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COLUMN LIQUID CHROMATOGRAPHY FOR THE SIMULTANEOUS DETERMINATION OF THE ENANTIOMERS OF LOXOPROFEN SODIUM AND ITS METABOLITES IN HUMAN URINE

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SUMMARY

A method for simultaneously quantitating the enantiomers in the α-substituted propionic acid moiety of loxoprofen and its two monohydroxy metabolites, *trans*- and *cis*-alcohols, by column liquid chromatography was described. Loxoprofen and its metabolites were readily converted to the amides by condensation with a chiral reagent (1*S*)-1-(4-dimethylaminonaphthalen-1-yl)ethylamine. The diastereoisomers were separated on a normal-phase column with *n*-hexane—ethylacetate (68:32) as a mobile phase and detected with a fluorescence detector. The established method was applied to the determination of the enantiomers of these compounds in the urine of human subjects having received loxoprofen sodium. The results indicated that the three compounds were largely composed of the (2*S*)-isomer, in accordance with previous findings in rats.

INTRODUCTION

Drugs consisting of an enantiomeric mixture are now recognized to have pharmacokinetically different properties between their stereoisomers. Therefore, establishment of an analytical method for the stereospecific determination of these drugs is important for understanding their pharmacological actions.

Inhibitory activity of α-arylpropionic acid anti-inflammatory agents toward prostaglandin synthetase is known to reside predominantly in the (2*S*)-isomer [1—2]. However, the stereoselective inversion of (2*R*)- to (2*S*)-configurations has been shown to occur in several α-arylpropionic acid derivatives [3—7] and is claimed to be a reason for the pharmacologically equal potency between

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their enantiomers. Newly developed loxoprofen sodium, sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate, was suggested to undergo this unique inversion reaction on the basis of the established stereochemistry of the urinary metabolites in rats [8], and this has been confirmed by column liquid chromatography (LC) of the enantiomers of both the parent acid and its major metabolites (*trans*- and *cis*-alcohols) in the plasma of rats given (2*R*)-loxoprofen using a chiral reagent, (1*S*)-1-(4-dimethylaminonaphthalen-1-yl)-ethylamine (DANE), synthesized by a new procedure [9].

This paper describes the details of this LC method for the simultaneous determination of the enantiomeric pairs of the three compounds mentioned above and its successful application to the analysis of urine from human subjects dosed with loxoprofen sodium.

EXPERIMENTAL

Chemicals

The standard samples of loxoprofen sodium and *trans*- and *cis*-alcohols were synthesized by Terada et al. [10] in the Chemical Research Laboratories of Sankyo. DANE reagent was synthesized by the new method described in ref. 9 and its optical purity was more than 99%. All other reagents were analytical-reagent grade.

Chromatography

A Twincle® high-performance liquid chromatograph (Jasco, Tokyo, Japan) equipped with a μPorasil column (30 cm × 3.9 mm I.D., 10 μm particle size) (Waters Assoc., Milford, MA, U.S.A.) at room temperature and an FP-110 spectrofluorometer (Jasco) was used.

Liquid chromatography was performed with *n*-hexane—ethylacetate (68:32) as a mobile phase at a flow-rate of 1.7 ml/min with fluorescence detection (excitation at 313 nm and emission at 420 nm).

Sample preparation

Quantitative determination was performed after alkaline treatment of urine samples, since the parent acid and its monohydroxy metabolites were excreted largely as their alkali-labile ester glucuronides in human urine.

A 1-ml aliquot of urine sample was made alkaline with 1 ml of 1 *M* sodium hydroxide in a stoppered test-tube and allowed to stand at room temperature for 1 h. The hydrolysed mixture was acidified to pH 1.0—1.5 with 1.5 ml of 1 *M* hydrochloric acid and extracted with 10 ml of *n*-hexane—ethylacetate (68:32) by shaking for 5 min and centrifuging at 1000 *g* for 5 min. A 1.0-ml aliquot of the organic phase was transferred to a 10-ml conical tube and evaporated to dryness in vacuo at ca. 40°C. To the residue, respective dichloromethane—pyridine (10:1) solutions of DANE reagent (200 μg, 0.1 ml), 1-hydroxybenzotriazole (200 μg, 20 μl) and N,N′-dicyclohexylcarbodiimide (DCC, 200 μg, 0.1 ml) were added and the mixture was allowed to stand at room temperature for 1 h. After evaporation of the solvents, the residue was mixed with 0.5 ml of the mobile phase and 0.5 ml of 0.1 *M* hydrochloric acid, and stirred for 1 min using a Vortex® mixer. The organic phase was separated, dried over anhydrous sodium sulphate and a 20-μl aliquot was injected into the LC system.

