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GAS CHROMATOGRAPHIC–MASS FRAGMENTOGRAPHIC DETERMINATION OF PROPIVERINE AND ITS METABOLITES IN PLASMA AND URINE

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SUMMARY

1-Methyl-4-piperidyl diphenylpropoxyacetate hydrochloride has been developed clinically for the therapy of urinary bladder dysfunction. A gas chromatographic–mass fragmentographic method was developed for the determination of this drug and its seven metabolites in plasma and urine. The sample was first treated with a Sep-Pak C_{18} cartridge, the methanol eluate was evaporated to dryness, and the resulting residue was redissolved in distilled water. This solution was then extracted with chloroform and adjusted to pH 9.0 with 0.1 *M* sodium borate solution. The acidified aqueous layers were extracted with ethyl acetate. The chloroform layer, which contained non-polar metabolites, was concentrated to dryness, then subjected to trifluoroacetylation, decomposition and methylation. The extract from the plasma sample was trimethylsilylated. The dried residue of the ethyl acetate layer, which contained polar metabolites, was subjected to methylation, trifluoroacetylation and decomposition. Aliquots of each reactant solution were injected into the gas chromatograph–mass spectrometer and analysed by the selected-ion monitoring method using an internal standard. Detection was limited to 1–2 ng/ml of plasma and urine for each metabolite. A precise and sensitive assay for the determination of 1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride and its metabolites in plasma and urine was thus established, and it should prove useful in basic and clinical pharmacological studies.

INTRODUCTION

1-Methyl-4-piperidyl diphenylpropoxyacetate hydrochloride [P-4, propiverine] has been developed for the treatment of hypertonic functional states in the region of the bladder, such as pollakisuria, nocturia and nocturnal enuresis [1–7].

In a study of the metabolic fate of P-4, 1-methyl-4-piperidyl diphenylpropoxyacetate N-oxide [P-4(N→O)], 1-methyl-4-piperidyl benzilate (DPr-P-4), 1-methyl-4-piperidylbenzilate N-oxide [DPr-P-4(N→O)], 4-piperidyl diphenyl-

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propoxyacetate (DM-P-4), ω-1-hydroxypropoxy benzilic acid lactone [ω-1-OH-Pr-BA(L)], ω-1-hydroxypropoxy benzilic acid (ω-1-OH-Pr-BA) and benzilic acid (BA) were isolated and identified as major metabolites. The chemical structures of P-4 and these metabolites are illustrated in Fig. 1.

Although the basic and clinical applications of P-4 at a dose of 5–30 mg per body to dogs or humans have been investigated, pharmacokinetic studies have been limited by the lack of a specific and sensitive assay. Therefore, various methods using gas chromatography (GC), high-performance liquid chromatography (HPLC) or high-performance thin-layer chromatography (HPTLC) were first examined. However, none of these was suitable because of their low sensitivity and poor selectivity.

Accordingly, a gas chromatographic–mass fragmentographic (GC–MF) method was then investigated, and a specific, precise and sensitive method was developed for measuring P-4 and its metabolites at a concentration of 1–2 ng/ml of plasma and urine.

EXPERIMENTAL

Materials

P-4 was obtained from VEB Sachsisches Serumwerk Dresden (Dresden, G.D.R.) and its metabolites, P-4(N→O), DPr-P-4, DPr-P-4(N→O), DM-P-4, ω-1-OH-Pr-BA(L), ω-1-OH-Pr-BA and the internal standard, *n*-butyl O-propylbenzilate (Bu-Pr-BA), were synthesized and purified in our laboratory. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine for the silylating solvent were purchased from Pierce (Rockford, IL, U.S.A.). BA, trifluoroacetic acid anhydride (TFAA) and other chemicals used were all purchased from Wako (Osaka, Japan). Chloroform, ethyl acetate and methanol were of liquid chromatographic reagent grade.

Extraction procedure

Blood samples were collected in heparinized containers and centrifuged at 5°C for 15 min (2000 *g*) to separate the plasma. The plasma and urine then were frozen until analysis.

