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DETERMINATION OF THE THROMBOXANE SYNTHETASE INHIBITOR 6-(1-IMIDAZOLYLMETHYL)-5,6,7,8-TETRAHYDRONAPHTHALENE-2-CARBOXYLIC ACID (DP-1904) IN HUMAN PLASMA AND URINE USING SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of 6-(1-imidazolylmethyl)-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid hydrochloride hemihydrate (DP-1904), a potent and long-acting thromboxane synthetase inhibitor, in human plasma and urine has been developed. DP-1904 and an internal standard were extracted from plasma and urine by means of a Sep-Pak C_{18} cartridge. The methanol eluate was evaporated and the residue was chromatographed on a reversed-phase column using tetrahydrofuran–0.5% potassium dihydrogen phosphate solution (pH 3.0) (1:16) as the mobile phase at a flow-rate of 1.2 ml/min. Ultraviolet detection at 240 nm resulted in limits of detection of 50 ng/ml for plasma and 1.0 μg/ml for urine. The method showed satisfactory accuracy and precision. The method was applied to the determination of DP-1904 in plasma and urine samples from a normal human volunteer who had received a 200-mg oral dose of the drug. DP-1904 was rapidly absorbed from the gastrointestinal tract and had a half-life of ca. 30 min. The primary route of elimination was renal, with ca. 60% of the dose being excreted in the urine in the unchanged form within 48 h.

INTRODUCTION

6-(1-Imidazolylmethyl)-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid hydrochloride hemihydrate (DP-1904) (Fig. 1) is a novel thromboxane synthetase inhibitor with a long duration of action. It is well known that thromboxane A_2 is produced in large amounts when platelets are activated by a variety of agonists such as collagen and ADP. This metabolite of arachidonic acid, via the cyclooxygenase and thromboxane synthetase pathway, is a potent platelet agonist and a vasoconstrictor [1–4]. Therefore, DP-1904 has potential in the treatment of var-

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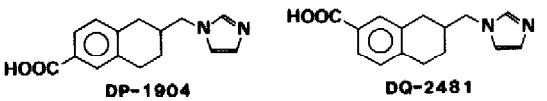

Fig. 1. Structures of DP-1904 and DQ-2481.

ious cardiovascular disorders where vasospasm or thrombosis may be contributing factors [5].

DP-1904 is currently under clinical investigation in Japan, and a sensitive and selective method for its determination in biological fluids is needed in order to study its absorption, elimination and metabolism in humans.

This paper reports a high-performance liquid chromatographic (HPLC) method for the determination of DP-1904 in human plasma and urine. Octadecylsilyl-bonded silica gel is used for the extraction of DP-1904 from plasma and urine and has sufficient specificity and sensitivity to determine 50 ng/ml of DP-1904 in plasma and 1.0 μg/ml in urine. The method was applied to evaluate the pharmacokinetics of DP-1904 after oral doses.

EXPERIMENTAL

Materials

DP-1904 and the internal standard used for the plasma and urine assay, 6-(1-imidazolylmethyl)-5,6,7,8-tetrahydronaphthalene-3-carboxylic acid hydrochloride (DQ-2481) (Fig. 1) were synthesized in the Research Institute of Daiichi Seiyaku (Tokyo, Japan) [6,7]. Stock solutions of DP-1904 (100 mg) and DQ-2481 (23 mg) in methanol (100 ml) were prepared. Intermediate and working standard solutions were prepared by diluting the stock solution with distilled water. Both the stock solution and the working standard solution were stable for at least 3 months when stored at 4°C. All other chemicals were of analytical-reagent grade and used without further purification.

A Sep-Pak C_{18} cartridge was obtained from Waters Assoc. (Milford, MA, U.S.A.) and activated prior to use by passage of methanol (2×5 ml) and distilled water (2×5 ml).

