protonated and neutral substrates. The following rate law may be written:

$$k_{obs} = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-] + k_{HA}[HA] + k_A[A^-])\, f_{BH^+} \quad \text{(Eq. 9)}$$

where f_{BH^+} and f_B represent the fractions of protonated and neutral chlordiazepoxide at a given hydrogen-ion concentration; pKa = 4.6–4.8 measured in 50% polyethylene glycol 300 at 80°.

The term $k_{OH^-}[OH^-]f_{BH^+}$, Eqs. 4 and 9, may be expressed as the kinetically equivalent $k_{H_2O}f_B$, as pointed out previously (8, 9).

Nonlinear dependency of the rate constant with buffer concentration in the 1.7–4.5 pH range was seen, which is likely the result of a change in the rate-limiting step with an increase of buffer in solution (11–14).

An extraction procedure was developed as a comparative method for the quantitative TLC process. The technique consisted of extraction of the decomposition products into methylene chloride from acidified chlordiazepoxide followed by UV analysis of the acidic aqueous layer.

The quantitative TLC method worked well for chlordiazepoxide, with initially discovered discrepancies being the consequence of a lack of control of the buffer concentration and occasional precipitation of the substance from solution upon cooling during the streaking procedure. When these factors were controlled, results were obtained in accord with those from the extraction procedure.

The work presented here corroborates applicability of the TLC technique to molecules that do not lend themselves to kinetic analysis by standard differential UV techniques. Thus, the delays occasionally encountered in assay development may be circumvented. Although this procedure is not the ultimate in simplicity, it affords ready availability of stability results to the formulator as well as to the kineticist.

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Simultaneous Determination of Phenylbutazone and Oxyphenbutazone in Plasma by High-Speed Liquid Chromatography

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Abstract □ A sensitive, specific, high-speed liquid chromatographic procedure is described for the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone, in plasma. Acidified plasma is partitioned with cyclohexane–ether (1:1) containing the 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde as an internal standard. The organic extract is reduced to dryness, the resulting residue is redissolved in chloroform, and aliquots of this solution are chromatographed on an adsorption column, using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at 35°. Use of a UV detector permits quantitative analysis of samples containing less than 0.25 μg/ml of phenylbutazone or oxyphenbutazone.

Keyphrases □ Phenylbutazone–oxyphenbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Oxyphenbutazone–phenylbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Liquid chromatography, high speed—simultaneous determination in plasma of phenylbutazone and oxyphenbutazone

Several spectrophotometric procedures (1–6) and a GLC method (7) have been reported for the determination of phenylbutazone in biological fluids; however, fewer methods have been described for the estimation of two known metabolites (1) of this drug, oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine].

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 and specific method for estimating phenylbutazone in plasma has been met by recently reported high-speed liquid chromatographic (HSLC) (8) and GLC procedures (9), the development of HSLC and GLC methods for measuring oxyphenbutazone in plasma, following single doses of phenylbutazone, was undertaken for pharmacokinetic studies under investigation in this laboratory (10).

The HSLC procedure described compares favorably with the recently reported GLC method (11). It possesses adequate sensitivity for single-dose studies, is somewhat less complex than the GLC procedure, and permits the simultaneous determination of both phenylbutazone and oxyphenbutazone from a single plasma extract.

EXPERIMENTAL

Materials—Complimentary samples of phenylbutazone[1], oxyphenbutazone[2], and hydroxyphenylbutazone[2] were used as received. Tetrahydrofuran[3], freshly distilled and dried over molecular sieves[3], and spectrograde *n*-hexane[4] were used as solvents. The 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde, used as the internal standard, was prepared by the reported procedure (12), mp 264–266° [lit. (12) mp 264°].

Chromatographic Procedure—Details of the modified constant-pressure high-speed liquid chromatograph[5] used in this study were previously reported (13). The chromatograph was equipped with a stop-flow injection port[5], a constant-temperature water bath[6], and a fixed-wavelength (254 nm) UV absorbance detector[5].

