Rapid LC–ESI-MS–MS Method for the Simultaneous Determination of Clopidogrel and its Carboxylic Acid Metabolite in Human Plasma

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Abstract

A high throughput liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) method is developed for the simultaneous estimation of clopidogrel (SR25990C) and its carboxylic acid metabolite (SR26334) in human plasma using glimepiride as internal standard. The extraction of SR25990C, its metabolite, and IS from the plasma (0.3 mL) involves treatment with phosphoric acid followed by solid-phase extraction (SPE). Sample preparation by this method yields clean extracts with quantitative and consistent mean recoveries of 98.05%, 85.45%, and 105.72% for SR25990C, SR26334, and IS, respectively. The SPE eluate without drying and reconstitution is analyzed by LC–MS–MS, operating in the positive ion and selective reaction monitoring mode. The injection volume is 2 µL with a total chromatographic run time of 5.0 min. The method response is linear over the dynamic range of 0.25 to 25.0 ng/mL for SR25990C and 50.0 to 6000.0 ng/mL for SR26334, with correlation coefficients of $r \geq 0.9989$ and 0.9984, respectively. The method is validated to demonstrate its specificity, linearity, accuracy, precision, recovery, matrix effect, dilution integrity, and stability studies. It is applied to study the bioavailability of 75 mg clopidogrel mesylate tablets in 16 human subjects with satisfactory results.

Introduction

Clopidogrel (SR25990C) is a potent antiplatelet drug and is used for the prevention of atherosclerotic events in patients with recent stroke, myocardial infarction, or cardiovascular disease (1–3). SR25990C inhibits platelet aggregation ex-vivo induced by ADP, low concentrations of thrombin, or by collagen (4). It is not active in-vitro, and a biotransformation by the liver is necessary to allow the expression of its antiaggregating activity (5). Thus, SR25990C can be considered as a precursor of an active metabolite. It is rapidly absorbed and undergoes extensive hepatic biotransformation forming an active thiol metabolite from 2-oxo-clopidogrel—a key intermediate metabolite (3) and an inactive carboxylic acid derivative (SR26334), which is a major circulating metabolite (85%) of SR25990C (6). In humans, very low levels of the parent compound and its active metabolite are detectable in plasma sample even after multiple oral doses (7).

Most of the bioanalytical methods described in literature determine either SR25990C or its carboxylic acid metabolite in plasma. Lagorce et al. (8) have reported a gas chromatography–mass spectrometry (GC–MS) method involving a derivatization step to quantitate the inactive carboxylic acid metabolite of SR25990C in human plasma and serum with a lower limit of quantitation (LLOQ) of 5 ng/mL. Another more sensitive GC–MS method has been reported by Caplain et al. (9) for SR26334 in human plasma with an LLOQ of 1 ng/mL. Two methods, based on high-performance liquid chromatography (HPLC)-UV and LC–MS (10–13) detection, have been described for the determination of SR26334 in plasma employing liquid–liquid extraction. However, they suffer from several drawbacks such as low sensitivity, long chromatographic run times, or tedious extraction procedure.

Several LC–MS–MS (14–17) methods have been published to quantitate the unchanged SR25990C in human plasma. Very few methods are available for simultaneous quantitation of SR25990C and its major circulating metabolite (SR26334) in plasma. One such method which merits comparison with the present study is of Taubert et al. (18), in which they used LC–MS–MS for simultaneous estimation of SR25990C and its carboxylic acid metabolite in human plasma with an LLOQ of 0.5 ng/mL and 500 ng/mL, respectively. The method was mainly intended to assess the platelet aggregation in healthy human volunteers for 600 mg SR25990C. A summary of methods developed for SR25990C and/or its active metabolite in plasma is given in Table I.

Thus, the aim of present study was to develop and validate a simple, reproducible, and high throughput bioanalytical method

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Table I. Comparison of Analytical Methods Developed for the Determination of SR25990C and/or SR26334 in Human Plasma*

Sr. no.	Extraction procedure (human plasma volume)	Chromatography column [length (mm) × i.d. (mm), particle size (µ)]	Elution; mobile phase; injection volume	Analytical run time	Detection Technique	LLOQ	Reference no.
1	LLE with $CHCl_3$ (0.5 mL)	Nova-pack C8 (250 × 4.6, 4)	Isocratic; 30mM K_2HPO_4–THF–ACN, pH 3.0 (79:2:19, v/v/v); 25 µL	12 min	HPLC–UV for SR26334	0.2 µg/mL	11
2	LLE with CH_2Cl_3 (0.25 mL)	Luna C18 (75 × 4.6, 3)	Isocratic; ACN–H_2O–FA (60:40:0.1, v/v/v); 10 µL	12 min	LC–MS–MS	20 ng/mL for SR26334	12
3	SPE with Hypercarb cartridge (0.8 mL)	Hypercarb PGC (50 × 3.0, 5)	Isocratic; MeOH–0.1% TFA in H_2O (70:30, v/v); —	7.5 min	LC–MS–MS	100 ng/mL for SR26334	13
4	LLE with DEE–hexane (0.5 mL)	Waters Symmetry C8 (150 × 4.6, 5)	Isocratic; 5mM AF–MeOH (5:95, v/v); 10 µL	2.5 min	LC–MS–MS	5 pg/mL for SR25990C	15
5	LLE with DEE (0.3 mL)	Luna C8 (50 × 2.0, 5)	Gradient; ACN (0.1% FA)–H_2O (0.1% FA); 10 µL	3.0 min	LC–MS–MS	10 pg/mL for SR25990C	16
6	LLE with Pentane (0.5 mL)	Hypersil GOLD C18 (150 × 2.1, 5)	Isocratic; 10mM AA–ACN (15:85, v/v); 20 µL	3.0 min	LC–MS–MS	10 pg/mL for SR25990C	17
7	SPE with HLB cartridge (0.3 mL)	Zorbax SB C18 (75 × 4.6, 3.5)	Isocratic; 0.1% FA in 10mM AF–ACN (10:90, v/v); 2 µL	5.0 min	LC–MS–MS	0.25 ng/mL for SR25990C and 50 ng/mL for SR26334	Present Method

* PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid-phase extraction; ACN: acetonitrile; AF: ammonium formate; FA: formic acid; TFA: trifluoroacetic acid; DEE: diethyl ether; THF: tetrahydrofuran; AA: ammonium acetate.

based on mass spectrometry detection for simultaneous quantitation of SR25990C and its carboxylic acid metabolite in human plasma (0.3 mL). The method presents a simple and clean solid-phase extraction (SPE) procedure without drying and reconstitution. The positive ion electrospray ionization (ESI) mode selected for the present study gave high and consistent responses for both analytes. The analytes and IS were well separated with minimum matrix interference in a chromatographic run time of 5 min. The sensitivity achieved for SR25990C and SR26334 was sufficient to study the bioavailability of 75 mg clopidogrel mesylate tablets in sixteen healthy human volunteers.

Experimental

Chemicals and materials

Reference standards of clopidogrel bisulphate (99.65%), carboxylic acid metabolite of SR25990C (97.25%), and the internal standard glimepiride (99.29%) were obtained from Torrent Pharmaceuticals Ltd. (Gandhinagar, India). Ammonium formate of molecular biology grade was procured from Sigma-Aldrich (Steinheim, Germany), while formic acid (100%) and ortho phosphoric acid were of suprapure grade obtained from Merck (Darmstadt, Germany). HPLC-grade methanol and acetonitrile were purchased from Rankem (New Delhi, India). Purified water was prepared from Milli Q A10 gradient water purification system (Millipore, MA). For SPE, Oasis HLB cartridges (1 cc, 30 mg) were procured from Waters (Milford, MA). Control human plasma was acquired from Green Cross blood bank (Ahmedabad, India) and was stored at –20°C.

LC–MS–MS instrumentation and analytical conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a binary LC-10 ADvp pump, an autosampler (Model SIL- HTc), an on-line degasser (DGU-14A), and a temperature controller compartment for column (CTO-10Avp). LC was performed on a Zorbax SB C-18 analytical column (75 mm length, 4.6 mm internal diameter, and 3.5 µm particle size) from Agilent Technologies (Palo Alto, CA), and was maintained at 45°C in column oven. The mobile phase consisted of 0.1% formic acid in 10mM ammonium formate buffer–acetonitrile in ratio of 10:90

v/v. The flow of the mobile phase was maintained at 300 µL/min under isocratic conditions. The system backpressure was 75 bars. The samples were stored at 4°C in an autosampler prior to injection and the injection volume was set at 2 µL. The retention times for SR25990C, SR26334, and IS were 4.10, 2.60, and 3.00 min, respectively, with a total chromatographic run time of 5 min.

A triple-quadrupole mass spectrometer (model, Finnigan TSQ Quantum Discovery) from Thermo-Electron Corporation (San Jose, CA) was interfaced with a liquid chromatographic system. Ionization of analytes was carried out using ESI technique with positive polarity and selective reaction monitoring (SRM) mode. The spray capillary temperature was maintained at 350°C. The ion spray voltage was optimized and kept at 3500 V. Flow of the source gases (nitrogen) for continuous and uniform ions response was optimized at 40 psi for sheath gas and at 20 psi for auxiliary gas. SRM mode was used to quantitate selected precursor $(M)^+ \rightarrow$ product ions transitions at *m/z* 321.905 → 211.995 for SR25990C; *m/z* 307.911 → 198.109 for SR26334; and *m/z* 491.082 → 351.904 for IS. The selected product ion spectrum for SR25990C, SR26334, and IS is presented in Figures 1A, 1B, and 1C, respectively. Argon was used as a collision gas and maintained at 1.5 mTorr pressure. Compound-dependent parameters like scan width, first quadrupole resolution (Q1PW), and third quadrupole resolution (Q3PW) were set at 0.500 u, 0.7 u, and 0.7 u for the analytes and IS, respectively. The tube lens offset for selected precursor ions of SR25990C, SR26334, and IS were controlled at 102 V, 91 V, and 120 V, respectively. For consistent and stable response of product ions, the collision energy was optimized at 21 V, 21 V, and 18 V for SR25990C, SR26334, and IS, respectively. A divert valve was set in the waste mode up to 1.4 min; thereafter, the flow was diverted to the load mode for MS detection of analytes and IS. The Lcquan software version 2.0 was used to control the LC–MS–MS system, as well as for data acquisition, peak integration, and result processing.

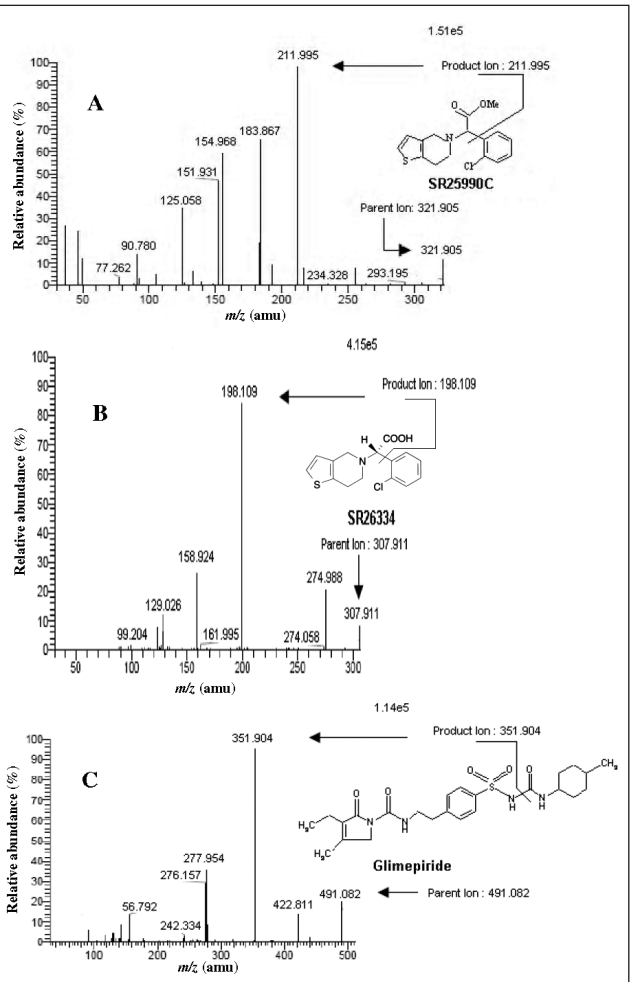

Figure 1. Q3 MS Product ion spectra of SR25990C (A), SR26334 (B), and IS (C).

