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Article

Enantiomeric Separation of Verapamil and its Active Metabolite, Norverapamil, and Simultaneous Quantification in Human Plasma by LC–ESI-MS-MS

Puran Singhal[1], Manish Yadav[1]*, Serge Winter[2], Swati Guttikar[1], Dharmesh Patel[1], Malcolm Mills[1] and Pranav S. Shrivastav[3]*

[1]Bioanalytical Research Department, Veeda Clinical Research, Ambawadi, Ahmedabad- 380015, India, [2]Novartis Pharma AG, Fabrikstrasse 14, WSJ-153.3.02.3, CH-4002 Basel, Switzerland, and [3]Chemistry Department, School of Sciences, Gujarat University, Navrangpura, Ahmedabad-380009, India

*Authors to whom correspondence should be addressed. Email: pranav_shrivastav@yahoo.com; ysmanish@yahoo.com

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A simple, selective and high-throughput liquid chromatography–tandem mass spectrometry method has been developed and validated for the chromatographic separation and quantification of (*R*)- and (*S*)-enantiomers of verapamil and its active metabolite, norverapamil, in human plasma. All four analytes along with deuterated internal standards (D_6-verapamil and D_6-norverapamil) were extracted from 50 μL human plasma by liquid–liquid extraction. Separation was achieved on a Chiralcel OD-RH (150 × 4.6 mm, 5 μm) analytical column with resolution factors of 1.4 and 1.9 for (*R*)- and (*S*)-enantiomers of verapamil and norverapamil, respectively. A mobile phase consisting of 0.05% trifluoroacetic acid in water–acetonitrile (70:30, v/v) afforded capacity factors of 2.45, 3.05, 2.27 and 3.13 for (*R*)- and (*S*)-enantiomers of verapamil and norverapamil, respectively. Detection was carried out on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ion modes. The method was validated over the concentration range of 1.0–250.0 ng/mL for all four analytes. Absolute recovery for the analytes ranged from 91.1 to 108.1%. Matrix factors calculated at three quality control levels varied from 0.96–1.07. The method was successfully applied to a pharmacokinetic study in 18 healthy Indian males after oral administration of a 240-mg verapamil tablet formulation under fasting conditions.

Introduction

Verapamil [VER, (RS)-2-(3,4-dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl]-(methyl) amino}-2-prop-2-yl-pentanenitrile] is an L-type calcium channel-blocking agent, used in the treatment of hypertension, angina pectoris, cardiac arrhythmia and, more recently, cluster headaches (1, 2). It is a synthetic paraverin derivate, belonging to the phenylalkylamine class. It is available in conventional tablet form in doses ranging from 40 to 180 mg and in a slow release form in doses of 120 and 240 mg. VER has one asymmetric carbon and thus exists in two enantiomeric forms. It is administered as a racemic mixture of (*R*)- and (*S*)-enantiomers, which show different pharmacodynamics and pharmacokinetic properties; (*S*)-VER is 10–20 times more potent than (*R*)-VER in slowing cardiac A-V conduction velocity in humans, dogs and rabbits (3–5). After oral administration, VER is almost completely absorbed (~90%) from the gastrointestinal tract; however, it has low bioavailability (10–20%) due to extensive hepatic first pass metabolism. The therapeutic concentration of the drug in human plasma ranges from 20–500 ng/mL with a half life of 3–7 h. VER is metabolized in human liver to give 6–12 metabolites, which are primarily excreted through the kidneys (6). The *N*-demethylated metabolite, norverapamil (NOR) is the major metabolite and shows approximately 20% of the efficacy of VER with regard to the vasodilation effect, but shows no antiarrhythmic activity (7).

Various analytical techniques, such as high-performance liquid chromatography with UV detector (HPLC–UV), gas chromatography with nitrogen-phosphorous detector (GC–NPD), micellar liquid chromatography and liquid chromatography–tandem mass spectrometry (LC–MS-MS), have been used to determine VER in blood (8), human plasma (8–12), rat plasma (13, 14), urine and serum samples (15). The simultaneous determination of VER and its metabolites is a subject of several reports (16–25). Largely, separation techniques have been developed based on liquid chromatography with UV (16, 17) and fluorescence detection (FLD) (18–22) to determine VER and NOR in different biological fluids. Ceccato *et al.* (20) developed an automated method for the determination of VER and NOR with on-line sample preparation using dialysis, followed by clean-up and enrichment of the dialysate on a pre-column and subsequent HPLC–FLD analysis. A rapid and simple achiral and chiral HPLC–FLD assay has been developed for the determination of VER and its seven metabolites in serum samples (21). Two achiral reversed-phase columns [shielded hydrophobic phase (Hisep) C18 and NovaPak C18] were used for simultaneous separation of all the analytes with both off-line and on-line solid-phase extraction (SPE). Further, an α1-acid glycoprotein (AGP) column was used to demonstrate chiral separation of all metabolite fractions with chromatographic resolution values sufficient for quantitative analysis of all metabolite enantiomers. Unadkat and co-workers (23) presented a rapid SPE-HPLC method to quantify [^{11}C]-VER and its [^{11}C]-metabolites in human and macaque plasma. Two methods based on LC–MS-MS analysis of VER and its metabolites in biological fluids have also been described (24, 25). Richter *et al.* (24) developed a rapid and sensitive method to determine VER and its four primary and three secondary metabolites in plasma and intestinal perfusate. A multidimensional on-line sample preparation of VER and six of its metabolites from cell cultures and urine is reported by a molecularly imprinted polymer (MIP) coupled to LC–MS (25). The multidimensional approach removed large matrix interferents such as proteins from the sample, while the selectivity of the MIP enabled cleanup of the smaller samples.

