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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF A NEW NON-STEROIDAL ANTI-INFLAMMATORY AGENT, 2-(10,11-DIHYDRO-10-OXODIBENZO[*b,f*]THIEPIN-2-YL)PROPIONIC ACID, IN HUMAN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic method for the quantitation of a new anti-inflammatory agent, 2-(10,11-dihydro-10-oxodibenzo[*b,f*]thiepin-2-yl)propionic acid (CN-100; I), has been developed. The assay consists in extracting samples containing I and mefenamic acid, the internal standard, under acidic conditions and analysis by reversed-phase chromatography using ultraviolet detection at 330 nm. Preliminary plasma concentration–time and cumulative urinary excretion profiles from a healthy subject following oral administration of the tablet formulation are presented. This method is simple, sensitive and reproducible and is applicable to studies of the pharmacokinetic behaviour of I in humans.

INTRODUCTION

Propionic acid derivatives (ibuprofen, ketoprofen, fenoprofen and naproxen) are anti-inflammatory agents frequently used for the treatment of rheumatic disorders [1,2]. These derivatives are now the main drugs used for symptomatic relief in patients with rheumatoid arthritis [2], mainly because of the significantly lower incidence of side-effects compared with other anti-inflammatory agents such as aspirin [2,3]. However, there have been striking individual variations in response to different propionic acid derivatives among patients with rheumatoid arthritis [4,5]. It may therefore be necessary to try several drugs before finding the right one for a particular patient, and new anti-inflammatory agents are desirable.

2-(10,11-Dihydro-10-oxodibenzo[*b,f*]thiepin-2-yl)propionic acid (CN-100; I) is a newly developed non-steroidal anti-inflammatory agent (Nippon Chemipha,

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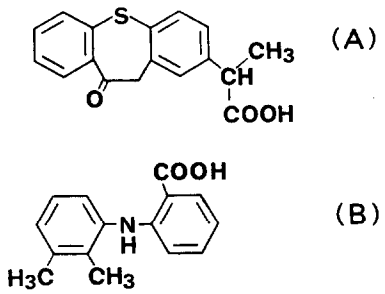

Fig. 1. Structures of (A) I and (B) mefenamic acid, internal standard.

Tokyo, Japan). It has been proved to be a compound with anti-inflammatory, analgesic and antipyretic properties in animals using several experimental test procedures [6]. In order to achieve optimal benefit from any new drug, it is first necessary to understand its disposition characteristics in the human body, and it is therefore essential to establish an effective assay method. This paper describes a rapid, sensitive and reliable high-performance liquid chromatographic (HPLC) assay for determining I in human plasma and urine. The method is considered to be applicable to pharmacokinetic studies.

EXPERIMENTAL

Chemicals and reagents

I and mefenamic acid as an internal standard (Fig. 1) were donated by Nippon Chemipha. Salicylic acid and indomethacin were purchased from Sigma (St. Louis, MO, U.S.A.). Sulindac and diflunisal were supplied by Banyu Pharmaceutical (Tokyo, Japan), ibuprofen and flurbiprofen by Kaken Pharmaceutical (Tokyo, Japan), alclofenac by Chugai Pharmaceutical (Tokyo, Japan), diclofenac sodium by Nippon Ciba-Geigy (Hyougo, Japan), fenoprofen calcium by Yamanouchi Pharmaceutical (Tokyo, Japan), ketoprofen by Nippon Rhodia (Tokyo, Japan), naproxen by Tanabe Pharmaceutical (Osaka, Japan), piroxicam by Pfizer Taito (Tokyo, Japan) and tolmetin sodium by Dainippon Pharmaceutical (Osaka, Japan).

Acetonitrile and methanol were of HPLC grade and dichloromethane, acetic acid and sodium acetate were of analytical-reagent grade. All of these chemicals were purchased from Wako (Osaka, Japan).

Standard stock solutions containing 200 μg/ml I and mefenamic acid were prepared in methanol and stored in the dark at room temperature when not in use. All calibration graphs were prepared using spiked plasma or urine of the required concentrations.