The plasma or urine samples (each 1.0 ml) were diluted to 2.0 ml with 5 m*M* KH_2PO_4, then applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.), which was activated before use with 5-ml volumes of methanol, distilled water and 5 m*M* KH_2PO_4, in that order, washed with 3 ml of distilled water, then eluted with 5 ml of methanol. The methanol eluate was dried under a stream of nitrogen gas, and the resulting residue was redissolved in 0.2 ml of methanol, then diluted with 2.0 ml of distilled water. This solution was adjusted to pH 9.0 with 0.2 ml of 0.1 *M* $Na_2B_4O_7$ and extracted with 20 ml of chloroform at room temperature for 10 min.

This extraction was repeated, and the combined chloroform extracts containing P-4, P-4(N→O), DPr-P-4, DPr-P-4(N→O), DM-P-4 and ω-1-OH-Pr-BA(L) were evaporated to dryness at water temperature (<20°C), transferred to a 10-ml test-tube by washing with chloroform and dried under nitrogen gas.

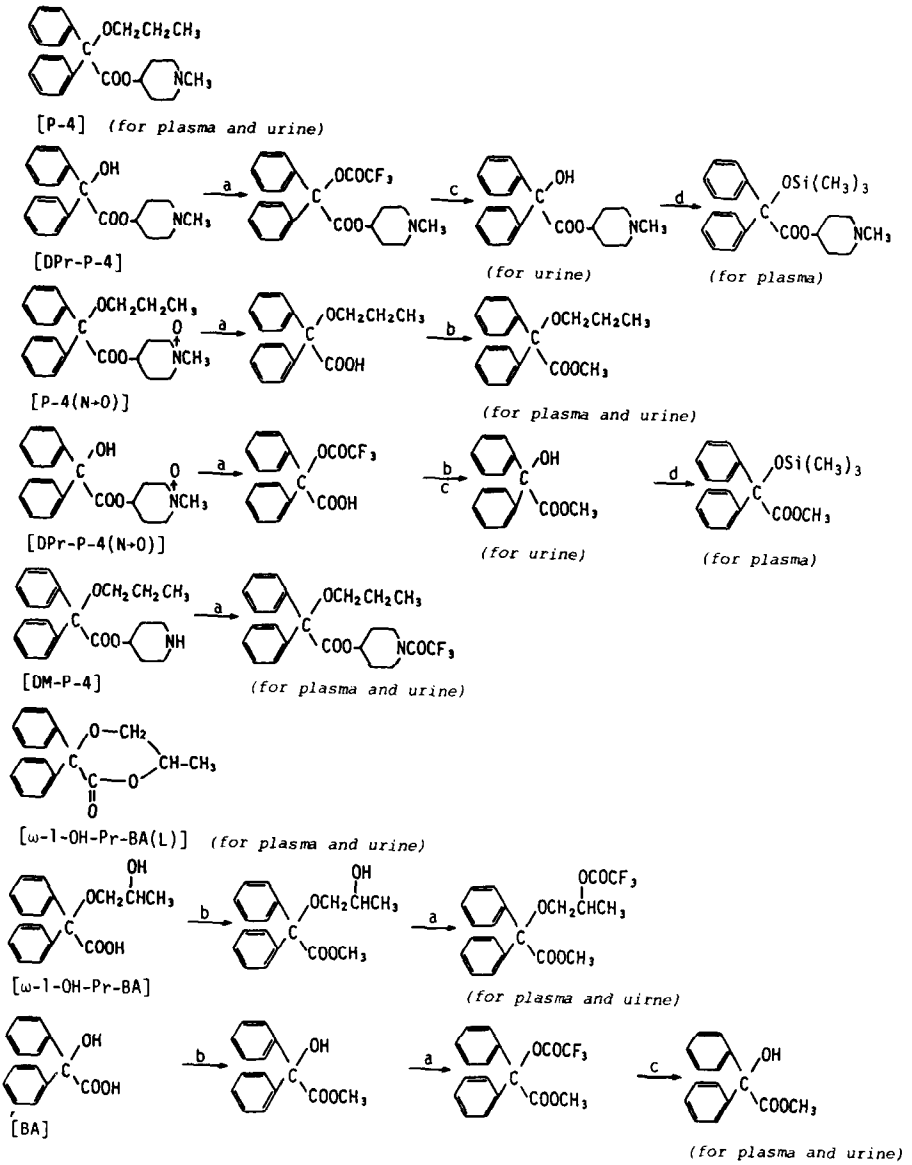

Fig. 1. Derivatization procedures of P-4 and its metabolites: (a) trifluoroacetylation; (b) methylation; (c) decomposition; (d) trimethylsilylation.

The aqueous layer, containing ω-1-OH-Pr-BA and BA separated from the chloroform layer, was acidified to pH 3–4 with 1 *M* hydrochloric acid, then extracted with 40 ml of ethyl acetate by vigorous shaking at room temperature for 20 min. The ethyl acetate layer was evaporated at 25 °C, transferred to a 10-ml test-tube by washing with methanol and dried under nitrogen gas at 25 °C.

Derivatization

The chloroform extract was subjected to trifluoroacetylation at room temperature for 30 min by addition of 0.3 ml of a mixed solution of TFAA and dichlo-

romethane (1:2, v/v). The residue obtained after drying under nitrogen gas at 5°C was next methylated at room temperature for 30 min by addition of 0.2 ml of diethyl ether solution containing saturated diazomethane. The reactant solution was dried under nitrogen gas at 5°C, then dissolved in 0.2 ml of methanol using an ultrasonic generator, and allowed to stand at room temperature for 30 min to decompose. The resulting solution was dried under nitrogen at 5°C, and the residue was stored at −20°C overnight or longer, then