Standard and quality control sample preparation

Separate standard stock solutions of clopidogrel bisulphate (250 µg/mL, equivalent to SR25990C), carboxylic acid metabolite (2.5 mg/mL), and glimepiride (1.0 mg/mL) were prepared by dissolving their requisite amounts in methanol. The intermediate solution of SR25990C (2.5 µg/mL), SR26334 (500 µg/mL), and glimepiride (300 µg/mL) were prepared by appropriate dilution of stock solution with methanol–water (50:50 v/v).

Combined working solutions for calibration were made at 5, 10, 40, 100, 140, 200, 300, 400, and 500 ng/mL concentration for SR25990C and 1, 2, 7, 15, 30, 60, 80, 100, and 120 µg/mL for SR26334 in methanol–water (50:50 v/v) from respective intermediate solutions. Similarly, combined working solutions for quality control (QC) samples were prepared at three levels, viz. 15 ng/mL (low quality control, LQC), 120 ng/mL (medium quality control, MQC), and 360 ng/mL (high quality control, HQC) for SR25990C and at 3, 50, and 90 µg/mL for SR26334, respectively. The working solution of IS, 3.0 µg/mL, was prepared from its intermediate stock solution in the same diluent. All the standard stock, intermediate, and working solutions were stored at 2–8°C until use.

Plasma standards of SR25990C and SR26334 were prepared by spiking 15 µL of the appropriate spiking stock solutions into 285 µL blank human plasma to yield calibration concentrations of 0.25, 0.5, 2.0, 5.0, 7.0, 10.0, 15.0, 20.0, and 25.0 ng/mL for SR25990C and 50, 100, 350, 750, 1500, 3000, 4000, 5000, and 6000 ng/mL for SR26334. Similarly, the plasma QC samples were prepared at 0.75, 6.0, and 18.0 ng/mL and 150, 2500, and 4500 ng/mL for SR25990C and SR26334, respectively. To prepare blank plasma and zero standard (IS only), 40 µL and 15 µL of methanol–water (50:50 v/v) were added to 285 µL blank human plasma, respectively. All the previously mentioned samples, along with the subject samples, were stored at –70°C until use.

Sample extraction protocol

Plasma samples stored at –70°C were removed and thawed at room temperature for 30–45 min. Aliquots of 300 µL plasma samples were dispensed into micro-tubes and 25 µL of IS working solution (3 µg/mL) was added. The resulting samples were vortexed for 10 s and 500 µL of 1% ortho phosphoric acid in water (v/v) was added and vortexed again to mix using a vortex mixer before pipetting. The SPE was performed on HLB cartridges (1 cc, 30 mg), which exhibit both hydrophilic and lipophilic retention characteristics to retain the compound at ionized or unionized form, respectively. The samples were applied to the cartridges which were previously conditioned with 1 mL methanol followed by 1 mL of water. The plasma was

drained out under vacuum and the cartridges were washed with 2 mL of water followed by 1 mL of 5% (v/v) methanol to clean up the samples. Further, vacuum was applied for 2 min to remove the aqueous part and the analytes and IS retained on the cartridges were eluted with 1.0 mL acetonitrile. The eluates were transferred into 1-mL glass vials and 2 µL was used for injection in the LC–MS–MS system.

Bioanalytical method validation

The method was validated for specificity/selectivity, linearity, precision and accuracy (intra- and inter-day), recovery, matrix effect, dilution integrity, and stability study following the US FDA guidelines (19).

Specificity/selectivity was checked in 10 different lots of blank plasma which consisted of four blank plasmas in EDTA, four heparinised blank plasma, one lipemic, and one lot of haemolysed blank plasma. They were processed by the same extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and the IS. The results of these blank plasma samples were compared with spiked plasma samples at LLOQ levels (0.25 ng/mL for SR25990C; 50.0 ng/mL for SR26334) from the same lots.

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentrations of SR25990C and SR26334 were calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with the reciprocal of the drug concentration as a weighting factor (1/concentration, i.e. $1/x$); $y = mx + c$. The curve was also used to calculate the nominal concentration of quality control samples.

The intra-day precision and accuracy were evaluated by analysis of five replicates for SR25990C and its metabolite at four concentration levels (viz., LLOQ, LQC, MQC, and HQC) in the same analytical run. Inter-day precision and accuracy were calculated after repeated analysis in three different analytical runs. Mean and standard deviation (SD) were obtained for calculated analyte concentration at each level. Accuracy and precision were evaluated in terms of relative error (%RE) and coefficient of variation (%CV), respectively, with respect to the nominal concentration.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher drug concentrations (more than ULOQ), which may be encountered during real subject sample analysis. This is to ensure that the dilution of sample with same matrix does not have any impact on the actual results. To carry out this experiment, double the ULOQ concentration was diluted 4 times in blank human plasma. Their back-calculated concentrations were obtained by applying the dilution factor of 4.

The percentage recovery of both the analytes was determined by comparing the mean area of five replicates each of extracted quality control samples (LQC, MQC, and HQC) with mean area of freshly prepared un-extracted QC samples (spiked with aqueous spiking stock solution in extracted plasma).

To study the effect of plasma matrix on analyte quantification with respect to consistency in signal suppression/enhancement, matrix effect was checked in six different lots of plasma at LQC and HQC levels. These lots of plasma were comprised of four lots of normal control heparinised plasma, one lot of lipemic control heparinised plasma and one lot of haemolysed control heparinised plasma. Three samples each of LQC and HQC were prepared from different lots of plasma (total 36 QC samples) and checked for the relative error in all the QC samples.

