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Simultaneous determination of loxoprofen and its diastereomeric alcohol metabolites in human plasma and urine by a simple HPLC-UV detection method

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Abstract

A simple, reliable HPLC-UV detection method was developed for the simultaneous determination of loxoprofen and its metabolites (i.e. *trans*- and *cis*-alcohol metabolites), in human plasma and urine samples. The method involves the addition of a ketoprofen (internal standard) solution in methanol, zinc sulfate solution and acetonitrile to plasma and urine samples, followed by centrifugation. An aliquot of the supernatant was evaporated to dryness, and the residue reconstituted in a mobile phase (acetonitrile:water = 35:65 v/v, pH 3.0). An aliquot of the solution was then directly injected into the HPLC system. Separations were performed on octadecylsilica column (250 × 4.5 mm, 5 μm) with a guard column (3.2 × 1.5 cm, 7 μm) at ambient temperature. Loxoprofen and the metabolites in the eluent were monitored at 220 nm (a.u.f.s. 0.005). Coefficients of variations (CV%) and recoveries for loxoprofen and its metabolites were below 10 and over 96%, respectively, in the 200 ~ 15 000 ng ml^{-1} range for plasma and 500 ~ 50 000 ng ml^{-1} range for urine. Calibration curves for all the compounds in the plasma and urine were linear over the above-mentioned concentration ranges with a common correlation coefficient of 0.999. The detection limit of the present method was 100 ng for all the compounds. These results indicate that the present method is very simple and readily applicable to routine bioavailability studies of these compounds with an acceptable sensitivity.

Keywords: HPLC-UV detection; Simultaneous determination; Loxoprofen; Alcohol metabolites; Human plasma and urine

1. Introduction

Loxoprofen, (±)-2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate (Fig. 1), a 2-phenylpropionate non-steroidal anti-inflammatory

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agent (NSAID) has marked analgesic and antipyretic activities and relatively weak gastrointestinal ulcerogenicity [1]. However, loxoprofen itself is not the major in vivo inhibitor of prostaglandin synthesis. Its pharmacological effect is derived from its metabolites [2]. The loxoprofen molecule contains two chiral centers and is marketed in the form of a racemic mixture, composed of four individual enantiomers. The cyclopentanone moiety in each enantiomer is stereoselectively reduced, resulting in eight optically active alcohol metabolites [3]. This reductive metabolism and the subsequent glucuronide conjugation of the products is thought to be the major metabolic pathway of loxoprofen in humans [4]. Of the eight alcohol metabolites, an enantiomer of *trans*-alcohol (Fig. 1), (2S)-[4-(trans)-(1′*R*,2′S)-2′-hydroxycyclopentylmethyl)-phenyl]propionic acid is known to be the most pharmacologically active [2]. The *trans*-alcohol (Fig. 1) is also derived from the metabolic inversion of the enantiomers of *cis*-alcohol (Fig. 1), (±)-2-[4-(*cis*)-(1′*R*,2′*R* + 1S′,2S′)-2′-hydroxycyclopentylmethyl)-phenyl]propionic acid.

It appears to be necessary to quantify all the metabolites in the plasma and urine in order to obtain precise information concerning the pharmacokinetics and/or pharmacodynamic behavior of loxoprofen. However, such enantioselective metabolism in humans is very rapid and highly preferential [5]. In comparative pharmacokinetic studies or bioequivalence tests, therefore, it might still be of practical value to evaluate the parent acid (loxoprofen) and the two alcohol metabolites.

In early clinical studies, loxoprofen and the two diasteromeric alcohol metabolites in plasma and urine were determined by gas chromatography combined with electron-impact ionization mass spectrometry (GC-MS) [6]. However, the overall analytical procedure was tedious and, moreover, only a partial resolution of the two diastereomeric alcohols (i.e. *trans*- and *cis*-alcohols) could be achieved. As a result, the GC-MS method is generally considered to be inadequate for routine analysis in non-specialized laboratories. Two high performance liquid chromatography (HPLC) methods with fluorescence detection for the quantitative analysis of loxoprofen and its reductive metabolites in biological samples have also been reported [7,8]. In one of those methods [7], loxoprofen and its metabolites are coupled with a chiral reagent, and the corresponding diasteroamides are separated in a normal-phase mode. In the other method [8], loxoprofen and its alcohol metabolites are coupled with an achiral reagent, and the separation of stereoisomer is performed, using the reversed-phase mode. In spite of reasonably good sensitivity of these HPLC methods (e.g. 1 ng for loxoprofen and 2 ng for the alcohol metabolites [7]), the coupling reagents used in these procedures are not available commercially. Moreover, a considerable amount of time is needed for the fluorescence tagging (coupling) in both methods. The objective