Enantiomeric separation on different chiral columns and quantification of VER and its active metabolite, NOR, is also described by several researchers using HPLC (5, 26–33) and LC–MS-MS (34, 35). Chu *et al.* (26) determined enantiomers of VER and NOR in serum using coupled achiral-chiral HPLC. The Hisep column was used to separate VER and NOR from the plasma and the eluents were then selectively transferred to the chiral α1-AGP column, where the enantiomers were stereochemically resolved in 20 min. An HPLC method with column switching (from a reversed-phase Inertsil ODS-2 column to a chiral ovomucoid column) for on-line determination and resolution of VER enantiomers was described by Oda *et al.* (28). In another method (30), VER and NOR were first separated on a diol silica column, and further enantiomeric resolution was achieved on an amylase-based Chiralpak AD column, followed by HPLC–FLD analysis. The calibration curves were linear in the range of 2.5–100 ng/mL for VER enantiomers and 5.0–100 ng/mL for NOR enantiomers. Asafu-Adjaye *et al.* (32) developed a sensitive and selective method for the stereospecific determination of VER and NOR enantiomers in urine. Sample preparation was carried out on a membrane-based SPE, and separation was achieved on a single chiral column (cellulose-based reversed phase, Chiralcel OD-R) in 30 min. Two promising LC–MS-MS methods have been demonstrated for enantiomeric resolution and simultaneous quantification of VER and NOR in human plasma (34) and rat plasma (35). Hedeland *et al.* (34) reported a stereoselective bioanalytical method for the simultaneous quantification of the enantiomers of VER and NOR in human plasma. Sample processing was done with 500 μL plasma by employing liquid–liquid extraction (LLE) in a hexane–2-butanol mixture under alkaline conditions. The separation was achieved on a Chiral-AGP column within 25 min and the enantiomers of VER and NOR were quantified at levels down to 50 pg and 60 pg/500 μL plasma sample, respectively. An enantioselective micro-method (35) for simultaneous analysis of VER and NOR was developed, validated and applied to the study of the kinetic disposition after the administration of a single dose of racemic-VER to male Wistar rats. The analytes were extracted from 100 μL of rat plasma using *n*-hexane and the enantiomers were resolved on a Chiralpak AD column in 18 min. The calibration curves were linear from 1.0–250 ng/mL for the enantiomers of VER and NOR.

The objective of the present study was to chromatographically separate both the enantiomers of verapamil and norverapamil in a single run and to develop a sensitive, rugged and efficient LC–ESI-MS-MS method. The proposed method exhibited excellent performance in terms of selectivity, ruggedness and efficiency (15 min) with a simple sample preparation. The validated method employs a very low plasma volume (50 μL) for processing and has higher sensitivity (1.0 ng/mL) than other procedures (26–33) for enantiomeric determination. Moreover, the total analysis time (extraction and chromatography) is shorter than existing methods. Chromatographic separation was achieved for both the enantiomers of VER and NOR. Also, the on-column loading of analytes at the lower limit of quantification (LLOQ) was only 2.5 pg per sample injection volume. This is significantly less, which helps to maintain the column efficiency for more injections. Ion suppression and enhancement was investigated by a post-column infusion experiment and the results for relative and absolute matrix effects were acceptable at all three quality control (QC) levels. The method ensured estimation of both enantiomers up to 24 h with desired accuracy and precision for a pharmacokinetic study in healthy Indian males.

Experimental

Chemicals and materials

Reference standards of (*R*)-VER (99.8%), (*S*)-VER (98%), (*R*)-NOR (99.7%) (S)-NOR (98%), (*R*)- and (*S*)-VER d_6 [98%, internal standard 1 (IS-1)], and (*R*)- and (*S*)-NOR d_6 (98%, IS-2) were procured from Toronto Research Chemicals (Toronto, Canada). HPLC-grade methanol, acetonitrile, diethyl ether and *n*-hexane were purchased from Merck Specialities (Mumbai, India), and trifluoroacetic acid (TFA) was purchased from Spectrochem (Ahmedabad, India). Water used in the entire analysis was procured from Spectrochem. Blank human plasma was purchased from Supratech Micropath (Ahmedabad, India) and stored at −20°C until use.

Liquid chromatographic conditions

A Shimadzu HPLC (Tokyo, Japan) consisting of an LC-10AD pump, a CTO10 ASVP column oven and an SIL HTc autosampler was used for setting the reverse-phase liquid chromatographic conditions. The separation of (*R*)- and (*S*)-enantiomers of VER and NOR was performed on a Chiralcel OD-RH (150 × 4.6 mm, 5 μm particle size) column and was maintained at 40 ± 0.5°C in a column oven. The mobile phase consisted of 0.05% (v/v) TFA in water–acetonitrile (70:30, v/v ratio). For isocratic elution, the flow rate of the mobile phase was kept at 0.6 mL/min with 70% flow splitting; flow directed to the ion-spray interface was equivalent to 180 μL/min. The total chromatographic run time was 15.0 min. The autosampler temperature was maintained at 5°C.

Mass spectrometric conditions

Ionization and detection of analytes and ISs was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API 2000 (Applied Biosystems, Canada) equipped with electrospray ionization and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for (*R*)- and (*S*)-VER (m/z 455.0 → 165.0), and (*R*)- and (*S*)-NOR (m/z 441.1 → 165.0), as shown in Figure 1, and their internal standards, IS-1 (m/z 461.0 → 165.0), and (*R*)- and IS-2 (m/z 447.0 → 165.0) (figure not shown). The source-dependent parameters maintained for enantiomers of both the analytes and their ISs were 4.00 kV (ion spray voltage); 50.0 psig (nebulizer gas, GS1); 60.0 psig (heater gas, GS-2); 400.0°C (ion source temperature); 18.0 psig (CUR gas); 4.0 psig (CAD gas); 600.0 ms (dwell time). The optimum values for compound-dependent parameters such as entrance potential (EP), focusing potential (FP) and cell exit potential (CXP) were set at 10, 400 and 7 V, respectively, for all analytes and their ISs. Declustering potential (DP) and collision energy (CE) were set at 50 and 40 V for (*R*)-VER, (*S*)-VER and IS-1, respectively; 25 and 35 V for (*R*)-NOR, (*S*)-NOR and IS-2, respectively. Quadrupoles 1 and 3