Instrumentation and chromatographic conditions

The HPLC equipment consisted of an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a Waters WISP 710B autosampler system (Waters Assoc., Milford, MA, U.S.A.), a Uvilog 5IV variable-wavelength

ultraviolet detector (Oyobunko Kiki, Tokyo, Japan), and a Shimadzu C-R1B Chromatopac (Shimadzu, Kyoto, Japan). Reversed-phase HPLC separations were carried out with a Zorbax ODS column (250 mm × 4.6 mm I.D.), 5-μm particles (DuPont, Wilmington, DE, U.S.A.), and the UV wavelength was set at 330 nm with a sensitivity setting of 0.01–0.16 a.u.f.s. The mobile phase (acetonitrile–water–acetic acid, 70:29:1) was supplied at a flow-rate of 1.0 ml/min at room temperature.

Sample preparation

Plasma (1–2 ml) or urine (1 ml) in a 10-ml PTFE-lined screw-capped tube was mixed with 25 μl of the internal standard solution (200 μg/ml) for the analysis of plasma samples containing high drug concentrations (1.0–20 μg/ml), 50 μl for urine samples containing drug concentrations (1.0–50 μg/ml) or with 100 μl of another internal standard solution (2 μg/ml) for plasma samples containing low drug concentrations (0.05–1.0 μg/ml). To this sample mixture 1 ml of 1 *M* acetate buffer (pH 4.5) and 3 ml of dichloromethane were added, and the tube was shaken manually using a vortex mixer for 1 min. After centrifugation at 1500 *g* for 10 min, the upper aqueous layer was removed by aspiration with a pasteur pipette. Dichloromethane was then transferred into a small glass tube and reconstituted with 100 μl of the mobile phase, and 20–40 μl of this solution were injected on to the chromatograph through the autosampler.

Pharmacokinetic study

A healthy male volunteer participated in the preliminary pharmacokinetic study of I after giving written informed consent. A single 150-mg dose of I in film-coated tablet form (3 × 50 mg) was administered orally after an overnight fast. Multiple venous blood samples were drawn over the following 24 h, and urine samples were collected at 0–1, 1–3, 3–6, 6–12 and 12–24 h after dosing. Blood samples were immediately centrifuged and the plasma samples were separated. Plasma and urine samples were stored at −20°C until taken for analysis.

RESULTS

Representative chromatograms obtained from plasma and urine extracts are shown in Fig. 2. The retention times of I and mefenamic acid were 5.1 and 8.2 min, respectively. Both exhibited symmetrical peaks with baseline resolution. No interfering peaks from endogenous component(s) of plasma or urine from a normal subject were observed. We also observed no interfering peaks when the blank plasma or urine extract was analysed (Fig. 2).

The absolute recoveries of I and the internal standard from plasma and urine were assessed by comparing the peak heights obtained from the direct injection of the standard stock solutions of the compounds with those of the drug-free plasma or urine spiked with the respective drugs taken through the complete procedure. The extraction recoveries from plasma averaged 93.8 and 89.0% for I at concentrations of 0.5 and 5 μg/ml, respectively, and 100.2% for the internal

