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# Validation of a liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of Sotrastaurin and its metabolite N-desmethyl-sotrastaurin in human blood

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ABSTRACT

A liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method was validated for the quantification of Sotrastaurin (AEB071) and N-desmethyl-sotrastaurin in human blood. The validation of the analytical procedure was performed according to the latest Food and Drug Administration (FDA) "Guidance for Industry, Bioanalytical Method Validation". Chromatographic separation was performed using an RP $C_{18}$ (50 mm × 4.6 mm, 5 μm) column at 40 ± 3.0 °C with a mobile phase consisted of 2 mM ammonium acetate in water (pH 4.5):methanol:acetonitrile (25:15:60, v/v) of a flow rate of 1 mL/min followed by quantification with tandem mass spectrometer, operated in electrospray ionization (ESI) positive ion mode and applying multiple reaction monitoring (MRM). The validated method described in this paper presents high absolute recovery, with a sensitivity of 3.00 ng/mL as lower limit of quantitation using a sample volume of 300 μL, low inter-run bias and variability (for Sotrastaurin, −4.4 to 0.4% and 1.8 to 2.5% and for N-desmethyl-sotrastaurin, ranged from 1.6 to 2.3% and 2.7 to 3.9%, respectively) with a short runtime of 3.5 min. The method was validated using $K_3$EDTA as specific anticoagulant and cross-validated using Li-Heparin and Na-Heparin. The method was specific for Sotrastaurin and N-desmethyl-sotrastaurin within the given criteria of acceptance (apparent peak area for Sotrastaurin and N-desmethyl-sotrastaurin in zero samples ≤20% of mean peak area at LLOQ) in human blood. The method was fully validated for the quantitative determination of Sotrastaurin and its metabolite N-desmethyl-sotrastaurin in human blood between the range of 3.00 ng/mL and 1200 ng/mL.


## 1. Introduction

Sotrastaurin (AEB071, 3-(1H-indol-3-yl)-4-[2-(4-methyl-1-piperazinyl)-4-quinazolinyl]-1H-pyrrole-2,5-dione acetate (1:1)) is an immunosuppressant that inhibits protein kinase C (PKC) [1]. PKC plays a critical role in the regulation of immune cell function. Drugs that are highly specific inhibitors of PKC are considered potentially useful for preventing allograft rejection after organ transplantation, as well as treating autoimmune and other inflammatory diseases [2]. The efficacy of Sotrastaurin after oral administration was demonstrated in rodent and non-human primate (NHP) organ transplant models by prolonging allograft survival [3–7]. Sotrastaurin is currently in clinical development for the prevention of acute rejection after solid organ transplantation as well as for the treatment of psoriasis [8–11].

The pharmacokinetics of Sotrastaurin was investigated in preclinical and clinical studies. Following oral (20 mg/kg) and intravenous (5 mg/kg) administration of Sotrastaurin, blood concentrations at 8 h were 70 and 18 ng/mL, respectively, in rats, and 98 and 84 ng/mL, respectively, in NHPs. The oral bioavailability was >30% and 18% in rats and NHPs, respectively [2]. In clinical trials conducted in renal transplant patients, Sotrastaurin was administered at doses of 200–300 mg twice daily. At these two dose levels, respectively, steady-state Sotrastaurin predose blood concentrations averaged 600 and 900 ng/mL and Cmax averaged 1600 and 2400 ng/mL. Sotrastaurin is primarily metabolized through CYP3A4. Of the metabolites identified in man, only the N-desmethyl metabolite is pharmacologically active but its blood levels are low, generally <5% those of the parent compound. The elimination half-life of Sotrastaurin averages 6 h [12,13].

To provide bioanalytical support for drug development pharmacokinetic studies, an LLOQ of 3 ng/mL was chosen. This fulfilled the bioanalytical method criteria for an LLOQ, namely the S/N (signal-to-noise) should be at least 10:1 with an imprecision of no more

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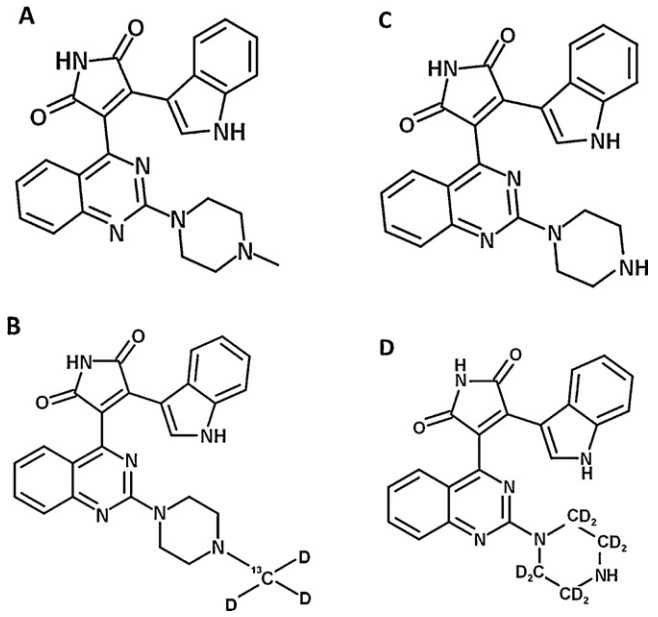