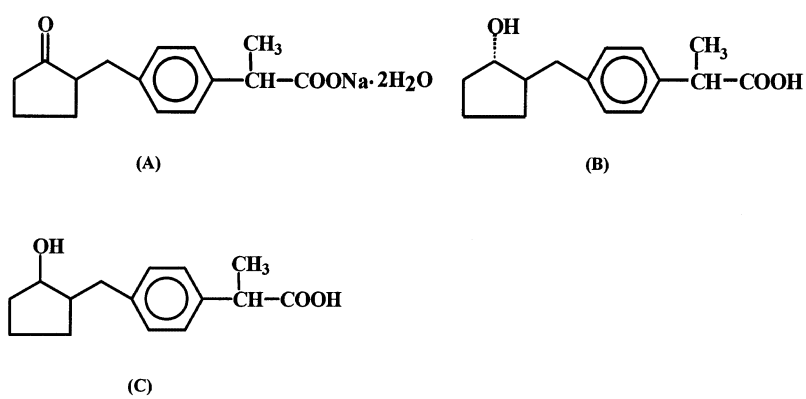

Fig. 1. Chemical structures of loxoprofen dihydrate (A), *trans*- (B) and *cis*-alcohol metabolites (C).

of this study, therefore, was to develop a simple, convenient HPLC method with an adequate sensitivity which could be used for the routine assay of loxoprofen and its alcohol metabolites in plasma and urine samples following the oral administration of loxoprofen to human subjects.

2. Experimental

2.1. Materials

Loxoprofen was supplied from Dae Wha Pharmaceutical Co. (Seoul, Korea) and Loxonin® tablets (60 mg/tablet as loxoprofen anhydrous) were supplied from Dong Wha Pharmaceutical Co. (Seoul, Korea), respectively. The two alcohol metabolites of loxoprofen, (±)-2-[4-(trans)-(1′*S*,2′*S*)-2′-hydroxycyclopentylmethyl)-phenyl]propionic acid (trans-alcohol) and (±)-2-[4-(*cis*)-(1′*R*,2′*S*)-2′-hydroxycyclopentylmethyl)-phenyl]propionic acid (*cis*-alchol) were synthesized and characterized by NMR and HPLC (>95% purity). Ketoprofen (internal standard) was purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). The other reagents used were of analytical grade or better, and used without further purification.

2.2. High performance liquid chromatography (HPLC) analysis

The HPLC system consisted of a Gilson HPLC system (Villiers-le-Bel, France) equipped with 321 pump, UV/VIS-151 detector, 234 autoinjector and Gilson Unipoint system software. Separations were performed on an octadecylsilica column (250 × 4.5 mm, 5 μm particle size, LUNA, Phenomenex®, Torrance, CA) with a guard column (3.2 × 1.5 cm, 7 μm particle size, P.J. Cobert Associates, INC. St Louis, MO) at ambient temperature.

The mobile phase used for the analysis of loxoprofen and its metabolites in plasma and urine was a mixture of acetonitrile and water in a volume ratio of 35:65, adjusted to pH 3.0 with phosphoric acid. The mixture was degassed prior to use and delivered at a flow rate of 1.2 ml/min. Loxoprofen and its metabolites in the eluent were monitored spectrophotometrically at 220 nm (a.u.f.s. 0.005).

