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SIMULTANEOUS DETERMINATION OF PHENYLBUTAZONE AND ITS METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TERUYOSHI MARUNAKA*, TOSHIAKI SHIBATA, YOSHINORI MINAMI and YUKIHIKO UMENO

Research Laboratory, Taiho Pharmaceutical Co. Ltd., Kawauchi-cho, Tokushima 771-01 (Japan)

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SUMMARY

A high-performance liquid chromatographic method was developed for the simultaneous determination of phenylbutazone and its metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone, in plasma and urine. Samples were acidified with hydrochloric acid and extracted with benzene—cyclohexane (1:1, v/v). The extract was redissolved in methanol and chromatographed on a μBondapak C_{18} column using a mobile phase of methanol—0.01 *M* sodium acetate buffer (pH 4.0) in a linear gradient (50 to 100% methanol at 5%/min; flow-rate 2.0 ml/min) in a high-performance liquid chromatograph equipped with an ultraviolet absorbance detector (254 nm). The detection limit for phenylbutazone, oxyphenbutazone and for γ-hydroxyphenylbutazone was 0.05 μg/ml.

A precise and sensitive assay for the determination of phenylbutazone and its metabolites was established.

INTRODUCTION

Burns et al. [1] reported an ultraviolet (UV) method for the determination in plasma of phenylbutazone, which is widely used as an antihistaminic agent. Many studies using this UV method were then reported [2—9]. However, the method had many problems in sensitivity and in specificity of determination.

Recently, several methods using gas—liquid chromatography (GLC) [10—15] and high-performance liquid chromatography (HPLC) [16, 17] have been reported as having improved sensitivity and precision for the determination of phenylbutazone and its metabolites.

Midha et al. [12] determined phenylbutazone and oxyphenbutazone as methylated derivatives by GLC, but each compound gave two peaks on the chromatograph. Bruce et al. [13] determined simultaneously phenylbutazone

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and a heptaflurobutyrated derivative of oxyphenbutazone by GLC. Tanimura et al. [14] determined phenylbutazone and trimethylsilylated derivatives of oxyphenbutazone and γ-hydroxyphenylbutazone by GLC. This method was a two-step analysis, phenylbutazone being determined first and then the trimethylsilylated derivatives of two metabolites.

Pound and co-workers [16, 17], on the other hand, determined phenylbutazone and oxyphenbutazone by HPLC using an adsorption chromatography column.

We examined the simultaneous determination of phenylbutazone and its metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone, in plasma and urine by HPLC using a reversed-phase chromatographic column. This report describes the precise and sensitive assay of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in plasma and urine.

EXPERIMENTAL

Materials and reagents

Phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were obtained from Esteve Products (Barcelona, Spain). Other chemicals used were all purchased from Wako Pure Chemicals (Osaka, Japan). Benzene, cyclohexane, chloroform and methanol were of liquid-chromatography grade.

A 0.01 *M* sodium acetate buffer (pH 4.0) used as a mobile phase for HPLC was prepared by dissolving 1.36 g of sodium acetate trihydrate and 2.36 ml of acetic acid in 1 l of ion-exchanged water.

Instruments

A Shimadzu Model LC-2 high-performance liquid chromatograph (Kyoto, Japan) equipped with a Model SIL-1A high-pressure injection valve, a Model UVD-2 detector (UV, 254 nm) and a Model GRE-2 gradient elution equipment was used.

A μBondapak C_{18} chromatographic column (30 cm × 4 mm I.D., 8—10 μm particle size) (Waters Assoc., Milford, MA, U.S.A.) was used for the separation and a pre-column (Permaphase ODS, 5 cm × 2.1 mm I.D., Shimadzu) was connected between the separation column and the injector. The mobile phase was a linear gradient (5%/min) of 50% methanol in 0.01 *M* sodium acetate buffer (pH 4.0) as the initial concentration and 100% methanol as the final concentration; the flow-rate was 2.0 ml/min. The column was maintained at room temperature, and compounds thus eluted were recorded by the detector at a constant wavelength of 254 nm; the attenuator was set at 0.04 a.u.f.s. Peak areas were determined by a Shimadzu Chromatopac Model 1A apparatus.

A JEOL Model JMS-01SG-2 mass spectrometer with an electron impact ion source (Tokyo, Japan) was used for identification of phenylbutazone and its metabolites. The mass spectrometric analysis was carried out under the following conditions: ionization energy 75 eV, ionization current 200 μA, and accelerating voltage 10 kV.