The adsorption column[7] (100 cm × 1.8 mm i.d., 304 stainless steel) was dry packed by a procedure similar to the one described by Kirkland (14), then coiled (radius 15 cm), and fitted horizontally into the water bath of the instrument. Analyses were performed using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at a flow rate of 60 ml/hr (475 psi) and a temperature of 35°. The mobile phase was "degassed" by applying vacuum (about 100 mm) to the solvent reservoir for approximately 1 min before use. Extraction residues were redissolved in chloroform, and 10-μl aliquots of this solution were injected directly on-column with a 25-μl syringe[8] using a stop-flow injection technique.

Preparation of Standard Solutions—Standard solutions of phenylbutazone and oxyphenbutazone were prepared by dissolving a sample of the drug substance, accurately weighed, in 95% ethanol (1 ml) in a 100-ml volumetric flask and then bringing the solution to volume with phosphate buffer (pH 7.2). Aliquots of each solution were mixed and further diluted with water to produce a final solution containing the desired concentration of phenylbutazone and oxyphenbutazone.

Spiked plasma solutions were prepared by transferring an aliquot (1.0 ml) of the aqueous phenylbutazone–oxyphenbutazone solution to a 5-ml volumetric flask, bringing the solution to volume with citrated human plasma, and mixing thoroughly.

Analytical Procedure—An aliquot (1.0 ml) of the plasma sample was transferred to a 10-ml screw-capped centrifuge tube, 1 *M* hydrochloric acid (1 ml) and a solution of the internal standard (15 – 75 μg/ml, accurately weighed) in cyclohexane–ether (1:1) (6.0 ml) were added, and the tube was tumbled[9] for 15 min at 30 rpm. The tube was then centrifuged for 2–3 min at 3000 rpm, and the upper organic layer was transferred, with a Pasteur pipet, to a 15-ml conical screw-capped centrifuge tube.

The solvent was evaporated under nitrogen in a constant-temperature (60°) water bath. Chloroform (1 ml), which was used to rinse the inner walls of the tubes, was also evaporated. Aliquots (10 μl) of the solution, prepared by promptly redissolving the resulting residue in 50–200 μl of chloroform to give an anticipated drug concentration of 35–200 ng/μl, were chromatographed.

Peak height ratios were calculated by dividing the peak height of phenylbutazone or oxyphenbutazone by the peak height of the internal standard. The amount of each drug substance was then calculated from the respective calibration curve.

[1] Mount Royal Chemicals, Montreal, Quebec, Canada.
[2] Ciba-Geigy Pharmaceuticals, Canada.
[3] Fisher Scientific, Montreal, Quebec, Canada.
[4] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[5] Model 4000, Varian Aerograph, Palo Alto, Calif.
[6] Model 3084, Labline Industries, Melrose Park, Ill.
[7] Sil-X adsorbent, Perkin-Elmer Corp., Norwalk, Conn.
[8] Model 702N, Hamilton Co., Reno, Nev.
[9] Multi-Rotator, Scientific Industries Ltd., Springfield, Mass.