RESULTS AND DISCUSSION

Quantitation conditions

DANE reagent was introduced by Goto et al. [11] as a highly fluorescent chiral reagent for determining the enantiomers of naproxen in rabbit serum [7]. For the present purpose of simultaneously determining all the epimers of loxoprofen and its two monohydroxy metabolites with high precision, further examinations were necessary for establishing the quantitative conditions.

Fig. 1 shows the chemical structures of the compounds studied and their reaction with DANE reagent. The reaction products were isolated and confirmed to be the corresponding amide derivatives on the basis of the observed molecular ions in their mass spectra.

For establishing the derivatizing conditions, the catalysts of DCC and 1-hydroxybenzotriazole were primarily examined in order to determine the optimal quantities. Various amounts of DCC were added to the reaction mixture of loxoprofen (20 μg), *trans*- and *cis*-alcohols (20 μg, each), DANE

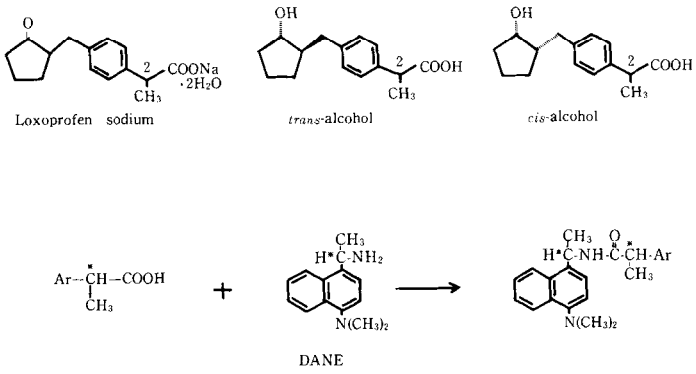

Fig. 1. Chemical structures of loxoprofen and its monohydroxy metabolites and their reaction with DANE reagent.

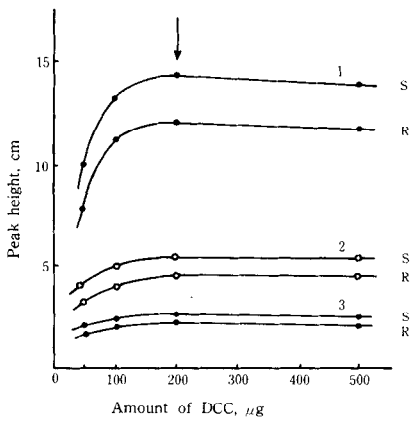

Fig. 2. Effect of concentration of N,N′-dicyclohexylcarbodiimide (DCC) on the formation of diastereomeric amides. (1) Loxoprofen, (2) *cis*-alcohol and (3) *trans*-alcohol; 20 μg each. 200 μg 1-Hydroxybenzotriazole added.