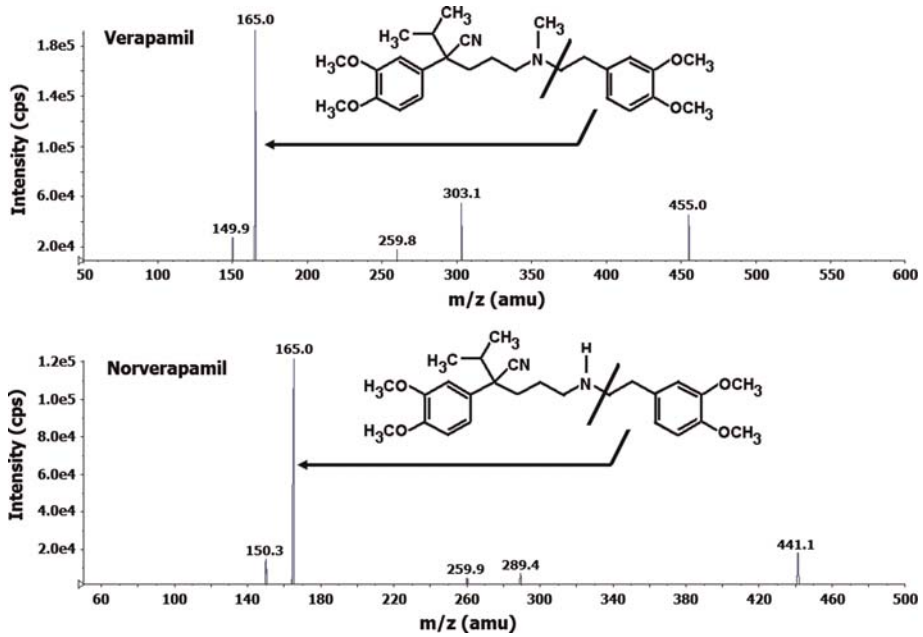

Figure 1. Product ion mass spectra in positive ionization mode: (*R*)- and (*S*)-enantiomers of VER (*m/z* 455.0 → 165.0, scan range 50–600 amu) (A); (*R*)- and (*S*)-enantiomers of NOR (*m/z* 441.1 → 165.0, scan range 50–500 amu) (B).

were maintained at unit mass resolution. Analyst software version 1.4.1 was used to control all parameters of LC and MS.

Standard stock solution, calibration standards and QC sample preparation

The standard stock solution of 250 μg/mL of (*R*)- and (*S*)-isomers of VER and NOR was prepared by dissolving requisite amounts in methanol. Calibration standards and QC samples were prepared by spiking (2% of total volume of blank plasma) blank plasma with working solutions (500 ng/mL) in methanol. Calibration curve standards were made at 1.0, 2.0, 12.5, 37.5, 125, 187.5, 225 and 250 ng/mL concentrations for all four analytes, and QC samples were prepared at three levels: 215 ng/mL [high quality control (HQC)], 112.5 ng/mL [medium quality control (MQC)], 3.0 ng/mL [low quality control (LQC)] and 1.0 ng/mL (LLOQ QC) for the analytes. Stock solutions (100 μg/mL) of IS-1 and IS-2 were prepared by dissolving requisite amounts in methanol. Furthermore, a combined working solution of ISs (1.0 μg/mL) for spiking was prepared in methanol. All solutions (standard stock, calibration standards and QC samples) were stored at 2–8°C until use.

Protocol for sample preparation

Before analysis, all frozen subject samples, calibration standards and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 50 μL of spiked plasma sample, 50 μL of mixed IS was added and vortexed for 10 s. To these samples, 2.0 mL of *n*-hexane–diethyl ether (50:50, v/v) extraction solvent was added and the samples were extracted on an extractor at 32 × g for 5 min. Centrifugation of the samples was done at 3,200 × g at 10°C. The supernatant was separated and evaporated to dryness under nitrogen at 40 ± 5°C. The dried samples were reconstituted with 100 μL of reconstitution solution [0.05% TFA–acetonitrile (30:70, v/v)] and 5 μL was used for injection in the chromatographic system.

Method validation procedures

The method validation was performed as per the United States Food and Drug Administration (USFDA) and European Medicines Agency (EMEA) guidelines (36, 37). The system suitability experiment was performed by injecting six consecutive injections using an aqueous standard mixture of (*R*)- and (*S*)-enantiomers of VER and NOR, IS-1 and IS-2 at the start of each batch during the method validation. System performance was checked by injecting one extracted LLOQ sample of the analyte along with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the autosampler was evaluated by injecting the solutions in the following sequence: aqueous sample at upper limit of quantification (ULOQ) [(*R*)- and (*S*)-enantiomers of VER and NOR] → aqueous sample at LLOQ → mobile phase → extracted sample at LLOQ [(*R*)- and (*S*)-enantiomers of VER and NOR] → extracted blank plasma → extracted sample at ULOQ [(*R*)- and (*S*)-enantiomers of VER and NOR] → extracted blank plasma → extracted sample at ULOQ [(*R*)- and (*S*)-enantiomers of VER and NOR] → extracted blank plasma.

The linearity of the method was determined by analysis of six calibration curves containing eight non-zero concentrations. The ratio of the area response for enantiomer to IS was used for regression analysis. Each calibration curve was analyzed individually by using least-squares weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma.

The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15.0%.

The selectivity of the method towards endogenous plasma matrix components was assessed in ten batches (seven normal K3 EDTA plasma, one hemolyzed, one lipidemic and one heparinised) of blank human plasma. A check for interference due to commonly used medications (paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen) in human volunteers was done for ionization (ion suppression/enhancement), analytical recovery (precision and accuracy) and chromatographic interference (interference with MRM of drug and IS). Their stock solutions (100 μg/mL) were prepared by dissolving requisite amounts in methanol. Furthermore, working solutions (100 ng/mL) of each drug were prepared in the mobile phase, spiked in plasma and analyzed under the same conditions at LQC and HQC levels.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples of (*R*)- and (*S*)-enantiomers of VER and NOR was performed on the same day. The run consisted of a calibration curve and five replicates of LLOQ QC, LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within ± 15.0% except LLOQ, for which it should be within ± 20.0%.

Ion suppression and enhancement effects on the MRM LC–MS-MS sensitivity were evaluated by the post-column analyte infusion experiment. A standard solution containing (*R*)- and (*S*)-enantiomers of VER and NOR (at MQC level) was infused post-column into the mobile phase at 10 μL/min, employing inline infusion pump. Aliquots of 5 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS-MS chromatogram was acquired for the analytes. Any dip in the baseline upon injection of double blank plasma (without IS) indicates ion suppression, while a peak at the retention time of analytes indicates ion enhancement.

The extraction efficiency (process efficiency/absolute recovery) and absolute matrix effect were evaluated at HQC, MQC and LQC levels in six replicates. Absolute recovery was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of neat standard solutions (in mobile phase) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions (in mobile phase). The assessment of relative ME was based on direct comparison of the MS-MS responses (peak areas) of the analytes spiked into extracts originating from different lots of plasma. The variability in these responses, expressed as %CV, was considered to be the measure of relative ME.

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison standards at LQC and HQC levels. Stock solutions of analytes and ISs were checked for short-term stability at room temperature and long-term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ± 10.0%. Autosampler stability (wet extract), dry extract, bench top (at room temperature) and freeze-thaw stability were performed at LQC and HQC using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at −20 and −70°C) and thawing (without warming) at room temperature. Long-term stability of spiked plasma samples stored at −20 and −70°C was also studied at both of these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed QC samples was within ± 15.0% (37).