**Fig. 1.** Structures of reference standards and stable labeled internal standards. (A) Sotrastaurin; (B) Sotrastaurin stable labeled internal standard; (C) N-desmethyl-sotrastaurin; (D) N-desmethyl-sotrastaurin stable labeled internal standard.

than ±20% at LLOQ and no more than ±15% above the LLOQ. The LLOQ depends on the ULOQ as well since after injection of a ULOQ sample the carryover of the analyte should be less than 20% of the LLOQ response. It also addressed the clinical pharmacokinetic needs for single-dose profiling studies which require quantification of low concentrations in the terminal phase to determine the drug's elimination half-life.

There is no previously established and published validated method to quantify Sotrastaurin and N-desmethyl-sotrastaurin in human blood. The aim of this study was to validate a specific and sensitive LC–MS/MS method for the quantification of Sotrastaurin and N-desmethyl-sotrastaurin at nano/micro gram level in human blood meeting the acceptance criteria for method validation [14].

## 2. Experimental

### 2.1. Chemicals and reagents

All the reference standards and stable labeled internal standards were obtained from Novartis Pharma, AG; Basel, Switzerland. For parent drug, the reference standard was Sotrastaurin ($C_{25}H_{22}N_6O_2$; molecular weight 438.46; purity 100%) acetate salt with a salt to base ratio of 1.137 and the internal standard was [$^{13}CD_3$]Sotrastaurin ($C_{24}{}^{13}CH_{19}D_3N_6O_2$; molecular weight 442.46; purity 98.3%). For the active metabolite, the reference standard was N-desmethyl-sotrastaurin ($C_{24}H_{20}N_6O_2$; molecular weight 424.39; purity 98.6%) dihydrochloride salt with a salt to base ratio of 1.172. The internal standard was [$D_8$]N-desmethyl-sotrastaurin ($C_{24}H_{12}D_8N_6O_2$; molecular weight 432.5; purity >84%). The chemical structures of the parent drug and the metabolite are depicted in Fig. 1.

All solvents and reagents were of analytical grade and were used without further purification. Methanol and acetonitrile were obtained from J.T. Baker. Propanol, formic acid, acetic acid and pH 10 buffer were obtained from Merck. Ethyl acetate and water of HPLC grade were obtained from Spectrochem; and ammonium acetate was obtained from Rankem.

### 2.2. HPLC operating conditions

The HPLC system (Shimadzu, Japan) consisted of LC-20ADVP pumps, CTO-10ASVP column oven, SIL-HTC auto sampler and DGU-20A3 degasser. Chromatographic separation was performed using a Gemini RP C18 column (150 mm × 4.6 mm, 5 μm, Phenomenex Inc., Torrance, USA) at 40 ± 3.0 °C. The mobile phase consisted of 2 mM ammonium acetate in water (pH 4.5):methanol:acetonitrile (25:15:60, v/v) with a flow rate of 1 mL/min, 90% flow splitting (100 μL/mL to MS) and 5 μL injection volume. The retention time was 1.5 and 2.10 min for the metabolite and parent, respectively; and total run time was 3.5 min.

### 2.3. Mass spectrometer operating conditions

The mass spectrometer (MS), API 3000 (Applied Biosystems, MDS Sciex) was operated in ESI positive ion mode applying MRM. The source temperature was 450 °C and the ion spray voltage (ISV) was set to 5500 V. The different gas pressures of nebulizer gas, curtain gas and collision gas were set at 8, 6 and 5 psi, respectively. The entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) for Sotrastaurin were 10, 64, 35 and 13 V and for N-desmethyl-sotrastaurin these were 10, 64, 37 and 13 V, respectively. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions, $m/z$ 439.4 → 382.3 (Sotrastaurin), 425.2 → 382.3 (N-desmethyl-sotrastaurin), 443.4 → 382.1 ([$^{13}CD_3$]Sotrastaurin), and 433.2 → 387.4 ([$D_8$]N-desmethyl-sotrastaurin) (Fig. 2). The LC–MS/MS system was controlled by Analyst 1.4.2 software (Applied Biosystems, and Foster City, CA, USA).