Stability experiments were performed to evaluate the analyte stability in stock solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability sample of analytes and IS with the area response of sample prepared from fresh stock solutions. Bench top stability (room temperature stability), refrigerated stability of extracted sample, freeze thaw stability, and long-term stability were performed at LQC, MQC, and HQC levels using six replicates at each level.

Bioavailability study design

The design of the study was comprised of "A randomized, open label, single dose bioavailability study of 75 mg clopidogrel mesylate tablets in sixteen normal healthy subjects under fasting condition". All the subjects were informed of the aim and risk involved in the study and written consent was obtained. The work was approved and subject to review by Institutional Ethics

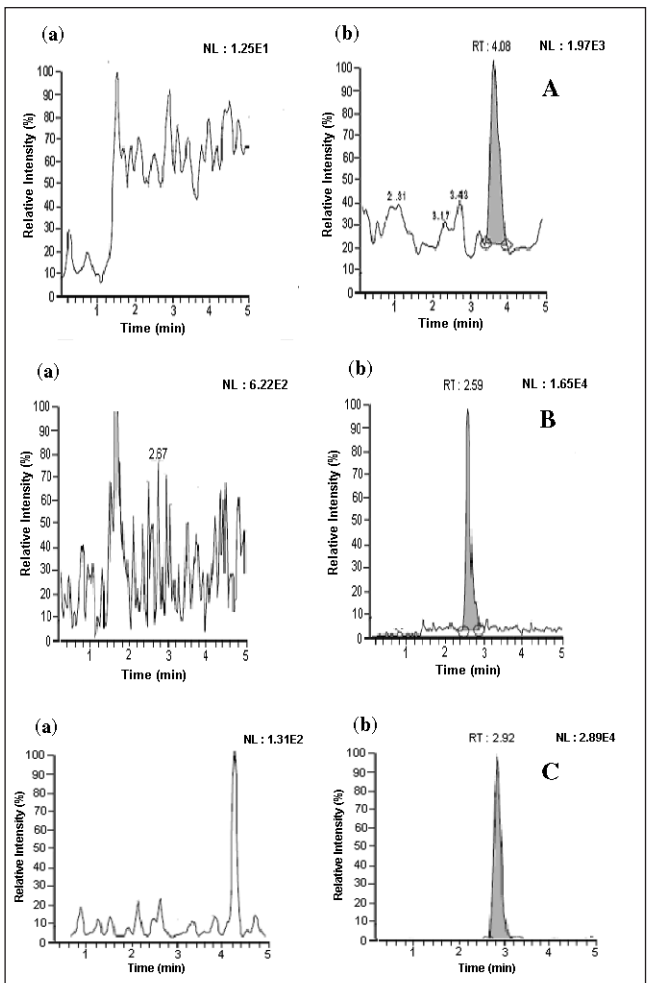

Figure 2. Representative ion chromatograms of blank plasma (a) and LLOQ (b) for SR25990C (A), SR26334 (B), and IS (C).

Committee, an independent body comprised of five members which includes a lawyer, medical doctor, social worker, pharmacologist, and an academician. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice guidelines (20). Health check-up for all subjects was done by general physical examination, ECG, and laboratory tests like hematology, biochemistry, and urine examination. All subjects were negative for HIV, HBSAg, and HCV tests. They were orally administered a single dose of test formulation after recommended wash-out period with 240 mL of water. Drinking water was not allowed and supine position was restricted 2 h post-dose. Standardized meals were provided as per schedule. Blood samples were collected in vacutainers containing heparin as an anticoagulant before (0.00 h) and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 3.00, 4.00, 6.00, 8.00, 12.0, 16.0, 20.0, 24.0, 30.0, and 36.0 h of administration of drug. Blood samples were centrifuged at 3200 rpm for 10 min and plasma was separated, stored at –20ºC until use.

Results and Discussion

Method development

The aim of this work was to develop and validate a rapid, rugged, and adequately sensitive assay for the quantitative extraction and simultaneous estimation of SR25990C and its carboxylic acid metabolite in therapeutic concentration range for the analysis of routine samples. To realize this, sample extraction procedure, chromatographic conditions, and MS detection parameters were optimized. To obtain better method specificity and reproducibility, the mass spectrometry was initiated using ESI as the ionization technique in the SRM mode. Also, to keep the method simple, it was decided to work in either positive or negative mode for both the analytes and IS. Due to the basic nature of SR25990C and IS, the response in the positive mode was much higher compared to the negative mode. Careful manipulation of mass spectrometry parameters helped in achieving a stable and consistent response in the positive mode for both the analytes and IS. To accomplish this, it was found that tube lens offset is an important parameter. Thus, it was optimized at 102, 91, and 120 V for SR25990C, its metabolite, and glimepiride, respectively. Further, to enhance the response of SR25990C and its metabolite, the ion spray voltage was raised from 2800 V (kept initially) to 3500 V. Auxiliary and sheath gas were optimized at 20 and 40 psig, respectively. SR25990C, its metabolite, and IS gave predominant precursor ions at *m/z* 321.905, 307.911, and 491.082, respectively, in full scan mode. By optimizing collision energy (21 V and 18 V) to precursor ions in Q2 MS, most abundant product ions obtained at *m/z* 211.995, 198.109, and 351.904 for the analytes and IS, respectively.