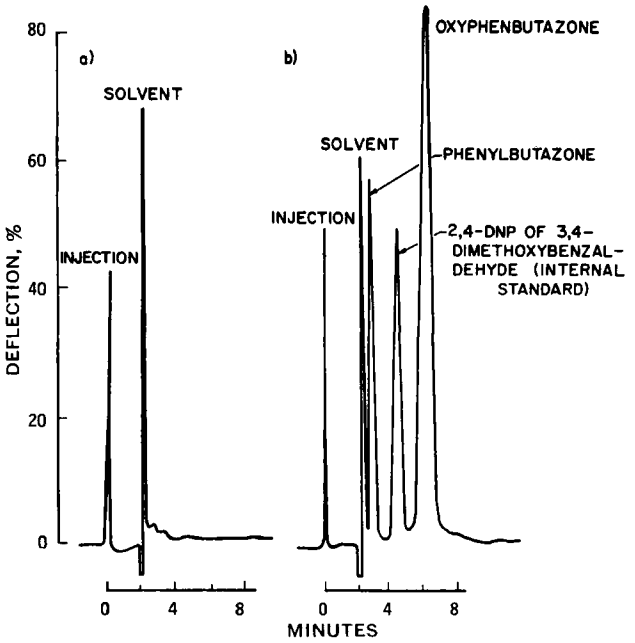

Figure 1—*High-speed chromatograms of human plasma extracts. Key: a, control plasma; and b, plasma containing 2.0 μg/ml phenylbutazone, 2.1 μg/ml internal standard, and 7.7 μg/ml oxyphenbutazone. The conditions were: 1 m × 1.8 mm i.d. column using a mobile phase of 0.002% acetic acid, 23.0% tetrahydrofuran in* n*-hexane at a flow rate of 60 ml/hr at 35°, and a detector attenuation of 0.04 absorbance unit full scale.*

RESULTS AND DISCUSSION

Figure 1*b* illustrates the chromatogram obtained from the analysis of a plasma sample containing 2.0 μg/ml of phenylbutazone, 2.1 μg/ml of internal standard, and 7.7 μg/ml of oxyphenbutazone, using the described chromatographic system. This figure repre-

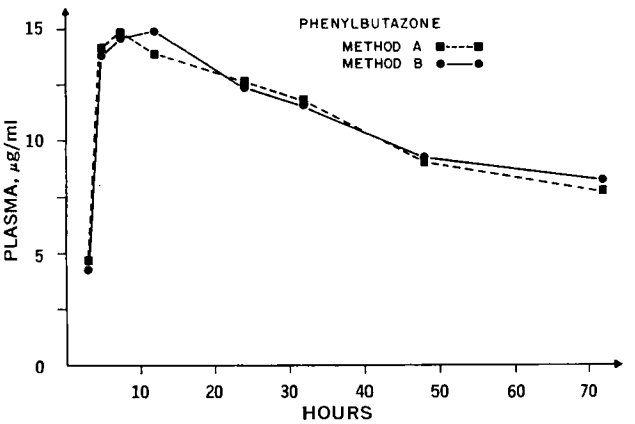

Figure 2—*Phenylbutazone concentration in plasma of human volunteer following single oral dose of 2 × 100-mg phenylbutazone tablets.*

Table I—Analysis of Spiked Phenylbutazone–Oxyphenbutazone Plasma Samples[a]

Sample	Phenylbutazone, μg/ml			Oxyphenbutazone, μg/ml		
	Calc.	Found	Recovery, %	Calc.	Found	Recovery, %
1	6.53	6.49	99.39	6.46	6.38	98.76
2	4.13	4.11	99.52	4.30	4.31	100.23
3	8.49	8.61	101.41	8.88	8.99	101.24
4	7.78	7.61	97.81	7.74	7.57	97.80
5	1.58	1.57	99.37	1.86	1.84	98.92
6	0.23	0.22	99.13	0.31	0.31	98.73
Mean			99.44			99.28
RSD, %			±1.17			±1.26

[a] Average analysis of duplicate aliquots.

sents an on-column injection of 0.4 μg of phenylbutazone and 1.5 μg of oxyphenbutazone. Analysis is achieved in less than 8 min. Although a small amount of interference does occur with the phenylbutazone peak, no compounds that interfere with oxyphenbutazone are extracted from plasma (Fig. 1*a*). The chromatogram shown in Fig. 1*a* is typical of the blank obtained from plasma samples from six volunteers.

Adsorption chromatography was chosen because it offers the advantage of rapid analyses and excellent column stability. A single column was used throughout this study. A simple solvent system of *n*-hexane modified with 23.0% tetrahydrofuran was used as a mobile phase. The addition of a small amount (0.002%) of acetic acid to the mobile phase reduced the slight tendency of the peaks to tail. Operation at 35° reduced column back pressure (475 psi) and improved column efficiency.