### 2.4. Standard stock solution preparation

Weighing was performed using microbalance (model MX 5, Mettler Toledo). The standard stock solution was prepared by dissolving Sotrastaurin, or N-desmethyl-sotrastaurin in methanol to obtain a target concentration of 1.00 mg/mL. For the stable labeled internal standards, the stock solutions were prepared in methanol to obtain target concentration of 0.200 mg/mL and 1.696 μg/mL for Sotrastaurin and N-desmethyl-sotrastaurin, respectively. Working solutions were made by diluting the stock solution with methanol:water (50:50, v/v). For Sotrastaurin and N-desmethyl-sotrastaurin the working concentrations were in the range of 3.00–1200 ng/mL. For [$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin the working concentration were 500 and 585 ng/mL, respectively. The standard stock solutions and working solutions were all stored at 5 ± 3 °C in polypropylene tubes. Calibration standards (C standards) and quality control (QC) samples were freshly spiked for each batch with blank human blood. Human blank blood batches ($K_3$EDTA, Li-, Na-Heparin) were obtained from Supratech Micropath Laboratory & Research Institute, Ahmedabad (India). Blood batches containing $K_3$EDTA were used throughout the validation except for the anticoagulant effect experiment. During the anticoagulant effect experiment QC samples were prepared from blood batches containing Li- and Na-Heparin as well and back-calculated using the calibration curve prepared from Cs of $K_3$EDTA blood batch.

Throughout the validation C standards were prepared from the same pooled human blood ($K_3$EDTA) on all analysis runs. QC samples were prepared from different batches of human blood ($K_3$EDTA) for each analysis run. Nine concentrations of C standards were prepared in the range of 3.00 ng/mL (lower limit of quantification, LLOQ) to 1200 ng/mL (upper limit of quantification, ULOQ). The concentrations were 3.00, 6.00, 15.0, 30.0, 75.0, 150, 300, 600 and 1200 ng/mL. Four concentrations of QC samples were

