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High-performance liquid chromatographic assay for the simultaneous determination of lansoprazole enantiomers and metabolites in human liver microsomes

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Abstract

In this study, a simple, sensitive and enantioselective HPLC method was developed for the simultaneous determination of lansoprazole enantiomers: a proton pump inhibitor, and its major metabolites: 5-hydroxylansoprazole and lansoprazole sulfone in human liver microsomes. After extraction from the microsomal incubation mixture with a diethyl ether–dichloromethane (7:3, v/v) mixture, analytes were measured by reversed-phase HPLC on a Chiralcel® OD-R column. Detection was made using an ultraviolet absorbance detector set at a wavelength of 285 nm. The mobile phase consisted of a methanol–water (75:25, v/v) mixture. At a flow-rate of 0.5 ml/min, the total run time was 35 min. The limit of quantification for both lansoprazole enantiomers was 0.25 μ*M* and for the metabolites 0.13 μ*M*. The method is suitable for the analysis of lansoprazole enantiomers and its metabolites from human microsomal liver incubations.

Keywords: Enantiomer separation; Lansoprazole; 5-Hydroxylansoprazole; Lansoprazole sulfone

1. Introduction

Lansoprazole is a substituted benzimidazole derivative, which inhibits gastric acid secretion acting via an interaction with (H^+/K^+)-ATPase, a gastric proton pump, in the secretory membrane of the parietal cell [1,2]. This drug represents a useful agent for the treatment of acid-related disorders such as gastric ulcer, duodenal ulcer, the Zollinger–Ellison syndrome and other hyper-secretory diseases [3]. Lansoprazole has an asymmetric sulfur atom in the molecule and thus has two enantiomers: (+)- and (−)-lansoprazole (Fig. 1).

Recently, it has been reported that many chiral agents show enantioselective pharmacokinetics and/or pharmacodynamics [4,5] and such differences have been regarded important in the development of new drugs from the clinical pharmacological point of view. Laboratory evaluations such as pharmacological effect, toxicity and pharmacokinetics for individual enantiomers are necessary to judge whether to

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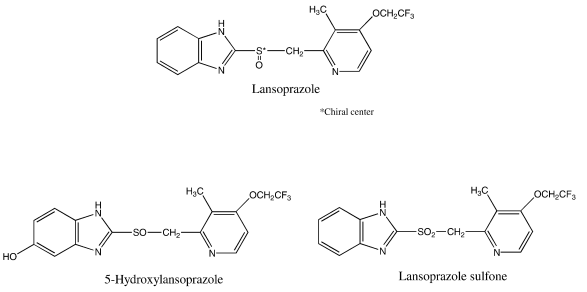

Fig. 1. Chemical structures of lansoprazole enantiomers and their main metabolites, 5-hydroxylansoprazole and lansoprazole sulfone.

develop a drug candidate as an enantiomer or not. For such evaluation, the establishment of a reliable analytical method for determination of enantiomers is in great demand.

Lansoprazole is metabolized extensively by the liver, and its primary metabolites found in serum are hydroxylansoprazole and lansoprazole sulfone [6–8]. Pharmacokinetic studies of lansoprazole enantiomers have been performed [9], but difference in the biotransformation of the enantiomers so far has not been studied sufficiently in vitro to confirm the involvement of specific CYP isoforms in the various metabolic pathways.

We have already reported a method for determination of lansoprazole enantiomers in human serum and microsomes [9], and there are other methods published for the separation of lansoprazole enantiomers [10,11]. However, there has as yet been no report on the determination of lansoprazole enantiomers and its metabolites simultaneously.

Therefore, we developed an analytical method to determine simultaneously lansoprazole enantiomers and its metabolites by high-performance liquid chromatography on a Chiralcel OD-R column.

2. Experimental

2.1. Chemicals

Racemic lansoprazole, hydroxylansoprazole and lansoprazole sulfone were a generous gift from Takeda Chemical Industry, Osaka, Japan and the internal standard (isobutyl 4-hydroxybenzoate) was purchased from Tokyo Kasei Industry, Tokyo, Japan. The human liver microsomes were purchased from Genest Corp., Woburn, MA. All other chemicals and solvents were of analytical reagent or chromatographic grade and obtained from common commercial sources.

2.2. Instrumentation and chromatographic conditions

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a pump (LC-10ADvp), an automatic injector (SIL-10ADvp), a UV detector (SPD-10AVvp), a column oven (CTO-10Acvp) and a LC-Workstation (CLASS-VP ver.5.02). A Chiralcel® OD-R column (250×4.6 mm I.D., 5 μm in particle size; Daicel Chemical Ind., Tokyo, Japan) was used for separation of the enantiomers and metabolites. The mobile phase consisted of methanol–water (75:25, v/v). The chromatographic runs were carried out at 30°C. Detection was performed at wavelength of 285 nm. The flow-rate was 0.5 ml/min.

