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Stereoselective high-performance liquid chromatographic assay for the determination of OPC-18790 enantiomers in human plasma and urine

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ABSTRACT

A high-performance liquid chromatographic assay method for the quantification of OPC-18790 enantiomers in human plasma and urine is described. A human plasma or urine was extracted with organic solvent under alkaline conditions following the addition of internal standard. The enantiomers and internal standard were then derivatized by reaction with the chiral reagent GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate), followed by octadecylsilica chromatographic separation of the diastereomeric products. The mobile phase consisted of acetonitrile–water (41:59). The fluorescence of the eluate was monitored at 355/405 nm. The lowest quantification limit of each enantiomer was 10 ng/ml in plasma and 0.1 μg/ml in urine. Both intra- and inter-day coefficients of variation were below 10%. The assay is sensitive, specific and applicable for stereoselective pharmacokinetic studies in human.

INTRODUCTION

OPC-18790, (±)-6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-2(1H)-quinolinone, is a novel positive inotropic agent [1] under development. OPC-18790 has a chiral centre and is used as the racemate (Fig. 1A). The *R*-(+)-isomer is about ten-fold more potent in increasing cardiovascular contractile force than its optical antipode, the *S*-(−)-isomer [2]. Therefore, the development of a chromatographic procedure for the quantitative analysis of OPC-18790 enantiomers in plasma or urine is necessary for pharmacokinetic studies of enantiomers after treatment with the racemic compound. 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Fig. 1B) is a commercially available chiral derivatizing reagent, and is known to react readily with primary and secondary amino groups. It can be applied under mild conditions and is easy to use compared with other reagents [3–8]. It has been aplied to the optical resolution of β-amino alcohols (*e.g.* β-blockers such as pro-

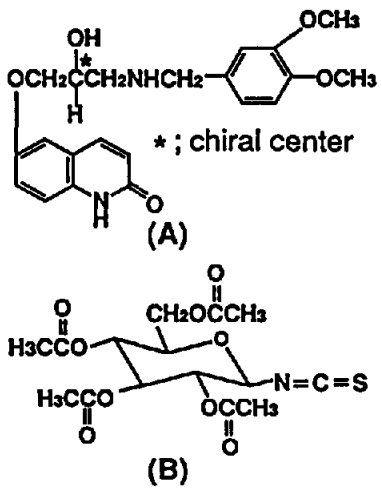

Fig. 1. Chemical structures of OPC-18790 (A) and GITC (B).

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pranolol and atenolol [8] as well as amino acids. Since, like β-blockers, OPC-18790 contains the β-amino alcohol structure inside the molecule, the chromatographic resolution of the corresponding diastereomers was considered likely. This paper describes the derivatization of OPC-18790 enantiomers with GITC followed by reversed-phase high-performance liquid chromatographic separation using fluorescence detection.

Furthermore, OPC-18790's own fluorescence enhanced the selectivity and sensitivity with no interference from any excess reagent added and without endogenous interference. The high derivatizing yield achieved almost equal sensitivity as the analysis of non-stereoselective quantification of OPC-18790 in human plasma and urine.

EXPERIMENTAL

Materials

OPC-18790 and internal standard *S*-(−)-6-[2-hydroxy-3-[(3-methoxybenzyl)amino]propoxy]-2(1H)-quinolinone were synthesized at the Second Factory of Otsuka Pharmaceutical (Tokushima, Japan). Chloroform, tris(hydroxymethyl) aminomethane, sodium chloride and acetic acid were analytical grade, while acetonitrile, methanol and tetrahydrofuran were HPLC grade. All the reagents described were purchased from Wako (Osaka, Japan). Bovine serum albumin (BSA, fraction V, 98–99%) was purchased from Sigma (St. Louis, MO, USA). Triethylamine was analytical grade, purchased from Aldrich (Milwaukee, WI, USA). GITC was purchased from Wako.