redissolved in 0.2 ml of methanol containing an internal standard (Bu-Pr-BA, 25 ng per 0.2 ml for plasma samples and 125 ng per 0.2 ml for urine samples) using an ultrasonic generator. The supernatant obtained after centrifugation at 5°C for 5 min (2000 *g*) was dried under nitrogen gas at 5°C. Then the residue obtained from the urine samples was dissolved in 50 μl of chloroform, and 1 μl of this solution was injected into the gas chromatograph–mass spectrometer.

The residue prepared from the plasma sample was further subjected to trimethylsilylation at 70°C for 30 min by addition of a freshly prepared solution of 50 μl of pyridine containing 20% BSTFA. Then 1 μl of the solution was injected.

The ethyl acetate extract was first methylated with 0.2 ml of saturated diazomethane in diethyl ether at room temperature for 30 min and then evaporated to dryness under nitrogen gas at 5°C. The resulting residue was next subjected to trifluoroacetylation at room temperature for 30 min by addition of 0.3 ml of TFAA and dichloromethane solution (1:2, v/v), and subsequently the solution was dried under nitrogen gas at 5°C and redissolved in 0.2 ml of methanol containing an internal standard (amounts added were the same as those for the chloroform extract). After centrifugation (5°C, 2000 *g*, 5 min), the supernatant was again evaporated to dryness under nitrogen gas at 5°C and dissolved in 50 μl of chloroform. Then 1 μl of the solution was subjected to GC–MF analysis.

Gas chromatography–mass fragmentography

A JEOL JMS DX-303 gas chromatograph–mass spectrometer, with an electron-impact (EI) ion source and equipped with a JMA-DA5100 data system (Tokyo, Japan), was used.

The chemical bonded-type fused-silica capillary column of the gas chromatograph was coated with methylsilicone (25 m × 0.33 mm I.D., Shimadzu, Kyoto, Japan) and was conditioned at 280°C for 24 h. The injector, separator and ion source temperatures were 280, 280 and 250°C, respectively. Analyses were carried out with an initial column temperature of 50°C and a temperature rise of 16°C/min to 300°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The split ratio was 20:1. This splitless injection was carried out using a moving needle.