2.3. Preparation of standards and quality control samples

Stock solutions of lansoprazole and metabolites were prepared by dissolving them separately in a volumetric flask, 14.8 mg of racemic lansoprazole and 2 mg of metabolite precisely weighed, in 20-ml methanol to yield a concentration of 2 m*M* for racemic lansoprazole and 0.13 m*M* for metabolites, respectively. Similarly, a stock solution of the I.S. was prepared by dissolving a precisely weighed amount of the substance in a volumetric flask to yield a concentration of 50 μ*M*. The stock solutions were stored in glass tubes at −20°C.

For the preparation of calibration standards, working solutions of analytes were produced by diluting appropriate volumes of stock solutions with methanol to yield concentrations of 1–800 μ*M* of racemic lansoprazole and 0.26–52 μ*M* of metabolites, respectively.

Calibration standards of 0.25, 0.5, 10, 25, 100 and 200 μ*M* of lansoprazole enantiomers and 0.13, 0.26, 1.3 and 26 μ*M* metabolites were obtained as follows. Appropriate volumes of working solutions were

added to glass tubes and evaporated to dryness under reduced pressure. The residue was reconstituted in 200 μl human liver microsomes mixtures (0.5 mg protein/ml) containing 100 m*M* potassium phosphate buffer (pH 7.4), 3 m*M* $MgCl_2$, 1 m*M* EDTA and 0.5% methanol at the final concentration indicated.

A second set of stock solutions, weighed separately, were used for preparation of quality control samples to yield concentrations of 0.25, 5 and 50 μ*M* of lansoprazole enantiomers and 0.13, 2.6 and 13 μ*M* of metabolites. Quality control samples were used to determine intra- and inter-day variability.

2.4. Sample preparation procedure

The in vitro metabolism of lansoprazole enantiomers was measured, based on adaptation of the method of Pearce et al. [12]. Appropriate volumes of stock solutions of lansoprazole enantiomers were added to glass tubes and evaporated to dryness under reduced pressure. The residue was reconstituted in 200 μl human liver microsome mixtures (0.5 mg protein/ml) containing 100 m*M* potassium phosphate buffer (pH 7.4), 3 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* NADP, 5 m*M* glucose 6-phosphate, 1 U/ml glucose 6-phosphate dehydrogenase, 0.5% methanol and 5 μ*M* racemic lansoprazole at the final concentration indicated. Pearce et al. [12] reported that if the final concentration of methanol in the incubation mixture was less than 2%, methanol did not inhibit the metabolism of lansoprazole. The samples were incubated at 37±1°C.

2.5. Sample extraction procedure [13]

A 200-μl volume of calibration standard, quality control or unknown samples were added to 100 μl of a 50-μ*M* solution of the I.S. and 4 ml of a diethyl ether–dichloromethane (7:3, v/v) mixture. The tubes were subjected to vortex for 2 min and centrifuged at 1000 *g* for 5 min at 4°C. After the organic phase was evaporated under a reduced pressure, the residue was immediately reconstituted in 100 μl of methanol and 20 μl of the aliquot was injected into the HPLC system.

2.6. Validation tests

2.6.1. Specificity

The interference of endogenous compounds was investigated by analysis of a drug-free human liver microsome sample.

2.6.2. Calibration and calculation procedures

Daily standard curves were constructed using a weighted (1/peak area ratio) least-squares linear regression analysis of the observed peak area ratios of lansoprazole enantiomers, metabolites and the I.S. [14] (drug to the internal standard). The unknown concentrations were calculated from the weighted linear regression equation of the peak area ratio against concentrations of the calibration curve.

2.6.3. Precision and accuracy of the assay

In order to evaluate the intra-day precision and accuracy, replicate samples were analyzed for each concentration on the same day. The inter-day validity was evaluated for 4 days. Intra- and inter-day assays were assessed using four spiked microsomal samples at the concentrations of 0.25, 5 and 50 μ*M* of lansoprazole enantiomers and 0.13, 2.6 and 13 μ*M* of lansoprazole metabolites. The accuracy was evaluated as percentage error [(found concentration−spiked concentration)/spiked concentration]×100%, and the precision was evaluated by the coefficient of variation (C.V. [(SD/mean)×100]%).