Sample preparation

Plasma. To 0.5 ml of human plasma in a 10-ml brown centrifuge tube were added 100 ng (in 20 μl of methanol) of internal standard, 100 μl of 1 *M* tris(hydroxymethyl)aminomethane (Tris) and 5.0 ml of chloroform. The mixture was shaken for 5 min on a mechanical shaker and centrifuged at 1800 *g* for 5 min. Then, a 4.0-ml aliquot of the organic phase was transferred to a brown conical tube and evaporated to dryness in a water bath at below 30°C under reduced pressure. To the residue, 100 μl of 0.2% triethylamine in tetrahydrofuran (prepared as needed) and 100 μl of 0.1% GITC in acetonitrile (prepared as needed) were added. After brief vortex mixing, the mixture was allowed to stand at room temperature while protected from light. After 15 min, the sample was evaporated to dryness under a nitrogen stream and reconstituted in 100 μl of acetonitrile–water–acetic acid (50:50:0.5, v/v/v) and a 40-μl aliquot was analysed by HPLC, protected from light.

Urine. To 0.1 ml of human urine in a 10-ml brown centrifuge tube was added 200 ng (in 20 μl of methanol) of internal standard, consecutively followed by 0.1 ml of control urine and 0.3 ml of 7% BSA in 0.9% saline. To the mixture, 100 μl of 1 *M* Tris and 5.0 ml of chloroform were added. Then the mixture was subjected to the same procedure as for the plasma sample until it was evaporated to dryness. The rest of the procedure was identical to the plasma preparation, except for the volume of GITC used and the increase in the reconstituted solvent to 200 μl. An aliquot of 20–40 μl was analysed by HPLC, protected from light.

High-performance liquid chromatography

The HPLC system consisted of a Model 510 pump, a WISP 710B (or 712) automatic sample injector with cooling system (Waters, Milford, MA, USA), an RF-535 fluorescence HPLC monitor as a detector and a Chromatopac C-R6A integrator (both from Shimadzu, Kyoto, Japan). The chromatographic separation of the diastereomers was achieved on a Develosil ODS-5 column (5 μm, 250 mm × 4.6 mm I.D.) (Nomura, Aichi, Japan). The mobile phase consisted of acetonitrile–water (41:59, v/v) and chromatographic runs were carried out at ambient temperature at a flow-rate of 1.0 ml/min. Detection was performed with an excitation wavelength of 355 nm and an emission wavelength of 405 nm.

Calibration curve

Calibration curves for plasma and urine were prepared by adding known amounts of racemic OPC-18790 to blank samples obtained from

healthy male volunteers. The concentrations of the spiked samples were 20, 40, 100, 400 and 1000 ng/ml for plasma, and 0.2, 1.0, 4.0, 20, 50 μg/ml for urine. The calibration curves were constructed by plotting the peak-area ratio of each diastereomeric derivative of OPC-18790 to that of internal standard derivative *versus* spiked concentration.

Extraction yield and derivatizing yield

The extraction yield from plasma or urine was estimated by comparing the peak area of derivatized OPC-18790 obtained after extraction of spiked samples with that obtained by direct injection of derivatives without extraction.

Derivatizing yield was evaluated by the remaining fraction of underivatized OPC-18790, which was measured by the non-stereoselective assay method [9]. It was calculated by making a comparison between the peak heights of OPC-18790 with and without the derivatizing process prepared from the same spiked plasma samples.

Accuracy and precision

Blank plasma or urine samples were obtained from healthy male volunteers. The samples were spiked with racemic OPC-18790 at concentrations of 20, 80 and 600 ng/ml (plasma) and 0.5, 10 and 40 μg/ml (urine) and stored at −20°C.

In order to evaluate the inter-day variations, replicate samples in each pooled sample were handled with the procedure described in the Sample preparation section and analysed on three different days. Intra-day variations were evaluated from the results obtained on the first experimental day.

The accuracy was evaluated as percentage error [(measured − added)/added] × 100 (%), and the precision was evaluated by the coefficients of variation [C.V. (%)].

Pharmacokinetic study and data analysis

Healthy male volunteers (phase I study at the Osaka Police Hospital, $n = 3$ per group) [9] were administered racemic OPC-18790 at 2.5 and 5 μg/kg/min by intravenous infusion for 1 h. Informed consent was obtained. The volunteers received a standard diet prepared by the hospital throughout the study period.