The mass spectrometer was operated under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3.0 kV; ion multiplier voltage, 1.6–2.0 kV. For the chloroform extract prepared from plasma,the mass fragment ions selected were the *m/z* 183 and 225 ions of P-4, P-4(N→O) and DM-P-4, the *m/z* 255 ion of DPr-P-4 and DPr-P-4(N→O), the *m/z* 182 ion of ω-1-OH-Pr-BA(L) and an additional *m/z* 155 ion of ω-1-OH-Pr-BA. For the chloroform extract prepared from urine, on the other hand, the *m/z*

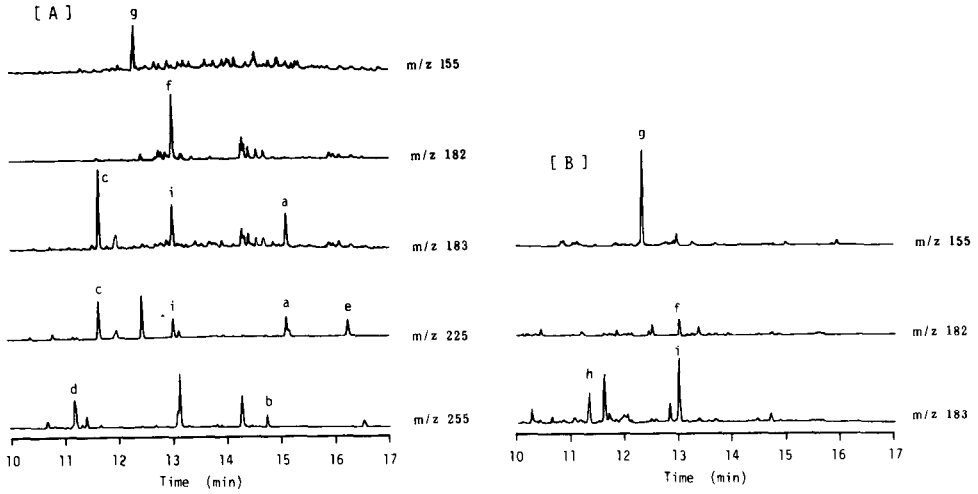

Fig. 2. Typical mass fragmentograms showing the separation of P-4, its metabolites and internal standard in beagle dog plasma prepared from (A) the chloroform extract and (b) the ethyl acetate extract. Peaks: a = P-4; b = DPr-P-4; c = P-4(N→O); d = DPr-P-4(N→O); e = DM-P-4; f = ω-1-OH-Pr-BA(L); g = ω-1-OH-Pr-BA; h = BA; i = internal standard (Bu-Pr-BA).

183 ion of P-4, P-4(N→O), DPr-P-4, DPr-P-4(N→O) and DM-P-4, the *m/z* 225 ion of P-4, P-4(N→O) and DM-P-4, the *m/z* 182 ion of ω-1-OH-Pr-BA(L) and an additional *m/z* 155 ion of ω-1-OH-Pr-BA were monitored. The selected fragment ions of ω-1-OH-Pr-BA and BA in the ethyl acetate extracts prepared from plasma and urine were the *m/z* 155 and 183 ions, respectively, and an additional *m/z* 182 ion of ω-1-OH-Pr-BA(L). The detected fragment ions of the internal standard, Bu-Pr-BA, were the *m/z* 183 and 225 ions for all samples.