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions with an aim to develop a simple sepa-

Table II. Back-Calculated Concentration of Calibration Standards (CS) from Respective Calibration Curves for SR25990C (A) and SR26334 (B)

Linearity	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9	Regression parameters		
Conc. (ng/mL)	0.250	0.500	2.000	5.000	7.000	10.000	15.000	20.000	25.000	Intercept	Slope	*r*
Table IIA. SR25990C												
1	0.266	0.379*	1.776	5.133	7.091	10.259	14.521	20.495	24.709	0.01863	0.13740	0.9995
2	0.246	0.495	1.913	5.145	7.271	9.954	15.503	20.255	23.968	0.00746	0.10656	0.9994
3	0.244	0.526	1.914	5.249	6.918	9.753	14.953	20.506	24.957	0.00836	0.14844	0.9997
4	0.282	0.547	1.632*	4.969	6.687	9.669	14.921	21.107	24.935	0.01516	0.16654	0.9989
5	0.274	0.495	1.877	4.907	6.847	9.875	15.061	20.847	24.567	0.00406	0.17444	0.9995
n	5	4	4	5	5	5	5	5	5			
Mean	0.262	0.515	1.870	5.081	6.963	9.902	14.938	20.642	24.627			
% Mean nominal conc.	104.99	103.09	93.50	101.61	99.47	99.02	99.58	103.21	98.51			
SD	0.017	0.025	0.065	0.140	0.225	0.228	0.379	0.335	0.402			
%CV	6.37	4.91	3.47	2.75	3.23	2.30	2.54	1.62	1.63			
Table IIB. SR26334												
1	58.478	96.794	310.022	745.087	1407.291	3155.068	3961.784	5010.804	6004.672	–0.07948	0.00973	0.9994
2	59.275	101.692	338.455	675.903	1403.794	2801.862	3858.859	5293.935	6216.224	–0.08644	0.00640	0.9984
3	56.781	98.168	331.476	767.032	1385.649	2860.079	3821.596	5265.598	6163.621	–0.10012	0.00878	0.9988
4	58.259	103.167	323.736	692.486	1405.318	2875.700	4121.621	5022.255	6147.459	–0.16329	0.00997	0.9993
5	43.843	95.179	396.153	796.564	1455.321	3081.520	4075.002	4806.001	6000.418	–0.01039	0.00732	0.9994
n	5	5	5	5	5	5	5	5	5			
Mean	55.327	99.000	339.958	735.415	1411.475	2954.846	3967.772	5079.719	6106.479			
% Mean nominal conc.	110.65	99.00	97.13	98.06	94.10	98.49	99.19	101.59	101.77			
SD	6.483	3.346	33.134	50.540	26.000	153.935	130.798	202.121	98.237			
%CV	11.72	3.38	9.75	6.87	1.84	5.21	3.30	3.98	1.61			

* Not included in calculation due to unexpected concentration.

Table III. Intra- and Inter-Day Precision and Accuracy of the Method

QC samples (ng/mL)	Intra-assay					Inter-assay				
	n	Mean conc. (ng/mL)*	RE (%)	SD	CV (%)	*n*	Mean conc. (ng/mL)†	RE (%)	SD	CV (%)
Table IIIA. SR25990C										
LLOQ 0.250	5	0.238	–4.80	0.036	15.07	15	0.250	0.00	0.025	10.18
LQC 0.750	5	0.751	0.13	0.067	8.91	15	0.758	1.07	0.068	8.94
MQC 6.000	5	6.121	2.02	0.256	4.18	15	6.061	1.02	0.265	4.38
HQC 18.000	5	18.503	2.79	0.275	1.49	15	18.424	2.36	0.391	2.12
Table IIIB. SR26334										
LLOQ 50	5	54.391	8.78	5.213	9.59	15	54.992	9.98	3.357	6.10
LQC 150	5	162.545	8.36	6.209	3.82	15	159.307	6.20	6.199	3.89
MQC 2500	5	2657.874	6.31	81.205	3.06	15	2652.500	6.10	124.933	4.71
HQC 4500	5	4955.199	10.12	150.534	3.04	15	4862.630	8.06	136.852	2.8

* Mean of five replicate observations at each concentration.
† Mean of fifteen observations recorded over 3 different analytical runs (5 replicates/run).

Table IV. Recovery of SR25990C (A), SR26334 (B), and IS (*n* = 5)

	LQC (0.75 ng/mL)	MQC (6.0 ng/mL)	HQC (18.0 ng/mL)	IS
Table IVA. SR25990C				
% Mean recovery within QC level	97.52	97.67	98.95	105.72
Recovery variability within QC level (%CV)	13.20	2.55	2.30	—
% Mean recovery between QC levels	98.05			—
Recovery variability between QC levels (%CV)	0.80			2.14
Table IVB. SR26334				
% Mean recovery within QC level	89.05	83.82	83.49	105.72
Recovery variability within QC level (%CV)	2.65	1.73	1.78	—
% Mean recovery between QC levels	85.45			—
Recovery variability between QC levels (%CV)	3.65			2.14

ration procedure with a short run time. Separation was tried using different mobile phases consisting of acetonitrile–methanol with acidic buffers to achieve better peak shape and reproducibility. Use of methanol in mobile phase resulted in better selectivity, but the response was not adequate for SR25990C and IS. Thus, acetonitrile was tested in varying proportions with acidic buffers. It was observed that a high content of acetonitrile in the mobile phase gave sufficient response with adequate selectivity. It was found that 0.1% formic acid in 10mM ammonium formate along with acetonitrile in ratio of 10:90 v/v was found most suitable as mobile phase. C18 stationary phases (Betasil C18 and Zorbax C18) with particle size less than 5 µm were most appropriate to achieve better selectivity, retention of compound, and separation of plasma matrix. Based on this criterion, Zorbax C18, 75 mm × 4.6 mm column, with 3.5 µm particle size was selected with a flow rate of 0.3 mL/min to get optimum results. The column oven temperature of 45°C was sufficient to obtain a symmetric shape for analyte peaks. The retention times of analytes and IS on the column followed the order SR25990C > IS > SR26334; this can be due to less polar nature of SR25990C compared to its metabolite for retention on C18 reverse phase column. The total LC run time per sample was only 5.0 min. To eliminate unwanted matrix components entering the MS system, a divert valve was set in the waste mode up to 1.4 min. The small injection volume of 2 µL reduced overloading of column with analytes thereby ensuring more number of injections on the same column. The maximum on-column loading (at ULOQ) of SR25990C and SR26334 per injection was 15 pg and 3600 pg, respectively.