Instruments and chromatographic conditions

The chromatographic system consisted of a high-performance liquid chromatograph (Model 655A, Hitachi, Tokyo, Japan), a sample injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop and a variable-wavelength UV detector (Model 655A, Hitachi). The absorbance range was 0.02 for plasma analysis and 0.08 for urine analysis. A reversed-phase TSK-GEL ODS-80 TM (5 μm) column (150×4.6 mm I.D.) (Toyo Soda, Tokyo, Japan) was used. A pre-column filter (Irika, Kyoto, Japan) was attached ahead of the analytical column. The mobile phase was a mixture of tetrahydrofuran and 0.5% potassium dihydrogen phosphate solution (adjusted to pH 3.0 with phosphoric acid) (1:16, v/v). The mobile phase was deaerated by applying reduced pressure and sonification before use. The operating conditions were as follows: flow-rate, 1.2 ml/min; de-

tection wavelength, 240 nm; volume injected, 10 μl. Under these conditions the retention times of DP-1904 and DQ-2481 were 6.1 and 10.0 min, respectively.

Assay procedure

Depending on the expected concentration of DP-1904, human plasma and urine were diluted appropriately with distilled water.

Plasma sample preparation. To neat or diluted plasma (1.0 ml) were added distilled water (3 ml) and internal standard (460 ng) and the mixture was vortexed and applied to a Sep-Pak C_{18} cartridge. After washing successively with water (6 ml) and *n*-hexane (5 ml), DP-1904 and DQ-2481 were eluted with methanol (3 ml). The eluate was evaporated to dryness under a stream of air in a water-bath maintained at approximately 30°C. The residue was dissolved in mobile phase (0.1 ml) and 10 μl of the solution obtained were injected into the HPLC system.

Urine sample preparation. The procedure was the same as that for plasma except that 4.6 μg of internal standard were used and the Sep-Pak C_{18} cartridge was washed with 10% aqueous methanol (8 ml) instead of distilled water.

Calibration graphs

Calibration graphs were prepared by adding different known amounts of DP-1904 to drug-free plasma and urine and carrying out the procedure described above. The peak-height ratio of DP-1904 to the internal standard was plotted against the concentration of DP-1904. The calibration graph for DP-1904 in plasma covered the concentration range 0.05–1.0 μg/ml and for urine 1.0–20 μg/ml.

Drug administration and sample collection

The studies were conducted on a healthy male volunteer who fasted for 12 h prior to and 4 h after dosing. Venous blood (7 ml) was collected from the subject into tubes containing heparin as anticoagulant at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 h. After centrifuging at 1800 *g* for 15 min, plasma was separated and frozen at −20°C until analysed. Urine was obtained at 0, 0–0.5, 0.5–1, 1–2, 2–4, 4–6, 6–8, 8–12, 12–24 and 24–48 h. Each urine specimen was mixed thoroughly, the volume was measured and an aliquot was frozen at −20°C until analysed.

Treatment of data

The plasma concentration of DP-1904 was plotted against time and pharmacokinetic parameters for the resulting curves were calculated using a curve-fitting programme [8]. A one-compartment open model with first-order absorption after a lag time was used to describe the plasma concentration of DP-1904 after oral administration. The area under the plasma concentration curve (AUC) from the time of administration to 24 h after dosing was calculated using the linear trapezoidal method.

RESULTS AND DISCUSSION

Chromatographic conditions

Various reversed-phase columns were tested for the separation of DP-1904 and the internal standard using a mixture of methanol and 0.5% potassium dihydro-

gen phosphate (pH 3.0) as the mobile phase. DP-1904 was eluted as a very broad and tailing peak when a Nucleosil 5 C_{18} column (250×6.0 mm I.D.) or a LiChrosorb RP-18 column (125×4.0 mm I.D.) was used. A typical chromatogram is shown in Fig. 2A. Further, an increase in the pH of the buffer solution resulted in the strong retention of DP-1904 on these columns and a much higher methanol content in mobile phase was needed for the elution of DP-1904 from the columns. This may be due to the strong interaction of the imidazole moiety of DP-1904 with the remaining free silanol groups on the reversed-phase column packing materials.