Although hydroxyphenylbutazone, the other known metabolite of phenylbutazone (1), is not completely resolved from oxyphenbutazone, this compound could be detected readily if it were present in a sample. Under these chromatographic conditions, retention times of 2.73, 4.33, 5.36, and 6.12 min are obtained for phenylbutazone, the internal standard, hydroxyphenylbutazone, and oxyphenbutazone, respectively. No hydroxyphenylbutazone has been detected in any of the single-dose studies performed in this laboratory (10).

Analyses were performed as described. To avoid degradation of the sample extracts (8), the residues were promptly redissolved in chloroform (50–200 μl) and chromatographed. No decomposition of the drug substances was detected. Chloroform was chosen as the solvent for redissolving the sample residues since the internal standard, phenylbutazone, and oxyphenbutazone are all readily soluble in this solvent, the injection of 10 μl of chloroform does not affect the resolution of the separation, and the vapor pressure of chloroform is high enough to prevent excessive loss due to evaporation between injections.

Quantitation was performed by peak height measurement to avoid the problems associated with the integration of the small broad peaks frequently encountered in HSLC (8). Previous work in this laboratory (8, 15) has indicated that peak height data compare favorably with those obtained by digital integration.

The 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde was used as an internal standard. It was added directly with the extracting solvent before partitioning the samples, thereby reducing errors caused during transfer and evaporation procedures. The use of 2,4-dinitrophenylhydrazine derivatives as internal standards shows particular promise due to their ease of synthesis, stability, and intense UV absorbance at 254 nm. Judicious selection of the ketone or aldehyde reagent makes it possible to prepare internal standards with the required retention time for a particular separation.

Comparable values for linearity and sample recoveries (>98%) were obtained for both phenylbutazone and oxyphenbutazone from spiked solutions prepared with either plasma or water. The use of water instead of plasma facilitated the preparation of synthetic solutions used to prepare and check the calibration curves. Calibration curves for phenylbutazone and oxyphenbutazone were obtained by plotting the response *versus* weight ratio of drug–internal standard for a series of synthetic aqueous mixtures of both drugs. Both plots were straight lines ($y = mx$) over the concentration range of from 0.230 to 37.64 μg/ml for phenylbutazone or from 0.315 to 43.37 μg/ml for oxyphenbutazone. Mean slope values of 1.146 and 0.460 were obtained for phenylbutazone and oxyphenbutazone, respectively. These curves were checked daily, and the difference between the calculated and found response ratios of the standard solutions was never more than 2%.

The precision of this procedure is reflected by the data obtained from the analysis of six aliquots of each of two spiked plasma samples. Recovery values of 0.228 ± 0.004 and 0.311 ± 0.004 μg/ml were obtained from the analysis of samples containing 0.230 and 0.315 μg/ml of phenylbutazone and oxyphenbutazone, respectively. Similarly, values of 7.61 ± 0.12 and 7.57 ± 0.09 μg/ml were obtained for samples containing 7.78 μg/ml of phenylbutazone and 7.61 μg/ml of oxyphenbutazone. These results were obtained using a detector attenuation of 0.04 absorbance unit full scale (aufs). At maximum detector sensitivity (0.005 aufs), the on-column injection of approximately 15 ng (0.075 μg/ml of plasma) of phenylbutazone would produce a readily detectable (10% of full scale) recorder response. The minimum detectable amount of oxyphenbutazone would be somewhat higher (20 ng/injection).

The results from the analysis of six spiked plasma samples reflect the accuracy of the procedure (Table I). Mean recovery values of 99.44 ± 1.17 and 99.28 ± 1.26% were obtained for phenylbutazone and oxyphenbutazone, respectively. All six plasma blanks used contained a small, relatively constant amount of extraneous material which produced some interference with the phenylbutazone peak. The chromatogram shown in Fig. 1*a* is typical. The phenylbutazone data shown in Table I were corrected by subtracting the value of the plasma blank from the height of the phenylbutazone peak before calculations were performed.

