Journal of Chromatography, 89 (1974) 23–30

CHROM. 7079

ANALYSIS OF PHENYLBUTAZONE IN PLASMA BY HIGH-SPEED LIQUID CHROMATOGRAPHY

N. J. POUND*, I. J. McGILVERAY and R. W. SEARS

Pharmaceutical Chemistry Division, Drug Research Laboratories, Health Protection Branch, Ottawa, Ontario K1A OL2 (Canada)

(Received October 1st, 1973)

SUMMARY

A sensitive, specific method for the determination of phenylbutazone in plasma in the presence of metabolites is described. The *n*-hexane extract of acidified plasma, redissolved in chloroform, is chromatographed on a SIL-X column using a mobile phase of 10% tetrahydrofuran in *n*-hexane on a high-speed liquid chromatograph fitted with ultraviolet absorbance detector. Quantitation of plasma samples containing less than 2 μg/ml of phenylbutazone is reported, using the 2,4-dinitrophenylhydrazone of benzaldehyde as an internal standard. Detection is limited to approximately 0.2 μg/ml. The retention times for the metabolites are such that they do not interfere in the procedure. For comparison, results determined by both electronic integration and peak-height measurements are quoted.

INTRODUCTION

The existing methods for the assay of phenylbutazone in blood or plasma, while adequate for therapeutic levels (40–100 μg/ml) are not ideal for biopharmaceutical studies in which single doses may be compared. The classical method of Burns *et al.*[1], in which the drug is extracted from acidified plasma by *n*-heptane, then back extracted into alkali to be measured by ultraviolet (UV) absorbance at 265 nm, suffers from a lack of sensitivity (>10 μg/ml) and specificity. Hermann[2] and later Van Petten *et al.*[3], by means of buffers and use of a mixed 1,2-dichloroethane–*n*-heptane solvent in the first extraction, removed most of the oxyphen butazone metabolite; however, background remained high (sensitivity 5 μg/ml in our hands). Wallace[4] reported a procedure based on permanganate oxidation which Jänchen and Levy[5] adapted for small samples. The UV background at 314 nm was minimized in this method with a sensitivity limit of approximately 2 μg/ml; but, although oxyphenbutazone does not interfere, any of the side-chain hydroxymetabolite (hydroxyphenylbutazone) present cannot be differentiated from the parent drug[5]. Thus, there is a requirement for more specific sensitive methods for measurement of phenylbutazone and its major metabolites. This report describes a high-speed liquid chromatographic (HSLC) analysis of phenylbutazone in the presence of these metabolites.

* To whom inquiries should be directed.

EXPERIMENTAL

Materials

Complementary samples of phenylbutazone (Mount Royal Chemicals, Montreal, Canada), oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine] (both from Ciba-Geigy, Montreal, Canada) were used as received. Tetrahydrofuran, freshly distilled and dried over Davison® Molecular sieves, Type 3A (both from Fisher Scientific, Montreal, Canada), and *n*-hexane (Caladon Labs., Georgetown, Canada) were used as solvents. The 2,4-dinitrophenylhydrazone of benzaldehyde, which was used as the internal standard, was prepared by the reported procedure[6], m.p. 234–236°; ref. 6, 237°.

Chromatographic procedure

Details of the modified constant-pressure high-speed liquid chromatograph (Varian Aerograph, Palo Alto, Calif., U.S.A., Model 4000) used in this study have been previously reported[7]. The chromatograph was equipped with a stop-flow injection port, a fixed-wavelength (254 nm) UV absorbance detector, attenuated to 0.04 absorbance units full scale (a.u.f.s.), and a digital integrator (Vidar, Mountain View, Calif., U.S.A., Model 6300).

The column (100 cm × 1.8 mm I.D.; 304 stainless steel) was dry-packed with SIL-X® (Perkin-Elmer, Norwalk, Conn., U.S.A.) adsorbent by a procedure similar to the one described by Kirkland[8], 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% glacial acetic acid and 10.0% tetrahydrofuran in *n*-hexane at a flow-rate of 60 ml/h (475 p.s.i.) and a temperature of 35°. The mobile phase was "degassed" by applying vacuum (*ca.* 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 (Hamilton, Reno, Nev., U.S.A.) using a stop-flow injection technique.

