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Enantioselective analysis of sotalol in plasma by reversed-phase high-performance liquid chromatography using diastereomeric derivatives

W.D. Hooper*, P.V. Baker

Department of Medicine, The University of Queensland, Clinical Sciences Building, Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia

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Abstract

A procedure for the concurrent determination of the (+)- and (−)-enantiomers of sotalol in plasma using high-performance liquid chromatography of diastereomeric derivatives is described. Sotalol is extracted from a 0.5-ml aliquot of plasma at pH 9.3 using ethyl acetate. Atenolol is used as the internal standard. The ethyl acetate is removed under vacuum, and the residue derivatized with *R*-(−)-1-(1-naphthyl)ethyl isocyanate (NEIC, 0.005% in chloroform) in the presence of trace quantities of carbonate buffer. The chloroform is removed, the residue reconstituted in mobile phase (acetonitrile–water, 39:61, v/v), and an aliquot injected into the HPLC column. A C_{18} trapping column is used to retain excess derivatizing reagent. While the derivatives are separated on a C_{18} analytical column with the isocratic mobile phase mentioned above at 1.5 ml/min, the column-switching allows back-flushing of the trapping column to prepare for the next injection. The derivatives were detected using a fluorescence detector with excitation wavelength 280 nm and emission wavelength 320 nm. The method was fully validated, and shown to have excellent linearity, specificity, sensitivity, accuracy and precision. It has been applied to the determination of (+)- and (−)-sotalol in plasma from twelve subjects dosed with racemic sotalol.

1. Introduction

Sotalol [4′-(2-isopropylamino-1-hydroxyethyl)-methanesulphonanilide hydrochloride; STL·HCl] is a unique antiarrhythmic agent that exerts nonselective β-adrenergic blockade and lengthens the duration of the cardiac action potential. Although discovered (1960) before propranolol, its unique combination of class II and class III antiarrhythmic actions was not fully appreciated until relatively recently [1]. It is used principally in the treatment of angina, hypertension and supraventricular and ventricular arrhythmias.

Like most β-adrenergic blockers, sotalol contains an asymmetric carbon atom and is currently marketed as the racemate. It has been shown that the (+)- and (−)-enantiomers have similar class III antiarrhythmic activities, while only the (−)-enantiomer contributes to β-blockade. For this reason, the (+)-isomer is currently being

* Corresponding author.

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evaluated as an antiarrhythmic agent lacking β-blocking properties.

While the question of enantioselectivity in the disposition of some β-blocking drugs, e.g. propranolol and metoprolol, has been studied extensively, studies on sotalol have been until recently hampered by the lack of appropriate analytical methods. Three recent reports gave enantioselective assays for sotalol in plasma. The method of Carr et al. [2] utilized diastereomeric derivatives with *S*-(+)-1-(1-naphthyl)ethyl isocyanate which were resolved under normal-phase conditions on a silica column. The method was applicable over the plasma concentration range of 50–5000 ng/ml for each enantiomer. Sallustio et al. [3] used a different derivatizing agent, *S*-(−)-α-methylbenzyl isocyanate, and reversed-phase chromatography, and their method was validated over the plasma concentration range of 25–2500 ng/ml for each enantiomer. Fiset et al. [4] used (−)-menthyl chloroformate for derivatization, reversed-phase chromatography, and showed satisfactory performance over the plasma concentration range of 12.5–2500 ng/ml for each enantiomer.

In the present report we describe a method which uses a simple column-switching approach to allow reversed-phase chromatography of the derivatives with *R*-(−)-1-(1-naphthyl)ethyl isocyanate (NEIC), which offers the advantages of being convenient, simple and sensitive, and which has been fully validated for use in clinical pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Racemic STL·HCl was obtained from Alphapharm, Brisbane, Australia. The enantiomers, (+)-STL and (−)-STL were obtained as a gift from Dr. B.C. Sallustio, Department of Clinical Pharmacology, Queen Elizabeth Hospital, Adelaide, Australia. Racemic atenolol (ATN) hydrochloride was used as internal standard in the assay for STL enantiomers and was obtained from ICI, Macclesfield, UK. The chiral derivatizing reagent, NEIC, was obtained from Tokyo Kasei, Tokyo, Japan. Hydroflumethiazide (3,4-dihydro-6-trifluoromethyl-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide: HFT) was used as internal standard for the assay of unresolved (i.e. [(+)+(−)]-STL), and was obtained from Sigma, St. Louis, MO, USA. Acetonitrile, methanol and ethyl acetate were HPLC grade; other solvents and chemicals were analytical grade.