To authenticate the ruggedness of the proposed method, it was evaluated on two precision and accuracy batches. The first batch was analyzed by two different analysts and the second batch was analyzed on different columns.

A dilution integrity experiment was conducted by diluting the stock solution prepared as a spiked standard at concentration of 500 ng/mL for (*R*)- and (*S*)-enantiomers of VER and NOR. The precision and accuracy for dilution integrity standards at 1/2th (250 ng/mL) and 1/10th (50 ng/mL) dilutions were determined by analyzing the samples against calibration curve standards.

Pharmacokinetic study

A pharmacokinetic study with A 240-mg VER tablet formulation (Isoptin SR, Ranbaxy Laboratories; India) was conducted in 18 healthy Indian males in the age group of 18–45 under fasting conditions. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA (38). A single oral dose of 240-mg drug was given to the volunteers with 240 mL of water. Blood samples were collected at 0.0 (pre-dose), 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12, 18 and 24 h after oral administration of the dose in labeled lithium heparin-vacuettes. An incurred sample re-analysis (ISR) was also conducted by computerized random selection of 20 subject samples. The results were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ± 20% (39).

Results and Discussion

Method validation results

Throughout the method validation, the precision (%CV) of the system suitability test was observed in the range of 0.2 to 0.8% for the retention time and 0.5 to 1.5% for the area response for all the analytes and ISs, which is not more than the acceptance criteria of 4%. The signal-to-noise (S/N) ratio for system performance was ≥ 60 for (*R*)- and (*S*)-enantiomers of VER and IS-1 and ≥ 50 for (*R*)- and (*S*)-enantiomers of NOR and IS-2. Carry-over evaluation was performed in each analytical run to ensure that the accuracy and precision of the proposed method were not affected. Negligible carryover (≤0.8%) was observed during the autosampler carryover experiment. No enhancement in the response was observed in extracted blank plasma (without ISs and analytes) after subsequent injection of the highest calibration standard (aqueous and extracted) at the retention times of (*R*)- and (*S*)-enantiomers of VER and NOR, IS-1 and IS-2, respectively.

Table I
Intra-Batch and Inter-Batch Precision and Accuracy for (*R*) & (*S*)-VER and (*R*) & (*S*)-NOR

Analyte	Quality control level	Intra-batch			Inter-batch		
		Mean concentration found (ng/mL)*	Precision (%CV)	Accuracy (%)	Mean concentration found (ng/mL)†	Precision (%CV)	Accuracy (%)
(*R*)-VER	LLOQ QC	1.03	1.4	103	1.02	2.8	102
	LQC	3.02	2.0	101	3.11	5.3	104
	MQC	115	1.7	102	113	2.9	101
	HQC	215	2.3	100	217	5.3	101
(*S*)-VER	LLOQ QC	1.04	0.7	104	1.03	4.1	103
	LQC	3.02	2.1	101	3.12	5.4	104
	MQC	116	1.3	103	115	2.7	102
	HQC	216	1.5	101	197	5.2	103
(*R*)-NOR	LLOQ QC	1.02	1.8	102	1.02	4.4	102
	LQC	3.03	1.7	101	3.09	5.4	103
	MQC	115	0.8	102	115	3.1	102
	HQC	214	1.7	100	221	5.0	102
(*S*)-NOR	LLOQ QC	1.03	2.1	103	1.03	4.1	103
	LQC	3.02	2.5	101	3.10	4.9	103
	MQC	116	1.6	103	115	3.2	102
	HQC	218	2.1	102	222	5.4	103

*Mean of five replicates at each concentration.
†Mean of five replicates for five precision and accuracy batches.

Table II
Extraction Efficiency (Absolute Recovery) of the analytes at Three QC Levels

	LQC				MQC				HQC			
	(*R*)-VER	(*S*)-VER	(*R*)-NOR	(*S*)-NOR	(*R*)-VER	(*S*)-VER	(*R*)-NOR	(*S*)-NOR	(*R*)-VER	(*S*)-VER	(*R*)-NOR	(*S*)-NOR
Mean area ratio ($n = 6$)	0.0264	0.0286	0.0300	0.0347	0.931	1.038	1.063	1.224	1.623	1.834	1.940	2.131
Recovery (%)	102.2	91.9	91.2	98.7	108.1	99.9	96.8	104.2	97.2	91.1	91.3	93.7
Precision (%CV)	1.3	0.2	0.4	0.3	1.3	1.8	1.6	1.1	0.8	0.4	1.0	0.8

The calibration curves were linear over the concentration range of 1.0–250 ng/mL for all four analytes with correlation coefficient $r^2 \geq 0.9988$ with a precision of $\leq 0.1\%$ (CV) for (*R*)- and (*S*)-enantiomers of VER and NOR. The equations for means ($n = 6$) of six calibration curves for (*R*)- and (*S*)-enantiomers of VER and NOR were $y = (0.0082 \pm 0.0002)x + (0.0045 \pm 0.0012)$, $y = (0.0086 \pm 0.0005)x + (0.0056 \pm 0.0014)$, $y = (0.0088 \pm 0.0003)x + (0.0057 \pm 0.0015)$ and $y = (0.0099 \pm 0.0005)x + (0.0044 \pm 0.0013)$, respectively. The standard deviation values for observed r^2 were 0.0007, 0.0005, 0.0004 and 0.0003 for (*R*)-VER, (*S*)-VER, (*R*)-NOR and (*S*)-NOR, respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 98 to 102% and 1.7 to 3.5% for (*R*)- and (*S*)-enantiomers of VER and NOR, respectively. The lowest concentration (LLOQ) in the standard curve for all four isomers was measured at an S/N of ≥ 50.

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC and LLOQ QC levels (Table I). The intra-batch %CV ranged from 0.8 to 2.5 and the accuracy was within 99.5 to 104.0%. For the inter-batch experiments, the precision varied from 2.7 to 5.4 and the accuracy was within 100.7 and 104.0% for all four analytes.