The acceptance criteria are not more than 15% deviation from the nominal value for accuracy and not more than 15% C.V. for precision [15].

2.6.4. Recovery

Extraction efficiency from microsomal samples, at the concentrations of 0.5, 5, 10, 25, 100, 200 μ*M* of lansoprazole enantiomers, 0.26, 1.3, 2.6, 13 μ*M* of lansoprazole metabolites and 0.5 μ*M* of I.S. were examined by comparing with the peak area equivalent to pure compounds dissolved in methanol.

2.6.5. Limits of quantification

The lower limit of quantification (LOQ) was defined as the concentration for which the coefficient of variation and accuracy were lower than 15% [15].

3. Results and discussion

3.1. Specificity

Typical chromatograms of extracts of lansoprazole enantiomers, metabolites and the I.S. from human liver microsome samples are shown in Fig. 2. The peaks of (+)- and (−)-lansoprazole were identified based on our previous report [9] after collecting each fraction.

Following the microsomal incubations, chromatographic peaks were detected with retention times corresponding to those of 5-hydroxylansoprazole (10.7 min), I.S. (12.3 min), lansoprazole sulfone (13.6 min), (+)-lansoprazole (15.7 min) and (−)-lansoprazole (18.1 min). The total run time was 35 min. There was no interference from extracted components of the microsomal incubation system with either lansoprazole enantiomers or lansoprazole metabolites.

3.2. Calibration curves

Standard curves for the lansoprazole enantiomers and metabolites were linear over the concentration ranges studied (0.25–200 μ*M* for lansoprazole enantiomers and 0.13–26 μ*M* for metabolites). Typical weighted linear regression equations for (+)-lansoprazole, (−)-lansoprazole, 5-hydroxylansoprazole and lansoprazole sulfone were $y = 0.187x + 0.005$ ($r^2 = 0.999$), $y = 0.189x + 0.000$ ($r^2 = 0.999$), $y = 0.098x - 0.004$ ($r^2 = 0.999$) and $y = 0.170x - 0.001$