The accuracy and precision of this method are further substantiated by the results presented in Figs. 2 and 3. Figure 2 illustrates the 72-hr plasma phenylbutazone profiles of a human subject [male, 90.8 kg (200 lb)] who had been administered 2 × 100-mg tablets of phenylbutazone. Samples of blood (10 ml) were withdrawn from the cubital vein by means of heparinized containers[10]. Blood samples were centrifuged, and the plasma was transferred and stored at −10°. Samples were stable for at least 2 weeks under these conditions (8, 9). The profiles shown in Fig. 2 were obtained using the procedure described in this article (Method B), which permits the simultaneous determination of both phenylbutazone and oxyphenbutazone, and a previously reported HSLC method (Method A) (8), which provided only for the determination of phenylbutazone. The two profiles compare favorably.

Phenylbutazone and oxyphenbutazone plasma levels of a male volunteer [90.8 kg (200 lb)] who had been given a 400-mg dose of phenylbutazone in solution were also monitored over 388 hr. Profiles obtained by both this HSLC procedure and a recently developed GLC (11) method are shown in Fig. 3. The two techniques compare favorably. Overall differences between the two procedures were 8.1 and 7.7% for phenylbutazone and oxyphenbutazone, respectively.

In conclusion, the HSLC procedure described here provides a rapid, sensitive, and precise method for the simultaneous determination of plasma levels of phenylbutazone and oxyphenbutazone. It offers the added advantage that since the second metabolite, hydroxyphenylbutazone, can be clearly distinguished, it can be em-

[10] Vacutainers, Beckton Dickinson & Co., Mississauga, Ontario, Canada.

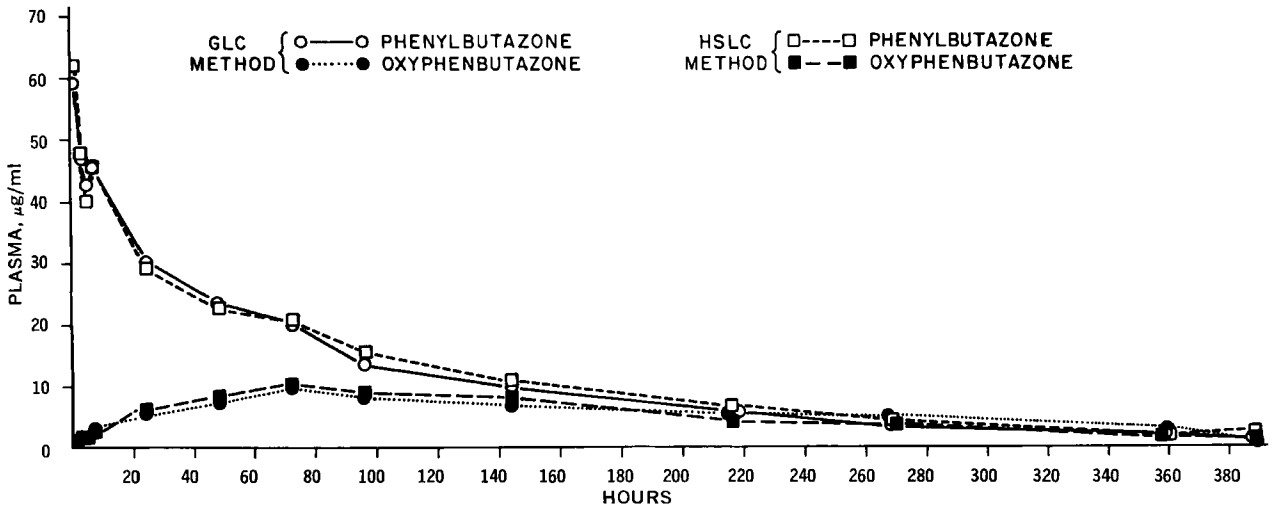

Figure 3—*Comparison of plasma levels determined by HSLC and GLC procedures (11) following a single oral dose of 400-mg solution of phenylbutazone to a volunteer.*

ployed for the analysis of phenylbutazone and oxyphenbutazone in multiple-dose as well as single-dose pharmacokinetic studies.

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