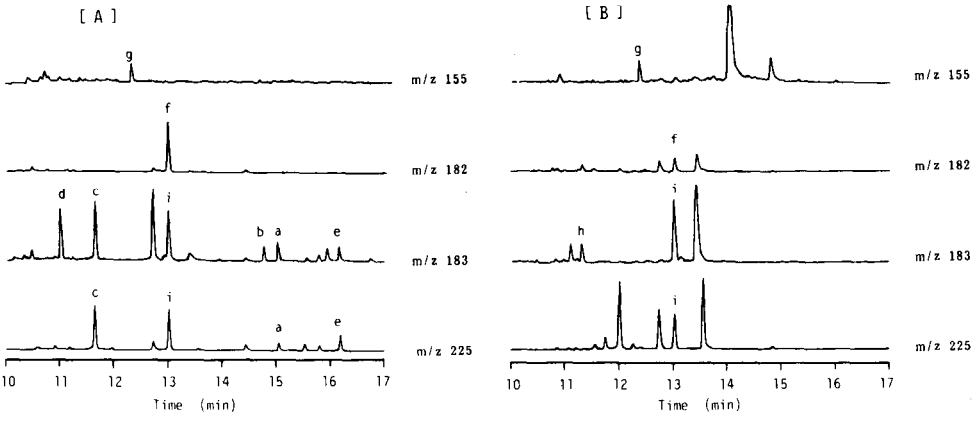

Fig. 3. Typical mass fragmentograms showing the separation of P-4, its metabolites and internal standard in beagle dog urine prepared from (A) the chloroform extract and (B) the ethyl acetate extract. Peaks as in Fig. 2.

Calibration curves

Calibration curves were prepared by adding known amounts of P-4 and its metabolites (2, 5, 10, 20 and 50 ng/ml for plasma and 20, 50, 100, 200 and 500 ng/ml for urine) to 1.0-ml aliquots of beagle dog plasma and urine, and then analysing the mixture using the same extraction procedure and derivatization.

Calibration curves for the determination of P-4 and its metabolites by GC–MF were obtained by plotting the ratio of the peak heights of the respective metabolites to that of the internal standard against the concentrations of these compounds. All of the calibration curves gave good results.

RESULTS AND DISCUSSION

Extraction procedure

Two independent assay procedures were investigated first on the basis of the physicochemical properties of P-4 and its metabolites. P-4, P-4(N→O), DPr-P-4, DPr-P-4(N→O), DM-P-4 and ω-1-OH-Pr-BA(L) could be recovered from aqueous solution with an organic solvent, while ω-1-OH-Pr-BA and BA remained in the aqueous layer. In the subsequent extraction examinations at several pH values, however, ω-1-OH-Pr-BA and BA in the aqueous layer prepared from the plasma samples resulted in low recoveries because of apparent co-precipitation with protein in the aqueous layer.

Therefore, preparation using a Sep-Pak C_{18} cartridge was carried out in the first step of the extraction procedure for both the plasma and urine samples. A Sep-Pak C_{18} cartridge must be activated with methanol, distilled water and 5 m*M* KH_2PO_4 in that order before use. The sample, diluted with 5 m*M* KH_2PO_4, was applied to this activated cartridge, washed with distilled water and eluted with methanol. This step gave quantitative recoveries of P-4 and its metabolites, and furthermore showed no disturbance of the protein against recoveries in the subsequent extraction procedure.

Of the extraction procedures tested for the diluted sample after preparation by Sep-Pak C_{18} cartridge, the following procedure was found to be the most reliable with the highest recovery for all compounds, and no decomposition was observed. The extraction with chloroform from an aqueous solution, adjusted to pH 9.0 with sodium borate solution, was employed for quantitative separation of P-4, P-4(N→O), DPr-P-4, DPr-P-4(N→O), DM-P-4 and ω-1-OH-Pr-BA(L) from ω-1-OH-Pr-BA and BA. The addition of 1 *M* hydrochloric acid to the aqueous layer containing the remaining ω-1-OH-Pr-BA and BA, adjustment to pH 3–4, and subsequent extraction with ethyl acetate resulted in good, constant recoveries. Each extract was then derivatizated.

Derivatization

In determining the concentrations of P-4 and its metabolites by GC–MF, it was possible to analyse P-4, DPr-P-4 and ω-1-OH-Pr-BA(L) directly, whereas other metabolites had to be derivatized because of their non-volatility or adsorption on the column packing. In particular, the N-oxide metabolites of P-4(N→O)

and DPr-P-4(N→O) were decomposed by the heat of the injection port to P-4 and DPr-P-4, respectively.