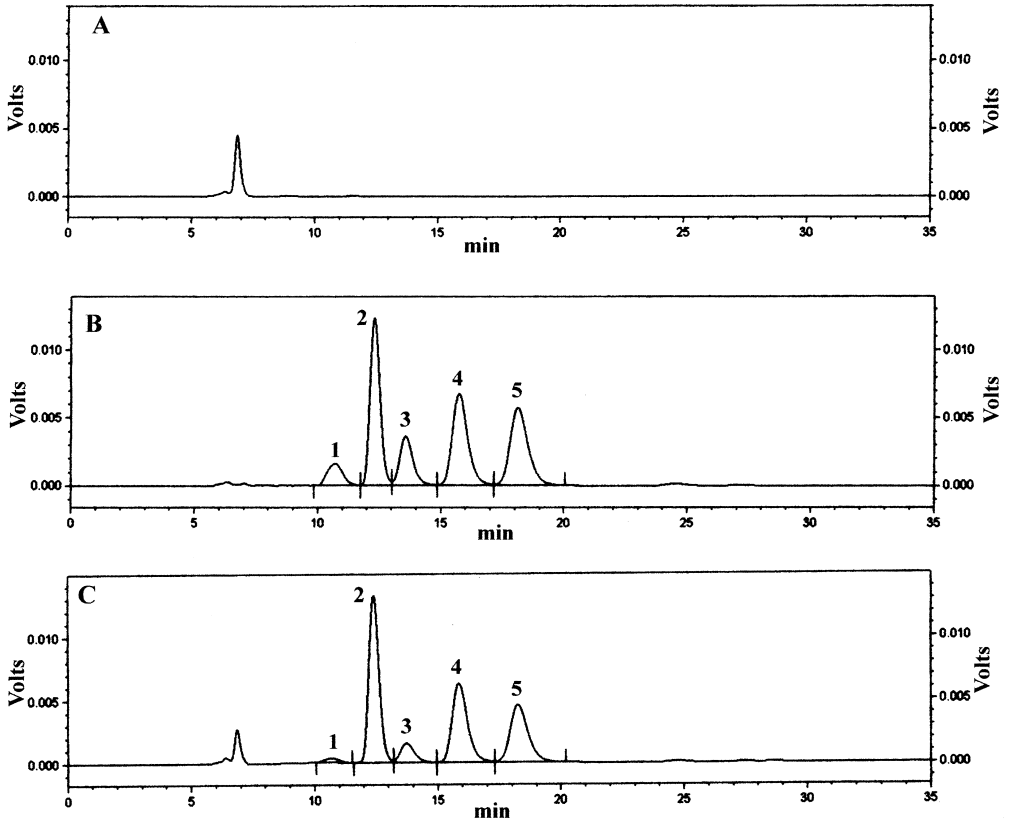

Fig. 2. Typical chromatograms after microsomal incubation for 20 min without substrate (A), of standard solution containing 5 μ*M* of racemic lansoprazole and 2.6 μ*M* of each metabolite (B), and after incubation of human liver microsomes for 20 min in the presence of 5 μ*M* of racemic lansoprazole (Peaks: 1, 5-hydroxylansoprazole; 2, I.S.; 3, lansoprazole sulfone; 4, (+)-lansoprazole; 5, (−)-lansoprazole).

Table 1
Intra-day and inter-day variation for lansoprazole enantiomers in human liver microsomes

Compound	Concentration in spiked sample (μM)	Intra-day ($n=4$)			Inter-day ($n=4$)		
		Concentration found (Mean±SD)	C.V. (%)	Accuracy (%)	Concentration found (Mean±SD)	C.V. (%)	Accuracy (%)
(+)-Lansoprazole	0.25	0.226±0.002	0.9	−9.6	0.226±0.003	1.3	−9.6
	5	5.273±0.293	5.6	5.5	5.265±0.057	1.1	5.3
	50	51.374±1.075	2.1	2.7	51.235±0.797	1.6	2.5
(−)-Lansoprazole	0.25	0.225±0.004	1.8	−10.0	0.224±0.012	5.4	−10.4
	5	5.203±0.269	5.2	4.1	5.198±0.070	1.3	4.0
	50	51.294±1.080	2.1	2.6	51.219±0.803	1.6	2.4

($r^2=0.999$), respectively, y being the peak area ratio and x the concentration in μM.

3.3. Precision and accuracy of the assay

Intra-day and inter-day precision and accuracy of the method were determined for lansoprazole enantiomers (Table 1) and for metabolites (Table 2). Table 1 shows that C.V. was 0.9–5.6% for (+)-lansoprazole and 1.3–5.4% for (−)-lansoprazole at different concentrations. In addition, the accuracy was estimated to be within 9.6% for (+)-lansoprazole and 10.4% for (−)-lansoprazole. Similar results were obtained for metabolites, as shown in Table 2. The C.V. was 1.3–7.3% for 5-hydroxylansoprazole and 1.6–7.3% for lansoprazole sulfone; the accuracy was within 5.0% for 5-hydroxylansoprazole and 3.0% for lansoprazole sulfone. These data suggest that the method is accurate and reproducible for the determination of lansoprazole enantiomers and its metabolites in human liver microsomal samples.

3.4. Recovery

The recovery following the extraction procedure was determined by comparing peak areas of stock solutions of analytes in methanol directly injected into the system, with those of extracted samples from human liver microsomes. The I.S. recovery was determined at the concentration used in the assay procedure. The mean analytical recoveries of (+)-lansoprazole, (−)-lansoprazole (Table 3), 5-hydroxylansoprazole and lansoprazole sulfone (Table 4)ranged from 70.3 to 75.8%, 70.0 to 75.7%, 70.1 to 73.7% and 72.2 to 77.5%, respectively. The recovery of the I.S. was 71.9%. Each recovery was acceptable for the determination of each substance.

Table 2
Intra-day and inter-day variation for metabolites in human liver microsomes

Compound	Concentration in spiked sample (μM)	Intra-day ($n=4$)			Inter-day ($n=4$)		
		Concentration found (Mean±SD)	C.V. (%)	Accuracy (%)	Concentration found (Mean±SD)	C.V. (%)	Accuracy (%)
5-Hydroxy lansoprazole	0.13	0.135±0.006	4.3	4.0	0.137±0.007	4.9	5.0
	2.6	2.564±0.188	7.3	−1.4	2.600±0.115	4.4	0.0
	13	12.890±0.166	1.3	−0.8	13.377±0.422	3.2	2.9
Lansoprazole sulfone	0.13	0.131±0.006	4.4	1.1	0.133±0.007	5.1	2.0
	2.6	2.583±0.190	7.3	−0.7	2.589±0.081	3.1	−0.4
	13	13.030±0.204	1.6	0.2	13.390±0.315	2.4	3.0

Table 3
Analytical recoveries of lansoprazole enantiomers and I.S. from human liver microsomes (Mean±SD, $n=4$)

Compound	Concentration added (μM)	Recovery (%)
(+)-Lansoprazole	0.5	71.6±2.9
	5	70.3±3.5
	10	72.7±3.3
	25	72.0±2.7
	100	75.5±4.0
	200	75.8±6.0
(−)-Lansoprazole	0.5	74.2±2.9
	5	70.0±3.2
	10	72.5±3.3
	25	71.8±2.7
	100	75.4±4.1
	200	75.7±6.0
I.S.	25	71.9±5.2

3.5. Limits of quantification

The LOQ was defined as the lowest concentration that can be measured with acceptable precision and accuracy. As indicated in Section 2.6.5., the LOQ values was 0.25 μM for both lansoprazole enantiomers and 0.13 μM for both metabolites whose precision and accuracy were within the proposed criteria.