Sharp and well defined chromatographic peaks for DP-1904 and the internal standard were obtained on the 5 μm TSK-GEL ODS-80TM column (150×4.6 mm I.D.), where the free silanol groups were almost completely end-capped with trimethylchlorosilane (Fig. 2B). Therefore, studies to optimize the chromatographic conditions were conducted employing the TSK-GEL ODS-80TM column. Methanol, acetonitrile and tetrahydrofuran were tested as organic modifiers. The best separation of DP-1904 and DQ-2481 from endogenous interfering components in plasma and urine extracts was achieved by using tetrahydrofuran–0.5% potassium dihydrogen phosphate (pH 3.0) (1:16).

Clean-up of DP-1904 in human plasma and urine

Conventional methods for the determination of many drugs in plasma and urine involve a solvent extraction step, which is simple and economical, prior to the chromatographic step. However, this procedure proved not to be applicable to DP-1904 in plasma or urine because it is amphoteric and hardly extractable with organic solvents such as benzene, chloroform or ethyl acetate.

The use of bonded-phase packing materials has been applied to the extraction of drugs in plasma and urine with improvements in assay times, simplicity and recoveries [9,10]. Therefore, we decided to use a Sep-Pak C_{18} cartridge for the determination of DP-1904 in plasma and urine, as DP-1904 is readily adsorbed on this cartridge.

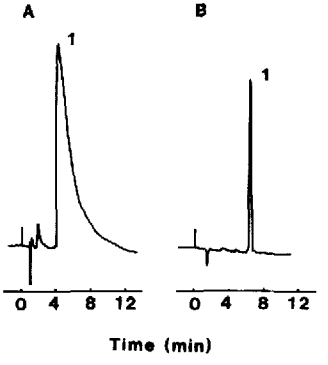

Fig. 2. Chromatograms of a standard sample of DP-1904 (peak 1) using methanol-0.5% KH_2PO_4 (pH 3.0) (1:1 for A, 1:3 for B) as the mobile phase at a flow-rate of 1.2 ml/min. Column: (A) LiChroCART RP-18 (125×4.0 mm I.D.); (B) TSK-GEL ODS-80TM (150×4.6 mm I.D.). Amount injected: (A) 1.0 μg; (B) 0.1 μg. Detection. 240 nm, 0.04 a.u.f.s.

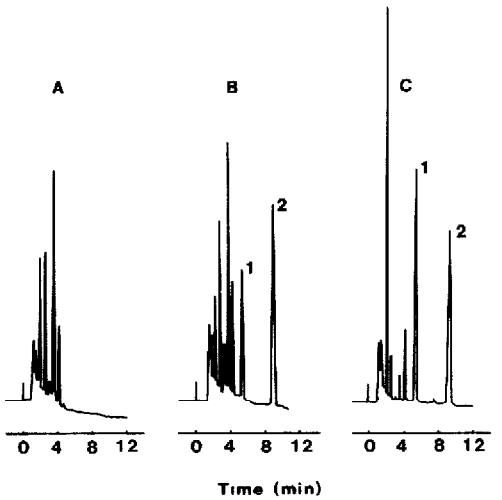

Fig. 3. Representative chromatograms of 1-ml plasma extracts obtained with (A) blank plasma, (B) blank plasma spiked with DP-1904 (200 ng) and internal standard (460 ng) and (C) plasma from a human volunteer after oral ingestion of a 200-mg tablet. Peaks: 1 = DP-1904; 2 = internal standard. Detection: 240 nm, 0.02 a.u.f.s.

TABLE I

ABSOLUTE RECOVERY OF DP-1904 ADDED TO HUMAN PLASMA

Concentration of DP-1904 (μg/ml)		Recovery (mean ± S.D., $n=7$) (%)	C.V. (%)
Added	Found		
0.050	0.0373	74 5 ± 4.25	5.71
0.200	0.150	74.8 ± 5.14	6.88
0.500	0.383	76 5 ± 2.50	3.26
1.000	0.769	76.9 ± 3.20	4.17

The cartridge was purged from water with *n*-hexane before elution of DP-1904 with methanol to facilitate evaporation of the eluate under a stream of air; DP-1904 and DQ-2481 were not eluted with *n*-hexane. Representative chromatograms for control human plasma, control plasma spiked with DP-1904 and internal standard and human plasma obtained 2 h after ingestion of a 200-mg tablet are shown in Fig. 3. DP-1904 and DQ-2481 were well resolved from endogenous materials in control plasma.