For simultaneous extraction of the analytes and IS from plasma, liquid–liquid extraction (LLE) using different solvents (viz., diethyl ether, ethyl acetate, dichloromethane, tetra butyl methyl ether) and their combinations in different ratios were examined. However, all efforts resulted in inconsistent recoveries, longer extraction time, and higher background. Thus, to get cleaner extracts with minimum matrix and quantitative recoveries, SPE was carried out using Oasis HLB cartridges (1 cc, 30 mg). A plasma volume of 0.3 mL was sufficient to obtain quantitative recovery for the analytes. The pre-treatment of sample with 1% ortho phosphoric acid helped in breaking the drug protein binding. The eluate (1 mL) was directly submitted to LC–MS–MS analysis without prior drying and reconstitution. The SPE method gave quantitative recoveries and higher selectivity for the analytes compared to LLE.

Ideally, an internal standard should mirror the analytes in as many ways as possible. It should track the analyte during extraction and compensate for any analyte on the column and any inconsistent response due to matrix effects. Glimepiride,

though belonging to a different class, was found most appropriate as IS in the present study. There was no significant effect of IS on analyte recovery, ion suppression/enhancement, or sensitivity. The results of method validation support the use of glimepiride and were acceptable in this study based on FDA guidelines.

Specificity/selectivity and sensitivity

The chromatograms (Figures 2A, 2B, and 2C) of blank plasma and the peak response of SR25990C (0.25 ng/mL), SR26334 (50 ng/mL) at LLOQ, and IS demonstrate the selectivity and sensitivity of the method. The ion chromatograms showed good peak shape for the analytes and IS. No endogenous interferences were found at the retention times of SR25990C (4.08 min), SR26334 (2.59 min), and IS (2.92 min). The total chromatographic run time of 5.0 min makes it suitable for routine sample analysis. The area observed at the retention time of SR25990C and SR26334 was less than 20% of their LLOQ area, whereas it was less than 5% IS area in the blank plasma samples.

Linearity, precision, and accuracy, dilution integrity

The SR25990C calibration curves were linear from 0.25 to 25.0 ng/mL with correlation coefficient $r \geq 0.9989$, while for carboxylic acid metabolite the linear dynamic range was from 50 to 6000.0 ng/L with correlation coefficient of $r \geq 0.9984$ between five calibration curves. The observed mean back calculated concentration at each level was within 93.50 to 104.99% and 94.10 to 110.65% of their nominal concentration for SR25990C and SR26334, respectively. The accuracy and precision values observed for the back-calculated concentrations, along with the intercept, slope, and correlation coefficients (r) of five linearities from CS-1 to CS-9 are presented in Tables IIA and IIB.

Table V. Matrix Effect of SR25990C (A) and SR26334 (B)

Blank plasma lots	Lot-1*	Lot-2*	Lot-3*	Lot-4*	Lot-5†	Lot-6‡
Table VA. SR25990C						
LQC (0.75 ng/mL)						
Mean calc. conc. ng/mL	0.761	0.733	0.774	0.745	0.712	0.747
% Relative error	1.52	–2.25	3.24	–0.69	–5.05	–0.40
HQC (18.0 ng/mL)						
Mean calc. conc. ng/mL	16.797	17.075	16.797	16.536	17.281	16.936
% Relative error	–6.68	–5.15	–6.68	–8.13	–3.99	–5.91
Table VB. SR26334						
LQC (150 ng/mL)						
Mean calc. conc. ng/mL	156.435	154.841	153.679	166.304	166.620	164.255
% Relative error	4.29	3.23	2.45	10.87	11.08	9.50
HQC (4500 ng/mL)						
Mean calc. conc. ng/mL	4340.791	4535.048	4393.891	4593.565	4524.013	4686.528
% Relative error	–3.54	0.78	–2.36	2.08	0.53	4.15

* Normal control heparinised plasma.
† Lipemic control heparinised plasma.
‡ Haemolysed control heparinised plasma.

Table VI. Stability Experiments of SR25990C (A) and SR26334 (B) in Plasma

Stability experiments	Storage condition	QC level	Mean comparison sample conc. found (ng/mL)	%CV	Mean stability sample conc. found (ng/mL)	%CV	% Mean change at QC level
Table VIA. SR25990C							
Bench top in plasma	Room temperature (6 h)	LQC (0.75)	0.735	10.92	0.800	11.29	8.84
		HQC (18.00)	18.100	1.60	17.933	3.74	–0.92
Process (extracted sample)	Autosampler (4°C, for 24 h)	LQC (0.75)	0.788	2.37	0.810	2.43	2.79
		HQC (18.00)	18.670	2.15	19.156	1.66	2.60
Freeze and thaw stability	After 3rd FT cycle at –70°C	LQC (0.75)	0.788	5.37	0.813	5.10	3.17
		HQC (18.00)	18.670	2.15	19.390	1.82	3.86
Long term stability in human plasma	For 84 days at –70°C	LQC (0.75)	0.735	7.15	0.687	4.88	–6.53
		MQC (6.00)	5.796	3.10	5.592	2.40	–3.52
		HQC (18.00)	17.554	1.32	17.092	2.57	–2.63
Table VIB. SR26334							
Bench top in plasma	Room temperature (6 h)	LQC (150.00)	160.660	2.46	163.910	3.29	2.02
		HQC (4500.00)	4808.866	1.55	5006.565	1.07	4.11
Process (extracted sample)	Autosampler (4°C, for 24 h)	LQC (150.00)	153.567	3.55	160.346	3.06	4.41
		HQC (4500.00)	4823.824	3.02	4907.134	2.83	1.73
Freeze and thaw stability	After 3rd FT cycle at –70°C	LQC (150.00)	153.567	3.55	164.384	4.24	7.04
		HQC (4500.00)	4823.820	3.02	4967.890	3.20	2.99
Long term stability in human plasma	For 84 days at –70°C	LQC (150.00)	143.522	4.14	145.631	3.55	1.47
		MQC (2500.00)	2395.236	1.82	2410.887	1.05	0.65
		HQC (4500.00)	4460.071	1.54	4422.692	2.27	–0.84

Intra-day and inter-day precision was less than 9.0% at the three QC levels for both the analytes. The precision values calculated at LLOQ level were 15.07% and 9.59% (intra-day); 10.18% and 6.10% (inter-day) for SR25990C and SR26334, respectively. Intra- and inter-day accuracy values expressed in terms of %RE were within –5.0 to 11.0% for both the analytes as shown in Tables IIIA and IIIB.