2.3. Quantitation of loxoprofen and its alcohol metabolites in plasma standard samples

Loxoprofen and its metabolites, the *cis*- and *trans*-alcohols (as the free acids) were added to drug-free plasma to give final plasma concentrations of 200 ~ 15 000 ng ml^{-1}. To 500 μl of the plasma sample, 50 μl of the internal standard (i.e. ketoprofen, 10 μg ml^{-1} in methanol), 50 μl of 10% zinc sulfate solution and 750 μl of acetonitrile were added with mixing (vortexing for 2 min for each step) and the resulting solution was centrifuged at 3000 rpm for 10 min. An 1 ml aliquot of the supernatant was evaporated to dryness under vacuum using a Speed-Vac concentrator (Savant, Holbrook, NY). The residue was then reconstituted in 150 μl of the mobile phase and an aliquot (100 μl) was directly injected into the HPLC system.

The peak height ratio of loxoprofen and its alcohol metabolites over the internal standard (ketoprofen) were used as assay parameters. The peak height ratio was plotted against the corresponding concentrations of loxoprofen and its diastereomeric alcohols in the plasma sample. Standard calibration curves were obtained from least-squares linear regression analysis of the plots.

2.4. Quantitation of loxoprofen and its alcohol metabolites in urine standard samples

Loxoprofen and its metabolites, the *cis*- and *trans*-alcohols (as the free acids) were added to drug-free 5-fold diluted urine to give final concentrations of 500 ~ 50 000 ng ml^{-1}. To 250 μl of the urine sample, 50 μl of internal standard (i.e. ketoprofen, 10 μg ml^{-1} in methanol) and 125 μl of 1M NaOH were added and the solution vortexed for 1 min. The purpose of the 1 M NaOH was to convert, through hydrolysis, the excreted ester–glucuronides to the corresponding free-acids. Af-

ter this procedure, 750 μl of acetonitrile was added and, after standing for 30 min the urine sample was neutralized by the addition of 250 μl of 1 M HCl. The mixture was centrifuged at 3000 rpm for 10 min and an aliquot of the supernatant was evaporated to dryness under vacuum using a Speed-Vac concentrator (Savant, Holbrook, NY). The residue was then reconstituted in the same mobile phase and an aliquot (100 μl) was directly injected into the HPLC system. Standard calibration curves were obtained in a manner identical to the case for the standard plasma samples.

2.5. In vivo human bioavailability study

One tablet of Loxonin® (60 mg as loxoprofen anhydrous) was administered orally to 10 healthy, Korean male subjects with 200 ml of water. Blood samples (5 ml) were collected from a forearm venipuncture at 10, 20, 30, 60, 90, 120, 180, 240 and 360 min, and then centrifuged at 3000 rpm for 15 min to obtain plasma samples. Urine samples were collected at 1, 2, 6, 8, 12 h after the administration. Plasma and urine samples were stored at −20°C until used for the HPLC procedure described in Section 2.3 and Section 2.4. The areas under the plasma concentration–time curve from time zero to 360 min (AUC) for loxoprofen and its metabolites were calculated by the standard trapezoidal method, and the maximum plasma concentrations (C_{max}) and time to reach the concentrations (T_{max}) for these compounds were compiled from the respective plasma concentration-time curves.

3. Results and discussion

3.1. Method development

In the previous clinical studies, loxoprofen in the plasma [9] and urine [10] was determined by HPLC method with UV detection. In the present study, we tried to extend these methods to the routine assay of loxoprofen and its major metabolites in the plasma and urine samples. Identical column (C18) was used for the separation of the compounds in the present study. The length of the column, composition of the mobile phase and wavelength of the UV detector were modified in order to make the separation of these compounds clearer. As the result of the modification, these compounds could be clearly separated (Fig. 2).

3.2. Quantitation of loxoprofen and its diastereomeric alcohols in the plasma

Fig. 2 depicts typical chromatograms for the blank plasma, blank plasma spiked with loxoprofen and its metabolites, and plasma sample taken from a human subject 1 h after the oral administration of Loxonin®. There were no apparent plasma components which interfered with the peaks corresponding to loxoprofen, its metabolites and internal standard (Fig. 2). Tables 1 and 2 show inter- and intra-day variations of the assay for the plasma samples. The coefficients of variations (CV%) were below 10% for 200 ~ 15 000 ng ml^{-1} of loxoprofen and its metabolites indicating that the present method is fairly precise for these compounds in the plasma. The recoveries of these compounds, when calculated from Tables 1 and 2 were over 96% indicating that the present method is accurate. Calibration curves for the three compounds were linear over the range of 200 ~ 15 000 ng ml^{-1} with a correlation coefficient of 0.999.