Preparation of standard solutions

Phenylbutazone, accurately weighed, was dissolved in 95% ethanol (1 ml) in a 100-ml volumetric flask and brought to volume with phosphate buffer (pH 7.2). An aliquot of this solution was further diluted with water to produce a final solution of the desired concentration. Spiked plasma solutions (1.75–40 μg/ml) were prepared by transferring an aliquot (1.0 ml) of the aqueous phenylbutazone solution to a 5-ml volumetric flask. The flask was then brought to volume with citrated human plasma and the resulting solution mixed thoroughly.

General procedure

An aliquot (1.0 ml) of the plasma sample was transferred to a 10-ml screw-cap centrifuge tube, 1 *M* hydrochloric acid (1 ml) and a solution of the internal standard (1.5–5 μg/ml, accurately weighed) in *n*-hexane (6.0 ml) were added, and the tube tumbled on a Multi-Rotator® (Scientific Industries, Springfield, Mass., U.S.A.) for 15 min at 30 rpm. The tubes were then centrifuged for 2–3 min at 3000 rpm and the upper *n*-hexane fraction transferred with a pasteur pipette to a 15-ml conical screw-

capped centrifuge tube. The *n*-hexane was evaporated under nitrogen in a constant-temperature (60°) water-bath. The inner walls of the tubes were rinsed with chloroform (1 ml) which was also evaporated. The resulting residue was promptly redissolved in 50–200 μl of chloroform to give an anticipated phenylbutazone concentration of 30–200 ng/μl. Aliquots (10.0 μl) of this solution were chromatographed.

The amount of phenylbutazone in the sample was determined from the following expression:

$$W_p = \frac{R \cdot W_I}{m}$$

where

W_p = micrograms of phenylbutazone
R = response ratio of phenylbutazone to internal standard
W_I = weight of internal standard
m = slope of calibration curve (Fig. 2)

RESULTS AND DISCUSSION

Fig. 1b illustrates the chromatogram obtained from the analysis of a plasma sample containing 5.5 μg/ml of phenylbutazone using the system described above. This represents an on-column injection of 0.7 μg of internal standard and 1.1 μg of phenylbutazone. An analysis time of less than 7 min is achieved. No interfering compounds are extracted from plasma with *n*-hexane (Fig. 1a). Samples for this study were prepared from plasma collected from six different subjects. No extraneous compounds were encountered in any of these samples.

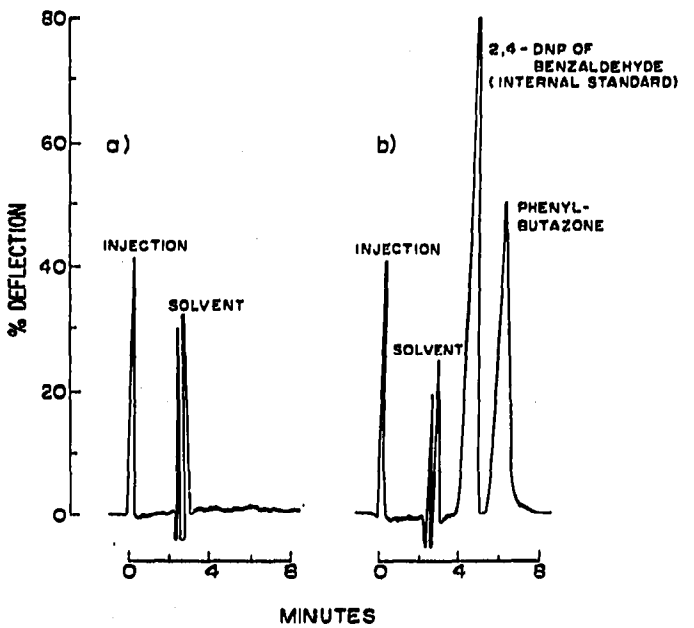

Fig. 1. High-speed liquid chromatogram of human plasma extract: (a) from blank plasma; (b) from plasma spiked with phenylbutazone (5.5 μg/ml).