2.2. Measurement of STL enantiomers in plasma

(±)-STL·HCl (11.34 mg) was dissolved in methanol (10 ml) to give a solution of 1.0 mg STL (free base)/ml. Appropriate dilutions with methanol gave a range of working solutions from 25.0 μg/ml to 0.1 μg/ml. A working internal standard solution (10.0 μg ATN/ml in methanol) was prepared. Plasma standards with final (±)-STL concentrations of 0.02, 0.05, 0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 μg/ml were prepared by dispensing aliquots of internal standard solution and appropriate quantities of STL in methanol into Pyrex screw-cap tubes of 15 ml, evaporating to dryness, and adding drug-free human plasma (0.5 ml). Carbonate buffer (1.0 *M*, pH 9.3, 0.2 ml) and ethyl acetate (6.0 ml) were added to the plasma standards or to aliquots (0.5 ml) of plasma samples to which internal standard had been added. The tubes were capped and shaken for 4 min. After centrifuging (4 min, 1000 *g*) the organic layer was transferred to a clean glass tube and evaporated to dryness using a 200H SpeedVac Concentrator (Savant, Farmingdale, NY, USA). Carbonate buffer (1.0 *M*, pH 9.3, 2.0 μl) and NEIC (0.005% in chloroform, 0.2 ml prepared freshly on the day of use) was added to the dry residue. The tubes were capped immediately and after being vortex-mixed for 15 s they were left to stand at room temperature for 1 h. The chloroform was evaporated under a stream of air and the residue was redissolved with vortex-mixing (10 s) in acetonitrile–water (39:61, v/v, 0.2 ml). The tubes were again capped and after standing at room temperature for another

hour, aliquots (10 μl) were injected into the HPLC column.

A schematic representation of the HPLC column-switching system is given in Fig. 1. A Model 6000A solvent delivery system (Pump 1; Waters, Milford, MA, USA) delivered an isocratic mobile phase (acetonitrile–water (39:61, v/v), Reservoir A) at 1.5 ml/min. Aliquots of sample extracts were injected by an ETP Kortec K65B automated sample injector (ICI, Dingley, Australia) via port 1 onto a 7.5 cm × 3.9 mm I.D. stainless steel Nova-Pak 4 μm C_{18} trapping column, which was connected across ports 3 and 6 of a Model E2C6W electrically activated switching-valve (Valco, Houston, TX, USA). The switching-valve was controlled by the external time events of the Model CR4-A computing integrator (Shimadzu, Auburn, NSW, Australia). Separation of the STL derivatives was achieved with a 10 cm × 8 mm I.D. Nova-Pak 4 μm C_{18} cartridge in an RCM-100 Radial Compression Module (Waters), connected to port 2, and the eluent was monitored with a Model RF-551 fluorescence detector (Shimadzu) operating at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. The trapping column was purged by a solvent gradient formed by a Model 2360 Gradient Programmer (Isco, Lincoln, NE, USA) and delivered by a second Model 6000A solvent delivery system (Pump 2), via port 4. The gradient program was started by a signal from the sample injector. At the time of injection the solvent delivered by Pump 2 consisted of acetonitrile–water (50:50, v/v, Reservoirs B and C). After 3 min the solvent gradient was stepped to acetonitrile only (Reservoir B) for a period of 12 min, then stepped back to acetonitrile–water (50:50, v/v) for a further 7.5 min. Finally, the trapping column was equilibrated for 7.5 min with mobile phase drawn from Reservoir A. The two external time events of the integrator were restarted by a signal from the sample injector at the time of sample injection. After 5.7 min the first time event signal turned the switching-valve from position 1 to position 2. In this position the isocratic phase delivered by Pump 1 by-passed the trapping column while this column was back-flushed with solvent delivered by Pump 2. At 29.9 min after injection the second time event signal returned the valve to position 1 ready for the next injection.