3.3. Quantitation of loxoprofen and its diastereomeric alcohols in the urine

Fig. 3 depicts typical chromatograms for the blank urine, blank urine spiked with loxoprofen and its metabolites, and a urine sample taken from a human subject 1-2 h after the oral administration of Loxonin®. There were no apparent urine components that interfered with the peaks corresponding to loxoprofen, its metabolites and internal standard (Fig. 3). Tables 3 and 4 show inter- and intra-day variations of the assay for the urine samples. Coefficients of variations (CV%) and recovery were below 10% and above 97.8%, respectively, for 500 ~ 50 000 ng ml^{-1} of loxoprofen and its metabolites indicating that the present method is fairly precise and accurate also for the urine samples. Calibration curves for the three compounds were linear over the range of 500 ~

Table 1
Within-day variations of loxoprofen, *trans*-alcohol and *cis*-alcohol in plasma ($n = 5$) [a]

Spiked Concentration ($\mu g\ ml^{-1}$)	Loxoprofen			*trans*-alcohol			*cis*-alcohol		
	Mean found Concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found Concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found Concentration ($\mu g\ ml^{-1}$)	SD	CV (%)
0.2	0.21	0.01	4.11	0.21	0.01	5.05	0.21	0.01	4.55
0.5	0.50	0.02	4.56	0.48	0.02	4.35	0.51	0.03	5.01
1	0.98	0.05	5.32	0.98	0.01	1.25	1.02	0.06	5.94
5	5.00	0.09	1.85	5.05	0.23	4.64	4.91	0.20	4.09
7.5	7.51	0.12	1.58	7.52	0.12	1.55	7.55	0.18	2.37
10	10.00	0.23	2.33	9.96	0.29	2.95	10.01	0.21	2.10

[a] SD, standard deviation, CV, coefficient of variation.

Table 2
Day to day variations of loxoprofen, *trans*-alcohol and *cis*-alcohol in plasma ($n = 3$)[a]

Spiked concentration (μg ml^{-1})	Loxoprofen			*trans*-alcohol			*cis*-alcohol		
	Mean found concentration (μg ml^{-1})	SD	CV (%)	Mean found concentration (μg ml^{-1})	SD	CV (%)	Mean founded concentration (μg ml^{-1})	SD	CV (%)
0.2	0.22	0.01	3.23	0.21	0.01	2.75	0.21	0.01	4.66
0.5	0.53	0.03	4.97	0.52	0.02	3.30	0.51	0.01	1.18
1	1.04	0.08	8.13	1.06	0.07	6.71	1.06	0.03	2.51
5	4.89	0.35	7.10	4.90	0.29	5.83	4.89	0.23	4.79
7.5	7.39	0.59	8.04	7.40	0.38	5.15	7.42	0.41	5.55
10	10.13	0.72	7.14	10.12	0.78	7.73	10.11	0.66	6.51

[a] SD, standard deviation, CV, coefficient of variation.

Table 3
Within-day variations of loxoprofen, *trans*-alcohol and *cis*-alcohol in urine ($n = 5$) [a]

Spiked Concentration ($\mu g\ ml^{-1}$)	Loxoprofen			*trans*-alcohol			*cis*-alcohol		
	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)
0.5	0.53	0.01	1.89	0.52	0.01	1.90	0.49	0.04	7.48
2	2.02	0.16	7.70	1.80	0.02	1.29	1.88	0.15	7.92
10	9.89	0.20	2.02	9.79	0.11	1.08	9.78	0.49	5.02
20	20.04	0.51	2.57	20.50	0.55	2.66	20.10	0.90	4.35
40	40.09	0.83	2.07	40.19	0.87	2.17	41.16	3.41	8.28
50	49.94	0.78	1.56	49.70	0.37	0.74	49.07	2.67	5.44

[a] SD, standard deviation, CV, coefficient of variation.