The absolute recovery of DP-1904 from plasma was determined by comparing the peak heights of extracted standards with those of known concentrations injected. The recoveries ranged from 74.5 to 76.9% over the concentration range 0.05–1.0 μg/ml (Table I). In addition, the mean recovery for 0.460 μg/ml of the internal standard was 77.8 ± 2.48% ($n=7$). The within-day precision of the procedure is shown by the coefficient of variation (C.V.) for the seven independent samples at each fortified level, and was excellent, the highest value being 4.08% (Table II). Further, the concentrations found were within 10% of the theoretical

values. The minimum quantifiable level, i.e., the lowest fortified level with a C.V. of 3.80% was 50 ng/ml. The detection limit for DP-1904, based on a signal-to-noise ratio of 4:1, was 10 ng/ml. Calibration graphs obtained over an 8-month period were linear and reproducible with mean ± standard error values for the constants in the regression equation of $y = (3.769 \pm 0.230)x - (0.0011 \pm 0.0541)$ and with a correlation coefficient of > 0.998 ($n = 10$). The between-day coefficient of variation of the slope of the calibration graph was 6.1%.

The clean-up procedure employed for plasma samples proved not to be satisfactory for urine samples because interfering peaks appeared on the chromatograms of urine extracts. In order to remove these interfering endogenous components, the Sep-Pak C_{18} cartridge was washed with 10% methanol prior to elution of DP-1904 with methanol. Typical chromatograms of blank human urine, blank human urine spiked with DP-1904 and internal standard and a 6–8 h urine collection after ingestion of a 200-mg tablet are shown in Fig. 4. Evaluation of the blank urine samples indicated that this modified assay procedure gave chromatograms with minimum interferences with the elution of DP-1904 and the internal standard.

Validation of the urine assay is presented in Table III. It is evident that the modified assay procedure is satisfactory with respect to within-day precision and accuracy, although the recovery of DP-1904 from urine is slightly lower than that from plasma (Table IV). The minimum quantifiable level for urine was 1.0 μg/ml and the detection limit was 0.1 μg/ml (signal-to-noise ratio = 4). The least-squares linear regression equation was $y = (0.356 \pm 0.0137)x + (0.034 \pm 0.057)$ with a correlation coefficient of > 0.998 ($n = 10$). Over an 8-month period, the slopes of the calibration graphs were reproducible (C.V. = 3.8%).

Application of the method

The method was employed to determine DP-1904 in plasma and urine from a human male volunteer following an oral dose of 200 mg. After a lag time of 16 min, DP-1904 was absorbed rapidly from the gastrointestinal tract. The plasma concentration of DP-1904 reached a maximum value 1 h after dosing, then decreased rapidly with a half-life of 33 min (Fig. 5). The area under the plasma concentration–time curve, calculated by the linear trapezoidal method, was 8.65 h μg/ml.

Cumulative urinary excretion data for DP-1904 are presented in Fig. 6. About half of the dose given orally was recovered in the urine in the unchanged form within 48 h after dosing. The excretion of DP-1904 into urine was very rapid and almost completed within 6 h after administration.

In conclusion, C_{18} bonded silica gel has been successfully applied to the initial extraction step of the potent thromboxane synthetase inhibitor DP-1904 from human plasma and urine. The method is satisfactory with respect to sensitivity, precision and accuracy, and has proved to be particularly useful for pharmacokinetic studies of DP-1904 in human volunteers. The details of these studies will be reported elsewhere.

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF DP-1904 IN HUMAN PLASMA

Concentration of DP-1904 (μg/ml)		C.V. (%)
Added	Found*	
0.050	0.0519 ± 0.00197	3.80
0.200	0.196 ± 0.00801	4.08
0.500	0.503 ± 0.00754	1.50
1.000	0.999 ± 0.0270	2.70

*Mean ± S.D. ($n = 7$).