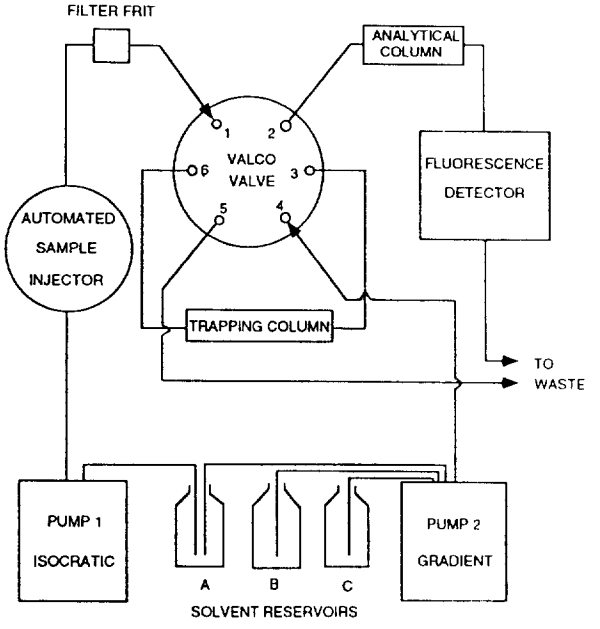

Fig. 1. Schematic representation of the HPLC column-switching system.

2.3. Method validation

Method validation generally followed the recommendations of Shah et al. [5]. Linearity was demonstrated on three separate occasions, using plasma standards in triplicate over the range of concentrations given above. Accuracy and precision, both intra-batch and inter-batch, were evaluated by repeated assays of spiked plasma standards. Specificity was established by assaying a selection ($n = 18$) of drug-free human plasma samples, and by assaying samples spiked with a selection of potentially interfering drugs. The limit of quantification was established as the lowest concentration on the standard curve at which acceptable accuracy and precision (both ≤ 20%) were achieved [5]. Extraction recovery, derivatization yield and the potential for interference of one STL enantiomer in the assay of the other were established as follows.

2.4. Extraction recovery and derivatization yield

For the determination of the extraction recovery and derivatization yield the HPLC system comprised a Model 6000A solvent delivery system, a 10 cm × 8 mm I.D. Nova-Pak 4 μm C_{18} cartridge contained in an RCM-100 Radial Compression Module, and a Model 470 fluorescence detector (all from Waters). The mobile phase [aqueous heptane sulfonate (1.0 g/l)–glacial acetic acid–acetonitrile, 840:5:160, v/v/v] was pumped at a flow-rate of 1.5 ml/min. The eluent was monitored with an excitation wavelength of 350 nm and an emission wavelength of 450 nm for a period of 9.5 min and then with an excitation wavelength of 230 nm and an emission wavelength of 310 nm for a further 5.5 min. Chromatograms were recorded with a CR3-A computing integrator (Shimadzu). Extraction recovery was determined by dispensing aliquots (0.1 ml) of racemic STL standard solutions in methanol into Pyrex screw-cap tubes of 15 ml to give final plasma concentrations of 0.1, 0.5 and 2.0 μg/ml. The methanol was evaporated to dryness and drug-free human plasma (0.5 ml), carbonate buffer (1.0 *M*, pH 9.3, 0.2 ml) and ethyl acetate (6.0 ml) were added. The tubes were capped and shaken for 4 min then centrifuged (4 min, 1000 *g*). A carefully measured amount (5.0 ml) of the organic layer was transferred to a clean tube and evaporated to dryness (Savant). To the residue was added internal standard solution (HFT, 20.0 μg/ml, 0.1 ml) and this was evaporated to dryness under a stream of air. The residue was reconstituted in mobile phase (0.2 ml) and 50 μl was injected into the HPLC column. Derivatization yield was determined by dispensing aliquots (0.1 ml) of racemic STL standard solutions at 1.0 μg/ml and 10.0 μg/ml (12 of each concentration) into 15 ml pyrex screw-cap tubes and evaporating to dryness. Six samples of each concentration were reconstituted in mobile phase (0.2 ml) with vortexing (10 sec), and aliquots (0.1 ml) were injected into the HPLC column. These samples represented underivatized STL. To the remaining two sets of six samples was added carbonate buffer (1.0 *M*, pH 9.3, 2.0 μl) and 0.005% NEIC in chloroform (0.2 ml). The tubes were capped immediately, vortex-mixed for 15 s and allowed to stand at room temperature. After 1 h the chloroform was removed by evaporation and the residue was redissolved in acetonitrile–water (39:61). The tubes were recapped, vortex-mixed (10 s) and left to stand at room temperature for another hour. After evaporating to dryness, the residues were reconstituted in mobile phase (0.2 ml) and an aliquot (0.1 ml) was injected into the HPLC column. Earlier experiments showed that the STL derivatives were unstable when stored in the dry state, so the samples were derivatized and assayed in turn instead of being processed as a batch.