3.6. Application

This method was applied to evaluation of enantioselective metabolism of lansoprazole enantiomers by human liver microsomes. Fig. 3 shows the concentrations of lansoprazole enantiomers in human liver microsomes during incubation. After incubation for 120 min, (+)-lansoprazole concentrations were approximately 2-fold higher than those of (−)-lansoprazole. The intrinsic clearance (CL_{int}) determined for the disappearance of lansoprazole enantiomers was examined by the least-squares linear regression analysis [16,17]. The values of CL_{int} were 13.8 μl/min/mg protein for (+)-lansoprazole and 33.4 μl/min/mg protein for (−)-lansoprazole at 5 μM racemic lansoprazole ($n=2$).

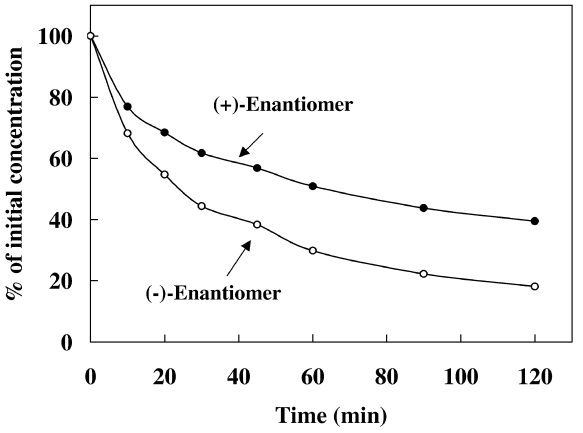

Fig. 3. Lansoprazole enantiomers (●, (+), ○, (−)) concentration–time profiles in human liver microsomes containing NADPH generating system during incubation. The substrate used was racemate and its initial concentration was 5 μM. Data are expressed as means obtained from duplicate determinations.

Fig. 4 shows the conversion of racemic lansoprazole into 5-hydroxylansoprazole and lansoprazole sulfone. The formation of both metabolites was almost linear for incubation times up to about 30 min.

Table 4
Analytical recoveries of metabolites from human liver microsomes (Mean±SD, $n=4$)

Metabolites	Concentration added (μM)	Recovery (%)
5-Hydroxylansoprazole	0.26	70.1±1.8
	1.3	72.8±5.3
	2.6	73.3±3.3
	13	73.7±4.7
Lansoprazole sulfone	0.26	72.2±4.8
	1.3	74.9±5.5
	2.6	75.8±3.9
	13	77.5±5.8

4. Conclusions

We developed a rapid and sensitive assay method for simultaneous HPLC determination of lansoprazole enantiomers and its metabolites; 5-hydroxylansoprazole and lansoprazole sulfone, in human liver microsomes.

This assay is suitable to study a possible stereoselective metabolism of lansoprazole enantiomers. The results of these investigations will be reported in a separate paper.

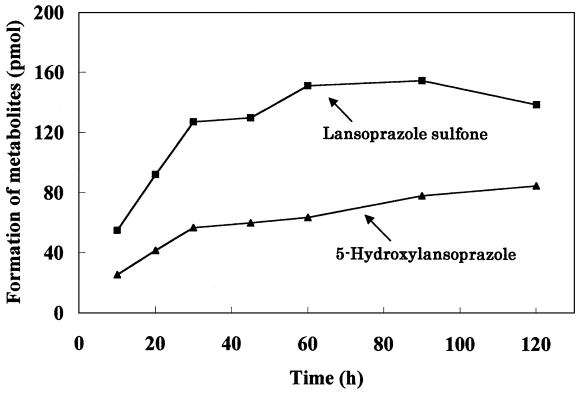

Fig. 4. Confirmation of the formation of 5-hydroxylansoprazole (▲) and lansoprazole sulfone (■) in human liver microsomes containing NADPH generating system during incubation. The substrate used was racemate and its initial concentration was 5 μ*M*. Data are expressed as means obtained from duplicate determinations.

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