Journal of Chromatography, 89 (1974) 17–22

CHROM. 7094

THE ASSAY OF PHENYLBUTAZONE IN HUMAN PLASMA BY A SPECIFIC AND SENSITIVE GAS–LIQUID CHROMATOGRAPHIC PROCEDURE

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(Received October 1st, 1973)

SUMMARY

A sensitive specific method is described for the determination of phenylbutazone in plasma. After simple solvent extraction from 1 ml of plasma the drug was estimated without interference from the known metabolites, oxyphenbutazone and the side-chain hydroxyphenylbutazone, by gas–liquid chromatography using a flame ionization detector. The procedure compared favourably with a less specific spectrophotometric method and was of sufficient sensitivity to determine plasma levels (<0.5 μg/ml) of the drug after single 200-mg doses.

INTRODUCTION

Phenylbutazone is one of the most widely prescribed antiarthritic drugs and although blood level studies have been described[1–3], the currently available methods for determination of the drug in plasma are not ideal for pharmacokinetic studies in which single doses may be compared. Burns *et al.*[1], who initiated metabolic studies of phenylbutazone, extracted plasma samples containing the drug with *n*-heptane, back extracted into alkali and measured the amount by UV absorbance at 265 nm. This method suffers from a lack of sensitivity (>10 μg/ml) and specificity. An improved extraction procedure developed by Hermann[2] and later by Van Petten *et al.*[3] removed most of the 1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine (oxyphenbutazone) metabolite by means of buffers and use of a mixed 1,2-dichloroethane–heptane solvent. However, the background was still significant (5–10 μg/ml limit). A permanganate oxidation procedure first reported by Wallace[4] was adapted for small samples recently by Jänchen and Levy[5]. Although oxyphenbutazone does not interfere with this procedure and the background at 314 nm can be minimized to give sensitivity limits of 2 μg/ml, any 1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)-pyrazolidine (hydroxyphenylbutazone) present can not be differentiated from the parent drug[5]. The objective of this study was to increase the sensitivity of the UV procedure and to develop a new, specific gas–liquid chromatographic (GLC) procedure for the measurement of single dose levels of phenylbutazone in plasma.

MATERIALS AND METHODS

Chemicals and reagents

Phenylbutazone and oxyphenbutazone were obtained from Ciba-Geigy Canada

Ltd. and hydroxyphenylbutazone from Dr. A. Sallmann of Ciba-Geigy, Basel, Switzerland. Spectral grade *n*-heptane (Caledon Labs. Ltd., Georgetown, Ont., Canada) was used throughout the determinations.

Spectrophotometric procedure

The method of Janchen and Levy[5] was employed with the following modifications.

1-ml plasma samples were shaken with 1 ml of *N* HCl and 6 ml of *n*-heptane for 15 min instead of 30. 4 ml of the heptane layer were treated as in the literature procedure except that the final incubating time was changed from 3 to 1 h. The absorbance was read at 314 nm with 1-cm microcuvets (1 ml) on a Model 500 Unicam spectrophotometer.

GLC procedure

A Perkin-Elmer Model F 11 gas chromatograph equipped with a flame ionization detector and a Perkin-Elmer Model 56 recorder was employed. The chromatographic column consisted of glass tubing 3 ft. × ¼ in. O.D. packed with 3% Apiezon L on 80–100 mesh Chromosorb W-HP supplied by Chromatographic Specialities, Brockville, Ont., Canada. The column was conditioned by injecting Silyl 8 (Pierce, Rockford, Ill., U.S.A.) and by maintaining the column at 300° for 18 h with low nitrogen flow.

The injection port and detector temperatures were 310 and 300°, respectively, and the column temperature was 230°. Compressed air and hydrogen flow-rates were adjusted to give maximum response. Nitrogen was used as the carrier gas at a flow-rate of 120 ml/min. (Retention time (t_R) 3.6 min for phenylbutazone and 6.0 min for diphenylphthalate.) The chart speed was 0.5 cm/min. The efficiency of the column was maintained by injecting 5 μl of Silyl 8 followed by 5 μl of Freon 113 (Pierce) each day before analysis.

Sample collection

Blood samples (10 ml) from two healthy human male subjects (170 and 200 lbs., respectively) were drawn into Vacutainers (Becton Dickinson Co., Mississagua, Ont., Canada) containing sodium citrate or heparin. The blood was centrifuged, plasma recovered and either analyzed immediately or stored at −15°.

Sample preparation

Add 1 ml of *N* HCl and 6 ml of *n*-heptane to 1 ml of heparinized plasma in a screw-cap centrifuge tube (15 ml). Secure the cap on the tube and mix the contents for 15 min on a rotorack before centrifugation for 5 min at 1200 × *g*. Transfer 2 ml of the heptane layer to another screw-cap tube (5 ml) and evaporate the solvent at 70° (water bath). Add 0.02 ml of ethyl acetate containing the external standard diphenylphthalate[6] (500 μg, *i.e.* 25 μg/μl) to the residue and mix on a vortex mixer. Inject 1-μl samples on to the gas chromatograph within 2 h.