For N-oxide metabolites of P-4(N→O) and DPr-P-4(N→O), decomposition [8, 9] or reaction [10] with acetic acid anhydride, thermal cleavage [11] or deoxygenation with reducing agents [12] were first investigated on the basis of previous reports. However, no satisfactory results could be obtained. During the investigation of several reactions or derivatizations, on the other hand, it was found that the piperidine moieties of P-4(N→O) and DPr-P-4(N→O) were cleaved by trifluoroacetylation with TFAA in dichloromethane to form the respective carboxylic acid derivatives, and the hydroxyl group of DPr-P-4(N→O) was trifluoroacetylated at the same time. This result was very useful and appropriate for the investigation by GC–MF of the derivatization procedure of these N-oxide metabolites, and also indicated the possibility of further derivatization. Thus, trifluoroacetylation was used in this first step of derivatization.

The metabolites with a carboxylic acid moiety, ω-1-OH-Pr-BA and BA, were subjected to methylation and subsequent trifluoroacetylation.

The trifluoroacetylated derivatives of DPr-P-4, DPr-P-4(N→O) and BA were not found to produce a higher detection sensitivity and a better separation by GC–MF. Accordingly, the procedure of decomposing the trifluoroacetyl group by methanol to the hydroxyl group was used in the derivatization procedure. Furthermore, DPr-P-4 and DPr-P-4(N→O) in the plasma extract were trimethylsilylated to obtain a clear separation from the plasma constituents.

All these derivatizations and decompositions gave quantitative results.

On the basis of the results and information described above, the following derivatization procedures were employed for P-4 and its metabolites. The chloroform extract containing P-4(N→O), DPr-P-4, DPr-P-4(N→O) and DM-P-4 was first trifluoroacetylated [this procedure was for DPr-P-4, DPr-P-4(N→O) and DM-P-4, whereas it is also the decomposition procedure for P-4(N→O) and DPr-P-4(N→O)], then methylated [this procedure was for P-4(N→O) and DPr-P-4(N→O)], and then decomposed with methanol [this procedure was for DPr-P-4 and DPr-P-4(N→O)]. The extract from the plasma sample was further trimethylsilylated [this procedure was for DPr-P-4 and DPr-P-4(N→O)].

On the other hand, the ethyl acetate extract containing ω-1-OH-Pr-BA and BA was first methylated, then trifluoroacetylated (these procedures were for ω-1-OH-Pr-BA and BA), and then decomposed with methanol (this procedure was for BA).

These derivatization procedures are summarized in Fig. 1. The trimethylsilyl derivatives were found to be stable at 5°C for one to two days, and methyl and trifluoroacetyl derivatives were stable at 5°C for several weeks. In addition, the same derivative was produced from DPr-P-4(N→O) and BA, but since the two compounds were well separated by the chloroform extraction, there is no interference in the quantitation. The internal standard was added in the final step, since the recoveries of P-4 and its metabolites under the present extraction and derivatization procedures were quantitative and constant. The procedure of drying under nitrogen gas had to be carefully carried out at 5°C, because of the volatile nature of the derivatives of P-4(N→O),DPr-P-4(N→O) and BA.

Gas chromatography–mass fragmentography

In the EI mass spectra of P-4 and its metabolites DPr-P-4, DPr-P-4(N→O), ω-1-OH-Pr-BA(L) and BA gave their respective molecular ions, but their relative intensities were very low, and other metabolites gave no molecular ions. Therefore, the following base peaks and characteristic major fragment ions were selected for GC–MF analysis, since no influences of biological constituents could be observed: the *m/z* 183 ion $[(C_6H_5)_2COH$, base peak$]^+$ of P-4, P-4(N→O) and DM-P-4, the *m/z* 225 ion of P-4 $[M-COOC_5H_9NCH_3]^+$, P-4-(N→O) $[M-COOCH_3$, base peak$]^+$ and DM-P-4 $[M-COOC_5H_9NCOCF_3]^+$, the *m/z* 255 ion of DPr-P-4 $[M-COOC_5H_9NCH_3$, base peak$]^+$ and DPr-P-4(N→O) $[M-COOCH_3$, base peak$]^+$, and the *m/z* 182 ion $[(C_6H_5)_2CO$, base peak$]^+$ of ω-1-OH-Pr-BA(L) were detected for analysis of the derivatized chloroform extract from plasma. For the determination of the derivatized chloroform extract from urine, the *m/z* 183 ion $[(C_6H_5)_2COH$, base peak$]^+$ of P-4, P-4(N→O), DPr-P-4, DPr-P-4(N→O) and DM-P-4, the *m/z* 225 ion of P-4 $[M-COOC_5H_9NCH_3]^+$, P-4(N→O) $[M-COOCH_3]^+$ and DM-P-4 $[M-COOC_5H_9NCOCF_3]^+$, and the *m/z* 182 ion $[(C_6H_5)_2CO$, base peak$]^+$ of ω-1-OH-Pr-BA(L) were selected. On the other hand, the *m/z* 155 ion $[C_5H_6F_3O_2$, base peak$]^+$ of ω-1-OH-Pr-BA and the *m/z* 183 ion $[(C_6H_5)_2COH]^+$ of BA were monitored for the assay of the derivatized samples of the ethyl acetate extract from plasma and urine.