TABLE III

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF DP-1904 IN HUMAN URINE

Concentration of DP-1904 (μg/ml)		C V. (%)
Added	Found*	
1.00	1.006 ± 0.045	4.49
2.00	2.035 ± 0.020	0.98
5.00	4.974 ± 0.123	2.48
10.0	10.02 ± 0.065	0.65

*Mean ± S.D. ($n = 6$).

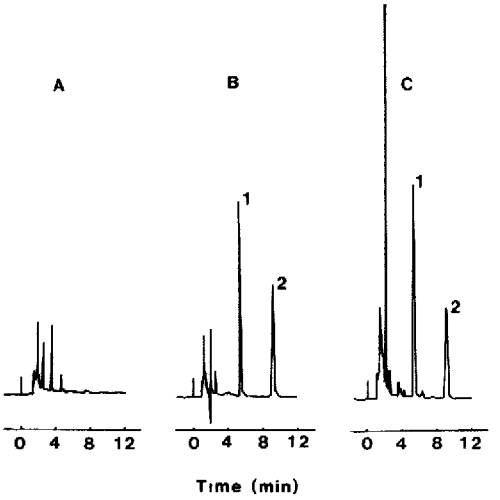

Fig. 4. Representative chromatograms of 1-ml urine extracts obtained with (A) blank urine, (B) blank urine spiked with DP-1904 (5.0 μg) and internal standard (4.6 μg) and (C) urine from a human volunteer after oral ingestion of a 200-mg tablet. Peaks: 1 = DP-1904; 2 = internal standard. Detection: 240 nm, 0.08 a.u.f.s.

TABLE IV

ABSOLUTE RECOVERY OF DP-1904 ADDED TO HUMAN URINE

Concentration of DP-1904 (μg/ml)		Recovery (mean $\pm$S.D., $n=6$) (%)	C.V. (%)
Added	Found		
1.00	0.616	61.6$\pm$3.05	4.96
2.00	1.416	70.8$\pm$2.68	3.79
5.00	3.208	64.2$\pm$3.15	4.91
10.0	6.369	63.7$\pm$1.96	3.07

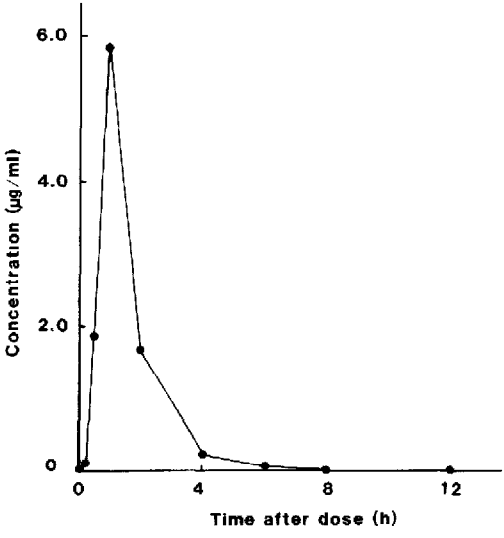

Fig. 5. Change in plasma level of DP-1904 after oral administration of 200 mg to a normal human volunteer.

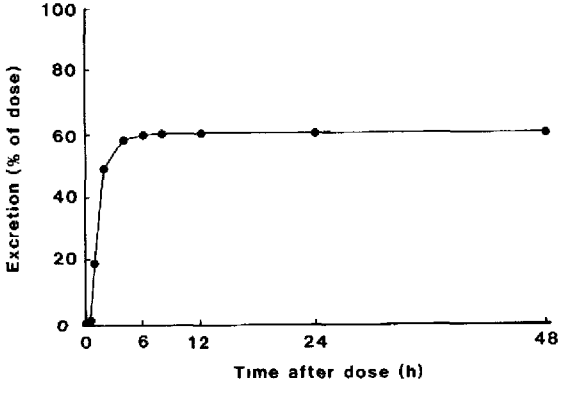

Fig. 6. Cumulative urinary excretion of DP-1904 following oral ingestion of a 200-mg tablet.

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