2.5. Assay of non-racemic mixtures

The effect of excess concentrations of one enantiomer on assay of the other was investigated by spiking plasma samples with (+)- and (−)-STL to give samples whose enantiomeric ratio varied from 1:20 to 20:1. Each of the "pure" enantiomers was demonstrated to contain approximately 1% contamination with its antipode, and this was allowed for in calculating the results of these experiments.

2.6. Assay of plasma samples

The utility of the method was demonstrated by assay of the enantiomers of STL in plasma samples from twelve individuals, each of whom took a 160-mg single dose of (±)-STL. Significance of the differences between the pharmacokinetic parameters for the two enantiomers was tested by parametric confidence interval analysis on the natural logarithms of the data (C_{max}, $t_{1/2}$, $AUC_{0-\infty}$) or by nonparametric confidence interval analysis (T_{max}). In all cases the 90% C.I. for the ratio of parameters [(+)-STL/(−)-STL] was calculated.

3. Results and discussion

The diastereomeric derivatives of (+)- and (−)-STL with NEIC were well resolved by

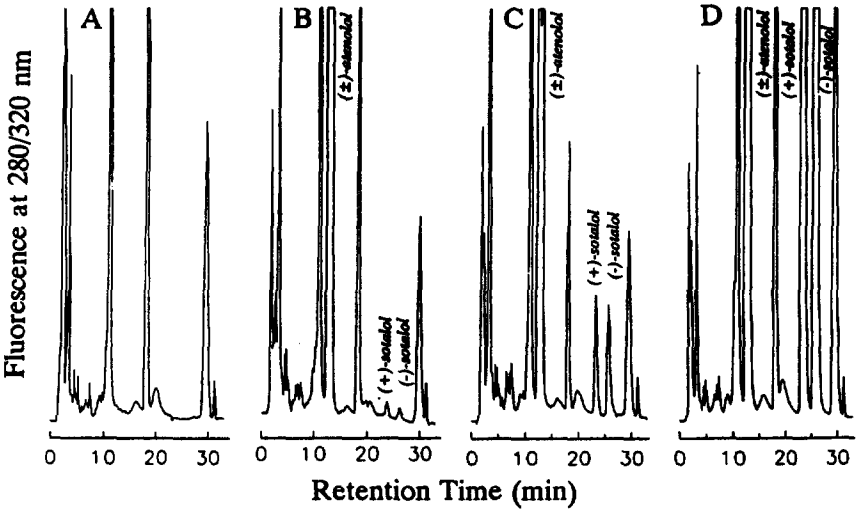

Fig. 2. Chromatograms obtained after analysis of (A) drug-free human plasma; and plasma spiked with (±)-atenolol (1.0 μg; B, C and D) and (±)-sotalol (B: 0.02 μg/ml; C: 0.2 μg/ml; D: 5.0 μg/ml).

reversed-phase chromatography on a C_{18} column (Fig. 2). There were no interfering peaks when samples containing no STL were assayed (Fig. 2A). Under these chromatographic conditions the enantiomers of ATN (as their diastereomeric NEIC derivatives) were incompletely resolved. While the integrator invariably recognized the two peaks (off scale in Fig. 2) and reported separate, approximately equal heights for them, we concluded that the sum of responses for ATN was the best value to use as the denominator when calculating peak-height ratios (PHR) for (+)- or (−)-STL, for their quantification.

Some previous workers have used *S*-(+)-NEIC to derivatize sotalol [2] or related compounds [7,8]. We showed that this reagent produced results quite comparable with those obtained using the *R*-(−)-enantiomer, and it seemed that either would have been satisfactory.