In GC–MF analysis, Bu-Pr-BA was chosen as an internal standard for multiple ion detection, because this compound showed a similar mass spectral pattern to those of P-4 and its metabolites and a suitable retention time. The detected mass fragment ions of the internal standard, Bu-Pr-BA, were the *m/z* 183 $[(C_6H_5)_2COH]^+$ and 225 $[M-COOC_4H_9$, base peak$]^+$ ions for all prepared samples.

For P-4, P-4(N→O), DM-P-4 and internal standard, two characteristic mass fragment ions were monitored simultaneously, since neither of the ions was separated clearly in some samples. None of the fragment ions of other metabolites detected had any appreciable influence on the biological constituents and gave an excellent separation on measurement by GC–MF.

Some of the ω-1-OH-Pr-BA(L) and ω-1-OH-Pr-BA at high concentrations were cleaved by the heat of the injection port to ω-1-OH-Pr-BA and ω-1-OH-Pr-BA(L), respectively. However, this problem was overcome by simultaneous monitoring of the *m/z* 155 ion of ω-1-OH-Pr-BA and the *m/z* 183 ion of ω-1-OH-Pr-BA(L) for all prepared samples, and by correction of the respective concentrations.

Typical GC–MF profiles of P-4, its metabolites and internal standard, Bu-Pr-BA, prepared from beagle dog plasma and urine following administration of P-4 are illustrated in Figs. 2 and 3. The retention times of P-4, P-4(N→O), DM-P-4 and ω-1-OH-Pr-BA(L) prepared from plasma and urine were 15.1, 11.4, 16.2 and 13.0 min, those of DPr-P-4 and DPr-P-4(N→O) prepared from plasma were 14.7 and 11.2 min, those of DPr-P-4 and DPr-P-4(N→O) prepared from urine were 14.8 and 11.0 min, those of ω-1-OH-Pr-BA and BA prepared from plasma

TABLE I

RECOVERIES OF P-4 AND ITS METABOLITES FROM PLASMA AND URINE

Amounts added are described in the text, and each value is the total mean (±S.D.) of three determinations for each added amount.

Metabolite	Recovery from plasma (%)	Recovery from urine (%)
P-4	84.8±3.1	96.8±1.7
P-4(N→O)	85.6±3.4	82.5±3.8
DPr-P-4	90.6±2.9	86.6±2.4
DPr-4(N→O)	86.2±2.6	83.1±3.1
DM-P-4	88.1±3.7	87.4±2.4
ω-1-OH-Pr-BA(L)	94.1±2.4	91.8±2.3
ω-1-OH-Pr-BA	95.4±1.8	94.7±1.6
BA	70.1±3.9	75.4±4.1

and urine were 11.3 and 12.3 min, and that of the internal standard, Bu-Pr-BA, was 13.1 min.