The extraction efficiency (absolute recovery) for all the analytes at LQC, MQC and HQC levels is presented in Table II. The absolute recovery obtained for (*R*)- and (*S*)-enantiomers of VER and NOR varied from 91.1 to 108.1% at all QC levels. The mean recovery for IS-1 and IS-2 at three QC levels was 98.5% and 102.1%, respectively. Absolute ME data at LQC, MQC and HQC levels is shown in Table III; the matrix factors across these QC levels ranged from 0.96 to 1.07 for all the analytes. Furthermore, the absence of relative ME is demonstrated by comparing the %CV values between different lots (sources) of plasma (spiked after extraction at four QC levels) samples. The precision results varied from 1.20 to 2.05% for different plasma lots for all the analytes.

The stability of the analytes and ISs in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged up to 24 h, while the stock solutions for long-term stability of the analytes and their respective ISs were stable for minimum of 129 days at refrigerated temperature below 8°C. (*R*)- and (*S*)-enantiomers of VER and NOR in control human plasma (bench top) at room temperature (25°C) was stable at least for 20 h and for minimum of three freeze and thaw cycles at −20 and −70°C. Spiked plasma samples stored at −20 and −70°C for the long-term stability experiment were found to be stable for a minimum period of 154 days. Dry extract stability of the spiked quality control samples stored at −20°C was determined up to 48 h. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5°C was determined up to 69 h without significant drug loss. The percentage change for different stability experiments in plasma at two QC levels was within ±5.7%, as shown in Table IV.

The results of ruggedness study for the (*R*)- and (*S*)-enantiomers of VER and NOR were well within the acceptance limit of 15% in precision (%CV) and 85.0 to 115.0% in mean accuracy. The precision and accuracy values for the ruggedness experiments (different analysts and columns) ranged

Table III
Matrix Effect Assessment at Different QC levels for All Four Analytes

Lot No.	LQC			MQC			HQC		
	Mean area ratio ($n = 6$)		MF‡	Mean area ratio ($n = 6$)		MF	Mean area ratio ($n = 6$)		MF
	A* (%CV)	B† (%CV)		A (%CV)	B (%CV)		A (%CV)	B (%CV)	
	(*R*)-VER								
1	0.0297 (3.1)	0.0317 (5.7)	1.06	1.003 (2.3)	1.074 (3.0)	1.07	1.823 (1.5)	1.922 (4.5)	1.05
2	0.0287 (2.7)	0.0309 (4.2)	1.07	1.006 (4.3)	1.081 (5.1)	1.07	1.904 (2.8)	1.874 (2.1)	0.98
3	0.0304 (2.9)	0.0311 (4.6)	1.02	1.010 (1.7)	1.031 (2.3)	1.02	1.888 (2.5)	1.834 (3.0)	0.97
	(*S*)-VER								
1	0.0381 (1.5)	0.0403 (3.2)	1.05	1.292 (1.1)	1.343 (2.7)	1.04	2.335 (3.2)	2.407 (2.4)	1.03
2	0.0373 (2.2)	0.0399 (3.0)	1.06	1.286 (1.6)	1.347 (2.2)	1.05	2.366 (1.9)	2.377 (2.8)	1.00
3	0.0382 (1.8)	0.0394 (1.9)	1.03	1.310 (0.9)	1.304 (1.7)	0.99	2.377 (2.0)	2.335 (1.6)	0.98
	(*R*)-NOR								
1	0.0315 (4.1)	0.0332 (5.5)	1.05	0.967 (4.5)	1.024 (4.1)	1.06	1.846 (2.2)	1.953 (4.5)	1.06
2	0.0336 (3.5)	0.0335 (3.7)	0.99	0.980 (2.4)	1.043 (5.0)	1.06	1.896(3.1)	1.870 (1.7)	0.99
3	0.0310 (3.9)	0.0328 (2.3)	1.06	0.994 (3.0)	0.989 (3.7)	0.99	1.895 (1.8)	1.835 (2.3)	0.97
	(*S*)-NOR								
1	0.0356 (2.7)	0.0345 (3.9)	0.97	1.232 (2.8)	1.252 (3.5)	1.02	2.257 (1.4)	2.345 (2.6)	1.04
2	0.0355 (2.0)	0.0340 (3.3)	0.96	1.248 (3.2)	1.267 (4.0)	1.02	2.292 (1.9)	2.314 (1.6)	1.01
3	0.0363 (3.1)	0.0370 (1.8)	1.02	1.238 (2.5)	1.254 (3.6)	1.01	2.280 (2.4)	2.231 (3.1)	0.98

*Mean area ratio (analyte/IS) response of three replicate samples prepared in mobile phase (neat samples).
†Mean area ratio (analyte/IS) response of three replicate samples prepared by spiking in extracted blank plasma.
‡MF: matrix factor = response of post spiked sample/response of neat sample.
CV: coefficient of variation

Table IV
Stability Data of (*R*) and (*S*)-VER and (*R*) and (*S*)-NOR under Various Conditions ($n = 6$)

QC level	(*R*)-VER		(*S*)-VER		(*R*)-NOR		(*S*)-NOR	
	Mean concentration found (ng/mL) ± SD	% Change*	Mean concentration found (ng/mL) ± SD	% Change	Mean conentration found (ng/mL) ± SD	% Change	Mean concentration found (ng/mL) ± SD	% Change
Bench top stability, 25°C								
HQC	204 ± 1.5	−5.2	207 ± 1.0	−3.6	208 ± 1.5	−3.1	204 ± 2.3	−5.2
LQC	2.83 ± 0.4	−5.7	2.85 ± 0.2	−5.1	2.86 ± 0.1	−4.7	2.84 ± 0.1	−5.7
Wet extract stability, 5°C								
HQC	216 ± 0.6	0.7	213 ± 1.0	−1.0	208 ± 1.5	−3.3	224 ± 2.1	4.2
LQC	3.11 ± 0.1	3.7	3.13 ± 0.4	4.3	3.10 ± 0.2	3.2	3.17 ± 0.5	5.7
Dry extract stability, −20°C								
HQC	216 ± 0.5	0.7	221 ± 4.9	2.6	221 ± 5.5	2.6	221 ± 5.1	2.8
LQC	3.08 ± 0.8	2.6	3.14 ± 0.5	4.7	3.10 ± 0.7	3.4	3.15 ± 0.6	5.1
Freeze and thaw stability, −20°C, 3 cycles								
HQC	213 ± 1.5	−1.0	208 ± 1.7	−3.1	209 ± 1.0	−2.6	214 ± 2.5	−0.5
LQC	3.01 ± 0.4	0.3	3.02 ± 0.0	0.7	3.01 ± 0.3	0.3	3.07 ± 0.3	2.4
Freeze and thaw stability, −70°C, 3 cycles								
HQC	213 ± 1.2	−1.0	209 ± 1.2	−2.6	207 ± 1.5	−3.6	218 ± 0.6	1.6
LQC	3.00 ± 0.4	0.0	3.03 ± 0.2	1.0	3.04 ± 0.2	1.3	3.08 ± 0.5	2.7
Long-term stability in plasma, −20°C								
HQC	216 ± 5.0	0.5	214 ± 3.2	−0.5	213 ± 3.3	−1.0	215 ± 4.4	0.0
LQC	3.10 ± 6	3.0	3.06 ± 0.7	2.0	3.01 ± 0.8	0.3	3.02 ± 1.0	0.3
Long-term stability in plasma, −70°C								
HQC	211 ± 1.6	−2.1	212 ± 4.1	−1.6	211 ± 0.9	−2.1	214 ± 3.8	−0.5
LQC	3.03 ± 1.1	1.0	3.02 ± 0.7	1.0	3.03 ± 0.9	1.0	2.97 ± 0.4	−1.0