The mean back-calculated concentrations for 1/4 dilution samples was within 85–115% of their nominal concentration, while the coefficient of variation (%CV) for this dilution was less than 3.7% for both the analytes.

Recovery and matrix effect in bioanalysis

The mean recovery found for SR25990C and its metabolite at three QC levels was 98.05% and 85.45% with precision values of 0.80% and 3.65%, respectively. The recovery for IS was 105.72% with %CV of 2.14. The data obtained at each level is given in Tables IVA and IVB. The quantitative and consistent recoveries obtained for analytes and IS supports the extraction procedure for its application to routine sample analysis.

During analysis with biological fluids, signal suppression or enhancement may occur in the mass spectrometer due to co-elution of some unknown matrix present in the fluid. Thus, evaluation of matrix effect constitutes an important and essential part of method validation, especially when quantifying analytes with LC–MS–MS for subject sample analysis. The observed %RE was within –5.05 and 10.87, and –8.13 and 4.15 at LQC and HQC levels, respectively, for SR25990C and SR26334 as presented in Tables VA and VB. This minor suppression and enhancement in the signal intensity does not affect the quantification of analytes and IS.

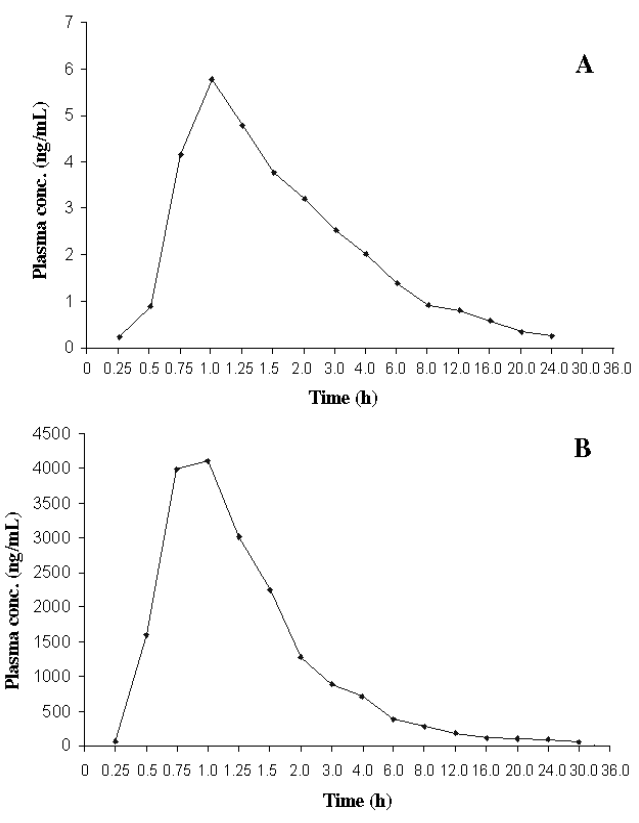

Figure 3. Mean pharmacokinetic profile of SR25990C (A) and SR26334 (B) after oral administration of 75 mg clopidogrel tablet to 16 healthy subjects.

Stability study

Stability experiments were performed to evaluate their stability in stock solutions and in plasma samples. The conditions which occurred during actual study sample analysis were simulated in method validation stability studies, such as: stock solution stability of SR25990C, its metabolite, and IS; stability in plasma at room temperature; extracted sample stability (process stability at 4°C); freeze thaw stability and long term stability at –70°C. Stock solution of SR25990C, SR26334, and IS were stable at room temperature for 6 h and at 2–8°C for 7 days with mean percent change within ± 5%. SR25990C and SR26334 in control human plasma were stable for at least 6 h at room temperature; up to 24 h (process stability) in the autosampler maintained at 4°C and for minimum three freeze and thaw cycles. The long term stability was also established for 84 days at –70°C. The observed and acceptable percent change for the stability experiments are compiled in Tables VIA and VIB.

Application of the method on human subjects

The proposed validated method was successfully applied to a bioavailability study of 75 mg clopidogrel mesylate tablet for the assay of SR25990C and its carboxylic acid metabolite in 16 healthy subject samples under fasting condition. The samples were processed based on the proposed extraction protocol. The method was sensitive enough to monitor plasma concentration of SR25990C and its metabolite up to 24 and 30 h, respectively. In all approximately 600 samples including the calibration, QC and volunteer samples were run and analyzed in 4 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for the treatment under fasting condition is presented in Figures 3A and 3B. The important parameters of the study (viz., C_{max}, AUC_{0-t}, AUC_{0-inf}, T_{max}, K_{el}, and $t_{1/2}$) were calculated for SR25990C and its metabolite and their mean values are summarized in Table VII. Thus, the assay procedure for SR25990C and its metabolite in human plasma samples demonstrates the precision and sensitivity needed for the pharmacokinetic studies of this drug.

Table VII. The Main Pharmacokinetic Parameters of SR25990C and SR26334 After Oral Administration of 75 mg Clopidogrel Tablet to Healthy Indian Volunteers Under Fasted Condition*

Pharmacokinetic parameters	SR25990C	SR26334
T_{max} (h)	1.19 (± 0.921)	1.10 (± 0.86)
C_{max} (ng/mL)	5.850 (± 4.171)	4101.869 (± 197.121)
AUC_{0-36} (h. ng/mL)	27.909 (± 12.171)	10730.074 (± 4819.511)
AUC_{0-inf} (h. ng/mL)	27.965 (± 12.226)	10739.080 (± 5167.871)
$t_{1/2}$ (h)	5.820 (± 4.201)	5.42 (± 1.51)
K_{el} (L/h)	0.119 (± 0.045)	0.128 (± 0.040)

* Mean (± SD).

