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# Short Communication

# Determination of enantiomeric concentrations of a 2,5-diaryltetrahydrofuran (L-668,750), a platelet-activating factor receptor antagonist, in rat plasma using a chiral $\alpha_1$-acid glycoprotein high-performance liquid chromatographic column

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ABSTRACT

Racemic sulfonylated 2,5-diaryltetrahydrofuran [L-668,750, (±)-*trans*-2-[3-methoxy-5-(2-hydroxy)ethylsulfonyl-4-*n*-propoxy]-phenyl-5-(3,4,5-trimethoxyphenyl)-tetrahydrofuran, I] is a potent, specific and orally active platelet-activating factor (PAF) receptor antagonist. Its (−)-(2*S*,5*S*) enantiomer [L-680,573, (*S*)-I] exhibited higher PAF antagonistic potency than the (+)-(2*R*,5*R*) enantiomer [L-680,574, (*R*)-I] *in vitro* and in animal models. For assay of drug concentrations in plasma of rats dosed intravenously or orally with tritium-labeled I, we have developed a high-performance liquid chromatographic (HPLC) method which directly resolved the two enantiomers. The column contained $\alpha_1$-acid glycoprotein as the chiral stationary phase and was eluted with phosphate buffer, methanol and ethanol at neutral pH. The concentration of each enantiomer in the plasma was then determined by reverse isotope dilution assay. Results showed that the plasma clearance rate of the more potent (*S*)-I enantiomer was more than ten-fold faster than that of the (*R*)-I enantiomer; the enantioselective clearance resulted in nearly ten-fold higher concentrations of the latter in plasma at all time points regardless of the dosing route. This paper describes the HPLC chiral resolution method and its application in plasma analysis.

## INTRODUCTION

Racemic L-668,750 [(±)-*trans*-2-[3-methoxy-5-(2-hydroxy)ethylsulfonyl-4-*n*-propoxy]phenyl-5-(3,4,5-trimethoxyphenyl)-tetrahydrofuran, I], a sulfonylated 2,5-diaryltetrahydrofuran, is a potent platelet-activating factor (PAF) receptor antagonist *in vitro* in isolated human and rat polymorphonuclear leukocytes and *in vivo* in rats [1] (Fig. 1). Of its two enantiomers, the levorotatory (−)-(2*S*,5*S*) enantiomer [(*S*)-I] was more active *in vitro* and *in vivo* in the rat than the dextrorotatory (+)-(2*R*,5*R*) isomer [(*R*)-I] [2,3]. During the preclinical evaluation of the (*S*)-I enantiomer, metabolism studies were carried out in the rat and also *in vitro* with rat hepatic microsomes using either the racemic compound I or the pure enantiomer (*S*)-I [4]. Pharmacokinetic studies

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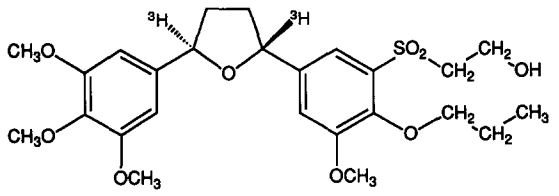

Fig. 1. Structure of L-668,750 (I). Radioactive I was tritium-labeled at the C-2 and C-5 positions of the tetrahydrofuran ring.

were also conducted with (*S*)-I in the rat and the dog [5]. During the course of these studies, it was observed that plasma concentrations of the parent drug were much higher when the compound was dosed as the racemate I instead of the pure enantiomer (*S*)-I. To investigate whether the observed difference might have been due to enantioselective metabolism of the isomers, we have developed a high-performance liquid chromatographic (HPLC) method for the direct resolution of the two enantiomers in biological samples after a racemic dose was administered to the animal. The method used a chiral $\alpha_1$-acid glycoprotein ($\alpha_1$-AGP) column [6–9]. This paper describes the method and its application in the quantification of the two enantiomers in plasma of rats dosed with the racemic drug.