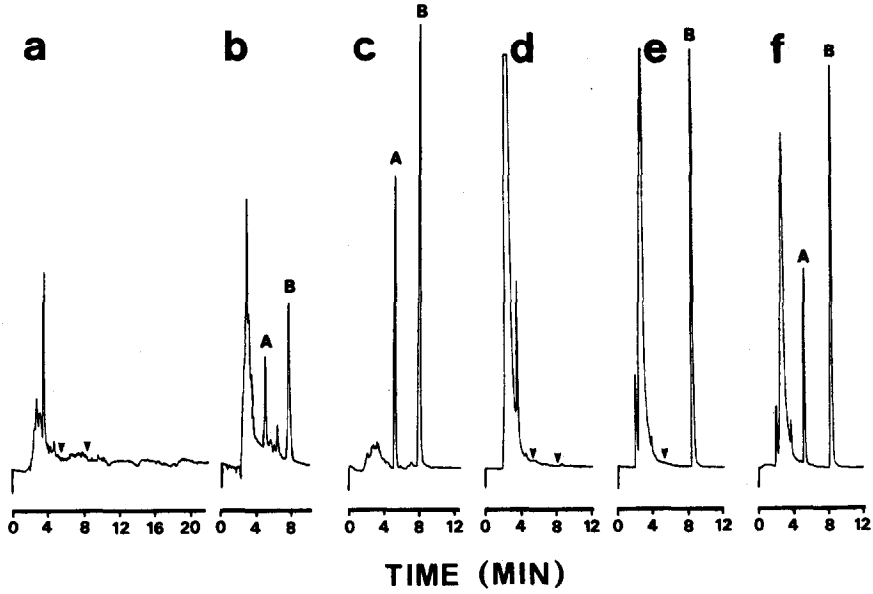

Fig. 2. Representative chromatograms of extracts from: (a) drug-free plasma; (b) plasma containing 0.152 μg/ml I derived from a healthy subject who had received 150 mg of I orally; (c) plasma containing 3.69 μg/ml I; (d) drug-free urine; (e) pre-dosing urine with the internal standard; (f) urine containing 2.69 μg/ml I derived from the same healthy subject as in (b). Peaks A and B indicate I and mefenamic acid, respectively. The arrows indicate the retention times of each of the analytes A and B.

standard at a concentration of 5 μg/ml. The recoveries from urine were much better than those from plasma (Table I).

As the plasma concentrations of I after the oral administration covered a wide concentration range, we constructed two calibration graphs when the samples were analyzed. The calibration graphs for plasma samples containing low (0.05–1.0 μg/ml) and high concentration ranges (1.0–20 μg/ml) were linear over the concentrations examined. The calibration graph for urine samples containing drug concentrations ranging from 1.0 to 50 μg/ml was also linear and passed through

TABLE I

EXTRACTION RECOVERIES OF I AND THE INTERNAL STANDARD (I.S.), MEFENAMIC ACID, FROM PLASMA AND URINE

Sample	Drug	Concentration (μg/ml)	n	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
Plasma	I	0.5	5	93.8 ± 6.5	6.9
		5	5	89.0 ± 4.8	5.4
	I.S.	5	5	100.2 ± 4.0	4.0
Urine	I	5	5	97.8 ± 1.2	1.3
		20	5	99.3 ± 2.7	2.7
	I.S.	10	10	99.6 ± 4.7	4.7

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF I IN PLASMA

Low-level range*				High-level range*			
Concentration of I given (μg/ml)	Concentration of I determined (mean ± S.D.) (μg/ml)	C.V. (%)	Relative error (%)	Concentration of I given (μg/ml)	Concentration of I determined (mean ± S.D.) (μg/ml)	C.V. (%)	Relative error (%)
Within-day-variation (n=5)							
0.05	0.0501 ± 0.0029	5.8	0.2	1	1.07 ± 0.045	4.2	7.0
0.1	0.107 ± 0.0013	1.2	7.0	2	2.08 ± 0.095	4.6	4.0
0.2	0.215 ± 0.0106	4.9	7.5	5	4.67 ± 0.312	6.7	−6.6
0.5	0.495 ± 0.0081	1.6	−1.0	10	9.81 ± 0.091	0.93	−1.9
1.0	0.999 ± 0.0223	2.2	0.1	20	20.18 ± 0.456	2.3	0.9
Day-to-day variation (n=5)							
0.1	0.107 ± 0.0103	9.6	7.0	1	0.989 ± 0.065	6.6	1.1
0.5	0.525 ± 0.0511	9.7	5.0	5	4.90 ± 0.299	6.1	2.0

*Mefenamic acid as the internal standard was added in amounts of 0.2 and 5 μg for low- and high-level ranges, respectively.

the origin. The correlation coefficients of the calibration graphs for plasma and urine analyses varied between 0.9980 and 0.9999.

To assess the precision of this analytical procedure, reproducibilities for both within-day and day-to-day variations were evaluated and the results are summarized in Table II. The coefficients of variation (C.V.) for different concentrations in the within-day study varied between 1.2 and 5.8% for plasma samples in the low concentration range (0.05–1.0 μg/ml), between 0.93 and 6.7% for those in the high concentration range (1.0–20 μg/ml) and between 1.3 and 4.8% for urine samples in the concentration range 1.0–50 μg/ml; the respective C.V.s in the day-to-day study varied between 6.1 and 9.7% for plasma samples containing low and high concentration ranges. The C.V. in the day-to-day study for urine samples was fairly similar to that for plasma. The accuracy was determined by comparing the given concentrations of the drug with those determined (Table II); the relative error ranged from −6.6 to 7.5% for plasma samples and from −4.2 to 9.5% for urine samples.

The detection limit was determined using a dilute solution of the analyte. I can be detected at levels as low as 0.02 μg/ml in plasma at a detector attenuation of 0.01 using 1-ml samples. The signal-to-noise ratio was greater than 5.