Calibration curves and quantitation

Stock solutions were prepared by suspending phenylbutazone (20 mg) in distilled water (50 ml) and dissolving the drug by dropwise addition of 5 *N* sodium hydroxide. 40 ml of 0.2 *M* phosphate buffer (pH 7.4) were added and the pH of the

solution was adjusted to 7.4 with 5 *N* HCl. The final volume was made up to 100 ml with phosphate buffer (pH 7.4) to yield a solution of 200 μg/ml.

Standard calibration curves were established by adding phenylbutazone in the range of 2–32 μg/ml to freshly collected plasma and proceeding as described under *GLC procedure*. Quantitation was performed by plotting the peak height ratio of drug/external standard against concentration to obtain a standard curve linear in the concentration range 0.5–200 μg/ml with a slope 0.2105±0.012.

RESULTS AND DISCUSSION

Although Jänchen and Levy[5] suggested that the color development of phenylbutazone oxidation product required 3 h incubating at 65°, it was observed that the color was reproducible in 1 h. Spectral grade heptane was also required in the procedure and each new batch should be checked for interfering materials. The precision of the method is illustrated in Table I, the overall coefficient of variation in the range 2–32 μg/ml is 4.04%.

TABLE I

ESTIMATION OF PHENYLBUTAZONE ADDED TO PLASMA BY SPECTROPHOTOMETRIC METHOD

Phenylbutazone added (μg/ml)	*Mean absorbance n= 7*	*S.D.*	*C.V.*
2.0	0.033	0.001	3.03
4.0	0.062	0.004	6.45
8.0	0.117	0.004	3.41
16.0	0.233	0.011	4.72
32.0	0.428	0.011	2.57
			Mean 4.04

The extractability of phenylbutazone from acidified plasma into heptane was checked using and UV absorption spectrophotometer. The extinction coefficient of the UV absorption maximum of phenylbutazone in heptane at 265 nm was sufficient to determine down to 10 μg/ml of phenylbutazone from plasma. The extraction efficiency of heptane for partitioning phenylbutazone from plasma was determined by means of this UV procedure. The recovery of the drug from acidified plasma (pH 1–2) was better than 90%.

The response of the flame ionization detector to phenylbutazone was linear with concentration in the range 0.5–200 μg/ml. Watson *et al.*[6] had found diphenylphthalate a useful reference standard in a procedure for determination of pharmaceutical purity of phenylbutazone. 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. Thus the external standard curve from the solvent and the plasma-recovered external standard curve were constructed by plotting peak height response ratios of phenylbutazone/diphenylphthalate *versus* concentration of phenylbutazone solutions containing a constant amount of diphenylphthalate as the reference

TABLE II
RECOVERY OF PHENYLBUTAZONE FROM WATER DETERMINED BY GLC ASSAY

Phenylbutazone added (μg/ml)	*Phenylbutazone recovered (μg/ml)*	*Recovery (%)*
2.0	1.85	92.5
4.0	3.79	94.7
8.0	8.42	105.2
16.0	14.34	89.6
32.0	30.27	94.6
		Mean 95.32 ± 6.19

TABLE III
RECOVERY OF PHENYLBUTAZONE FROM PLASMA DETERMINED BY GLC ASSAY

Phenylbutazone added (μg/ml)	*Phenylbutazome recovered (μg/ml)*	*Recovery (%)*
2.0	1.80	90.0
4.0	3.79	94.7
8.0	7.54	94.2
16.0	14.62	91.4
32.0	30.98	96.8
		Mean 93.42 ± 2.9

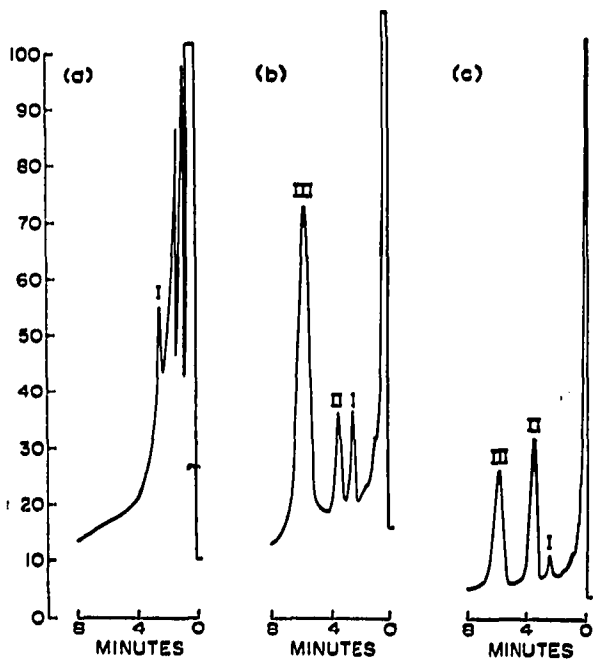

Fig. 1. Chromatograms of human plasma extracts. (a) Blank plasma, (b) control plasma to which 2.0 μg/ml of phenylbutazone and external standard are added, (c) plasma unknown from a human volunteer who received an oral dose of 200 mg of phenylbutazone. I = Peak from endogenous plasma material (t_R = 2.4 min); II = peak for phenylbutazone (t_R = 3.4 min); III = peak for external standard (t_R = 6.0 min).

standard per millilitre of ethylacetate. The overall recoveries of 2–32 μg of phenylbutazone from water and from plasma were of the order of 95.32±6.19 and 93.42±2.9%, respectively (Tables II and III).