Table 4
Day to day variation of loxoprofen, *trans*-alcohol and *cis*-alcohol in urine ($n = 3$) [a]

Spiked Concentration ($\mu g\ ml^{-1}$)	Loxoprofen			trans-alcohol			*cis*-alcohol		
	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)	Mean found concentration ($\mu g\ ml^{-1}$)	SD	CV (%)
0.5	0.49	0.04	7.47	0.47	0.05	9.91	0.51	0.04	8.59
2	1.96	0.17	8.78	1.80	0.12	6.83	2.07	0.17	8.43
10	10.23	0.42	4.08	10.43	0.69	6.57	9.81	0.24	2.48
20	20.14	0.85	4.21	19.62	0.49	2.51	20.09	0.66	3.30
40	38.93	1.12	2.88	40.89	1.43	3.50	40.08	1.09	2.71
50	50.75	1.03	2.03	49.40	0.45	0.91	49.92	0.85	1.70

[a] SD, standard deviation, CV, coefficient of variation.

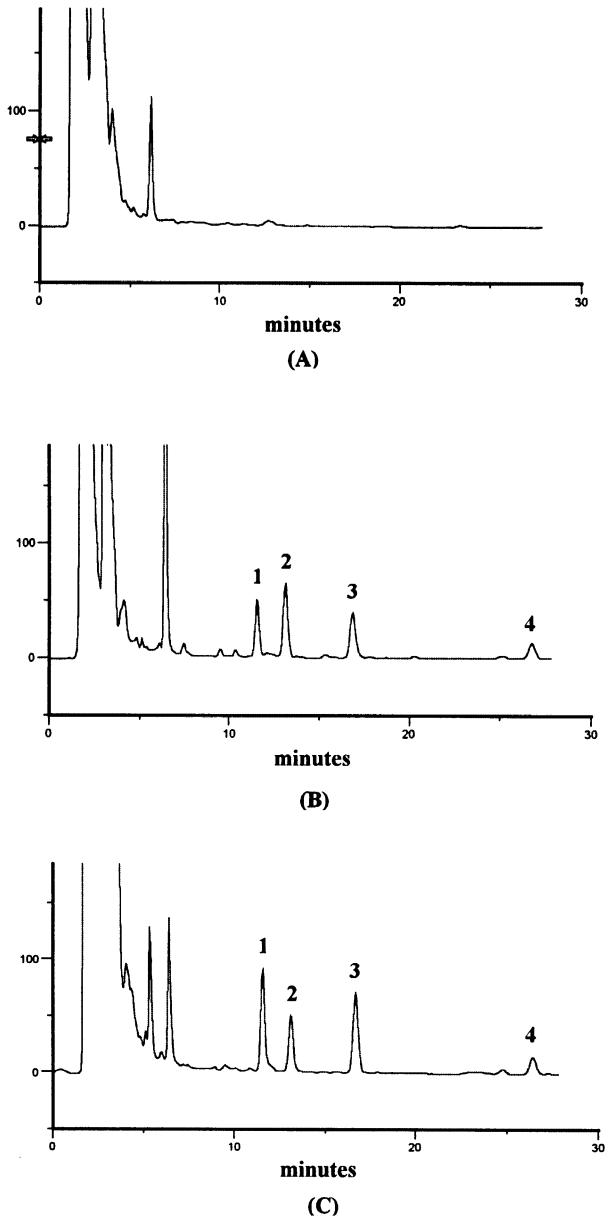

Fig. 2. HPLC chromatograms for blank plasma (A), blank plasma spiked with loxoprofen, *trans*-alcohol, *cis*-alcohol and internal standard (ketoprofen) (B), and a plasma sample obtained from a volunteer 1 h after the oral administration of a Loxonin® tablet (60 mg/tablet as loxoprofen anhydrous) (C): (1) *trans*-alcohol; (2) *cis*-alcohol; (3) loxoprofen; (4) ketoprofen.

50 000 ng ml^{-1} with a correlation coefficient of 0.999.