Possible interferences from selected non-steroidal anti-inflammatory agents (which might be administered concurrently with I) were tested. Although all anti-inflammatory drugs that we selected and evaluated show some absorbance intensity at 250 nm, alclofenac, fenoprofen, flurbiprofen and ibuprofen could not be detected at 330 nm under the present assay conditions. None of the other anti-inflammatory drugs, except for indomethacin, which appears at 330 nm, interferes with peaks of I and mefenamic acid. The capacity ratios for the selected compounds are summarized in Table III.

TABLE III

CAPACITY RATIOS FOR SELECTED NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Drug	Capacity ratio	Drug	Capacity ratio
Salicylic acid	0.361	Flurbiprofen*	1.045
Sulindac	0.455	I	1.156
Alclofenac*	0.553	Indomethacin	1.205
Tolmetin	0.554	Diclofenac	1.295
Ketoprofen	0.635	Ibuprofen*	1.566
Naproxen	0.693	Mefenamic acid	2.291
Fenoprofen*	0.980	Piroxicam**	⩾7.20
Diflunisal	1.020		

*These compounds show no absorbance intensity at 330 nm.
**Piroxicam was not eluted within 20 min under the described conditions.

Preliminary data on the clinical applicability of this proposed HPLC method for studying the pharmacokinetics of I are shown in Fig. 3, where the plasma concentration–time and cumulative urinary excretion–time data for I obtained from a healthy male volunteer are illustrated. Kinetic data analysed by using a two-compartment model with first-order absorption [7] are given in Table IV.

DISCUSSION

A simple, sensitive and reliable HPLC method has been developed for determining I in human biological fluids. During the development of the method, var-

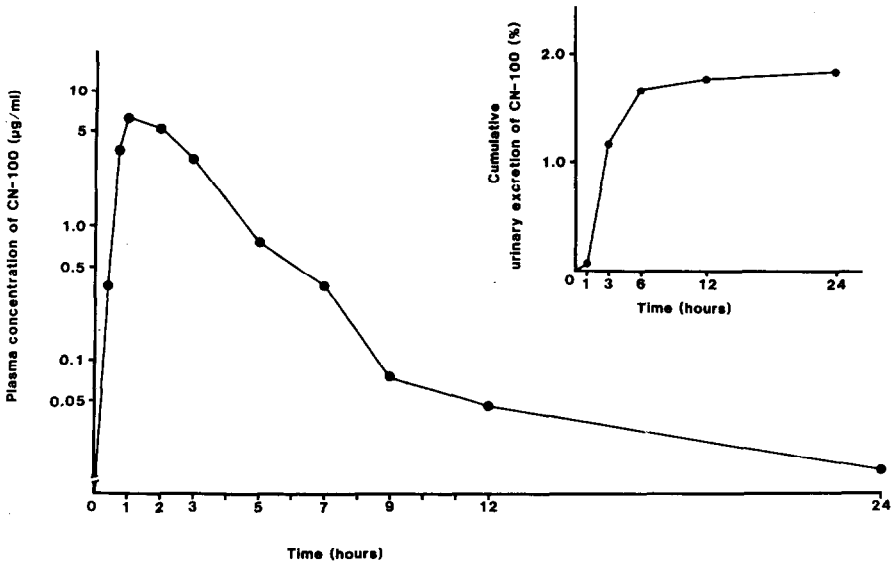

Fig. 3. Plasma concentration-time and cumulative urinary excretion-time profiles of I (CN-100) following an oral dose of 150 mg of I to a healthy subject.

TABLE IV

PHARMACOKINETIC PARAMETERS DERIVED FROM A HEALTHY SUBJECT WHO HAD RECEIVED A SINGLE ORAL ADMINISTRATION OF 150 mg OF I

Abbreviations: C_{max} = maximum plasma concentration; t_{max} = time to reach C_{max}; $t_{1/2\alpha}$ = distribution phase half-life; $t_{1/2\beta}$ = elimination phase half-life; K_a = absorption rate constant; t_0 = lag time elapsing prior to the start of first-order absorption; $[AUC]_0^\infty$ = area under the plasma concentration–time curve from zero to infinity; Cl_p/F = plasma clearance relative to the extent of availability (F) calculated as $Cl_p/F = dose/[AUC]_0^\infty$; V_c/F = apparent central distribution volume relative to the extent of F calculated as $V_c/F = dose/k_{10} \cdot [AUC]_0^\infty$; $V_{d_{ss}}/F$ = apparent distribution volume relative to the extent of F calculated as $V_{d_{ss}}/F = V_c \cdot \frac{k_{12}+k_{21}}{k_{21}}$; free $[X_u]_0^\infty$ = projected cumulative urinary excretion of free I from zero to infinity; Cl_R = renal clearance calculated as free $[X_u]_0^\infty/[AUC]_0^\infty$.