Extraction procedure

Blood samples were collected in heparinized containers and centrifuged to separate the plasma. The plasma (1.0 ml) was diluted with 1.0 ml of physiological saline, adjusted to pH 2.0 with 5 *N* HCl, and then shaken vigorously with 20 ml of benzene—cyclohexane (1:1, v/v) at room temperature for 20 min. After centrifugation at 2000 *g* for 5 min, the organic layer was separated. This extraction was repeated once more. The organic layer containing phenylbutazone and its metabolites was evaporated to a suitable volume under nitrogen gas at 30°C, then transferred to a test-tube (10 ml capacity) and dried under nitrogen gas. The residue was dissolved in 100 μl of methanol, and 20 μl of this solution were injected into the liquid chromatograph.

Urine was treated by the same procedure except that a 2.0-ml sample was used and then was extracted with 30 ml of benzene—cyclohexane.

Calibration curves

Calibration curves for the determination of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone by HPLC were prepared by adding known amounts of these compounds to plasma and urine, and assaying the mixture by the same extraction procedure; the peak areas were plotted against the concentrations of these compounds. As shown in Fig. 1, all these calibration curves were linear.

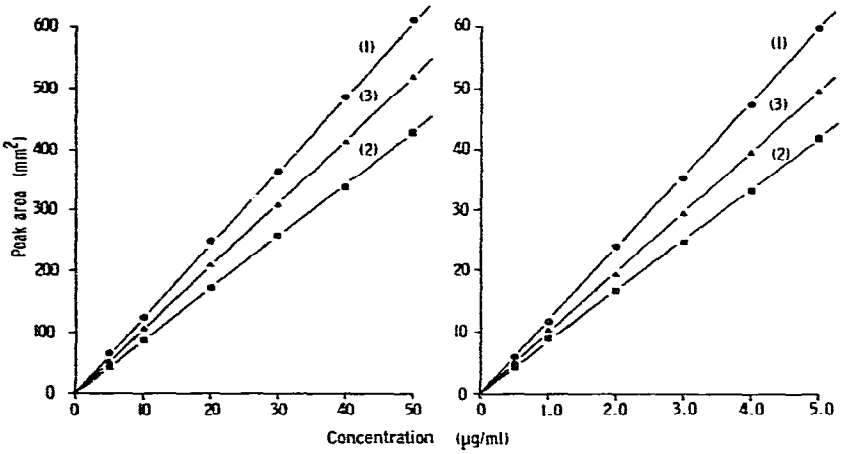

Fig. 1. Calibration curves of phenylbutazone (1), oxyphenbutazone (2) and γ-hydroxyphenylbutazone (3) extracted from plasma and urine. Calibration curves for the ranges 5—50 μg/ml and 0.5—5 μg/ml are presented.

RESULTS AND DISCUSSION

A reversed-phase chromatographic column, μBondapak C_{18}, was used for the separation. A mobile phase consisting of a linear gradient (0 to 100% methanol at 8%/min, flow-rate 2.0 ml/min) of methanol—water (mobile phase A), methanol—0.05 *M* KH_2PO_4 (mobile phase B) or methanol—0.01 *M* sodium acetate buffer (pH 4.0) (mobile phase C) was found to be suitable for the

separation of standard phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone. Furthermore, the separation patterns using a linear gradient (5%/min, flow-rate 2.0 ml/min) of 50% methanol as the initial concentration and 100% methanol as the final concentration in mobile phases A, B and C were investigated to obtain a satisfactory analysis time. As a result, the mobile phase C system showed good separation and analysis time.

Next, a suitable solvent for the extraction of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone from plasma and urine was investigated. Chloroform extracts under the acidic conditions of hydrochloric acid gave no interfering peaks from biological components on analysis using mobile phase A, but the same extract gave peaks due to biological components overlapping in elution time with phenylbutazone and oxyphenbutazone on analysis with mobile phase B or C. Benzene—cyclohexane (1:1, v/v) extracts under the acidic conditions of hydrochloric acid gave no interfering peaks on analysis with mobile phase A, B or C.

On the basis of the above results, benzene—cyclohexane (1:1, v/v) was used as extraction solvent for phenylbutazone and its metabolites from plasma and urine, and a linear gradient (50 to 100% methanol at 5%/min, flow-rate 2.0 ml/min) of methanol—0.01 *M* sodium acetate buffer (pH 4.0) was used as the mobile phase for reversed-phase HPLC for the subsequent experiments.