## EXPERIMENTAL

### *Chemicals*

Tritium-labeled racemic I was synthesized by reducing the 1,4-diaryl-1,4-diketone to 1,4-diol with $NaB(HT)_4$ followed by cyclization of the diol with trifluoroacetic acid [1–3]. Radiochemical purity was 98.8% by thin-layer chromatography and 99.0% by HPLC. Unlabeled I and the two pure enantiomers, (*S*)-I and (*R*)-I, were obtained from Drs. S. Sahoo and T. Biftu of Merck Reasearch Labs.

### *Apparatus*

The HPLC system consisted of two pumps (Spectroflow 400, ABI Analytical-Kratos Division, Foster City, CA, USA), a programmable absorbance detector, Spectroflow 783 with gradient controller (ABI Analytical-Kratos Division), a Rheodyne injector and a Nelson Analytical software package connected to an IBM-AT computer for data analysis. The column used was EnantioPac (100 mm × 4 mm I.D.), with $\alpha_1$-AGP bonded to 10-$\mu$m diethylaminoethyl silica gel (LKB Pharmacia, Uppsala, Sweden). A 10 mm × 4 mm I.D. guard column of the same packing material was used to protect the column.

### *Chromatographic conditions*

The mobile phase consisted of 0.004 *M* phosphate buffer (sodium phosphate), pH 7.0 containing 10% (v/v) methanol–ethanol (1:1, v/v). The solvents were filtered and degassed with helium before use. All chromatograms were monitored at 293 mm. A flow-rate of 0.25 ml/min was maintained in all analyses unless otherwise designated.

### *Plasma extraction*

Unlabeled I (30 $\mu$g) was added to plasma aliquots (80–200 $\mu$l) containing ~30 000 dpm of radioactivity. To each sample 1 ml of methanol was added to precipitate the plasma protein. The supernatant was separated by centrifugation (10 min, 1500 *g*) and dried under a stream of nitrogen at room temperature. It was then redissolved with 25 $\mu$l of methanol. A 5-$\mu$l aliquot was injected onto the EnantioPac column, and another 5-$\mu$l aliquot was analyzed for radioactivity by scintillation counting. For control plasma samples in which the assay was validated, [$^3$H]I (~30 000 dpm) and unlabeled I (30 $\mu$g) were added before the methanol precipitation step.

### *Radioactivity analysis*

All plasma samples were counted directly in Instagel (Packard, Downers Grove, IL, USA) using a Beckman Model LS 5000 scintillation counter (Beckman, Fullerton, CA, USA). Column eluate fractions of 0.3 ml were collected from all HPLC samples and counted to establish the radioactivity profile.

### *Standard curves*

Standard curves of (*S*)-I and (*R*)-I were established by chromatography of different amounts of I in duplicate: 2, 5, 10, 15 and 20 $\mu$g. Peak

areas of (*S*)-I and (*R*)-I were plotted against the concentration of each enantiomer calculated as 50% of the concentration of racemic I ($r^2 = 0.9928$ and 0.9929 for the two enantiomers, respectively).

*Reverse isotope dilution assay (RIDA) of enantiomers (S)-I and (R)-I*

Plasma samples from male rats dosed orally with [$^3$H]I at 1 mg/kg body weight were assayed using a modified HPLC–RIDA method [10]. To dilute the plasma samples, unlabeled I was added prior to solvent extraction as described above. Aliquots (5 $\mu$l) of the final extracts were analyzed by HPLC. The mass of each enantiomer was calculated by comparing the area under the peak as determined by the UV absorption *versus* the standard curves. Radioactivity in the column eluent corresponding to the UV absorbance peaks of the enantiomer was compared to the mass in order to calculate the specific acitivity of each enantiomer in the plasma extract. Concentrations ($\mu$g/ml) of each enantiomer were determined by comparing the specific acitivity of the recovered enantiomer against the initial specific activity of the dose.

## RESULTS AND DISCUSSION

Resolution of enantiomers by HPLC can be accomplished either directly or indirectly after chemical derivatization of the isomers with optically active reagents to form diastereoisomers. Direct resolution is usually achieved by use of a column containing a chiral stationary phase, or by a mobile phase with an added chiral modifier. Successful indirect resolution after derivatization is usually accomplished if the functional group to be derivatized is close to the asymmetric center. For the 2,5-diaryltetrahydrofurans, the two asymmetric centers at the tetrahydrofuran ring junctures do not possess readily derivatizable groups, and, as expected, attempts to derivatize hydroxyl functions away from the asymmetric centers failed to provide diastereoisomers sufficiently different for chromatographic resolution. We have therefore chosen to pursue the direct separation of racemic I by columns containing chemically bonded chiral phases.