3.4. Plasma profile and urinary excretion of loxoprofen and its diastereomeric alcohols following oral administration of Loxonin® tablet to human subjects

Fig. 4 depicts the temporal profiles for the plasma concentration (mean ± S.E.) of loxoprofen and its diasteromeric alcohol metabolites after the oral administration of Loxonin® to 10 adult volunteers. In general, loxoprofen in the plasma samples could be readily quantified between 10 min (the first sampling time, 1641 ± 829 ng ml^{-1}) and 360 min (the last sampling time, 460 ± 168 ng ml^{-1}) after the oral dosing. The highest plasma loxoprofen level (C_{max}) of 7827 (± 2073) ng ml^{-1} was found at 34 (± 8) min after the dose. The plasma concentrations of the *trans*- and *cis*-alcohol metabolites were consistently much lower compared to loxoprofen throughout the study. However, the metabolites in plasma could also be readily quantified between 10 min (for the *trans*-alcohol, 298 ± 195 ng ml^{-1}) or 20 min (for the *cis*-alcohol, 277 ± 91 ng ml^{-1}) to 360 min (301 ± 78 ng ml^{-1} for the *trans*-alcohol and 278 ± 50 ng ml^{-1} for the *cis*-alcohol). This suggests that the assay procedure established in the present study can be used for the routine assay of loxoprofen, as well as its *trans*- and *cis*-alcohol metabolites in human plasma samples following an oral administration of loxoprofen at a normal dose (60 mg) to human subjects. The calculated or compiled pharmacokinetic parameters (i.e. AUC, C_{max} and T_{max}) for loxoprofen and its metabolites are summarized in Table 5.

Fig. 5 depicts the temporal profiles for the cumulative urinary excretion (amount) of loxoprofen and its metabolites after an oral administration of Loxonin® to 10 adult volunteers. Each metabolite in the figure represents the sum of the respective free alcohol and the corresponding glucuronide-derived alcohol. The urinary excretion of loxoprofen and its metabolites in 12 h was equivalent to 58 ± 5.8% of the loxoprofen dose (60 mg).

4. Conclusion

A simple HPLC-UV detector method with reasonable precision and accuracy was developed for

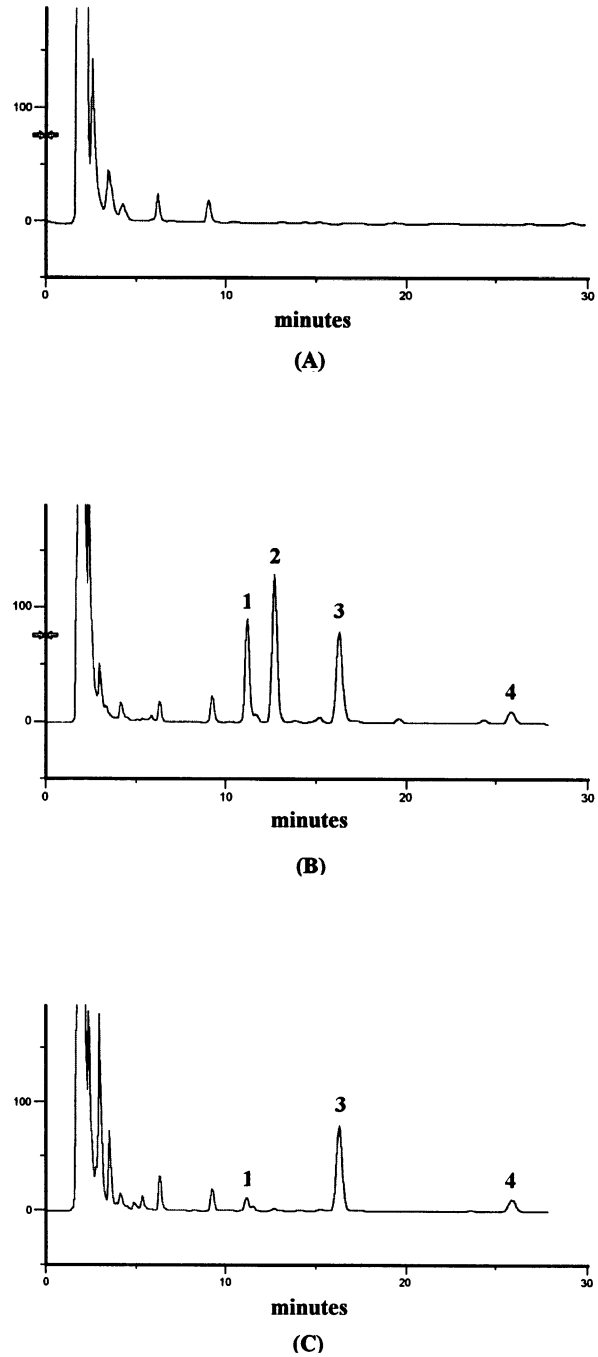

Fig. 3. HPLC chromatograms for blank urine (A), blank urine spiked with loxoprofen, *trans*-alcohol, *cis*-alcohol and internal standard (B), and a urine sample collected from a volunteer during 1–2 h after the oral administration of a Loxonin® tablet: (1) *trans*-alcohol; (2) *cis*-alcohol; (3) loxoprofen; (4) ketoprofen.