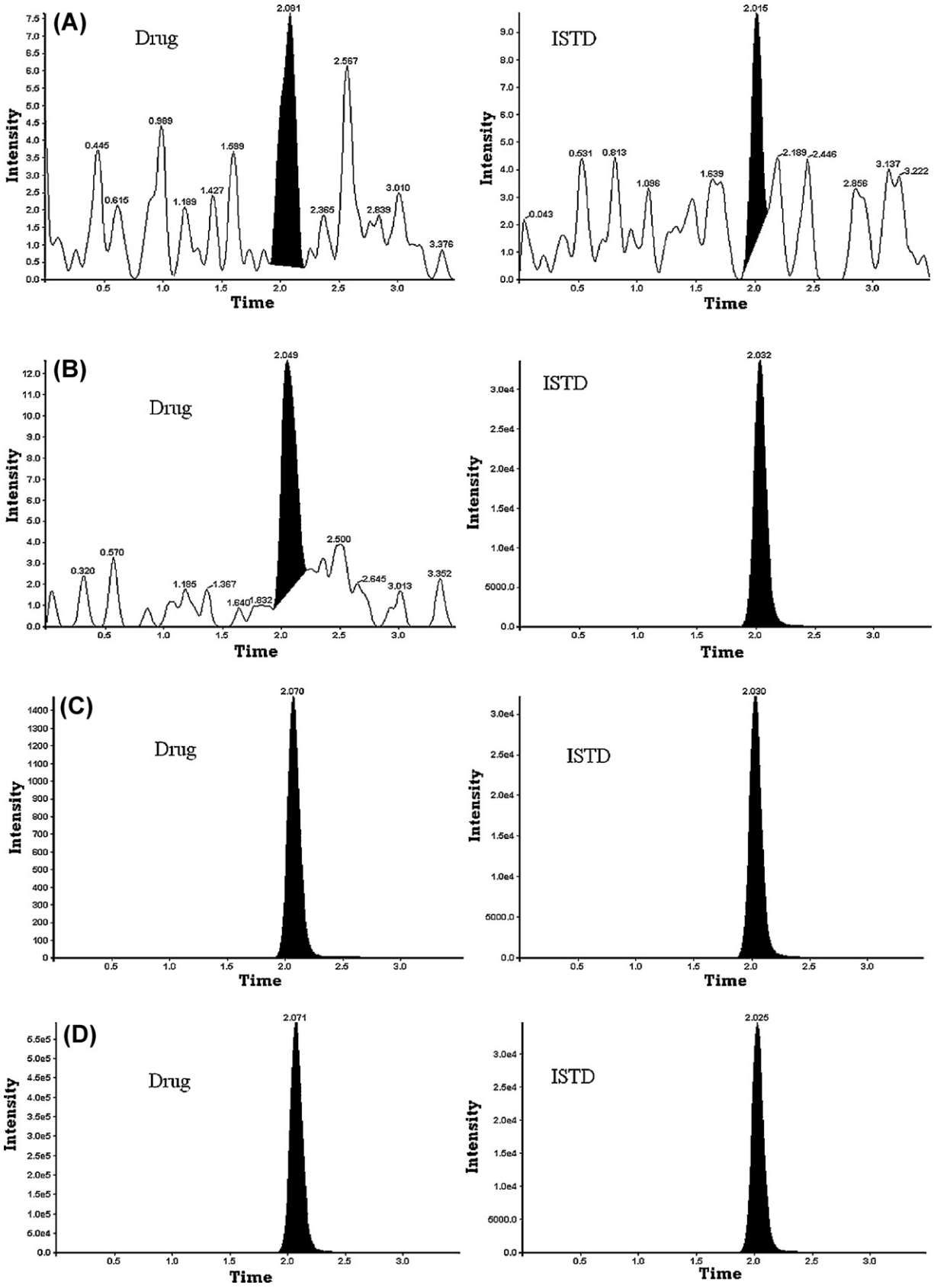


**Fig. 2.** Typical chromatogram of blank human blood extract (A), blank sample spiked with working concentration of ISTDs (B), with LLOQs (C), and ULOQs (D) of Sotrastaurin.

**Table 1**
Effect of anticoagulant on sotrastaurin and N-desmethyl-sotrastaurin in human blood.

| Anticoagulant (human blood) | Concentration (ng/mL) | Calculated concentrations (bias (%)) | Precision (%) |
|---|---|---|---|
| **Sotrastaurin** | | | |
| Lithium heparin | 9.00 (QCs low) | 8.59 (−4.6) | 1.5 |
| | 900 (QCs high) | 814 (−9.6) | 2.5 |
| Sodium heparin | 9.00 (QCs low) | 8.99 (−0.1) | 3.1 |
| | 900 (QCs high) | 820 (−8.9) | 6.2 |
| **N-desmethyl-sotrastaurin** | | | |
| Lithium heparin | 9.00 (QCs low) | 9.23 (2.6) | 1.9 |
| | 900 (QCs high) | 886 (−1.6) | 1.3 |
| Sodium heparin | 9.00 (QCs low) | 9.62 (6.9) | 3.1 |
| | 900 (QCs high) | 906 (0.7) | 6.7 |

QC, quality control.

prepared in the range of 3.00–900 ng/mL (LLOQ: 3.00 ng/mL, low QC: 9.00 ng/mL, mid QC: 45.0 ng/mL, high: 900 ng/mL).

### 2.5. Sample preparation

A 300 μL of blank human blood ($K_3$EDTA, Supratech Micropath Laboratory & Research Institute, Ahmedabad, India) aliquot was vortex-mixed with 50 μL ISTD dilution ([$^{13}CD_3$]Sotrastaurin at 500 ng/mL and [$D_8$]N-desmethyl-sotrastaurin at 585 ng/mL) to all sample tubes, except the standard blank (double blank). A 300 μL volume of pH 10.0 buffer (boric acid/potassium chloride/sodium hydroxide) was added to all the samples and vortex mixed for about 20 s, followed by the addition of 2.5 mL of ethyl acetate. The extraction was done at 40 rpm on a rotospin (Tarsons Products Pvt. Ltd., India) for 20 min followed by centrifugation (5810R, Eppendorf) for 5 min at 4000 rpm (10 ± 2 °C). About 2.0 mL of supernatant was transferred into disposable pre-labeled polypropylene tubes and evaporated to dryness under nitrogen gas using nitrogen evaporator (TurboVap LV, Caliper Life Sciences) at 40 ± 5 °C. The residue was reconstituted in 150 μL mobile phase, vortex mixed and transferred to glass auto-sampler vial (300 μL, Shimadzu) for the LC–MS/MS analysis.

### 2.6. Method validation

The method was validated to demonstrate the specificity, recovery, accuracy, precision, dilution, ruggedness, anticoagulant effect ($K_3$EDTA, Li-, Na-Heparin) and stability according to the latest US Food and Drug Administration (US FDA) Guidelines for Industry on Bioanalytical Method Validation [14].

Laboratory Information Management System, Watson LIMS software (version 7.3, Thermo Fisher Scientific, Philadelphia, PA, USA) was used for the calculations.

## 3. Results and discussion

### 3.1. Specificity

Typical chromatograms of blank sample (a), blank sample spiked with working concentration of ISTD's (b), LLOQ (c), and ULOQ (d) for Sotrastaurin and N-desmethyl-sotrastaurin are presented in Fig. 2.