The chromatograms showing the separation of benzene—cyclohexane extract from rat plasma control and from rat plasma at 4 h after oral administration of phenylbutazone (100 mg/kg) are shown in Fig. 2; those from rat urine control and from rat urine at 6—12 h are shown in Fig. 3. Analysis time was 15 min. The retention times for phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone under the present HPLC conditions were 7.0, 6.1 and 4.9 min, respectively. Each fraction eluting from the HPLC column was separately

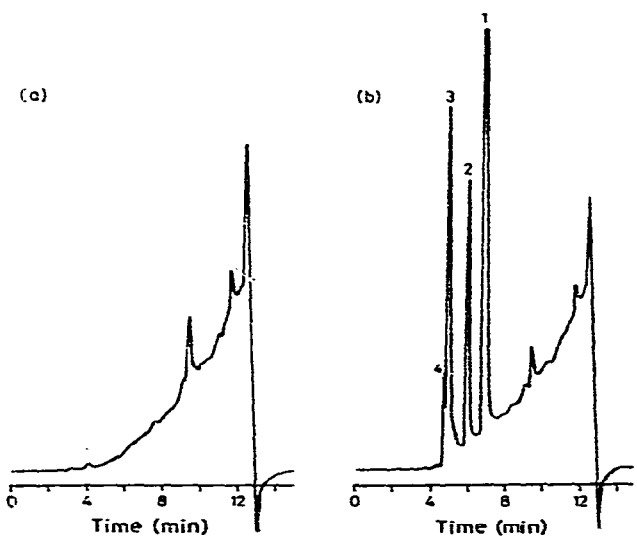

Fig. 2. Liquid chromatograms showing the separation of (a) control, and (b) phenylbutazone (1), oxyphenbutazone (2), γ-hydroxyphenylbutazone (3) and unknown metabolite (4) extracted from rat plasma. Details are described in the text.

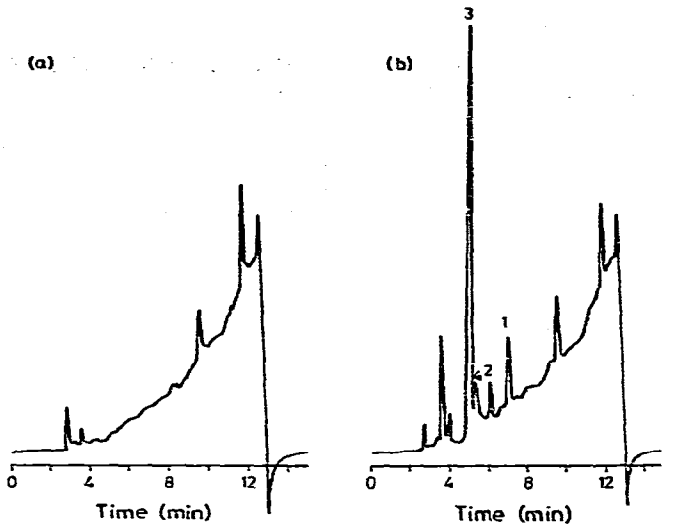

Fig. 3. Liquid chromatograms showing the separation of (a) control, and (b) phenylbutazone (1), oxyphenbutazone (2), γ-hydroxyphenylbutazone (3) and *p*,γ-dihydroxyphenylbutazone (4) extracted from rat urine. Details are described in the text.

collected and identified by mass spectrometry: phenylbutazone, *m/e* 308 (M^+), 252, 183, 105 and 77; oxyphenbutazone, *m/e* 324 (M^+), 268, 199, 93 and 77; γ-hydroxyphenylbutazone, *m/e* 324 (M^+), 309, 280, 266, 183, 83 and 77.

The detection limits under the present method were 0.05 μg/ml of plasma or urine for all compounds. The present method had an accuracy of ± 1.5—3.1% and a very good reproducibility.

Methanol was used as a sample solvent since phenylbutazone and its metabolites are all readily soluble in methanol and no decomposition at all was observed. Similar results were also obtained when water was used as a sample solvent.

Known amounts of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were added to the plasma and urine, and then the recovery for each compound was examined. As shown in Table I, the overall recoveries of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were 96.7 ± 1.7%, 93.1 ± 3.7% and 81.7 ± 4.2%, respectively.

In addition, *p*,γ-dihydroxyphenylbutazone has been found as a metabolite of phenylbutazone [18]. This metabolite showed a retention time of 5.4 min, and did not affect the separation of phenylbutazone and other metabolites. This corresponding peak was confirmed by gas chromatography—mass spectrometry as its trimethylsilylated derivative: *m/e* 556 (M^+), 541 ($M-CH_3$), 426, 334, 246, 181 and 73. This metabolite, however, could not be determined because of recovery problems under the present method.

The concentrations of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in plasma after oral administration of 100 mg/kg phenylbutazone to male Wister rats (180 g body weight) determined by the present method were compared with those determined by GLC [14]. The results obtained are shown in Fig. 4. The overall difference between the HPLC and GLC methods was 4.8%.

TABLE I

RECOVERIES ON EXTRACTION OF PHENYLBUTAZONE, OXYPHENBUTAZONE AND γ-HYDROXYPHENYLBUTAZONE FROM PLASMA AND URINE

Each value is the mean of five determinations.