Our initial approach to the development of this method did not involve the use of a trapping column and column-switching. The derivatizing reagent gave very large, late-eluting peaks which typically appeared in chromatograms from subsequent injections. Others have overcome these problems, at least in part, by using normal-phase chromatography on silica columns. We preferred to work with reversed phase, to avoid the use of water-immiscible mobile phases. Various attempts were made at sample clean-up to remove excess derivatizing reagent prior to injecting samples into the HPLC column, but none were successful. The only practicable approach in our hands was the column-switching technique described above. Previous workers have utilized NEIC at concentrations of 0.05% [2,6] or greater [7]. We observed some breakthrough of reagent from the trapping column, giving the characteristic large, late-eluting peaks, when this quantity of NEIC was used, but this problem was completely avoided by using 0.005% NEIC in chloroform. This was confirmed to be sufficient reagent to achieve virtually complete derivatization of both STL and ATN.

It is often stated that NEIC should be used in a dry environment, and that solutions of NEIC in chloroform should be kept over a drying agent (e.g. sodium sulphate). However, a serendipitous observation on an inadvertently moist residue revealed a markedly improved derivative yield during our assay development. Thereafter a 2-μl aliquot of carbonate buffer (1.0 *M*) was added to the dried extract from plasma samples, which ensured consistently complete derivatization.

Table 1
Intra-batch precision and accuracy for assay of (+)- and (−)-STL in plasma

STL enantiomer concentration (μg/ml)	Measured concentration (μg/ml)	
	(+)-STL	(−)-STL
0.010	0.010 (5.1; 3.3)	0.010 (3.0; 2.0)
0.025	0.024 (8.4; 5.7)	0.025 (3.7; 2.7)
0.050	0.049 (2.1; 2.4)	0.050 (1.6; 1.1)
0.10	0.100 (1.3; 1.0)	0.099 (0.7; 0.9)
0.25	0.252 (2.2; 1.5)	0.251 (1.9; 1.3)
0.5	0.495 (0.6; 1.0)	0.493 (0.5; 1.5)
1.0	1.01 (1.1; 1.0)	1.00 (1.0; 0.7)
2.5	2.57 (4.6; 3.2)	2.56 (4.4; 2.8)

Mean of three measurements of (+)- and (−)-STL obtained on one occasion; values in parentheses are relative coefficients of variation (precision) and mean relative errors (accuracy).

This finding was contrary to the experience of Piquette-Miller et al. when derivatizing acebutolol [8].

PHR was linear with respect to concentration of each enantiomer of STL over the range of 0.01–2.5 μg/ml. The r^2 values for calibration curves in triplicate were typically 0.997–0.999 for each enantiomer.

Intra-batch precision and accuracy were evaluated from assays of standards in triplicate over the calibration range, and are shown in Table 1. In all instances both precision and accuracy were within 10%.

Inter-batch precision and accuracy were assessed from twelve standard curves on separate occasions (in conjunction with the assay of subjects' plasma samples), over the same calibration range. The data are presented in Table 2, and again show both precision and accuracy within 10% in all instances.

The LOQ as defined above was accepted as being 0.01 μg/ml for each enantiomer (Table 1). The data suggested that a lower LOQ may have been achievable, but this was not pursued, since an LOQ of 0.01 μg/ml from a 0.5-ml aliquot of plasma was superior to the sensitivity of previously published methods, and easily met the requirements of clinical pharmacokinetic studies. A specimen chromatogram for a standard at the LOQ is given in Fig. 2B.

Table 2
Inter-batch precision and accuracy for assay of (+)- and (−)-STL in plasma

STL enantiomer concentration (μg/ml)	Measured concentration (μg/ml)	
	(+)-STL	(−)-STL
0.010	0.0099 (7.8; 6.0)	0.0099 (6.2; 4.0)
0.025	0.023 (5.7; 8.0)	0.025 (6.9; 4.8)
0.050	0.049 (4.6; 3.8)	0.049 (6.6; 4.8)
0.10	0.103 (3.5; 3.5)	0.102 (3.7; 3.2)
0.25	0.250 (3.8; 2.4)	0.250 (3.4; 2.6)
0.5	0.498 (3.4; 2.9)	0.490 (3.2; 2.9)
1.0	1.04 (2.4; 3.9)	1.02 (2.2; 2.6)
2.5	2.51 (3.4; 2.8)	2.48 (3.1; 2.5)

Mean of twelve measurements of (+)- and (−)-STL obtained on separate occasions; values in parentheses are relative coefficients of variation (precision) and mean relative errors (accuracy).