Separation of the (*R*) and (*S*) isomers of the racemic I was first attempted using a chiral cyclodextrin column (Cyclobond-I, Astec, Whippany, NJ, USA). No separation was obtained using the whole range of methanol-to-water ratio in the mobile phase. Another column tried was a Pirkle column (Pirkle-covalent bound D-Phenylglycine, Regis, Morton Grove, IL, USA). Some degree of separation was achieved with this column using a combination of hexane, methanol and *tert.*-butyl alcohol as the mobile phase. Hexane appeared to be necessary for achieving any resolution of the two enantiomers. Baseline separation, however, was never obtained. Resolution of the enantiomers was greatly improved ($R_s \geq 1$) when a column with chemically bonded bovine serum albumin (BSA) (Resolvosil, Macherey-Nagel, Düren, Germany) was used with mobile phase consisting of 2% isopropanol in 0.004 *M* phosphate buffer at pH 7.4, flow-rate 1 ml/min. Similar to the BSA column, another chiral column with a plasma protein $\alpha_1$-AGP (EnantioPac) as the stationary phase showed promising resolution. Partial resolution ($R_s = 0.7$) was achieved by using a combination of propanol, sodium phosphate buffer at pH 7, eluted at 0.3 ml/min. Maintaining a neutral pH gave slightly better resolution ($R_s = 0.8$). The buffer concentration of 0.004 *M* was optimum; increasing or decreasing this concentration only resulted in lower resolutions. Changing the organic phase from propanol to ethanol significantly improved the resolution ($R_s = 0.9$). A baseline separation was obtained when 5% ethanol was used and was improved when it was replaced with a 1:1 mixture of ethanol–methanol. The best resolution ($R_s = 1.20$) of the enantiomers was accomplished with 10% ethanol–methanol (1:1, v/v) in 0.004 *M* sodium phosphate buffer at pH 7.0 (Fig. 2). The EnantioPac column was thus chosen for the analysis of the plasma samples.

Standard curves, one for each enantiomer, were established as described in Experimental and used for the determination of enantiomer concentration in the plasma samples of male rats

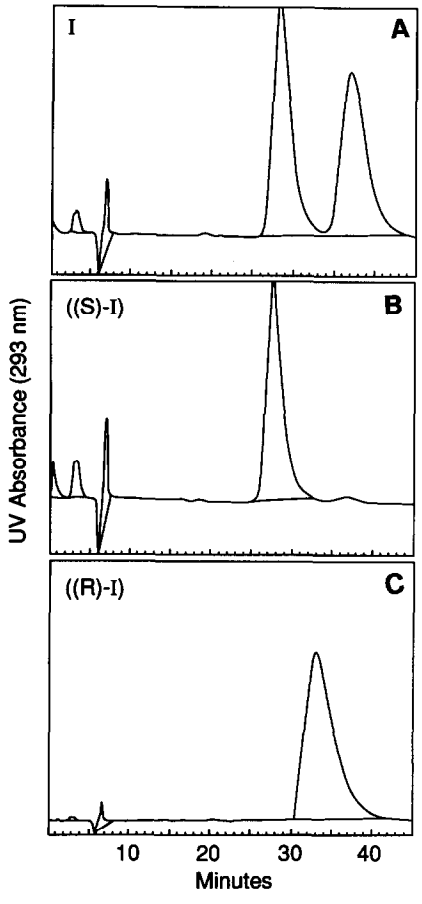


Fig. 2. (A) Resolution of standard racemic I and its enantiomers by a chiral $\alpha_1$-acid glycoprotein HPLC column (EnantioPac): (*S*)-I and (*R*)-I. The mobile phase was 0.004 *M* sodium phosphate buffer, pH 7.0, containing 10% ethanol–methanol (1:1, v/v). (B) (*S*)-I. (C) (*R*)-I.