Compound	Added (μg/ml)	Recovery from plasma (%)	Recovery from urine (%)
Phenylbutazone	50.00	96.8	97.1
	25.00	96.3	96.5
	5.00	96.9	96.1
	0.50	96.7	96.8
	0.10	97.3	96.9
Mean ± S.D.		96.7 ± 1.7	
Oxyphenbutazone	50.00	92.8	92.7
	25.00	93.1	93.0
	5.00	93.3	93.0
	0.50	92.9	93.2
	0.10	93.7	93.5
Mean ± S.D.		93.1 ± 3.7	
γ-Hydroxyphenylbutazone	50.00	79.9	80.7
	25.00	81.9	81.1
	5.00	82.4	81.9
	0.50	81.9	82.6
	0.10	82.1	82.9
Mean ± S.D.		81.7 ± 4.2	

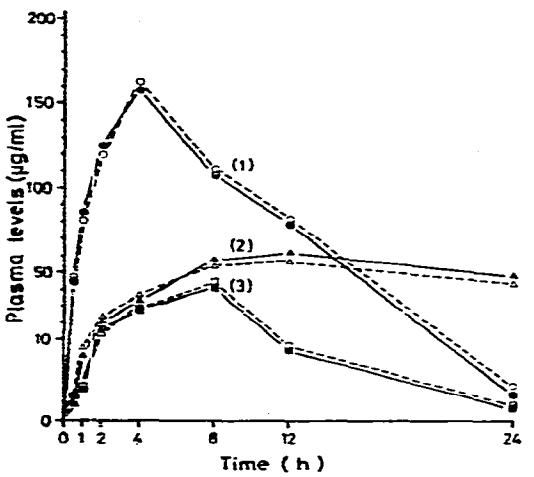

Fig. 4. Comparison of plasma levels of phenylbutazone (1), oxyphenbutazone (2) and γ-hydroxyphenylbutazone (3) determined by HPLC (●, ▲, ■) and GLC (○, △, □). The single oral dose given to male Wistar rats was 100 mg (0.3 mmol) phenylbutazone per kg.

The present assay method was then applied to the plasma and urine of other animals and man to which phenylbutazone and its metabolites were added. The results obtained for the chromatographic separation, recoveries, precision and sensitivities were in good agreement with those obtained with rat plasma and urine.

Furthermore, the conjugates of phenylbutazone and its metabolites [18] in urine could be determined using the same extraction procedure and under the same HPLC conditions as non-conjugated compounds after the following treatment. Sodium chloride was added to saturation to the aqueous layer after extraction of non-conjugated compounds with benzene—cyclohexane (1:1, v/v). The solution was extracted twice with fifteen volumes of ethyl acetate, and the organic layer containing the conjugates was evaporated to dryness under nitrogen gas. The residue was dissolved in 1/15 *M* sodium acetate buffer (pH 5.5), then 3000 units of β-glucuronidase and 10 units of sulfatase were added, and the mixture was incubated at 37°C for 3 h to convert the conjugates into non-conjugated compounds.

Phenylbutazone (100 mg/kg) was given orally to a beagle dog (9 kg body weight) and the concentrations of phenylbutazone and its metabolites, and their conjugates in urine, were determined using the present method. These results obtained are shown in Table II.

TABLE II

CUMULATIVE URINARY EXCRETION OF PHENYLBUTAZONE AND ITS METABOLITES, AND THEIR CONJUGATES AFTER ORAL ADMINISTRATION OF PHENYLBUTAZONE

Each value is the mean (± S.D.) of three determinations of molar per cent of dose. Dose administered was 100 mg/kg.

Compound	Time (h)			
	0—12	12—14	24—48	48—72
Phenylbutazone	0.42 ± 0.29	0.74 ± 0.16	1.35 ± 0.54	1.41 ± 0.52
Phenylbutazone conjugate	0.54 ± 0.32	1.01 ± 0.21	1.09 ± 0.18	1.09 ± 0.18
Oxyphenbutazone	0.00	0.05 ± 0.01	0.29 ± 0.10	0.29 ± 0.10
Oxyphenbutazone conjugate	0.39 ± 0.15	2.04 ± 0.44	3.39 ± 0.58	3.55 ± 0.62
γ-Hydroxyphenylbutazone	2.40 ± 1.60	3.78 ± 0.68	19.47 ± 0.60	20.70 ± 0.51
γ-Hydroxyphenylbutazone conjugate	0.13 ± 0.09	0.47 ± 0.16	1.01 ± 0.35	1.01 ± 0.35

The precision and sensitivity of the assay described appear to be satisfactory for the determination of the plasma and urine levels of phenylbutazone and its metabolites.

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