Table 3
β-Blocking drugs and metabolites tested for interference with the assay of (+)- and (−)-STL

Oxprenolol
Timolol
N-Desisopropyl propranolol
Propranolol glycol
4-Hydroxypropranolol
4-Methylpropranolol
Propranolol
Labetolol
Metoprolol
Pindolol
Practolol

The extraction recovery of STL, measured as the sum of enantiomers, was calculated as the ratio of the slopes of the regression lines for extracted versus non-extracted standards over the range 0.1–2.0 μg/ml, and was shown to be 80%. STL is sufficiently polar to pose difficulties in extraction unless conditions are optimized. We increased the recovery of STL from approximately 60% to 80% by halving the volume of carbonate buffer added to plasma, and keeping the volume of ethyl acetate relatively high in relation to the aqueous volume.

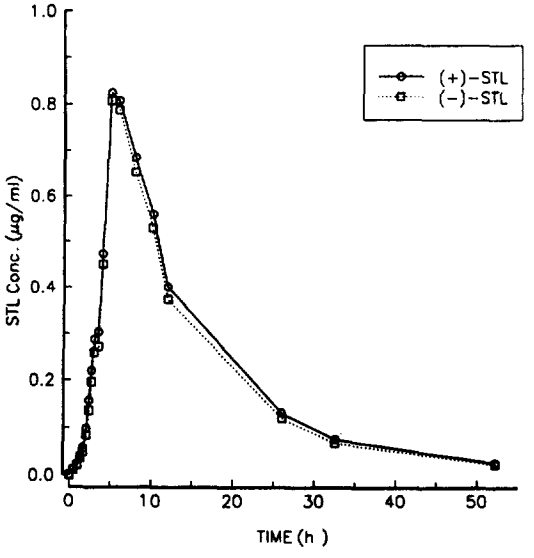

Fig. 3. Plasma concentration–time profiles for (+)- and (−)-sotalol in one subject who took a single oral 160-mg dose of (±)-sotalol hydrochloride.

The derivatization yield, measured in terms of quantity of [(+)+(−)]-STL remaining after treatment with NEIC, was shown to be ≥96% at both 1.0 μg/ml and 10.0 μg/ml.

Plasma samples from eighteen subjects taking no medication gave no peaks which would interfere chromatographically with either STL or ATN. There are no metabolites of STL which would be expected to interfere in the assay. The other drugs which were shown not to interfere in the assay are given in Table 3.

Assay of each enantiomer in the presence of up to a 20-fold excess of the other was shown to give results equivalent to those obtained with the respective pure enantiomer. There was thus no interference with the assay of either enantiomer by excess concentrations of the other, and the results using pure enantiomers were interpreted as showing the absence of racemization of sotalol in this assay.

The profiles of the plasma concentrations of the enantiomers of STL in one volunteer who took a single oral dose (160 mg) of (±)-STL·HCl are shown in Fig. 3. The close correspondence in the plasma concentration–time profiles of the enantiomers is apparent.

We have studied twelve volunteers in this single-dose study, and have shown no significant differences between the enantiomers for some pharmacokinetic parameters (C_{max}, T_{max}) or statistically significant differences of very small magnitude for others ($AUC_{0-\infty}$, $t_{1/2}$). These findings (Table 4) are consistent with the previous observations of the absence of clinically significant enantioselectivity in STL disposition in single-dose studies in volunteers [9,10] and in steady-state studies in treated patients [3,11].

Table 4
Mean (±S.D.) pharmacokinetic parameters for (+)- and (−)-STL in twelve subjects given a single oral dose of STL·HCl (160 mg)

Parameter	(+)-STL	(−)-STL	90% C.I.
C_{max} (μg/ml)	0.78 (0.26)	0.77 (0.25)	0.986–1.032
T_{max} (h)	4.01 (0.93)	4.01 (0.93)	1.000–1.000
$AUC_{0-\infty}$ (μg ml^{-1} h^{-1})	9.80 (2.14)	9.06 (1.95)	1.067–1.094
$t_{1/2}$ (h)	8.71 (1.27)	8.29 (1.21)	1.030–1.072

In summary, the present method for the enantioselective assay of STL in plasma is convenient, simple and sensitive, has been fully validated, and readily meets the requirements for clinical pharmacokinetic studies of the enantiomers of this drug.

Acknowledgements

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