On the day of the study, an indwelling catheter was inserted into an antecubital vein prior to the infusion. Venous blood samples were collected in heparinized tubes at 5, 10, 20, 30, 45, 60, 65, 70, 80, 90 and 105 min and 2, 2.5, 3, 4, 6, 8, 10, 12 and 24 h after the start of infusion from the antecubital vein of the other arm. Urine samples were collected once before drug administration and at intervals of 0–2, 2–4, 4–8, 8–12, 12–24 and 24–48 h after the start of infusion. The blood samples were centrifuged at 1800 *g* for 10 min to obtain plasma. Plasma and urine samples were stored at −20°C until analysis.

Assayed plasma concentration–time data for each enantiomer were used to determine the pharmacokinetic parameters by non-compartmental procedures.

In this study, C_{max} represents the concentration at the time the infusion was stopped. $T_{1/2}$ was defined as the half-life of the terminal phase and the area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule.

RESULTS AND DISCUSSION

Derivatization

The reaction time was roughly estimated to be less than 30 min from previous papers [4,6]. As preliminary trials, the time course study was conducted with a variable reaction time of 5–30 min. It was found that the derivatization of racemic OPC-18790 and internal standard with GITC was completed within 15 min [10]. The remaining fraction of underivatized OPC-18790 in plasma was 3.1% at 80 ng/ml and 1.6% at 600 ng/ml. The study was performed with a 15 min reaction time. This result shows that the derivatization was achieved with high yield within this reaction time, which confirmed the high selectivity and sensitivity of the assay.

Under the HPLC conditions described in this paper, only a single peak was obtained for the GITC derivative of authenic pure *S*-(−)- and *R*-

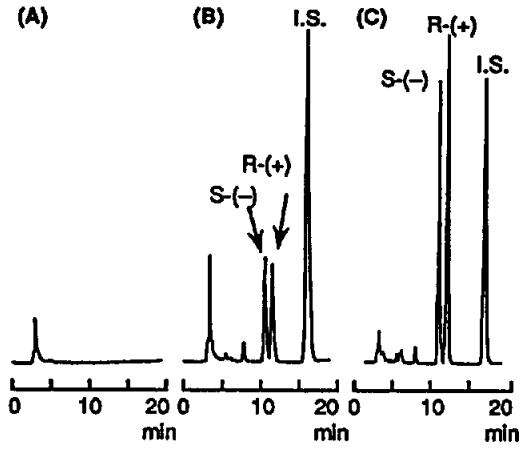

Fig. 2. Chromatograms of (A) a derivatized extract of a blank plasma sample, (B) a derivatized extract of a spiked plasma sample containing 100 ng/ml [*S*-(−) 50 ng/ml, *R*-(+) 50 ng/ml] OPC-18790 and internal standard and (C) a derivatized extract of a plasma at 30 min, following i.v. infusion of OPC-18790 racemate at 5 μg/kg/min for 1 h to a healthy human volunteer.

(+)-isomers, individually, of OPC-18790. It confirmed that no racemization occurs during the process of derivatization. In the chromatograms of derivatized racemic OPC-18790, the peak height of the *S*-(−)-derivative was slightly higher than that of the *R*-(+)-derivative, as shown in Figs. 2B and 3B.

In the preliminary study, when racemic OPC-18790 was derivatized with GITC, it often happened that the peak height of the *R*-(+)-derivative was higher than that of the *S*-(−)-derivative in the chromatograms. It looked as if the the *S*-(−)-isomer had been converted to *R*-(+)-isomer. However, in the isomer experiment on the same day, the GITC derivative of the authentic pure *S*-(−)-isomer of OPC-18790 gave a single peak only. This phenomenon was attributed to the instability of the derivatives, especially upon exposure to sunlight in the laboratory. This phenomenon was not observed when the reacted solvent was directly injected onto the column omitting the drying process in derivatization. However, the repeated direct injection of a large volume of the reacted solution containing alkali might damage the ODS column. Moreover, the large difference in solubility between the mobile phase and injection solvent causes peak shapes that are inappropriate for quantification. This makes it necessary to avoid direct injections of the reacted solvent. This instability could be prevented by protecting the derivatives from sunlight (*e.g.* with a blackout curtain) and by avoiding their prolonged direct contact with air.