The specificity of the method was determined by analyzing human blood prepared and extracted without addition of Sotrastaurin, ([$^{13}CD_3$]Sotrastaurin), N-desmethyl-sotrastaurin, and [$D_8$]N-desmethyl-sotrastaurin (using 3 replicates of blank samples) from 6 different batches of human blood. Specificity was assessed by comparing mean analytical response of Sotrastaurin, N-desmethyl-sotrastaurin, [$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin in the blank extracts ($n$ = 3 for each batch) at the retention time (RT) of Sotrastaurin, N-desmethyl-sotrastaurin, [$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin . The interference at the RT of analyte were not more than 20% (for Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin) of the mean of the LLOQ ($n$ = 6) response and by more than 5% (for ([$^{13}CD_3$]Sotrastaurin) and ([$D_8$]N-desmethyl-sotrastaurin)) of the mean internal standard ($n$ = 6) respectively. To analyze any potential contribution of [$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin to Sotrastaurin and N-desmethyl-sotrastaurin, respectively, three replicates of blank samples spiked at the working concentrations of ISTD in at least one batch of human blood were analyzed (three samples with ([$^{13}CD_3$]Sotrastaurin) and three samples with [$D_8$]N-desmethyl-sotrastaurin). Acceptance criteria were that the mean analytical response obtained at the retention time of Sotrastaurin and N-desmethyl-sotrastaurin should not interfere by more than 20% of the mean of the LLOQ calibration standard peak signals.

Since multiple analytes were determined, the specificity of each analyte to the other analytes in the assay was evaluated. Each analyte was added to at least one batch of blank human blood at the ULOQ concentration without addition of the other analytes ($n$ = 3). The expected retention time for each of the non-spiked analytes in the relevant mass channel was checked to ensure that no peaks were present. Specificity was assessed by comparing the mean apparent analytical response for the analytes (Sotrastaurin and N-desmethyl-sotrastaurin) and for the internal standards ([$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin) in blank samples to the mean analytical response obtained for samples spiked with a concentration of the analytes at the LLOQ, and with the internal standards at the working concentration (zero samples).

The specificity for the analytes (Sotrastaurin and N-desmethyl-sotrastaurin) and for the internal standards ([$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin) was within acceptable limits. The mean percentage of interference for Sotrastaurin and N-desmethyl-sotrastaurin was 0.4–1.1% and 0.4–1.0%, respectively (acceptance criteria <20%). Furthermore, no contribution from the internal standards ([$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin) was observed (% of interference: 0; acceptance criteria <5%). The method was specific for Sotrastaurin and N-desmethyl-sotrastaurin within the given criteria of acceptance (apparent peak area for Sotrastaurin and N-desmethyl-sotrastaurin in zero samples ≤20% of mean peak area at LLOQ) in human blood. The method was validated using $K_3$EDTA as specific anticoagulant and no significant difference were obtained when different anticoagulants (Li-Heparin or Na-Heparin) were used. The anticoagulant effect experiment biases and CV% are shown in Table 1.

### 3.2. Matrix effect and recovery

The matrix effect was investigated by calculating the ISTD normalized matrix factor (MF) (MF = (Analyte response$_{\text{presence of matrix}}$/ISTD response$_{\text{presence of matrix}}$)/

(Analyte response$_{\text{absence of matrix}}$/ISTD response$_{\text{absence of matrix}}$))
for one human blood batch for three replicates and at three concentration levels (low, mid and high) for Sotrastaurin, N-desmethyl-sotrastaurin. The coefficient of variation for MF should be within ±15% at each concentration level and also between the mean MF for each concentration level. The ISTD normalized MF for each analyte at the three concentration levels were 1.0 with the coefficient of variation of the matrix factors of 1.90 and 1.20% for Sotrastaurin and N-desmethyl-sotrastaurin, respectively.

The absolute recovery was calculated by the ratio of the analytical response obtained from analysis of extracted spiked matrix samples relative to the analytical response obtained from analysis of the extracted blank samples spiked after extraction with reference solutions. Recovery studies were conducted as well as at three different concentrations of QC levels (low, mid, high). The mean recovery of the analytes and the internal standards were required to be at a precision of <15%. The range of recovery for Sotrastaurin was 112.1–119.2% and for N-desmethyl-sotrastaurin is 107.5–115.8%. The overall mean recovery of Sotrastaurin and N-desmethyl-sotrastaurin was 115.9% and 110.4% with a precision of 3.1 and 4.2%, respectively. The mean recovery of internal standards ([$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin) was 105.8% and 107.8%.

### 3.3. Carryover

The carryover is the observed response of the analyte(s) and internal standard(s) in an injected blank sample following the injection of an ULOQ sample or working solution for the internal standards. The extent of any carryover is assessed in one validation run by injecting a series of two blank samples directly after the injection of the ULOQ analyte sample or working solution of the internal standard.

Mean response observed in extract of blank matrix at the retention time of Sotrastaurin, N-desmethyl-sotrastaurin, ([$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin), should not interfere with the analyte by more than 20% (for Sotrastaurin and N-desmethyl-sotrastaurin) of the mean of the LLOQ ($n = 2$, or accepted LLOQ in case of rejection of either of LLOQ) calibration standard peak signals and not by more than 5% (for ([$^{13}CD_3$]Sotrastaurin) and ([$D_8$]N-desmethyl-sotrastaurin)) of the mean internal standard peak signal ($n = 2$, or ISTD peak signal of accepted LLOQ in case of rejection of either of LLOQ), respectively. For Sotrastaurin the carryover for the first and second blank injected after an ULOQ sample was 4.1 and 2.2%, respectively. For desmethyl Sotrastaurin this was 2.8% and 1.4%. There was no carryover found [$^{13}CD_3$]Sotrastaurin, and [$D_8$]N-desmethyl-sotrastaurin.