Parameter	Value	Parameter	Value
C_{max} (μg/ml)	6.35	$[AUC]_0^\infty$ (μg·h/ml)	17.8
t_{max} (h)	1.0	Cl_p/F (1/h/kg)	0.115
$t_{1/2\alpha}$ (h)	0.942	V_c/F (1/kg)	0.172
$t_{1/2\beta}$ (h)	6.86	$V_{d_{ss}}/F$ (1/kg)	0.235
K_a (h^{-1})	1.718	Free $[X_u]_0^\infty$ (% of dose)	1.83
t_0 (h)	0.482	Cl_R (1/h/kg)	0.0021

ious extraction and HPLC conditions, UV wavelength, composition of mobile phase and selection of the internal standard were carefully assessed in order to establish the optimal assay conditions [8].

With respect to the selection of the UV wavelength, I possesses two absorbance peaks at 230 and 330 nm. However, around the first peak the chromatogram of blank plasma revealed an extremely high UV absorbance that interferes with the peak of I. Because of this interference, 330 nm, where the blank plasma did not interfere with the second peak of I, was adopted. In addition, possible interferences from other anti-inflammatory drugs was evaluated by using the UV wavelength of 330 nm. Only indomethacin interfered with the peak of I (Table III).

Mefenamic acid was eventually selected as the internal standard after comparison with other non-steroidal anti-inflammatory drugs including propionic acid derivatives. Although another propionic acid drug would be preferable as an internal standard owing to the structural similarity, all of the propionic acid derivatives tested (ketoprofen, ibuprofen, fenoprofen or naproxen) generally gave inconvenient retention times under the present assay conditions (Table III). In contrast, mefenamic acid gave an analytically acceptable result with regard not only to retention time but also recovery, and was therefore used throughout the study.

A mobile phase consisting of acetonitrile–water–acetic acid (70:29:1) was found to provide a good separation of I and the internal standard, mefenamic acid, and relatively short retention times. Although some small peaks that might be derived from possible endogenous substances from plasma could not be completely eliminated, they showed no significant or unacceptable interferences with the peaks of I and the internal standard (Fig. 2).

The extraction recoveries were considered sufficient for the analysis of I and

the internal standard (Table I). The results from the within-day and day-to-day analyses were satisfactory, implying a good reproducibility of precision and accuracy. In addition, excellent linearity was observed for the calibration graphs for plasma and urine samples ($r=0.9980$–0.9999).

The preliminary data on the pharmacokinetics of I obtained from a healthy subject who had received a single oral 150-mg dose of the drug suggested that the kinetic profile can be fitted to a two-compartment model with first-order absorption and elimination (Fig. 3). The preliminary kinetic data for I seem to indicate that it is likely to be extensively metabolized in the liver, as the renal clearance (Cl_R) was less than 2% of the total plasma clearance (Table IV). Using the present assay method, a more detailed pharmacokinetic study of I in humans is in progress.

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REFERENCES

1 J.R. Lewis, J. Am. Med. Assoc., 237 (1977) 1260.
2 F.D. Hart and E.C. Huskisson, Drugs, 27 (1984) 232.
3 E.C. Huskisson, J.A. Wojtulewski, H. Berry, J. Scott, F.D. Hart and H.W. Balme, Br. Med. J., 1 (1974) 176.
4 E.C. Huskisson, D.L. Woolf, H.W. Balme, J. Scott and S. Franklyn, Br. Med. J., 1 (1976) 1048.
5 C. Wasner, M.C. Britton, R.G. Kraines, R.L. Kaye, A.M. Bobrove and J.F. Fries, J. Am. Med. Assoc., 246 (1981) 2168.
6 Investigational Brochure CN-100, Nippon Chemipha, Tokyo, 1981.
7 K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, J. Pharmacobio-Dyn., 4 (1981) 879.
8 M. Ahnoff, M. Ervik, P.-O. Lagerström, B.-A. Persson and J. Vessman, J. Chromatogr., 340 (1985) 73.