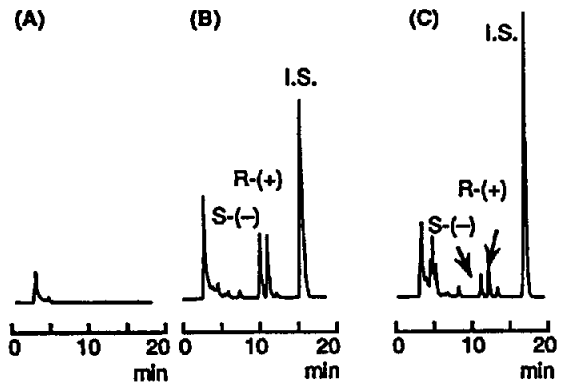

Fig. 3. Chromatograms of (A) a derivatized extract of blank urine, (B) a derivatized extract of a spiked urine sample containing 1 μg/ml [*S*-(−) 0.5 μg/ml, *R*-(+) 0.5 μg/ml] OPC-18790 racemate and internal standard and (C) a derivatized extract of a 24–48 h urine sample, following i.v. infusion of OPC-18790 racemate at 5 μg/kg/min for 1 h to a healthy human volunteer.

Chromatograms

Typical chromatograms of the extracted and derivatized diastereomeric products from the blank samples and the spiked with racemic OPC-18790 and internal standard are shown in Fig. 2A and B for plasma and Fig. 3A and B for urine. The chromatograms of human samples are shown in Fig. 2C for plasma (30 min) and Fig. 3C for urine (24–48 h) following the intravenous infusion of racemic OPC-18790 at 5 μg/kg/min for 1 h.

OPC-18790's own fluorescence enhanced the selectivity and sensitivity with no interference from excess reagent or endogenous substance in both plasma and urine, as shown in Figs. 2 and 3. The *S*-(−)- and *R*-(+)-diastereomeric derivatives eluted at about 11 and 12 min under chromatographic conditions, corresponding to *S*-(−)- and *R*-(+)-OPC-18790, as confirmed by derivatizing and injecting samples of authentic pure *S*-(−)- and *R*-(+)-OPC-18790.

Both diastereomeric derivatives were well sep-

arated. The capacity factors (k') for *S*-(−)- and *R*-(+)-derivatives of OPC-18790 and internal standard were 2.30, 2.63 and 4.14, respectively. The separation and resolution factors (α and R_s) were 1.14 and 1.01, respectively, for *S*-(−) and *R*-(+)-diastereomeric derivatives.

Extraction

Extraction yields for plasma ranged from 88.7 to 100.9% and from 90.2 to 110.3% for *S*-(−) and *R*-(+)-derivatives, respectively. Those for urine were from 88.8 to 95.4% and from 89.3 to 94.8% for *S*-(−)- and *R*-(+)-derivatives, respectively.

It was necessary to further modify the extraction process for urine compared with that of plasma to obtain constant extraction yields. The extraction recoveries varied greatly in urine samples, unlike plasma specimens. This suggested that the salt concentration in urine samples was extremely variable among samples compared with plasma samples. Therefore, 7% BSA in saline was added as an artificial plasma to give constant extraction yields in the urine samples.

Control urine in a volume of 0.1 ml was also added to the urine sample in expectation of a "buffer effect". Constant extraction yields for urine samples were also achieved in this way.

On the other hand, the concentrations of a few samples were below the lower quantification limit. The use of a 0.2-ml aliquot of the urine sample allowed the quantitative analysis of these low-level samples. It was important to standardize various conditions regardless of sample volumes, *e.g.* the extraction yield and the interfering peaks on chromatograms. Therefore, to samples in which the concentration was expected to be high 0.1 ml of control urine was added to make the final urine volume 0.2 ml, while 0.2 ml of sample were used from the beginning when the concentration was expected to be low. Consequently, this standardization of sample volumes avoided construction of two calibration curves.

Linearity, accuracy and precision and quantification limit

Typical calibration curves gave the following equations. Plasma: *S*-(−), $y = 0.00432x^{1.008}$, $r = 0.9999$; *R*-(+), $y = 0.00391x^{1.033}$, $r = 0.9997$, in the range 10–500 ng/ml. Urine: *S*-(−), $y = 0.4114x^{1.016}$, $r = 0.9998$; *R*-(+), $y = 0.4252x^{1.015}$, $r = 0.9998$, in the range of 0.1–25 μg/ml. Good linearity was observed between OPC-18790 derivatives/internal standard derivative peak-area ratios and concentrations. The use of power regression is necessary to cover the wide range of the concentration.