$$^{*}\%\text{Change} = \frac{\text{Mean stability samples (mean concentration found)} - \text{Mean comparison samples (nominal concentration)}}{\text{Mean comparison samples(nominal concentration)}} \times 100$$

from 0.5 to 2.2% and 99.5 to 104.1% for (*R*)- and (*S*)-VER and 0.8 to 3.0% and 98.3 to 104.0% for (*R*)- and (*S*)-NOR, respectively.

The dilution integrity experiment was performed to validate the dilution test to be performed on higher analyte concentrations above the ULOQ, which may be encountered during real subject sample analysis. The precision (%CV) and accuracy values for 1/2th and 1/10th dilution ranged from 0.9–2.2% and 95.4–98.2% for (*R*)- and (*S*)-enantiomers of VER and NOR, respectively.

Method development

Chromatographic resolution of (*R*)- and (*S*)-enantiomers of VER and NOR has been reported on different chiral stationary phases, based on amylose derivatives like Chiralpak AD (30, 35), cellulose-based Chiralcel OD (32), protein-based α1-AGP (26, 29, 31, 33, 34) and an ovomucoid (5, 28) column. Previous studies using chiral α1-AGP columns (26, 29) have observed an overlapping of the enantiomeric peaks of VER and NOR when

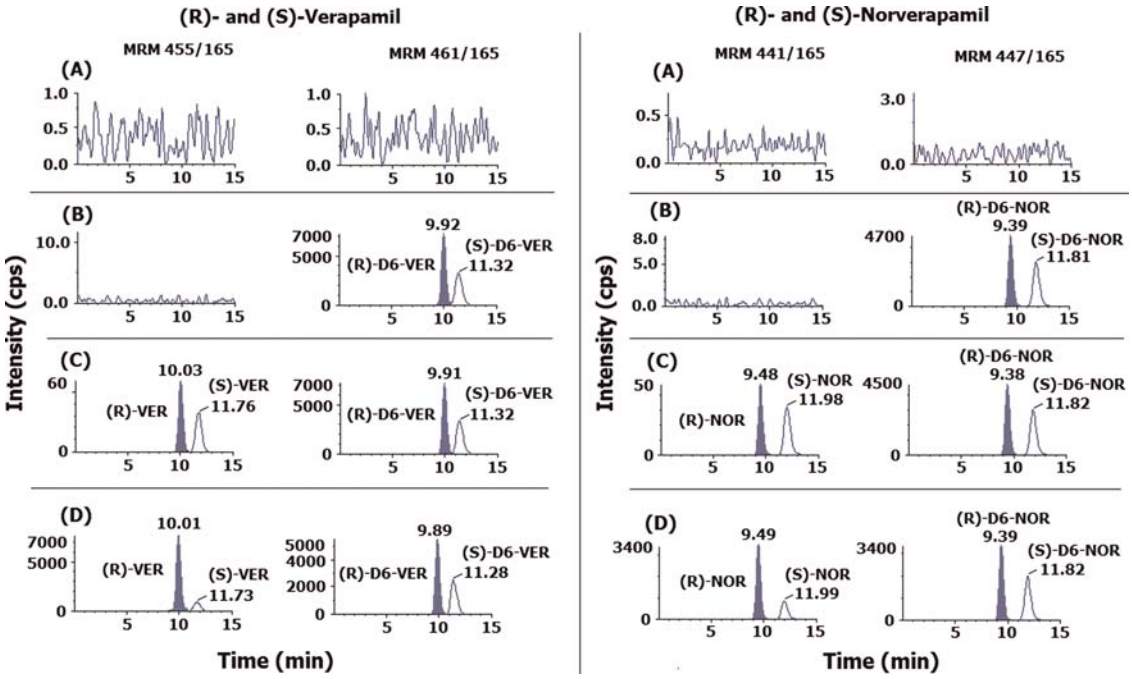

Figure 2. MRM ion-chromatograms of (*R*)- and (*S*)-enantiomers of VER and NOR: in double blank plasma (without analyte and IS) (A); in blank plasma fortified with IS (B); at LLOQ and IS (C); a real subject sample at C_{max} after administration of a 240-mg dose of verapamil (D).

