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Fully Automated Analysis of Phenylbutazone in Plasma and Urine

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Abstract □ A rapid, automated method for the determination of phenylbutazone in plasma and urine was developed. The method offers distinct advantages over earlier procedures and is particularly suitable for large-scale bioavailability studies.

Keyphrases □ Phenylbutazone—analysis, automated method, human plasma and urine □ Automated methods—analysis of phenylbutazone in human plasma and urine □ Anti-inflammatory agents—phenylbutazone, analysis, automated method, human plasma and urine

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) has been used extensively during the past 2 decades in the treatment of rheumatoid and other inflammatory conditions. Several methods have been developed for the analysis of the drug in body fluids in conjunction with investigations on its biological disposition.

The plasma levels of phenylbutazone were measured spectrophotometrically following its extraction (1), and several modifications were introduced (2, 3) to reduce the interference caused by oxyphenbutazone, the principal metabolite. The procedure also was modified for small samples (4). Another method based on hydrolysis of phenylbutazone to hydrazobenzene, with subsequent rearrangement to benzidine, was developed (5) and modified (6, 7). Also, phenylbutazone was oxidized to azobenzene, and the UV absorbance of the latter compound was measured (8, 9). Several workers (10–15) used GC for the assay of the drug in biological fluids or solid dosage forms. A method using liquid chromatography was reported recently (16).

Although most of these methods have merit in terms of specificity and/or sensitivity, they are rather time consuming. Ahuja *et al.* (17) developed an automated method capable of assaying solid dosage forms at the rate of 13 samples/hr. Unfortunately, their method lacks the sensitivity required for the analysis of phenylbutazone in biological fluids.

Analysis of plasma levels in conjunction with large-scale bioavailability assessments of phenylbutazone dosage forms after a therapeutic dose in humans requires speed, sensitivity, and specificity. The automated method presented here satisfies these criteria.

Table I—Standard Curve of Phenylbutazone from Aqueous Solutions[a]

Concentration, μg/ml	Peak Height[a]
0	0.0 ± 0.0
0.5	0.3 ± 0.1
1	0.6 ± 0.1
2	1.4 ± 0.2
3	2.5 ± 0.1
4	3.3 ± 0.2
5	4.1 ± 0.1
7	5.6 ± 0.3
10	8.0 ± 0.2
20	15.5 ± 0.4
30	25.4 ± 0.6
40	32.2 ± 0.5
50	38.5 ± 1.5
60	48.0 ± 2.7

[a] Each value represents the average ± *SD* of five determinations (arbitrary units).

EXPERIMENTAL

Reagents and Standards—All chemicals except heptane (chromatoquality) were reagent grade or of equivalent purity. The following were used: aminoacetic acid–hydrochloric acid buffer (pH 1.2), prepared by dissolving 25 g of aminoacetic acid and 50 ml of 6 *N* HCl in a final volume of 1000 ml of water; heptane–isopentyl alcohol (98.5:1.5); and 2.5 *N* NaOH.

The following standard solutions, preferably prepared daily, were used: (*a*) phenylbutazone, 1 or 10 mg/ml in 1.0 *N* NaOH; and (*b*) phenylbutazone, 2–80 μg/ml in plasma[1].

Procedure[2]—The flow diagram for the automated manifold is depicted in Fig. 1. At the beginning of the day's analyses, the re-

[1] Plasma was purchased from the American Red Cross and was mixed with varying amounts of phenylbutazone.

[2] The following equipment was used: Technicon sampler IV, Technicon proportioning pump III, Technicon continuous filter with hydrophobic filter paper (Product No. 518-3041), Beckman model DB-G grating spectrophotometer equipped with a 6-mm rectangular flowcell, and Technicon single-pen linearized recorder with linear-scale chart paper.