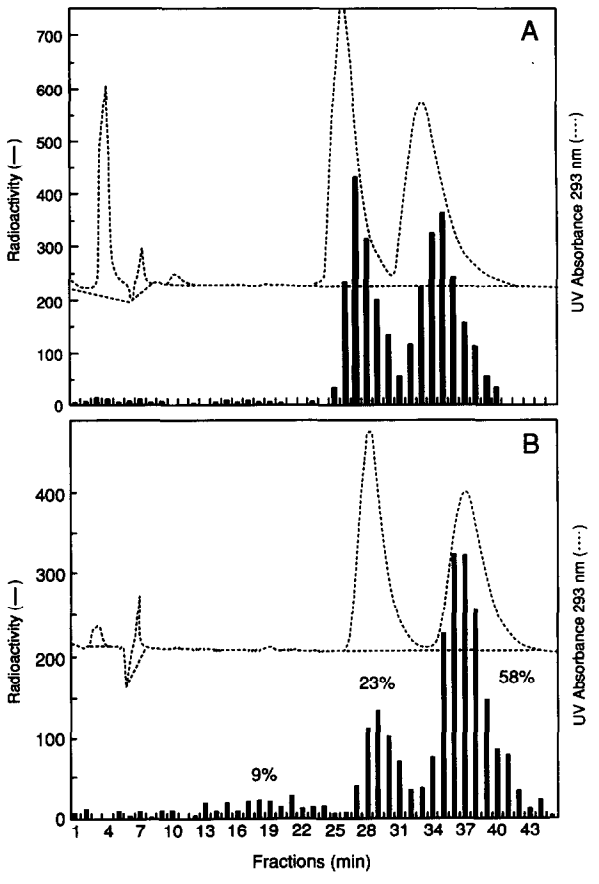


Fig. 3. (A) Control rat plasma spiked with unlabeled and tritium-labeled I. Chromatographic conditions as described for Fig. 2. Distribution of radioactivity between (*S*)-I and (*R*)-I was 47 and 53%, respectively. Quantification of spiked (*S*)-I and (*R*)-I (from racemic I) was 98 and 110%, respectively. (B) Radioactivity profiles of plasma of a male rats dosed intravenously with [$^3$H]I. Chromatographic conditions were identical to that in (A). Distribution of radioactivity between (*S*)-I and (*R*)-I was 23 and 58%, respectively. Column fractions eluting between 13 and 25 min, presumably metabolites, accounted for 9% of radioactivity recovered.

dosed intravenously or orally with the racemic compound I. To evaluate the RIDA method, both unlabeled and tritium-labeled I was spiked into plasma samples from control male rats. Following solvent extraction an aliquot was chromatographed, the concentration of each isomer calculated from UV absorbance area using the standard curve and the radioacitivity corresponding to the UV peak was compared to the original specific acitivity. The RIDA results of the enantiomers (*S*)-I and (*R*)-I were 98 and 110%, respectively, indicating a quantitative recovery in the analysis (Fig. 3A). Throughout the study standard samples were chromatographed to monitor the inter-day variation of standard curves (Table I).

In one of the preclinical metabolism studies, male Sprague–Dawley rats were dosed orally or intravenously with [$^3$H]I at 1 mg/kg body weight and blood was collected by orbital bleeding at 5, 15, 30 min and 1, 2, 4 and 6 h post dosing. Plasma samples from these animals were analyzed by RIDA in which unlabeled I was added as carrier prior to extraction of the plasma samples. The

TABLE I

DISTRIBUTION OF (*S*)-I AND (*R*)-I ENANTIOMERS ON THE BASIS OF UV ABSORPTION AS RESOLVED BY THE ENANTIOPAC COLUMN OF RACEMIC I

Analyses were performed over a three-week period. Distribution of each enantiomer was calculated as the ratio of the UV absorbance or radioactivity in the respective peak to that in both enantiomer peaks.