chromatographed together. This was resolved by coupling chiral α1-AGP column to an achiral (Hisep) column (26) or by acetylation of norverapamil to its *N*-acetyl derivative (29, 31). As reported previously (33), selection of the right column, its temperature, pH and composition of the mobile phase (aqueous and organic part) are important critera. Asafu-Adjaye *et al.* (32) described an SPE-HPLC method for baseline separation of VER and NOR enantiomers on a Chiralcel OD-R column in urine samples. Similarly, complete separation of enantiomers was achieved on a Chiralpak AD column by Mateus *et al.* (35). Thus, in the present study, both of these columns [Chiralcel OD-RH (150/250 × 4.6 mm, 5/10 μm) and Chiralpak AD (150/250 × 4.6 mm, 5/10 μm)] were investigated for enantioseparation of VER and NOR under isocratic conditions to obtain adequate response, sharp peak shape and a short analysis time. The separation was tried using various combinations of organic modifiers like methanol–acetonitrile (15–30%), acidic buffers (acetic acid–ammonium acetate and formic acid–ammonium formate) and additives like formic acid and TFA. Efficient separation of (*R*)- and (*S*)-VER was obtained on both the columns; however, baseline resolution of (*R*)- and (*S*)-NOR was not possible on the Chiralpak AD column (selectivity factor ~1.0). Better enantioselectivities for VER and NOR were achieved with acetonitrile, as reported previously (33). Initially, the run time was too high (> 25 min) with 15% acetonitrile content in the mobile phase. Optimum results in terms of sensitivity, efficiency and peak shape were achieved on the Chiralcel OD-RH (150 × 4.6 mm, 5 μm) using 0.05% TFA in water and acetonitrile (70:30, v/v) as the mobile phase. The concentration of TFA was critical in achieving the desired selectivity and sensitivity. The effect of temperature on the chromatograms was also studied from 20–40°C. An increase in temperature from 20 to 40°C helped to improve the selectivity factor ($\alpha = k_S/k_R$) from 1.19 to 1.24 for (*R*)- and (*S*)-VER and 1.30 to 1.38 for (*R*)- and (*S*)-NOR. The retention times for (*R*)- and (*S*)-enantiomers of VER and NOR were 10.3, 11.76, 9.48 and 11.98, respectively, with a total chromatographic run time of 15 min. The use of deuterated internal standards helped in LC–ESI-MS-MS recovery of the analytes. The reproducibility of retention times for the analytes, expressed as %CV, was ≤ 1% for 100 injections on the same column. The capacity factors (*k*), which describe the rate at which the analytes migrate through the column, were 2.45 and 3.05, and 2.27 and 3.13 for (*R*)- and (*S*)-enantiomers of VER and NOR, respectively, based on a dead time of 2.9 min. Resolution factors of 1.4 and 1.9 were achieved on a Chiralcel OD-RH (150 × 4.6 mm, 5 μm) analytical column for (*R*)- and (*S*)-enantiomers of VER and NOR, respectively. The number of theoretical plates obtained for (*R*)- and (*S*)-enantiomers of VER and NOR were 955, 1,550, 850 and 1,360, respectively.

Representative MRM chromatograms in Figure 2 of (*R*)- and (*S*)-enantiomers of VER and NOR in extracted blank plasma (without IS and analyte), blank plasma fortified with IS, analytes at LLOQ and an actual subject sample at C_{max} demonstrate the selectivity of the method. None of the commonly used medications by subjects showed interfering signals at the retention time of analytes or their ISs. Two other metabolites of VER with much lower molecular weights were also monitored, D617 (m/z 291.0 → 151.0) and D620 (m/z 277.0 → 151.0), in subject samples. However, both were undetected in the MRM of VER and NOR, as shown in Figure 2D. Results of the post-column infusion experiment in Figure 3 indicate no ion suppression or enhancement at the retention times of analytes and ISs. There was, however, some ion suppression and enhancement observed between 3 and 5 min, which did not affect the quantitation.

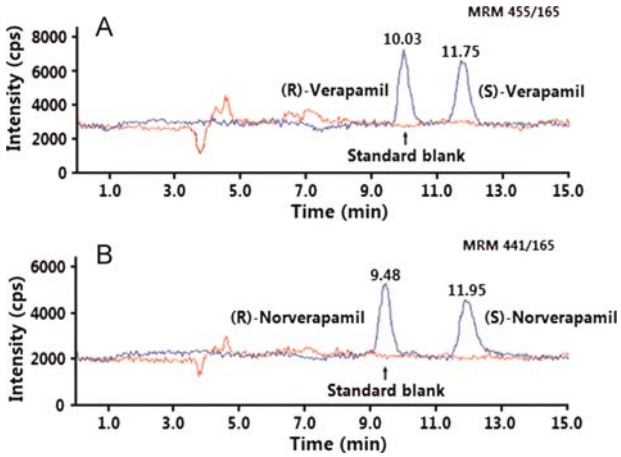

Figure 3. MRM LC–MS-MS chromatograms for post-column analyte infusion experiment: (*R*)- and (*S*)-enantiomers of VER (A); (*R*)- and (*S*)-enantiomers of NOR (B).

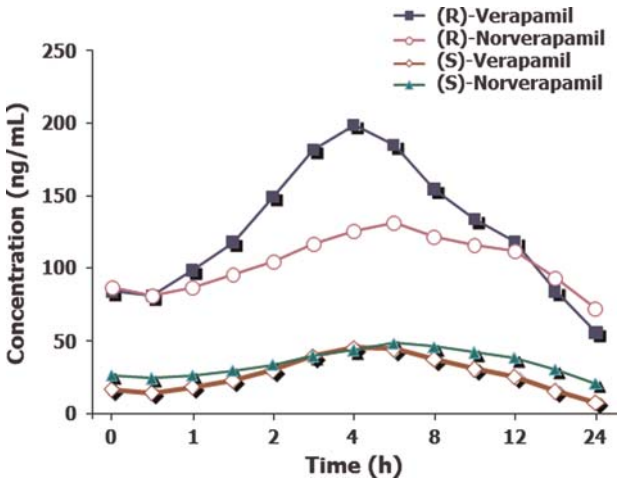

Figure 4. Mean plasma concentration-time profile of (*R*)- and (*S*)-enantiomers of VER and NOR after oral administration of a 240-mg verapamil tablet formulation to 18 healthy Indian males under fasting conditions.

The inherent selectivity of MS-MS detection was expected to be beneficial in developing a selective and sensitive method. The present study was conducted using electrospray ionization in the positive mode because this gave high intensity for (*R*)- and (*S*)-enantiomers of VER and NOR, IS-1 and IS-2, due to the presence of similar sites for protonation. Also, the use of trifluoroacetic acid in the mobile phase further augmented the response for protonated precursor ions $[M + H]^+$ at m/z 455 for (*R*)- and (*S*)-VER, m/z 441 for (*R*)- and (*S*)-NOR, m/z 461 for (*R*)- and (*S*)-VER-d_6 and m/z 441 for (*R*)- and (*S*)-NOR-d_6 in the Q1 MS full scan spectra. The most consistent and abundant product ions were found at m/z 165 (due to the cleavage of one of the nitrogen-carbon bonds) by applying collision energy of 40 and 35V for (*R*)- and (*S*)-enantiomers of VER and NOR, respectively, in Q3 MS. A dwell time of 600 ms was adequate for the analytes and ISs and no cross talk was observed between their MRMs.