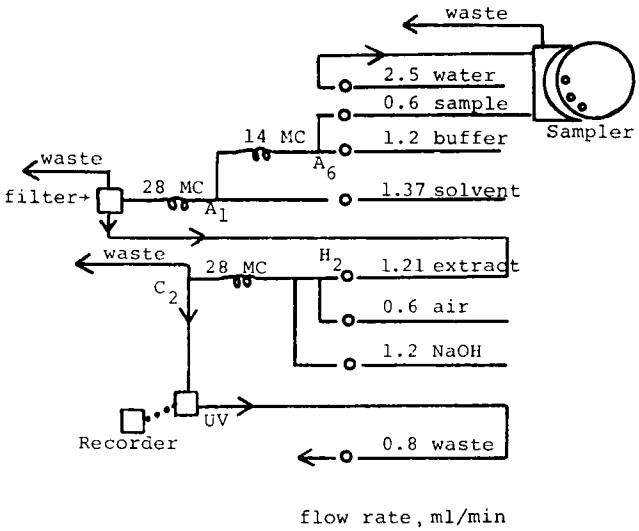

Figure 1—*Flow diagram of the automated analytical procedure for phenylbutazone. Key: 14 MC = 14-turn mixing coil; 28 MC = 28-turn mixing coil; and A_1, A_6, H_2, and C_2 = separators.*

agents were pumped through the system for at least 15 min and a reagent blank baseline was established.

Plasma samples containing phenylbutazone, normally in the range of 0–50 μg/ml, were analyzed, yielding the first standard curve of the day. The samples were aspirated with a proportioning pump from 2-ml plastic sample cups at the rate of 20/hr. Consecutive samples were separated by an aqueous wash, using a sample–wash ratio of 1:2. Under these conditions and with a sample flow rate of 0.6 ml/min, each assay required 0.6 ml of sample.

During these preliminary steps, the heparinized, frozen plasma samples containing unknown concentrations of phenylbutazone were allowed to stand at room temperature until thawing occurred. Particulate matter was removed by brief centrifugation, and the supernatant fluids were transferred to sample cups. The samples were aspirated into the flow system and analyzed at the rate of 20/hr. A detailed description of the method follows.

Samples and the pH 1.2 buffer were mixed in a 14-turn mixing coil. Subsequently, the buffered plasma and heptane–isopentyl alcohol (98.5:1.5) were mixed in a 28-turn mixing coil, where the extraction of phenylbutazone occurred. The stream of the mixed aqueous and organic phases was separated by being fed to the top side of a 2.5-cm (1-in.) wide strip of hydrophobic filter paper. Since this paper was moving at a rate of 2.5 cm (1 in.)/min, it was always providing a fresh surface for the separation. The organic phase was collected on the bottom side of the paper by aspiration, and it was fed once more through the pump. The flow was segmented with air and mixed with 2.5 *N* NaOH.

Mixing of the phases and extraction of phenylbutazone into the aqueous alkaline solution took place in a 28-turn mixing coil. The mixture was fed through a phase separator for the removal of air bubbles and the organic phase. Finally, the resulting aqueous solution containing the sodium salt of phenylbutazone was fed through a flowcell where the UV absorption of the solution at 265 nm was continuously monitored.

Polyethylene tubing was used between the first 28-turn coil and the continuous filter, as well as between the exit side of the continuous filter and the suction side of the pump. Solvent-resistant tubing[3] was used at all other points for streams containing organic solvent. Polyethylene tubing connected the phase separator with the flowcell. Tygon tubing was used at the remaining points of the system. Solvent-resistant sleevings[3] were used for all connections.

A series of plasma standards containing phenylbutazone (0–50 μg/ml) was assayed after every group of approximately 30 test samples. The values obtained with several standards at each concentration were averaged, and a calibration curve of peak height *versus* phenylbutazone concentration was prepared each day. The plasma blank samples gave average values of approximately 0.5 μg/ml. The blank values were subtracted from the standards before the latter data were tabulated.

The described method was applicable without modification to the analysis of phenylbutazone in urine.

[3] Acidflex, Technicon Inc., Tarrytown, N.Y.

Table II—Accuracy of Automated Analytical Procedure for Phenylbutazone

Concentration, μg/ml		Average Recovery, %	n[a]
Expected	Found (Average ± *SD*)		
2	1.6 ± 0.6	80	6
3	2.8 ± 0.4	93.3	6
4	4.0 ± 0.7	100	6
5	5.5 ± 0.6	110	6
7	7.0 ± 0.3	100	6
10	10.8 ± 0.5	108	6
20	21.9 ± 0.7	109.5	6
25	24.8 ± 1.9	99.2	10
40	38.0 ± 1.0	95	6
50	49.7 ± 2.0	99.4	11

[a] n = number of determinations.