During the development of this method various solvents for injection were evaluated and it was found that ethyl acetate gave the cleanest separation free from interfering substances. Fig. 1a shows a chromatogram obtained by treating fresh plasma containing no drug as described under *GLC procedure* but omitting the external standard. The single extraneous peak with a t_R of 2.4 min was observed in chromatograms of all human plasma samples. A chromatogram observed when the method was applied to spiked plasma containing 2 μg/ml of phenylbutazone is shown in Fig. 1b where it is evident that the extraneous peak I does not interfere with the

TABLE IV

ESTIMATION OF PHENYLBUTAZONE ADDED TO PLASMA BY GLC

Phenylbutazone added (μg/ml)	*Mean peak height ratio drug/standard n=4*	*S.D.*	*C.V.*
2.0	0.36	0.038	10.64
4.0	0.71	0.021	2.91
8.0	1.45	0.036	2.45
16.0	2.98	0.038	1.27
32.0	6.67	0.092	1.37
			Mean 3.73

$y = mx$
where $m = 0.2105 \pm 0.012$
$r^2 = 0.998$.

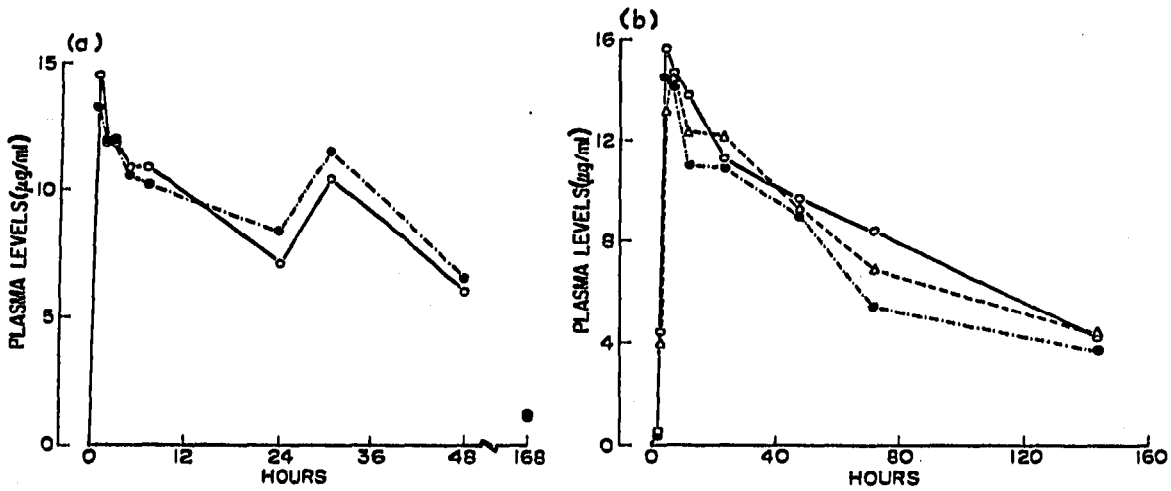

Fig. 2. Comparison of plasma levels determined by different analytical methods following single doses of phenylbutazone to volunteers. (a) 100-mg solution to subject I; (b) 200 mg (two 100-mg tablets) to subject II. O—O, GLC; ●-·-·-●, UV; △---△, HSLC.

peaks from phenylbutazone (II, $t_R = 3.4$ min) or diphenylphthalate (III, $t_R = 6.0$ min). The known metabolites oxyphenbutazone and hydroxyphenylbutazone do not interfere in the procedure. Oxyphenbutazone does not come through the column and the hydroxyphenylbutazone gives a peak of poor response with a t_R of 8.5 min.

The accuracy and precision of the GLC determination are demonstrated in Table IV. Four aliquots each of five solutions of phenylbutazone ranging from 2 to 32 μg/ml in plasma were treated as described under *GLC procedure*. The overall coefficient of variation (C.V.) was 3.73%.

Application of the method to plasma level determinations is demonstrated in Fig. 2 along with comparisons with other methods. A 100-mg dose of phenylbutazone was given in solution to a healthy male volunteer (170 lbs.), plasma withdrawn at intervals over 48 h and assayed for phenylbutazone using both the UV method and the GLC procedure. The methods compare favourably (Fig. 2a) with an overall 6.0% difference. A second male volunteer was given a 200-mg (two 100-mg tablets) dose, plasma levels over 144 h being compared by the UV, GLC and a recent high-speed liquid chromatographic (HSLC) procedure[7]. The comparison is shown in Fig. 2b, some scatter being evident between the methods but with acceptable variation (GLC to UV, 12.5%; GLC to HSLC 8.9% overall). It should be noted that the UV procedure does not differentiate phenylbutazone from all metabolites which may interfere in some assays.

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