Table I summarizes the precision (coefficients

TABLE I

INTRA-DAY VARIATIONS IN THE DETERMINATION OF *S*-(−) AND *R*-(+)-OPC18790 IN HUMAN PLASMA AND URINE

Enantiomer concentration	*n*	Coefficient of variation (%)		Error[a] (%)	
		S-(−)	*R*-(+)	*S*-(−)	*R*-(+)
Plasma					
10.0 ng/ml	5	3.88	5.77	−1.56	+2.18
40.0 ng/ml	5	0.73	1.01	+0.23	+3.01
300.0 ng/ml	4	0.39	0.74	−0.54	−0.91
Urine					
0.25 μg/ml	5	5.53	5.11	+3.44	+4.80
5.0 μg/ml	5	1.11	0.89	−1.46	−1.32
20.0 μg/ml	5	0.63	0.45	−1.53	−1.07

[a] Error (%) = [(measured − added)/added] × 100 (%).

TABLE II

INTER-DAY VARIATIONS IN THE DETERMINATION OF *S*-(−) AND *R*-(+)-OPC18790 IN HUMAN PLASMA AND URINE

Enantiomer concentration	*n*	Coefficient of variation (%)		Error[a] (%)	
		S-(−)	*R*-(+)	*S*-(−)	*R*-(+)
Plasma					
10.0 ng/ml	15	5.21	4.13	+1.67	+1.57
40.0 ng/ml	15	6.87	6.43	+2.71	+3.40
300.0 ng/ml	14	1.32	1.82	+0.78	+0.24
Urine					
0.25 μg/ml	15	5.14	4.26	−0.44	+1.48
5.0 μg/ml	15	1.79	1.86	−0.40	−0.07
20.0 μg/ml	15	1.92	1.78	−1.12	−1.10

[a] Error (%) = [(measured − added)/added] × 100 (%).

of variation) and accuracy as percentage error [(measured − added)/added · 100%] for intra-day assay. Table II shows the inter-day results. The intra-assay C.V. (%) value was less than 10% throughout the concentration range examined for both plasma and urine, as was the inter-assay C.V. (%) value. The percentage error of the assay was within ±5%.

The lower limits of quantification for both enantiomers were 10 ng/ml in plasma, using a 0.5-ml aliquot, and 0.1 μg/ml in urine, using a 0.1-ml aliquot, from the results of linearity and validation studies. Increased sample volume enhances the sensitivity of the assay, especially for urine.

Application

This stereoselective assay method was applied to pharmacokinetic studies. Fig. 4 shows the plasma concentration–time profiles of *S*-(−)- and *R*-(+)-OPC-18790 following intravenous infusion of the racemate at 2.5 and 5.0 μg/kg/min for 1 h. The concentration of *R*-(+)-isomer was almost equal to, or slightly higher than, that of *S*-(−)-isomer. Cumulative urinary excretions of *S*-(−)- and *R*-(+)-OPC-18790 following intravenous infusion of the racemate at 5.0 μg/kg/min for 1 h are depicted in Fig. 5. The cumulative excretions of *S*-(−)- and *R*-(+)-isomers amounted to 20.29% and 21.93%, respectively, which brings the cumulative excretion to 42.22%. There was no marked difference in plasma concentrations or urinary excretion of the two enantiomers. The relatively high degree of excretion can be largely attributed to the renal clearance of OPC-18790. Pharmacokinetic parameters are given in Table III. The C_{max} and AUC of the *R*-(+)-isomer were higher than those of the *S*-(−)-isomer, as expected from its more potent pharmacological effects.