RESULTS AND DISCUSSION

Standard Curves and Linearity—Aqueous solutions containing known amounts of phenylbutazone (0–60 μg/ml) were carried through the entire analysis. The blank samples, corresponding to the reagent blank, had a reading of zero. The data (Table I) gave a straight line for the entire range (r = 0.99). The smallest detectable concentration from aqueous solutions was 0.5 μg/ml.

Subsequently, standard curves in plasma were obtained. A typical curve was generated by analyzing phenylbutazone at concentrations of 0, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, and 80 μg/ml. Five determinations were made at each concentration, except at 40 μg/ml (three measurements). The resulting curve of peak height readings *versus* concentrations had the following regression equation: $y = 0.672x - 0.361$, and demonstrated good linearity in the expected range of drug concentrations (r = 0.99). During the analyses of unknowns, similar standard curves were generated daily, using at least five concentrations, and were used for the calculation of the results obtained on that day.

Precision—The data generated for the standard curve of phenylbutazone in plasma gave a valid estimate of the precision of the methodology. At 2 μg/ml, a relative standard deviation of 20% was obtained. However, at each point between 3 and 80 μg/ml, the relative standard deviation was equal to or less than 7%, indicating good precision.

Sensitivity—A panel of 18 subjects had average predose plasma levels of apparent phenylbutazone of 0.3 μg/ml. Most subjects had nondetectable levels; three of the 18 individuals had values of 0.5 μg/ml, and another three had 1.5 μg/ml. Based on the variability of these data, it was estimated that the lowest value that could be assayed reliably (*i.e.*, quantitatively distinguished from the blank) would be approximately 3 μg/ml. This sensitivity is comparable to,

Table III—Specificity of Automated Analytical Procedure for Phenylbutazone[a]

Concentration of Oxyphenbutazone, μg/ml	Average Peak Height, % of control	n[b]
0	100	2
2	97.1	4
5	93.1	4
10	97.1	4
20	81.6	2
30	109.4	4
40	98.0	2

[a] Each sample contained 10 μg/ml of phenylbutazone. The samples containing no oxyphenbutazone served as controls. [b] n = number of determinations.

Table IV—Interference by Salicylic Acid with the Accuracy of Automated Analytical Procedure for Phenylbutazone

Concentration, μg/ml		Peak Height, Average ± SD, % of control	n^a
Phenylbutazone	Salicylic Acid		
20	0	100.0 ± 5.0	20
20	20	111.3 ± 7.8	8
20	40	116.3 ± 7.8	10
20	80	136.2 ± 9.9	10

[a] n = number of determinations.

or better than, the one obtainable with the manual method of Burns *et al.* (1). At the same time, the automated method is four to five times faster (100–120 analyses/8-hr day).

Accuracy—Solutions of phenylbutazone in plasma ranging from 2 to 50 μg/ml were prepared and then analyzed in a blind manner. The data (Table II) demonstrated that the analytical procedure gave reliable results in the 3–50-μg/ml range, with an average recovery of 101.3%. A relatively low recovery (80%) was obtained at 2 μg/ml.

Specificity—Varying concentrations (2–40 μg/ml) of oxyphenbutazone, the principal metabolite of phenylbutazone, were added to plasma samples containing 10 μg/ml of phenylbutazone. Results of the analyses (Table III) demonstrate that the presence of oxyphenbutazone did not affect appreciably the plasma level values obtained for phenylbutazone. Similarly, when each sample contained 25 μg/ml of phenylbutazone, the 20-fold increase in the concentration of oxyphenbutazone caused only a 13% increase in the values of apparent phenylbutazone.

A separate experiment showed that as much as 50 μg/ml of oxyphenbutazone did not interfere with the analysis of 5 μg/ml of phenylbutazone. Thus, the automated method is considerably more specific than the original method of Burns *et al.* (1), which suffers from appreciable interference by oxyphenbutazone.

