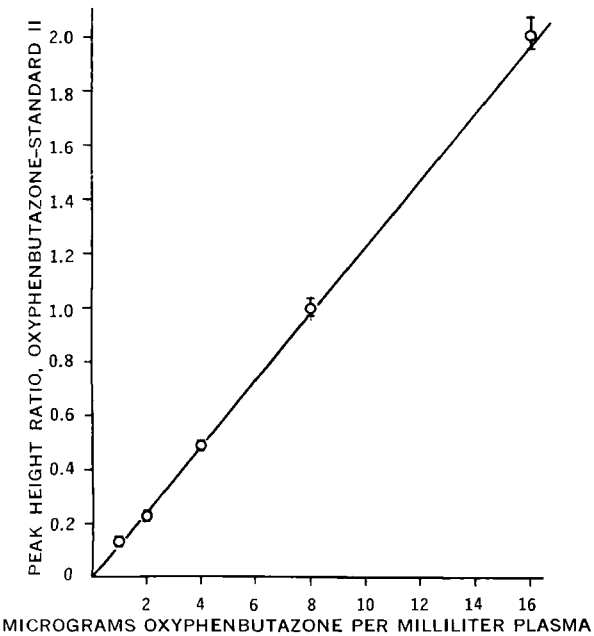


Figure 5—*Calibration curve of oxyphenbutazone extracted from human plasma. Each point is a mean of at least five determinations.*

Table I—Recovery of Oxyphenbutazone and Standard II from Plasma Determined by GLC Assay

Micrograms Added to 1 ml Plasma	*n*	Mean Micrograms Recovered	Mean Recovery, %	*SD* of Recovery, %
Recovery of Standard II				
1.11	5	0.77	69.72	2.37
Recovery of Oxyphenbutazone				
4.00	6	0.98	24.43	0.74
8.00	6	1.98	24.75	0.23
Mean 24.59 ± 0.55%				

There was no interference from hydroxyphenylbutazone because flash methylation of this metabolite gives peaks at 6.8, 8.4, 13.7, and 16.0 min—well separated from the methylated oxyphenbutazone (Fig. 3D). Figure 3C shows the chromatogram of a sample (72 hr) from a human subject who received 200 mg of phenylbutazone. The response of the flame-ionization detector to oxyphenbutazone was linear with concentration in the 1.0–16.0-μg/ml range. The peak height ratio of oxyphenbutazone and the internal standard (Standard II) was used as the index of detector performance for the second gas chromatograph and overall efficiency of the analytical procedure. The overall recovery of 4.0 and 8.0 μg of oxyphenbutazone and 1.11 μg of Standard II from plasma was of the order of 24.59 ± 0.55 and 69.72 ± 2.37%, respectively (Table I). The recovery of oxyphenbutazone in this procedure was low but was consistent.

Several attempts were made to improve the recovery of oxyphenbutazone by employing different solvents, such as dichloromethane and dichloroethane. There were large variations in the recovery, which were attributed to the breakdown of oxyphenbutazone and/or carryover of trace amounts of acid resulting in the inhibition of flash methylation. Ethylene dichloride, employed by Burns *et al.* (1), was also tested. No improvement in recovery of oxyphenbutazone was obtained, while the procedure became more difficult due to transference of ethylene dichloride (specific gravity 1.25). Figure 5 represents the calibration curve obtained by plotting the peak height ratio of oxyphenbutazone/Standard II *versus* the concentration of oxyphenbutazone. The plot is a straight line ($y = mx$) over the concentration range of 1.0–16.0 μg oxyphenbutazone/ml plasma. A mean slope value of 0.126 ± 0.002 ($r^2 = 1$) was obtained. Following single doses of up to 400 mg phenylbutazone, hydroxyphenylbutazone could not be detected. Calibration curves for oxyphenbutazone were also tested in the presence of various amounts of phenylbutazone and no change in slope was detected.