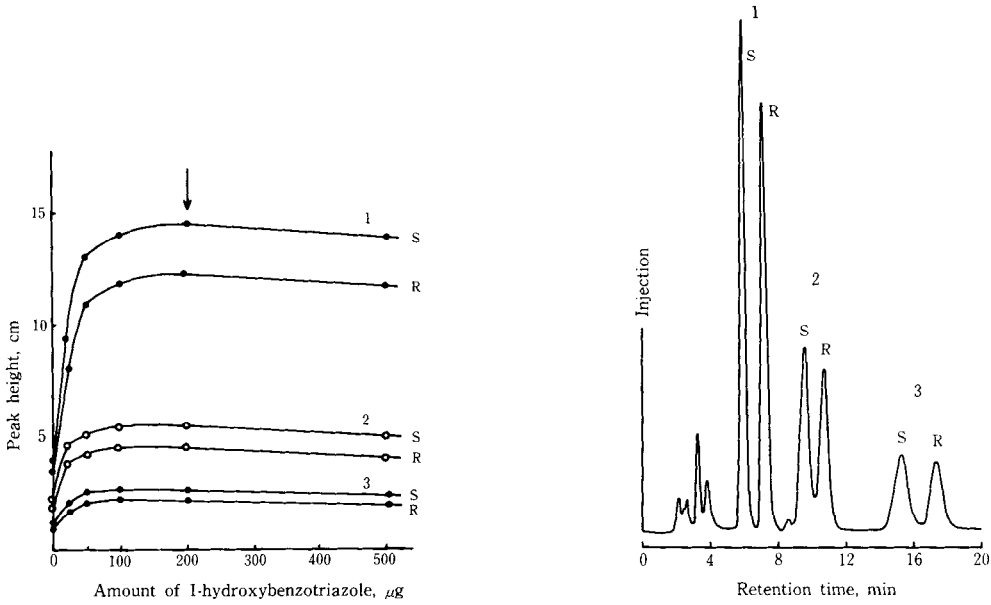

Fig. 3. Effect of concentration of 1-hydroxybenzotriazole on the formation of diastereomeric amides. (1) Loxoprofen, (2) *cis*-alcohol and (3) *trans*-alcohol; 20 μg each. 200 μg DCC added.

Fig. 4. Chromatogram of a mixture of diastereomeric amides of loxoprofen, *cis*-alcohol and *trans*-alcohol; 20 μg each. 200 μg 1-Hydroxybenzotriazole and 200 μg DCC added. Peaks: 1 = loxoprofen; 2 = *cis*-alcohol; 3 = *trans*-alcohol.

(100 μg) and 1-hydroxybenzotriazole (200 μg), and the respective amides formed were estimated. From the results shown in Fig. 2, the favourable amount of DCC was seen to be 200 μg. An optimal amount of 1-hydroxybenzotriazole was then examined using the same mixture as described above, except with DCC (200 μg) instead of 1-hydroxybenzotriazole, and determined to be 200 μg (Fig. 3). Addition of 1-hydroxybenzotriazole produced amide peaks that were three to five times higher compared to that without addition.

A chromatogram under optimal conditions is shown in Fig. 4. The three compounds were separated with preferable retention times and the respective enantiomeric pairs were resolved favourably. Separation (α) and resolution (R) factors for pairs of diastereoisomers were as follows: loxoprofen: $\alpha = 1.38$, $R = 2.59$; *trans*-alcohol: $\alpha = 1.34$, $R = 1.92$; *cis*-alcohol: $\alpha = 1.19$, $R = 1.47$. The α and R were calculated from the equations $\alpha = (t_2 - t_0)/(t_1 - t_0)$ and $R = 2(t_2 - t_1)/(W_1 + W_2)$, where t_1 and t_2 are retention times and t_0 is that of the unretained peak, and W_1 and W_2 are the bases of triangles derived from the peaks.

Calibration curves and recovery test

Standard samples of 2, 5, 10, 15 and 20 μg of loxoprofen, and *trans*- and *cis*-alcohols were derivatized and determined by LC according to the procedure described above. Calibration curves constructed from the peak heights showed good linearity passing through the origin, as shown in Fig. 5. The limit of

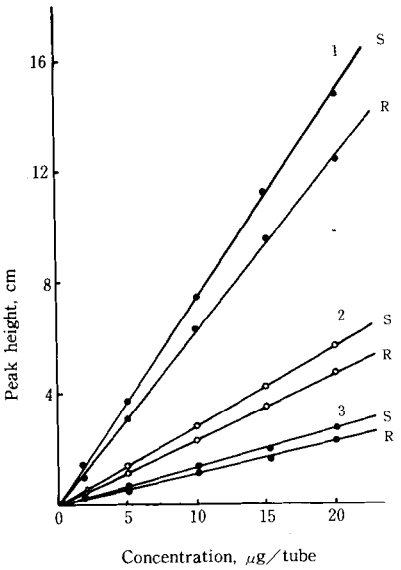

Fig. 5. Calibration curves for loxoprofen (1), *cis*-alcohol (2) and *trans*-alcohol (3).

quantitation in this system was found to be ca. 5 ng of loxoprofen and 10 ng each of *trans*- and *cis*-alcohols.