Table 5
Pharmacokinetic parameters (mean ± SD, $n = 10$) after oral administration of loxoprofen at a dose of 60 mg

Parameter	Loxoprofen	*trans*-alcohol	*cis*-alcohol
AUC (ng h ml^{-1})	12866 (± 2650)	5878 (± 1368)	2921 (± 1180)
C_{max} (ng ml^{-1})	7827 (± 2073)	1748 (± 387)	634 (± 174)
T_{max} (h)	0.56 (± 0.13)	0.88 (± 0.18)	1.08 (± 0.31)

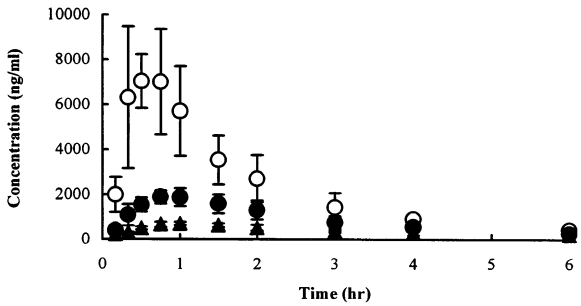

Fig. 4. Mean (± S.E.) plasma concentration-time profiles for loxoprofen (○), *trans*-alcohol (●) and *cis*-alcohol (▲) following oral administration of Loxonin® tablets to 10 subjects.

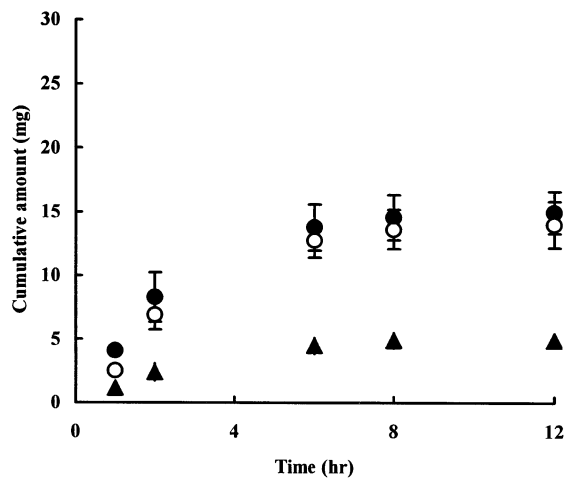

Fig. 5. Mean (± S.E.) cumulative amount of loxoprofen (●), *trans*-alcohol (○) and *cis*-alcohol (▲) excreted into urine following oral administration of Loxonin® tablets to 10 subjects. Assay was performed after the conversion of glucuronides to corresponding free alcohols by alkaline hydrolysis of the urine sample.

the simultaneous determination of loxoprofen and its diastereomeric alcohol metabolites (trans-, *cis*-alcohols) in human plasma and urine samples following oral administration of loxoprofen at a normal dose level (60 mg). Loxoprofen and the metabolites in plasma samples could be determined for the concentration range of 200 ~ 15 000 ng ml^{-1} using a 500 μl volume of the plasma sample by the present method. The method was also applicable to urine samples in the concentration range of 500 ~ 50 000 ng ml^{-1}. The method has poor sensitivity compared to HPLC-fluorescence detection methods [7,8], i.e. the detection limit of the present method for loxoprofen and alcohol metabolites is 100 ng, while that of the previous methods is 1 ~ 2 ng [7,8]. However, most importantly, the fluorescence tagging of plasma and urine samples is not necessary in the present study, and thus, the method is much faster and economical. In addition, the amount of mobile phase can be reduced substantially by adopting a reversed-phase mode of separation. In summary, the present method is clearly more convenient than the previously described HPLC-fluorescence detection methods [7,8].

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