Adsorption chromatography was chosen because it offers the advantage of rapid analyses and excellent column stability. A simple solvent system of *n*-hexane modified with 10% tetrahydrofuran was used as a mobile phase. The addition of a small amount (0.002%) of glacial acetic acid to the mobile phase produced a symmetrical phenylbutazone peak by reducing the slight tendency of this compound to tail. Operation at a column temperature of 35° reduced the column back pressure (475 p.s.i.) and modestly improved the efficiency of the column.

This chromatographic separation also eliminates any interference from either hydroxyphenylbutazone or oxyphenbutazone, two known metabolites of phenylbutazone[1], if either were present in the plasma. Under these chromatographic conditions retention times of 4.4, 5.8, 33.0, and 40.8 min are obtained for the internal standard, phenylbutazone, hydroxyphenylbutazone, and oxyphenbutazone, respectively. Neither hydroxyphenylbutazone nor oxyphenbutazone was detected in any of the biological samples that were analysed. This may be attributed to the low partition coefficient of these compounds in *n*-hexane and to the poor detector response partially caused by peak broadening of these highly retained compounds. Studies are presently in progress to develop analytical procedures for the analysis of oxyphenbutazone in biological samples.

Plasma samples were prepared by a modification of the partitioning procedure reported by McGilveray *et al.*[9]. Aliquots of plasma were treated with 1 *M* hydrochloric acid, to release the protein bound phenylbutazone, and partitioned for 15 min with *n*-hexane containing a known amount of internal standard. Previous procedures[1–3] used *n*-heptane or *n*-heptane modified with 2–3% of a polar solvent such as dichloromethane as an extraction solvent. *n*-Hexane was used in this procedure because the partition coefficient of phenylbutazone into *n*-hexane and *n*-heptane were comparable (*ca.* 98%), no interfering UV absorbing substances were extracted from the plasma with *n*-hexane, and the higher volatility of *n*-hexane simplified evaporation of the sample extracts.

Although refrigerated plasma samples containing phenylbutazone were found to be stable for several weeks, degradation of the evaporated extracts presented a problem initially. It was observed that degradation occurred when the residues were stored, even if refrigerated. Since this effect appeared to be random, it is suggested that traces of aqueous acid, which may have been carried over into some of the tubes, caused this degradation. This problem was eliminated by analyzing the samples within two to three hours following partitioning. Degradation, when present, is readily detected by the appearance in the spectrum of two, as yet unidentified, peaks with retention times of 7.1 and 11.6 min.

Chloroform was chosen as the solvent for redissolving the sample residues for injection since the internal standard and phenylbutazone are both 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 of the sample between injections.

The output of the UV detector is linear in absorbance units; therefore, for compounds that obey the Lambert-Beer law, the area under the peak is proportional to concentration, within the linear range of the detector. As a result, it is possible to quantitate the phenylbutazone in plasma by electronic integration and peak-height measurements. Quantitation by peak-height measurement is particularly applicable

to HSLC since solute peaks tend to be symmetrical. This study (Table I), along with previous work in this laboratory[10], and results reported by Roos[11] indicate that peak-height data compare favourably with that obtained by electronic integration. This is particularly true for the measurement of peaks with relatively long retention times. The integrator in use in this laboratory provides a maximum slope sensitivity setting of 0.2 μV/msec. Since the peak base width increases with retention time, the slope of small peaks is often too low to trip the integrator. Therefore, a significant percentage of the peak area of small, broad peaks may not be integrated. In this study, the on-column injection of less than 200 ng of phenylbutazone gave poor results when the detector was attenuated to 0.04 a.u.f.s. Although it is possible to reduce the detector attenuation by a factor of 8, *i.e.* 0.005 a.u.f.s., at this attenuation problems caused by detector noise and baseline drift frequently produce spurious results. An attenuation setting of 0.04 a.u.f.s. was used routinely in this procedure since it provided adequate sensitivity for the levels of phenylbutazone to be analyzed and minimized any interference caused by detector noise or drift. In order to analyze samples containing as little as 2 μg/ml of phenylbutazone, plasma samples containing less than 6 μg/ml of drug were extracted with *n*-hexane containing 25% of the normal amount of internal standard. The resulting sample residues were re-dissolved in 50 μl of chloroform. This provided a final concentration of at least 200 ng per injection. Using this procedure it was possible to quantitate routinely plasma samples containing as little as 1.75 μg/ml (*i.e.*, 350 ng per injection) of phenylbutazone by electronic integration.