### 3.4. Accuracy and precision

Intra-run and inter-run accuracies and precisions were evaluated by analysis of the QC samples at a minimum of four concentrations (3.00, 9.00, 45.0 and 900 ng/mL) analyzed each run. The deviation (bias) from the nominal value was used to evaluate the accuracy. The intra-run accuracy and precision were calculated as the mean bias and precision of all individual QC sample concentrations analyzed during a single validation run, respectively. The overall accuracy and precision (inter-run) were calculated as the mean bias and precision of all individual QC sample concentrations analyzed during the 3 validation runs. The acceptance criteria were mean bias within ±15% (±20% at LLOQ) of the nominal values and precision of <15% (<20% at LLOQ). For Sotrastaurin, the intra-run bias and precision across batches ranged from −5.1 to 4.7% and 0.3 to 4.6%, respectively Similarly the inter-run bias and precision ranged from −4.4 to 0.4% and 1.8 to 5.2%, respectively. Further for N-desmethyl-sotrastaurin, the intra-run bias and precision ranged from −3.4 to 5.7% and 1.0 to 4.4%, respectively. Similarly the inter-run bias and precision ranged from −1.6 to 2.3% and 2.7 to 3.9%, respectively. The values for the analyte(s) were within the acceptable range of 15% (±20% at LLOQ). According to these criteria, the analytical method used in this study could reproducibly measure the concentration of Sotrastaurin and N-desmethyl-sotrastaurin in human blood. The ruggedness was analyzed by (i) evaluating one validation batch using a different column, (ii) one validation batch using different equipment, and (iii) one validation batch performed by a different analyst. Four different concentrations of QC samples were used. Mean bias of all concentrations was within ±15% (±20% at LLOQ) of the nominal values with a precision of <15% (<20% at LLOQ). For Sotrastaurin, the intra-run bias and precision across batches during the ruggedness test ranged from −1.1 to −11.0% and 1.8 to 8.6%, respectively. Further for N-desmethyl-sotrastaurin, the intra-run bias and precision ranged from −7.0 to 3.3% and 1.5 to 5.6%, respectively.

**Table 2**
Daily variation of calibration parameters in species matrix (calibration parameters $a$ and $b$ of the calibration function $y = ax + b$ and coefficient of determination $r^2$ on each day of the validation).

| Run number | $a$ (slope) | $b$ (intercept) | $r^2$ |
|---|---|---|---|
| **Sotrastaurin** | | | |
| 1 | 0.0129 | 0.0014 | 0.9977 |
| 2 | 0.0140 | 0.0029 | 0.9971 |
| 4 | 0.0154 | 0.0007 | 0.9991 |
| Mean | 0.0141 | NA | 0.9980 |
| % CV | 9.2 | NA | 0.1 |
| **N-desmethyl-sotrastaurin** | | | |
| 1 | 0.0374 | 0.0100 | 0.9955 |
| 2 | 0.0397 | 0.0027 | 0.9984 |
| 4 | 0.0423 | 0.0083 | 0.9985 |
| Mean | 0.0398 | NA | 0.9975 |
| % CV | 6.3 | NA | 0.2 |

NA, not applicable; CV%, coefficient of variation percent.

### 3.5. Calibration curve

Calibration curves ($y = ax + b$), represented by the plots of the peak-area ratios ($y$) of the response for analyte(s) to the internal standard versus the concentration ($x$) of the calibration standards, were generated using weighted ($1/x^2$) linear least-squares regression as the simplest mathematical model for the current validation. The daily variation of the calibration curve parameters are presented in Table 2. The acceptance criteria of the C standards were within ±15% (±20% at LLOQ) deviation from nominal values for at least 75% (with a minimum of six different levels) of the non-zero samples. For Sotrastaurin mean bias of the C standards was −5.0 to −2.3% for LLOQ to ULOQ with the precision of 1.7 to 3.9 and for N-desmethyl-sotrastaurin it was found −5.0 to −2.0% with the precision of 4.1–4.5.

### 3.6. Dilution

A human blood QC sample at a concentration of 2400 ng/mL and volume of 0.150 mL was diluted by factors of 2-fold, 5-fold and 10-fold using blank human blood and analyzed for Sotrastaurin and N-desmethyl-sotrastaurin. The results of the analysis were within the acceptable range. For Sotrastaurin mean bias was found −10.0, −2.9 and 2.5%, respectively with the precision of 2.6, 0.7 and 1.6% for 2-fold, 5-fold and 10-fold dilution and for N-desmethyl-sotrastaurin it was found −9.6, −2.9 and 1.3%, respectively with the precision of 0.9, 1.3 and 1.6% for 2fold, 5fold and 10fold dilution.