Refrigerated plasma samples containing phenylbutazone have been reported to be stable for several weeks (8, 9). It was found that refrigerated plasma samples containing oxyphenbutazone were also stable for several weeks. However, evaporated extracts

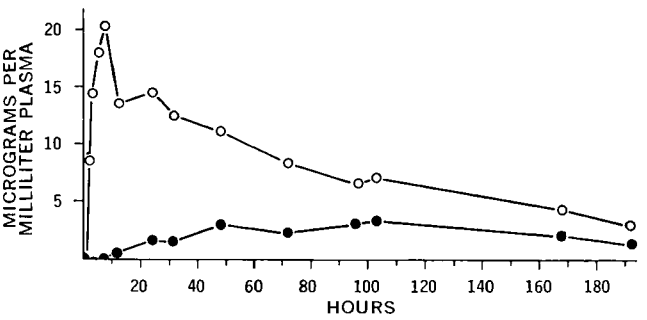


Figure 6—*Phenylbutazone and oxyphenbutazone concentrations in a human volunteer following single oral dose of 200 mg (2 × 100-mg tablets) phenylbutazone. Key: ○, phenylbutazone; and ●, oxyphenbutazone.*

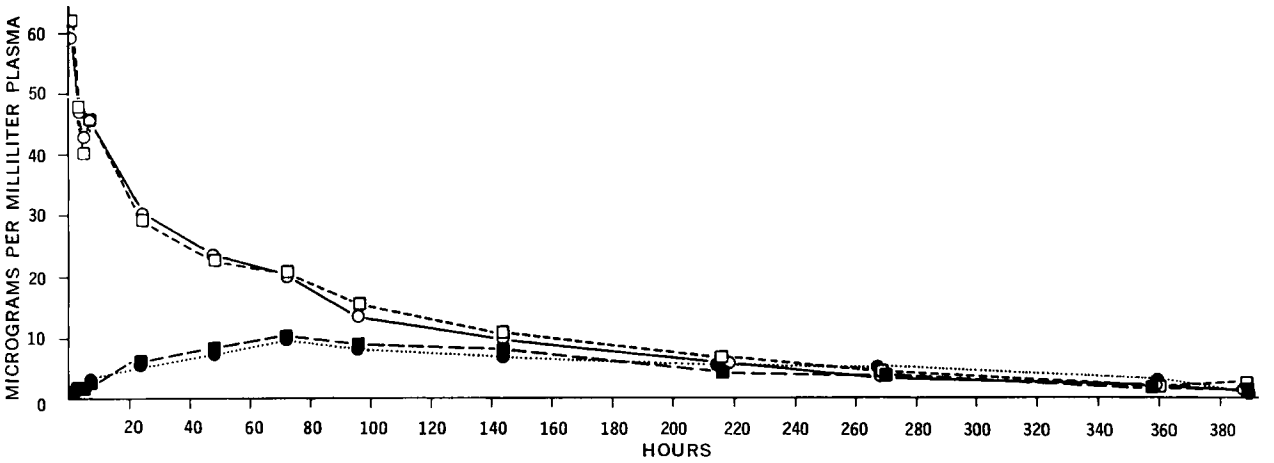


Figure 7—*Comparison of plasma levels determined by GLC method with those of a HSLC method following a single oral dose of 400-mg solution of phenylbutazone to a volunteer. Key:* ○, *phenylbutazone, GLC method;* ●, *oxyphenbutazone, GLC method;* □, *phenylbutazone, HSLC method; and* ■, *oxyphenbutazone, HSLC method.*

to which trimethylanilinium hydroxide had been added were unstable over 24 hr. The tubes containing evaporated extracts, when stored overnight in a freezer or in dry ice–acetone, showed lower amounts (4.7–5%) of oxyphenbutazone. Thus, extraction and GLC analysis of oxyphenbutazone should be completed on the same day.

Application of the method of plasma level determination of phenylbutazone and oxyphenbutazone by GLC is shown in Figs. 6 and 7, where it is compared with a recent HSLC procedure[15]. A 200-mg dose of phenylbutazone (2 × 100-mg tablets) was given to a healthy male volunteer [95.3 kg (210 lb)], and plasma was withdrawn at intervals over 192 hr and assayed for phenylbutazone and oxyphenbutazone using the GLC method (Fig. 6). A second male volunteer [90.8 kg (200 lb)] was given a 400-mg dose of phenylbutazone in solution, and plasma levels of phenylbutazone and oxyphenbutazone over 388 hr were compared by the GLC method and a recent HSLC procedure (Fig. 7). The methods compare favorably, with an overall 8.06% difference for phenylbutazone and 7.68% difference for oxyphenbutazone between the two methods.

The described GLC method is sensitive for the determination of phenylbutazone as well as oxyphenbutazone. It offers the advantage that determinations of the drug as well as its metabolite are made by extraction of the same plasma sample. It is a specific method, because the second metabolite hydroxyphenylbutazone is distinguished clearly; therefore, it can be employed for single- as well as multiple-dose pharmacokinetic studies.

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[x] To whom inquiries should be directed.