At a detector attenuation setting of 0.005 a.u.f.s. the injection of as little as 40 ng of phenylbutazone would produce a recorder response of approximately 10% full scale deflection. This would represent a minimum detectable amount of 0.2 μg/ml of phenylbutazone.

The 2,4-dinitrophenylhydrazone of benzaldehyde was used as an internal standard. It is added directly with the extracting solvent before partitioning the

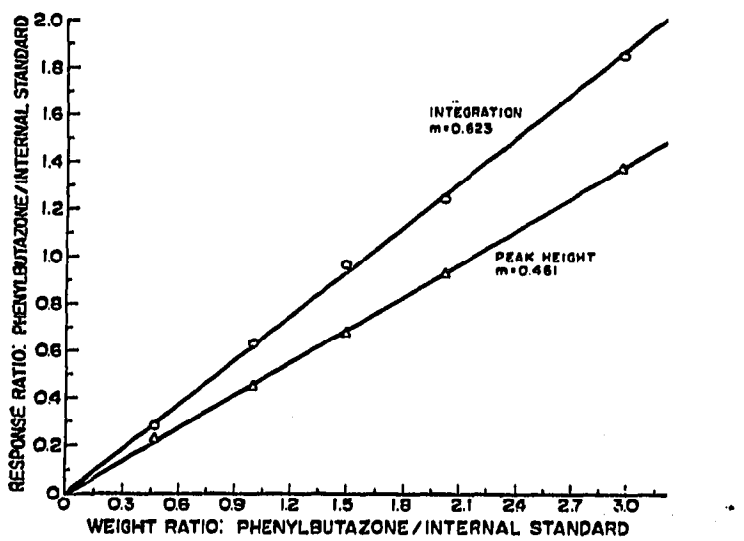

Fig. 2. Phenylbutazone/internal standard calibration curve.

samples. This reduces 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. Fig. 2 represents the calibration curves obtained by plotting the response *vs.* concentration ratios of phenylbutazone/internal standard obtained from the analysis of spiked plasma samples. Data determined by integration and peak-height measurements are shown. Both plots are straight lines ($y=mx$) over the concentration range of 1.8 to 41.7 μg/ml. Mean slope values of $0.623 \pm 3.8\%$ (correlation coefficient, $r=1.0$) and $0.461 \pm 4.7\%$ ($r=1.0$) were obtained by integration and peak-height measurements, respectively. These calibration curves were checked frequently over the course of the study and the drift from the curve was always less than the mean slope error.

Table I lists the results obtained from the analysis of a number of spiked plasma samples. These data reflect the accuracy and precision of this method. Mean recovery values of $99.9 \pm 1.7\%$ and $99.3 \pm 3.1\%$ were obtained by integration and peak-height measurement, respectively.

TABLE I

ANALYSIS OF SPIKED PHENYLBUTAZONE-PLASMA SAMPLES

Results from the analysis of duplicate aliquots are shown.

Sample number	*Phenylbutazone concentration (μg/ml)*				
	Theoretical	*Found**	*Recovery (%)*	*Found***	*Recovery (%)*
1	38.86	39.60	101.9	39.91	102.7
		39.83	102.5	39.61	101.9
2	29.00	28.42	98.0	27.73	95.6
		27.97	96.4	27.52	94.9
3	19.33	19.27	99.7	19.18	99.2
		19.27	99.7	19.05	98.6
4	19.30	19.88	103.0	19.40	100.5
		19.82	102.7	20.39	105.7
		19.28	99.9	19.13	99.1
		18.98	98.1	18.57	96.2
		19.72	102.2	19.93	103.3
	Mean***		101.2		101.0
	Coefficient of variation		±2.1%		±3.7%
5	6.96	7.14	102.6	7.03	101.0
		6.68	96.1	6.64	95.4
6	6.18	6.15	99.5	6.08	98.4
		6.04	97.7	6.02	97.4
7	3.10	3.19	102.9	3.14	101.3
		2.96	95.6	3.02	97.4
	Mean§		99.9		99.3
	Coefficient of variation		±1.7%		±3.1%

* Determined by integration.
** Determined by peak-height measurement.
*** Mean of five aliquots of the same sample.
§ Mean of 17 analyses.