| Compound | | (*S*)-I | (*R*)-I |
|---|---|---|---|
| Unlabeled I | Mean ± S.D. ($n=7$) | 0.50 ± 0.01 | 0.50 ± 0.01 |
| [$^3$H]I | Mean ± S.D. ($n=3$) | 0.50 ± 0.04 | 0.50 ± 0.04 |
| | Mean ± S.D. ($n=10$) | 0.50 ± 0.02 | 0.50 ± 0.02 |

extracted radioactivity was >90% of the total radioactivity in all plasma samples. HPLC analysis of the plasma extract resolved the radioactive enantiomers, (*S*)-I and (*R*)-I, whose specific activities were calculated on the basis of total radioactivity and mass eluted in the peak corresponding to each enantiomer. Typical radioactivity and UV absorbance profiles are shown in Fig. 3B. In this plasma sample collected 5 min after intravenous dosing, the radioactivity peaks corresponding to (*S*)-I and (*R*)-I accounted for 23 and 58% of the total plasma radioactivity, with the rest being metabolites as shown in Fig. 3B. The profile of UV absorbance of the plasma extract, however, was largely due to the unlabeled I added prior to extraction and was resolved into the two enantiomers at a ~1:1 ratio.

Results of RIDA on the concentration of (*S*)-I and (*R*)-I in plasma of male rats after a single intravenous or oral dose of I are shown in Table II. It is apparent that by both administration routes, concentrations of (*R*)-I in plasma were substantially higher than those of (*S*)-I at all time points, suggesting enantioselective pharmacokinetics in the disposition of the two isomers. The clearance rates of (*S*)-I and (*R*)-I, calculated as the ratio of the dose (0.5 mg/kg) to area under the plasma concentration *versus* time curve between 0 and 6 h after the intravenous dose, was 67 and 6 ml/min/kg, respectively. These results provided

TABLE II

PLASMA CONCENTRATIONS OF ENANTIOMERS, (*S*)-I AND (*R*)-I, IN MALE SPRAGUE–DAWLEY RATS DOSED ORALLY OR INTRAVENOUSLY WITH RACEMIC I

Rats were dosed with [$^3$H]I at 1 mg/kg body weight. The drug was prepared in PEG 400 at 1 mg/ml for the intravenous dose and in 0.5% methylcellulose at 0.4 mg/ml for the oral dose. Three animals were dosed at each time point. All samples were analyzed by RIDA. Values represent mean ± S.D. from three animals.

| Time post dose | Plasma concentration (μg/ml) | |
|---|---|---|
| | (*S*)-I | (*R*)-I |
| *Intravenous dose* | | |
| 5 min | 0.14 ± 0.03 | 0.79 ± 0.18 |
| 15 min | 0.10 ± 0.01 | 0.76 ± 0.10 |
| 30 min | 0.09 ± 0.03 | 0.78 ± 0.31 |
| 1 h | 0.02 ± 0.02 | 0.35 ± 0.27 |
| 2 h | 0.02 ± 0.00 | 0.42 ± 0.34 |
| 4 h | 0.01 ± 0.01 | 0.10 ± 0.09 |
| 6 h | 0.00 ± 0.00 | 0.05 ± 0.00 |
| *Oral dose* | | |
| 15 min | 0.03 ± 0.00 | 0.21 ± 0.02 |
| 30 min | 0.03 ± 0.02 | 0.34 ± 0.10 |
| 1 h | 0.07 ± 0.03 | 1.09 ± 0.41 |
| 2 h | 0.01 ± 0.00 | 0.14 ± 0.00 |
| 4 h | 0.01 ± 0.00 | 0.08 ± 0.07 |
| 6 h | 0.01 ± 0.01 | 0.03 ± 0.03 |

an explanation for the low plasma levels observed in rats when only the more active enantiomer (*S*)-I was administered intravenously or orally [5]. As a result of the more than ten-fold faster plasma clearance rate of (*S*)-I, this enantiomer was the minor radioactive component in the plasma at all time points studied, contributing to <20% of the unchanged parent I. In separate experiments, we have studied the *in vitro* metabolism of I by rat liver slices and freshly isolated hepatocytes. It was shown that (*S*)-I was preferentially metabolized in these systems. No interconversion of these enantiomers was observed *in vivo* and under the *in vitro* study conditions. These observations taken together with results from plasma binding studies of (*S*)-I and (*R*)-I indicated that the enantioselective pharma-

cokinetics may be largely due to the difference in the rate of metabolism of the enantiomers [(*S*)-I, (*R*)-I], which in turn may be largely due to the difference in binding to plasma proteins [(*S*)-I, (*R*)-I]. Result from these studies will be described in a separate publication [4].

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