Several methods have employed LLE with diethyl ether (26, 29), heptane (30), butanol in hexane (31, 34), pentane-methylene chloride (5) and hexane (35) for quantitative extraction of these enantiomers from biological matrices. In the present work, trials were conducted with diethyl ether, *n*-hexane, heptane and methylene chloride (alone and in combination) for the extraction of (*R*)- and (*S*)-enantiomers of VER and NOR and ISs. Plasma sample prepared in diethyl ether gave inconsistent recovery for NOR enantiomers with some ion suppression (>15% CV), while samples with *n*-hexane had mistiness in the final extracts after reconstitution. With heptane and methylene chloride, the recovery was not quantitative (50–60%), and thus, the mixture of these solvents in different volume ratios were tested. Cleaner extracts with quantitative and precise recovery for all four analytes and ISs were obtained in an *n*-hexane–diethyl ether (50:50, v/v) solvent mixture at all QC levels employing a 50-µL plasma sample.

Table V
ISR Data for (*R*) and (*S*)-VER and (*R*) and (*S*)- NOR

Sample No.	% Change*			
	(*R*)-VER	(*S*)-VER	(*R*)- NOR	(*S*)- NOR
1	−6.4	−7.7	−3.1	−4.4
2	−4.1	−1.9	−3.5	−5.0
3	2.6	−9.6	2.8	−2.0
4	4.3	8.2	2.5	5.9
5	−5.3	6.2	−4.2	−5.2
6	−2.6	−5.4	−3.6	−5.2
7	1.8	−10.9	0.4	−6.1
8	−1.5	−2.9	0.9	−0.5
9	2.2	2.7	8.3	4.1
10	5.3	−4.3	4.8	4.3
11	−2.6	−10.9	−2.8	−3.8
12	−7.3	−9.3	−5.3	−9.5
13	2.4	3.4	−0.4	3.9
14	−2.3	−8.8	−2.6	6.3
15	−5.2	−6.9	−6.9	−8.8
16	−7.2	−10.1	−4.9	−8.5
17	−3.5	5.2	−4.7	−0.4
18	−5.8	−9.8	−3.7	−2.6
19	0.9	−0.8	2.6	3.2
20	2.2	−1.3	8.4	1.7

$$^{*}\%\text{Change} = \frac{\text{Repeat value} - \text{Initial value}}{\text{Initial value}} \times 100$$

Application of the method in healthy human subjects

The validated method was successfully applied for the assay of (*R*)- and (*S*)-enantiomers of VER and NOR in healthy Indian males. Figure 4 shows the plasma concentration versus time profiles of (*R*)- and (*S*)-enantiomers of VER and NOR under fasting conditions. The profiles suggest that the (*S*)-isomer has a faster rate of metabolism than the (*R*)-isomer for VER and NOR, which is in concurrence with a previous report (34). The method was sensitive enough to monitor the plasma concentration up to 24 h. Approximately 500 samples, including the calibration and QC samples with volunteer samples, were run and analyzed during a period of 10 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The pharmacokinetic parameters; i.e., maximum plasma concentration (C_{max}, ng/mL) and time point for maximum plasma concentration (T_{max}, h) values for (*R*)-VER, (*S*)-VER, (*R*)-NOR and (*S*)-NOR were 205 ± 25/4.1 ± 1.7, 47 ± 12/4.0 ± 1.5, 142 ± 19/5.9 ± 1.1 and 52 ± 10 /6.0 ±

Table VI
Comparison of LC–MS-MS Methods for Simultaneous Analysis of (*R*)- and (*S*)-Enantiomers of VER and NOR in Plasma

Serial No.	Extraction procedure (plasma volume); reconstitution volume; internal standard; mean recovery at QC levels (%)	Column; elution process; mobile phase; flow rate; injection volume; maximum on-column loading at LLOQ	Linear dynamic range (ng/mL); analytical run time	Application	Ref. No.
1	LLE with 6 mL *n*-hexane–2-butanol mixture (500 µL, human plasma); 100 µL; D_3-verapamil; —	Chiral-AGP column (150 × 4.0 mm, 5 µm); isocratic; 20 mM ammonium acetate buffer, pH 7.4–acetonitrile (85:15, v/v); 0.6 mL/ min; 50 µL; 25pg	0.10–125 for (*R*)- and (*S*)-VER and 0.12–121 for (*R*)- and (*S*)-NOR; 25 min	*In vivo* intestinal perfusion study by Loc-I-Gut method with 8 healthy male volunteers	34
2	LLE with 2 mL *n*-hexane (100 µL, rat plasma); 200 µL; paroxetine; (*R*)-VER, 88.4; (*S*)-VER, 87.1; (*R*)-NOR, 71.5; (*S*)-NOR, 74.0	Chiralpak AD column (250 × 4.6 mm, 10 µm); isocratic; *n*-hexane–isopropanol–ethanol–diethylamine (88:6:6:0.1, v/v/v/v); 1.35 mL/ min; 130 µL; 65pg	1.0–250 for (*R*)- and (*S*)-VER and (*R*)- and (*S*)-NOR; 18 min	Enantioselective kinetic disposition of VER and NOR in rat plasma with a single dose of 10 mg/kg of racemic VER	35
3	LLE with 2 mL *n*-hexane–diethylether mixture (50 µL, human plasma); 100 µL; D_6-VER and D_6-NOR; (*R*)-VER, 102.5; (*S*)-VER, 94.3; (*R*)-NOR, 93.1; (*S*)-NOR, 98.8	Chiralcel OD-RH (150 × 4.6 mm, 5 µm) column; isocratic; 0.05% (v/v) trifluoroacetic acid in water–acetonitrile (70:30, v/v); 0.6 mL/ min; 5 µL; 2.5pg	1.0–250 for (*R*)- and (*S*)-VER and (*R*) and (*S*)-NOR; 15 min	Bioavailability study in 18 healthy Indian male volunteers with a 240-mg tablet formulation	Present Method

1.3, respectively. The percentage change in the randomly selected subject samples for incurred sample re-analysis (assay reproducibility) was 8.4 to −10.9% (Table V). Furthermore, there were no adverse events during the course of the study.

Conclusion

The proposed method successfully demonstrates chromatographic separation of (*R*)- and (*S*)-enantiomers of VER and NOR in human plasma with high resolution. The bioanalytical methodology for their simultaneous determination is highly specific, rugged and rapid for therapeutic drug monitoring. The method involved a simple and specific sample preparation by LLE followed by isocratic chromatographic separation in 15 min. The overall analysis time is more promising than several other reported procedures for both enantiomers (26, 29, 31, 32, 34). A detailed comparison of LC–MS methods for quantification of VER and NOR enantiomers in plasma is presented in Table VI. The established LLOQ and a wide linear dynamic range are adequate to conduct a pharmacokinetic study with 240-mg dose tablet formulations of VER in healthy human volunteers.

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