The plasma phenylbutazone profile of a human subject (male, 200 lbs.) who had been administered two 100-mg tablets of phenylbutazone (Butazolidin®; Ciba-Geigy, Montreal, Canada) is illustrated in Fig. 3. Blood was collected in heparinised 10-ml Vacutainers (Becton-Dickinson, Toronto, Canada), and the plasma, separated by centrifugation, was either analysed immediately or stored in a freezer (−15°).

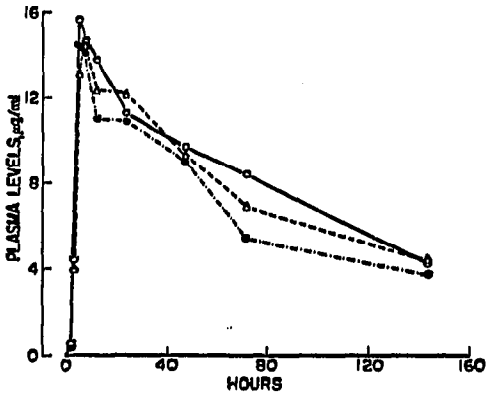

Fig. 3. Comparison of plasma levels by different analytical methods following single dose (two 100-mg tablets) of phenylbutazone. ○—○, GLC; ●-·-●, UV; △ - - -△, HSLC.

These results compare the plasma levels over 144 h determined by a UV procedure[5], a gas–liquid chromatographic (GLC) procedure[9] and the HSLC procedure described above. The overall difference between the HSLC and UV methods was 8.2% and that between the GLC and HSLC 8.9%. The UV determination would not distinguish between any side-chain hydroxymetabolite present and the parent drug and could contribute to the difference between levels estimated by this and the HSLC procedure. Two specific points —the 5 and 72 h levels— contribute greatly to the variations between the GLC and HSLC methods, no explanation being evident. Analysis of spiked plasma by the two chromatographic procedures gave greater precision (2% at 4 μg/ml level) and give greater confidence in the comparison.

This HSLC method is a sensitive procedure for the determination of phenylbutazone in plasma. It offers high specificity, since it differentiates the parent drug from the two known metabolites, and could form the basis of a method for metabolic studies.

REFERENCES

1 J. J. Burns, R. K. Rose, T. Chenkin, A. Goldman, A. Schulert and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, 109 (1953) 346.
2 B. Hermann, *Med. Exp.*, 1 (1960) 170.
3 G. R. van Petten, H. Feng, R. J. Withey and H. F. Lettau, *J. Clin. Pharmacol., J. New Drugs*, 11 (1971) 177.
4 J. E. Wallace, *J. Pharm. Sci.*, 57 (1968) 2053.
5 E. Jänchen and G. Levy, *Clin. Chem.*, 18 (1972) 984.
6 A. I. Vogel, *Elementary Practical Organic Chemistry*, Part II, Longmans, London, 1962, pp. 417 and 585.

7 N. J. Pound, R. W. Sears and A. G. Butterfield, *Anal. Chem.*, 45 (1973) 1001.
8 J. J. Kirkland, *J. Chromatogr. Sci.*, 9 (1971) 531.
9 I. J. McGilveray, K. K. Midha, R. Brien and L. Wilson, *J. Chromatogr.*, 89 (1974) 17.
10 N. J. Pound and R. W. Sears, *Can. J. Pharm. Sci.*, 8 (1973) 84.
11 R. W. Roos, *J. Pharm. Sci.*, 61 (1972) 1979.