Recovery, sensitivity and accuracy

Known amounts of P-4 and its metabolites were added to control plasma and urine of beagle dogs at concentrations of 2, 5, 10, 20 and 50 ng/ml for plasma and 20, 50, 100, 200 and 500 ng/ml for urine, and the samples were analysed. As summarized in Table I, the overall recoveries of each compound prepared from plasma and urine were 90.8% for P-4, 84.1% for P-4(N→O), 88.6% for DPr-P-4, 84.7% for DPr-4(N→O), 87.8% for DM-P-4, 93.0% for ω-1-OH-Pr-BA(L), 95.1% for ω-1-OH-Pr-BA and 72.8% for BA.

The detection limits for P-4 and its metabolites using the present method were 1–2 ng/ml of plasma and urine for each compound. The reproducibility of this method was ±2.1–3.3% at a concentration of 2–500 ng/ml for each metabolite.

Stability

The stabilities of P-4 and its metabolites were investigated. P-4 and DPr-P-4 in plasma and urine were N-oxidized to produce P-4(N→O) and DPr-P-4-(N→O), respectively, at above room temperature. However, no significant degradations of P-4 and DPr-P-4 in frozen plasma and urine samples were observed during a two-week period or longer, and these metabolites in samples standing at 5°C were stable over 24 h. Other metabolites were stable for several weeks at less than 5°C. On the basis of these results obtained, it was evident that samples must be collected at less than 5°C and immediately frozen until used for analysis.

Application

P-4 was given intravenously at a dose of 10 mg/kg to beagle dogs, and the time-course of changes in the concentrations of P-4 and its metabolites in plasma were measured by the present method (Fig. 4). DM-P-4 was not observed in this experiment.

The present method was also applied to other biological fluids. The results

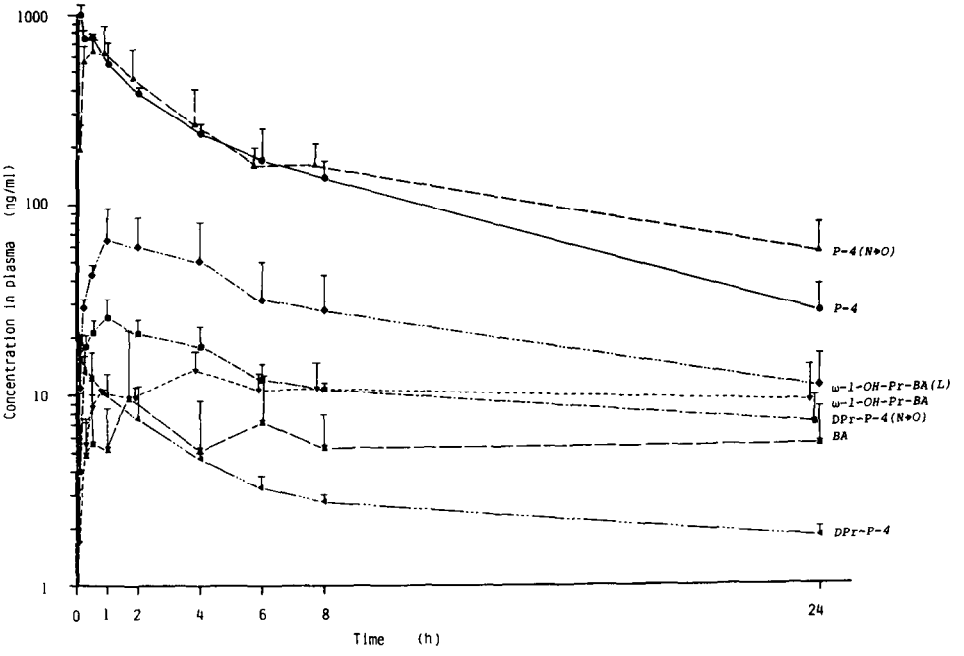

Fig. 4. Plasma levels of P-4 and its metabolites after intravenous administration of P-4 at a dose of 10 mg/kg to beagle dogs. Each point is the mean ± S.D. of three dogs.

obtained for the chromatographic separation, recovery, sensitivity and precision were in good agreement with those obtained with human plasma and urine. The specification, precision and sensitivity of this assay appear to be satisfactory for the determination of the plasma and urine levels of P-4 and its metabolites in basic and clinical pharmacological studies.

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