**Table 3**
Long term and freeze–thaw stability in human blood at −78 ± 8 °C and −20 ± 5 °C.

| | Nominal concentration (ng/mL) | No. of replicates | Measured concentrations (ng/mL) after 542 days −78 ± 8 °C | Mean bias (%) −78 ± 8 °C | Measured concentrations (ng/mL) after 47 days −20 ± 5 °C | Mean bias (%) −20 ± 5 °C |
|---|---|---|---|---|---|---|
| **Sotrastaurin** | | | | | | |
| Long term stability | 9.00 | 3 | 8.35 | −0.9 | 8.88 | −1.3 |
| | | | 9.25 | | 9.00 | |
| | | | 9.15 | | 9.48 | |
| | 900 | 3 | 923 | 1.4 | 858 | −5.4 |
| | | | 922 | | 828 | |
| | | | 895 | | 868 | |
| Freeze–thaw stability | 9.00 | 6 cycles | | −10.2 | | −5.0 |
| | 900 | | | −1.3 | | −5.4 |
| **N-desmethyl-sotrastaurin** | | | | | | |
| Long term stability | 9.00 | 3 | 7.78 | −7.6 | 8.27 | −6.8 |
| | | | 8.68 | | 8.44 | |
| | | | 8.51 | | 8.47 | |
| | 900 | 3 | 946 | 6.6 | 807 | −7.4 |
| | | | 955 | | 842 | |
| | | | 975 | | 849 | |
| Freeze–thaw stability | 9.00 | 6 cycles | | −10.9 | | 0.0 |
| | 900 | | | 1.8 | | −1.2 |

### 3.7. Stability

The stability of Sotrastaurin and N-desmethyl-sotrastaurin was assessed by analyzing the QC samples in the stock/diluted/spiking solutions, storing under different temperature conditions for different time lengths. Stability of the solutions is assessed at room temperature for at least 6 h as well as under the intended storage conditions. For short-term stability, QCs at 2 concentrations (low and high) were analyzed after a typical assay process time of from 4 to 24 h at room temperature. Long term stability was analyzed at −78 ± 8 °C. Samples analyzed before and after a storage period exceeding the time between the first sample collection and the last sample analysis. The short term stability of stock and diluted solutions of Sotrastaurin and N-desmethyl-sotrastaurin at ambient temperature was 7.5 h. Further, the long term stability at 5 ± 3 °C of the stock solutions of Sotrastaurin and N-desmethyl-sotrastaurin were 541 days, and for the diluted solutions, it was 36 days for both analyte(s). The internal standards, [$^{13}CD_3$]Sotrastaurin and [$D_8$]N-desmethyl-sotrastaurin, in stock solutions, were stable for about 8 h and 7 h respectively under ambient temperature. In diluted solutions these were stable for 7 h 50 min at ambient temperature. At low temperature (5 ± 3 °C) these compounds were stable for 541 days and for diluted solutions it was 59 days for both internal standards(s). Post preparative stability was analyzed at the beginning of a run and after a storage period on the autosampler covering the anticipated run time. Human blood post preparative stability in extracts at 5 ± 3 °C was assessed for 1 day and passed. No further assessment was needed since this period covers the needed time frame. Samples subjected to freeze–thaw (6 cycles), were stable under −20 ± 5 °C and −78 ± 8 °C (Table 3). At room temperature the spiked blood samples were stable for 10 h.

The long term stability of Sotrastaurin and N-desmethyl-sotrastaurin in spiked samples were stable for up to 542 days at −78 ± 8 °C and only 47 days at 20 ± 5 °C in species matrix. The long term stability of Sotrastaurin and N-desmethyl-sotrastaurin at −78 ± 8 °C in true samples (Incurred Sample Stability – ISS) have been proven for about 533 days (Table 4). The stability results were found to be acceptable as per the regulatory guidance [14].