Interference by salicylic acid was tested by analyzing plasma samples to which 20 μg/ml of phenylbutazone and 0, 20, 40, or 80 μg/ml of salicylic acid had been added. A 2-mg/ml stock solution of salicylic acid in 0.025 *N* NaOH was used. The data (Table IV) gave the regression line of $y = 14.1 + 0.063x$ (peak height *versus* concentration), indicating that each 10 μg/ml of the salicylic acid present in the plasma would be assayed as 0.63 μg/ml of apparent phenylbutazone. The magnitude of this interference depends on the relative proportions of the two drugs and, in practice, should not be troublesome.

In all likelihood, a single dose of a salicylate taken inadvertently during a bioavailability study would not interfere materially with the results of the phenylbutazone assay. On the other hand, steady-state salicylate levels, resulting from chronic intake prior to a phenylbutazone study, would appear as unusually high zero-time levels. Under these conditions, the subject would probably be eliminated from the study.

Human Plasma Level Studies—The described automated analytical method was used in several human studies. Plasma concentrations of phenylbutazone[4] were followed for up to 336 hr after a single oral dose. The data obtained in a representative study[5] were reported in preliminary form (18).

Analysis of Phenylbutazone in Urine—A standard curve was generated by analyzing phenylbutazone in urine at concentrations of 0, 2, 4, 6, 10, 20, 30, and 40 μg/ml. Ten determinations were made at each concentration between 4 and 40 μg/ml. The resulting curve of peak height readings *versus* concentrations showed good linearity ($r = 0.99$) with the following regression line: $y = 0.609x - 0.512$.

REFERENCES

(1) J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **109**, 346(1953).

(2) B. Herrmann, *Med. Exp.*, **1**, 170(1959).

(3) G. R. Van Petten, H. Feng, R. J. Withey, and H. F. Lettau, *J. Clin. Pharmacol.*, **11**, 177(1971).

(4) W. Fuchs, *Muench. Med. Wochenschr.*, **107**, 1267(1965).

(5) R. Pulver, *Schweiz. Med. Wochenschr.*, **80**, 308(1950).

(6) D. G. Moss, *J. Clin. Pathol.*, **1**, 344(1954).

(7) E. A. Tophøj, *Acta Med. Scand.*, **160**, 197(1958).

(8) J. E. Wallace, *J. Pharm. Sci.*, **57**, 2053(1968).

(9) E. Jähnchen and G. Levy, *Clin. Chem.*, **18**, 984(1972).

(10) R. Perego, E. Martinelli, and P. C. Vanoni, *J. Chromatogr.*, **54**, 280(1971).

(11) J. R. Watson, F. Matsui, R. C. Lawrence, and P. M. J. McConnell, *ibid.*, **76**, 141(1973).

(12) I. J. McGilveray, K. K. Midha, R. Brien, and L. Wilson, *ibid.*, **89**, 17(1974).

(13) R. B. Bruce, W. R. Maynard, and L. K. Dunning, *J. Pharm. Sci.*, **63**, 446(1974).

(14) K. K. Midha, I. J. McGilveray, and C. Charette, *ibid.*, **63**, 1234(1974).

(15) O. M. Bakke, G. H. Draffan, and D. S. Davies, *Xenobiotica*, **4**, 237(1974).

(16) N. J. Pound, I. J. McGilveray, and R. W. Sears, *J. Chromatogr.*, **89**, 23(1974).

(17) S. Ahuja, C. Spitzer, and F. R. Brofazi, in "Technicon Symposium, 1967, Automation in Analytical Chemistry," vol. 1, Medlad, White Plains, N.Y., 1968, p. 467.

(18) G. Lukas, M. B. Maggio-Cavaliere, C. B. Borman, and J. D. Arnold, *J. Clin. Pharmacol.*, **14**, 397(1974).

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[4] Butazolidin, Ciba-Geigy Corp., Ardsley, N.Y.

[5] G. Lukas, M. B. Maggio-Cavaliere, C. B. Borman, and J. D. Arnold, to be published.