The stereoselective method in this study was

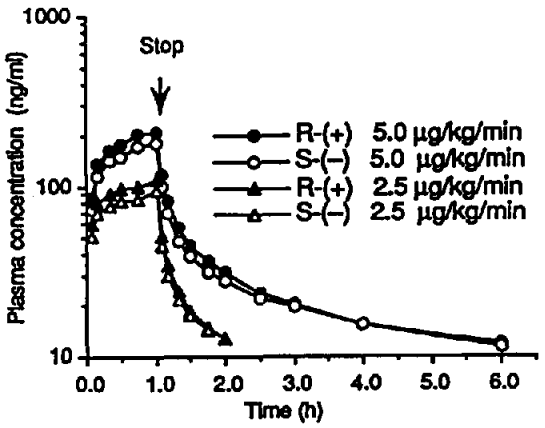

Fig. 4. Plasma concentration–time profiles of *S*-(−)- and *R*-(+)-OPC 18790 following i.v. infusion of OPC-18790 racemate at 2.5 and 5 μg/kg/min for 1 h. Each point represents the mean of values from three volunteers.

TABLE III

PHARMACOKINETIC PARAMETERS FOLLOWING INTRAVENOUS INFUSION OF OPC-18790 RACEMATE AT 5 μg/kg/min FOR 1 h TO HEALTHY HUMAN VOLUNTEERS

Parameter	Isomer	Values		
		Subject A	Subject B	Subject C
C_{max} (ng/ml)	S-(−)	180.7	166.9	193.3
	R-(+)	215.5	191.4	228.0
$t_{1/2}$	S-(−)	2.84	4.29	4.21
	R-(+)	2.75	4.14	4.00
$AUC_{0-6\,h}$ (ng ml^{-1} h)	S-(−)	262.5	260.5	260.0
	R-(+)	306.7	288.6	297.8
R/S ratio (AUC)		1.17	1.11	1.15
R/S ratio (urinary excretion 0–48 h)		1.09	1.06	1.08

verified by comparing it with the non-stereoselective method [9]. Fig. 6 shows the correlation between the stereoselective and non-stereoselective analytical methods, which are validated by using human plasma after administration of OPC-18790 racemate. The deviation was less than 10%, indicating the applicability of the stereoselective assay.

It is known from preclinical studies that there is no interconversion between the enantiomers in rat, dog and monkey [10]. However, there are differences of various kinds and among animal species between S-(−)- and R-(+)-isomers in terms of pharmacokinetics. Therefore, this sensitive and specific assay method could be applied usefully in the future, *e.g.* in patient studies, protein-binding studies, etc.

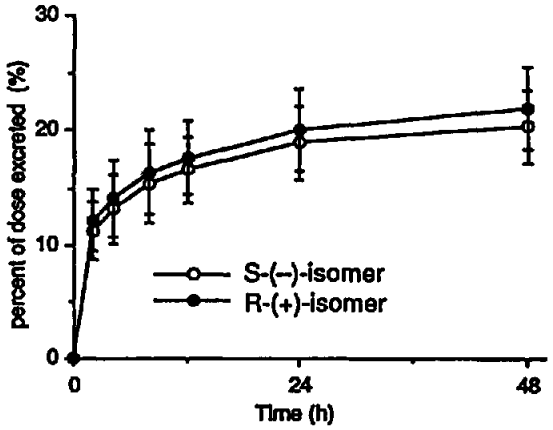

Fig. 5. Cumulative urinary excretion of S-(−)- and R-(+)-OPC-18790 following i.v. infusion of OPC-18790 racemate at 5μg/kg/min for 1 h. Points and bars represent the mean ± S.D. of values from three volunteers.

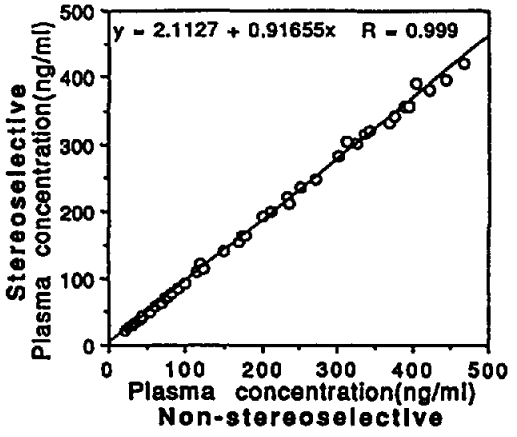

Fig. 6. Correlation between the plasma OPC-18790 concentrations obtained by the stereoselective analytical method and those obtained by the non-stereoselective analytical method. This result was obtained from human plasma samples following i.v. infusion of OPC-18790 racemate at 5 μg/kg/mn for 1 h to healthy human volunteers.

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