**Table 4**
Long term stability of incurred human blood samples at −78 ± 8 °C for 533 days.

| Sr. No | Incurred sample stability, Sotrastaurin | | | Incurred sample stability, N-Desmethyl-Sotrastaurin | | |
|---|---|---|---|---|---|---|
| | Conc. (ng/mL) | | | Conc. (ng/mL) | | |
| | Before storage | After storage | % difference | Before storage | After storage | % difference |
| 1 | 189 | 177 | −6.3 | 18.4 | 16.8 | −8.7 |
| 2 | 1880 | 1650 | −12.2 | 58.4 | 64.9 | 11.1 |
| 3 | 1720 | 1750 | 1.7 | 62.7 | 67.9 | 8.3 |
| 4 | 1510 | 1340 | −11.3 | 64.7 | 70.5 | 9 |
| 5 | 197 | 182 | −7.6 | 19.6 | 16.5 | −15.8 |
| 6 | 66 | 65.9 | −0.2 | 11 | 11.5 | 4.5 |
| 7 | 1530 | 1360 | −11.1 | 54 | 48.7 | −9.8 |
| 8 | 177 | 171 | −3.4 | 14.1 | 13.1 | −7.1 |
| 9 | 1830 | 1390 | −24 | 77.3 | 69.2 | −10.5 |
| 10 | 1700 | 1590 | −6.5 | 87 | 83.7 | −3.8 |
| 11 | 177 | 165 | −6.8 | 15.3 | 15.5 | 1.3 |
| 12 | 121 | 114 | −5.8 | 10.5 | 8.9 | −15.2 |
| 13 | 1580 | 1290 | −18.4 | 68.5 | 57 | −16.8 |
| 14 | 1340 | 1220 | −9 | 61.7 | 63.8 | 3.4 |
| 15 | 2560 | 2280 | −10.9 | 64 | 77.8 | 21.6 |
| 16 | 90 | 78 | −13.3 | 12.7 | 11.5 | −9.4 |
| 17 | 1670 | 1630 | −2.4 | 80.3 | 85.3 | 6.2 |
| 18 | 154 | 165 | 7.1 | 16.8 | 16.1 | −4.2 |
| 19 | 2530 | 2170 | −14.2 | 97.6 | 123 | 26 |
| 20 | 2380 | 2120 | −10.9 | 51.5 | 62.3 | 21 |

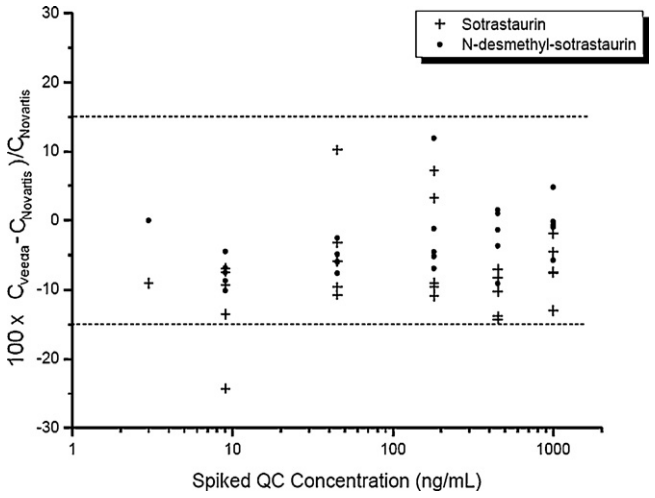


**Fig. 3.** Cross-checked results on spiked QCs between the reference site (Novartis) and the test site (Veeda).

### 3.8. Cross-validation

A cross-validation was carried out by both the reference (Novartis Basel) and the test (Veeda) laboratories (validation described in this article) using spiked QC samples. The reference laboratory has validated the method first. The method details of the reference laboratory are not described here.

QC samples were prepared at six different concentration levels for Sotrastaurin and N-desmethyl-sotrastaurin (3.00, 9.00, 45.0, 180, 450 and 1000 ng/mL) for five replicates at each level by the reference lab. The spiked QC samples were measured by both the reference and the test laboratories. The normalized differences were calculated for Sotrastaurin and N-desmethyl-sotrastaurin based on the following formula $100 \times (C_{Veeda} - C_{Novartis})/C_{Novartis}$. The result is presented in Fig. 3. The results were within the acceptance criteria (the normalized differences of at least 2/3 of the samples should be within ±20%) for both analytes.

## 4. Conclusions

The method is suitable for the routine analysis of Sotrastaurin and N-desmethyl-sotrastaurin in human blood over the range of 3.00 (LLOQ) to 1200 ng/mL using a sample volume of 0.300 mL. The method is specific for Sotrastaurin and N-desmethyl-sotrastaurin within the given criteria for acceptance in human blood. The precision and accuracy were found to be within acceptable limits. The method was validated using $K_3$EDTA as specific anticoagulant and cross-validated with Li-Heparin and Na-Heparin. The method is precise and accurate for 2-fold, 5-fold and 10-fold dilution of samples. The method ruggedness was demonstrated using different columns of the same make and type, different analysts and different equipment. Recovery is 112.1–119.2% for Sotrastaurin and 107.5–115.8% for N-desmethyl-sotrastaurin. Mean ISTD normalized matrix factor is 1.0 for Sotrastaurin and N-desmethyl-sotrastaurin. The incurred sample stability for Sotrastaurin and N-desmethyl-sotrastaurin was demonstrated for 533 days at $-78 \pm 8\,^{\circ}C$.

The fully validated method has also been cross validated by the testing and reference laboratories.

## Disclosure

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