Conclusion

The objective of this work was to develop a simple, high throughput, and sensitive method to simultaneously estimate SR25990C and its active metabolite in human plasma, especially in the absorption and elimination phase after oral administration of 75 mg SR25990C tablets. The advantage of using SPE is that it gives cleaner and consistent extracts with minimum matrix effect. Unlike most LLE methods (10,11,15–17) which require the extraction solvent to be evaporated and reconstituted, the present method involves a single extraction step, followed by direct injection of SPE eluate for LC–MS–MS analysis to give a short turnaround time per sample. Moreover, the sensitivity of this method is better compared to the work reported by Taubert et al. (18) for simultaneous estimation of SR25990C and SR26334. Also, the chromatographic run time of 5.0 min makes it possible to analyze 200 samples in a day. From the results of the validation parameters, we can conclude that the method can be very useful for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples with desired precision, accuracy, and high throughput.

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References

1. T. Richter, T.E. Murdter, G. Heinkele, J. Pleiss, S. Tatzel, M. Schwab, M. Eichelbaum, and U.M. Zanger. Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J. Pharm. Exp. Ther.* **308:** 189–97 (2004).
2. P. Savi and J.M. Herbert. Clopidogrel and ticlopidine: P2Y12 adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis. *Semin. Thromb. Hemost.* **31(2):** 174–83 (2005).
3. J.M. Pereillo, M. Maftouh, A. Andrieu, M.F. Uzabiaga, O. Fedeli, P. Savi, M. Pascal, J.M. Herbert, J.P. Maffrand, and C. Picard. Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Met. Disp.* **30(11):** 1288–95 (2002).
4. CAPRIE Steering Committee. A randomized, blinded, trial of clopidogrel versus aspirin in patients at risk of ischemic events (CAPRIE). *Lancet* **348:** 1329–39 (1996).
5. P. Savi, J.M. Herbert, A.M. Pflieger, F. Dol, D. Delebassee, J. Combalbert, G. Defreyn, and J.P. Maffrand. Importance of hepatic metabolism in the antiaggregating activity of the thienopyridine clopidogrel. *Biochem. Pharmacol.* **44:** 527–32 (1992).
6. M. Reist, M. Roy-De Vos, J.P. Montseny, J.M. Mayer, P.A. Carrupt, Y. Berger, and B. Testa. Very slow chiral inversion of clopidogrel in rats: A pharmacokinetic and mechanistic investigation. *Drug Metab. Dispos.* **28(12):** 1405–10 (2000).
7. M.C. Guillin, G. Bonnet, J. Sissmann, J. Necciari, and J.P. Dickinson. Pharmacodynamics and pharmacokinetics of the novel antiplatelet agent, clopidogrel, in the young and the elderly with and without symptomatic atherosclerosis. *Eur. Heart J.* **17(Suppl):** 161 (1996).
8. P. Lagorce, Y. Perez, J. Ortiz, J. Necciari, and F. Bressolle. Assay method for the carboxylic acid metabolite of clopidogrel in human plasma by gas chromatography–mass spectrometry. J. *Chromatogr. B* **720(1–2):** 107–17 (1998).
9. H. Caplain, M.D. Francois, C. Gaud, and J. Necciari. Pharmacokinetics of clopidogrel. *Semin. Thromb. Hemost.* **25(2):** 25–8 (1999).
10. S.S. Singh, K. Sharma, D. Barot, P.R. Mohan, and V.B. Lohray. Estimation of carboxylic acid metabolite of clopidogrel in Wistar rat plasma by HPLC and its application to a pharmacokinetic study. *J. Chromatogr. B* **821(2):** 173–80 (2005).
11. E. Souri, H. Jalalizadeh, A. Kebriaee-Zadeh, M. Shekarchi, and A. Dalvandi. Validated HPLC method for determination of carboxylic acid metabolite of clopidogrel in human plasma and its application to a pharmacokinetic study. *Biomed. Chromatogr.* **20(12):** 1309–14 (2006).
12. H. Ksycinska, P. Rudzki, and M. Bukowska-Kiliszek. Determination of clopidogrel metabolite (SR26334) in human plasma by LC–MS. *J. Pharm. Biomed. Anal.* **41(2):** 533–9 (2006).
13. A. Mitakos and I. Panderi. Determination of the carboxylic acid metabolite of clopidogrel in human plasma by liquid chromatography–electrospray ionization mass spectrometry. *Anal. Chim. Acta* **505(1):** 107–14 (2004).
14. A. Lainesse, Y. Ozalp, H. Wong, and R.S. Alpan. Bioequivalence study of clopidogrel bisulfate film-coated tablets *Arzneimittelforschung* **54(9a):** 600–4 (2004).
15. R.V. Nigori, V.N. Kandikere, M. Shukla, K. Mudigonda, S. Maurya, and R. Boosi. Quantification of clopidogrel in human plasma by sensitive liquid chromatography–tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **20(11):** 1695–700 (2006).
16. A. Robinson, J. Hillis, C. Neal, and A.C. Leary. The validation of a bioanalytical method for the determination of clopidogrel in human plasma. *J. Chromatogr. B* **848(2):** 344–54 (2007).
17. B.S. Shin and S.D. Yoo. Determination of clopidogrel in human plasma by liquid chromatography–tandem mass spectrometry: application to a clinical pharmacokinetic study. *Biomed. Chromatogr.*, in press.
18. D. Taubert, A. Kastrati, S. Harlfinger, O. Gorchakova, A. Lazar, N.V. Beckerath, A. Schomig, and E. Schomig. Pharmacokinetics of clopidogrel after administration of a high loading dose. *Thromb. Haemost.* **92(2):** 311–316 (2004).
19. Guidance for Industry: Bioanalytical method validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.
20. Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), April 1996.

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