Recoveries of loxoprofen and the two alcohols from human urine were examined after addition of 25, 50 and 100 μg, respectively, to control urine (1.0 ml), followed by extraction, derivatization and LC determination according to the procedure described above. Observed mean values of the recoveries were > 99% for loxoprofen, 95% for *trans*-alcohol and 94% for *cis*-alcohol, with standard deviations (n = 5) of less than 3, 4 and 3%, respectively.

TABLE I

URINARY EXCRETION OF ENANTIOMERS OF THE PARENT ACID, *trans*- AND *cis*-ALCOHOLS FOLLOWING AN 80-mg ORAL DOSE OF LOXOPROFEN SODIUM TO HEALTHY MALE ADULTS

Compound	Enantiomer*	Amount excreted in urine (mg) (n = 2)				
		0—2 h	2—4 h	4—8 h	8—12 h	Total
Parent acid	*S*	7.99	4.82	3.36	1.09	17.26
	R	7.00	1.76	N.D.**	N.D.	8.76
	Percentage *S*	53	73	100	100	66
trans-Alcohol	*S*	5.62	4.61	2.64	N.D.	12.87
	R	2.64	0.61	N.D.	N.D.	3.25
	Percentage *S*	68	88	100		80
cis-Alcohol	*S*	3.94	2.93	1.11	N.D.	7.98
	R	0.85	0.37	N.D.	N.D.	1.22
	Percentage *S*	82	89	100		87

*Enantiomers on the asymmetric carbon in the propionic side-chain.
**N.D. = Not detected.

Stereospecific determination of loxoprofen and its monohydroxy metabolites in human urine

Urine samples of two healthy male adults in the phase I trial [12] of loxoprofen sodium were used for enantiomer determination. An 80-mg oral dose was administered and urine was collected at given intervals. The results obtained are given in Table I and typical chromatograms of the urine sample are shown in Fig. 6. Metabolites are expressed as mg equiv. of loxoprofen.

Urinary excretion was almost complete 12 h after administration, accounting for 55—70% of the total dosage and decreasing in the order loxoprofen > *trans*-alcohol > *cis*-alcohol. Relative content of the (2*S*)-epimers of the three compounds increased with time, giving 73—89% in 2—4-h urine and almost 100% in 4—8-h urine. *cis*-Alcohol showed relatively higher ratios of the (2*S*)-isomer in the 2—4-h urine.

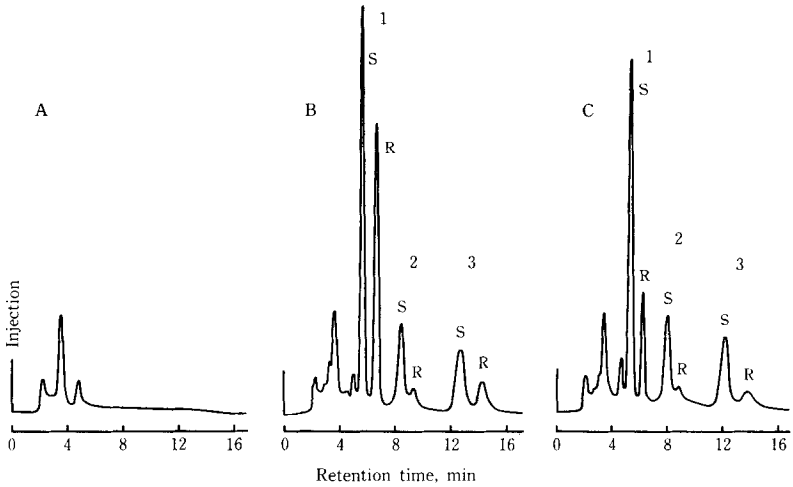

Fig. 6. Chromatograms of loxoprofen and its monohydroxy metabolites in human urine after extraction and derivatization with DANE reagent. (A) Control urine; (B) 0—2 h and (C) 2—4 h urine after oral administration of 80 mg of loxoprofen sodium. Peaks: 1 = loxoprofen; 2 = *cis*-alcohol; 3 = *trans*-alcohol.

The authors have previously reported that the parent acid and its monohydroxy metabolites in the plasma of rats dosed with (2*R*)-loxoprofen showed rapid inversion to each (2*S*)-isomer and not vice versa [9]. The present results strongly suggest the occurrence of the same stereoselective inversion reaction in humans, and this unique metabolic reaction is obviously favourable for the pharmacological potency of loxoprofen sodium in humans.

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