



ORIGINAL ARTICLE

# Development of a sensitive and rapid method for quantitation of (*S*)-(–)- and (*R*)-(+)-metoprolol in human plasma by chiral LC–ESI–MS/MS

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**Abstract** A selective, sensitive and high throughput liquid chromatography-tandem mass spectrometry (LC–ESI–MS/MS) method has been developed for separation and quantification of metoprolol enantiomers on a chiral Lux Amylose-2 (250 mm × 4.6 mm, 5 μm) column. Solid phase extraction of (*S*)-(–)- and (*R*)-(+)-metoprolol and *rac*-metoprolol-d6 as an internal standard (IS) was achieved on Lichrosep DVB HL cartridges employing 200 μL human plasma. Both the analytes were chromatographically separated with a resolution factor of 2.24 using 15 mM ammonium acetate in water, pH 5.0 and 0.1% (v/v) diethyl amine in acetonitrile (50:50, v/v) as the mobile phase within 7.0 min. The precursor → product ion transitions for the enantiomers and IS were monitored in the multiple reaction monitoring and positive ionization mode. The method was validated over the concentration range of 0.500–500 ng/mL for both the enantiomers. Matrix effect was assessed by post-column analyte infusion experiment and the mean extraction recovery was greater than 94.0% for both the enantiomers at all quality control levels. The stability of analytes was evaluated in plasma and whole blood under different storage conditions. The method was successfully applied to a clinical study in 14 healthy volunteers after oral administration of 200 mg metoprolol tablet under fasting conditions. The assay reproducibility is shown by reanalysis of 68 incurred samples. The suitability of the developed method was assessed in comparison with

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different chromatographic methods developed for stereoselective analysis of metoprolol in biological matrices.

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## 1. Introduction

Enantiomeric study of drugs and/or its metabolites is of growing interest in the field of pharmaceutical and biomedical analysis.  $\beta$ -blockers or  $\beta$ -adrenergic blocking agents are one of the most explored pharmaceuticals for their stereochemical impact on pharmacodynamics and pharmacokinetics. Majority of  $\beta$ -blockers are marketed as racemic mixtures and hence enantiomeric analysis in biological fluids is essential to understand their stereoselective implications, therapeutic use and also in toxicological studies [1,2].

Metoprolol, a selective  $\beta$ -adrenoceptor antagonist, is used in clinical practice in the racemic form for the treatment of hypertension, angina pectoris and for several other cardiovascular diseases [3–5]. The (*S*)-(-)-metoprolol has significantly higher  $\beta$ -adrenergic receptor affinity (about 500-fold) compared to its (*R*)-(+)-antipode. In humans, the absorption of metoprolol is rapid and complete. Plasma levels after oral administration are almost 50% of levels attained following intravenous administration, indicating ~50% first-pass metabolism. A small fraction of the drug (~12%) is bound to human serum albumin and has a plasma half-life of 3–7 h. It is primarily metabolized by CYP2D6 enzymes and exhibits stereoselective metabolism that is essentially dependent on oxidation phenotype. The three principal metabolic pathways of metoprolol include (a) *O*-dealkylation to give *O*-desmethyl metoprolol, which undergoes rapid oxidation to form an acid metabolite, (b)  $\alpha$ -hydroxylation to form  $\alpha$ -hydroxy metoprolol and (c) oxidative deamination to give *N*-dealkyl metoprolol. About 85% of the administered drug is excreted in the urine as metabolites, along with small amounts of unchanged parent drug (less than 5%). The stereoselectivity in metoprolol pharmacokinetics is observed with higher plasma concentration of (*S*)-(-)-metoprolol (*S*/*R*-metoprolol ratio >1) and higher renal excretion of (*R*)-(+)-metoprolol in healthy volunteers and hypertensive patients after oral dose of *rac*-metoprolol [6,7].

Numerous methods have been developed for enantioselective determination of metoprolol in biological samples using different analytical techniques, such as capillary electrophoresis [8], GC–MS [9], HPLC with UV [10] and fluorescence detection [7,11–21], and LC–MS/MS [22,23]. These methods can be characterized based on two different approaches (i) direct methods, which involve use of chiral stationary phase [7,10,11–14,17–23] and (ii) indirect methods, employing derivatization with chiral reagents [9,15,16,19]. The choice of a particular approach in bioanalysis is dictated by several factors which include (a) the required assay sensitivity, (b) ready availability, purity and stability of chiral derivatizing agent, (c) efficiency and ease of derivatization, (d) suitable chiral stationary phase, (e) simple and easy optimization of chromatographic conditions and (f) overall analysis time (extraction and chromatography). This is specifically intended to facilitate application of the method in routine analysis of real samples. Lanchote et al. [19] have compared direct

enantioselective separation of metoprolol enantiomers on chiral stationary phase (Chiralpak AD and Chiralcel OD-H columns) and indirect separation based on the formation of diastereomeric derivatives with *S*-(-)-menthyl chloroformate by HPLC. They concluded that the direct method with Chiralpak AD was more sensitive compared to the indirect approach, although both the methods demonstrated interchangeable use in the pharmacokinetic investigation. Mistry et al. [20] carried out a stereoselective HPLC-fluorescence assay for the enantiomers of metoprolol and diastereoisomers of its hydroxyl metabolite on Chirobiotic T bonded phase column. The analytes were extracted from 1.0 mL plasma sample by solid phase extraction (SPE) and the calibration curve was established from 0.5 to 50 ng/mL for metoprolol enantiomers. So far very few LC–MS/MS based methods are available in literature for the analysis of metoprolol enantiomers in biological matrices. Jensen et al. [22] developed and validated a stereoselective LC–MS/MS assay using Chirobiotic T column for quantification of *S*- and *R*-metoprolol in human plasma. The linear dynamic range was established from 0.5 to 50 ng/mL and the run time for the method was 11.0 min. A human dried blood spot (DBS) sampling with LC–MS/MS for enantioselective determination of metoprolol and its metabolite has also been described [23]. This is a highly rapid method (3.0 min); however, the sensitivity of the method was 2.5 ng/mL. A detailed comparative summary of different chromatographic methods developed for metoprolol enantiomers in biological samples is presented in Table 1.

The objective of the present study was to separate both the isomers chromatographically and to develop a simple and reliable LC–ESI–MS/MS method based on direct approach for their quantitation in human plasma. The proposed method exhibited superior performance in terms of sensitivity, dynamic concentration range, selectivity, ruggedness and efficiency (7.0 min per sample) due to cleaner extracts with a simple and straight forward sample extraction protocol. It ensured the estimation of both the isomers up to 24 h with desired accuracy and precision to support a bioequivalence study in healthy Indian volunteers. Additionally, this is the first report on successful demonstration of assay reproducibility through incurred sample reanalysis for metoprolol enantiomers.

## 2. Experimental

### 2.1. Chemicals and materials

Reference standards of *S*-(-)-metoprolol (99.0%) and *R*-(+)-metoprolol (99.0%) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada), while *rac*-metoprolol-d6 (99.6%) used as an internal standard (IS) was from TLC Pharmachem Inc. (Ontario, Canada). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker S.A. de C.V. (Ecatepec, Mexico). Analytical reagent grade glacial acetic acid, formic acid, sodium hydroxide, ammonium

**Table 1** Comparative summary of chromatographic methods developed for *S*(-)-metoprolol and *R*(+)-metoprolol in biological matrices.

Methods	Technique; column	Matrix; sample volume ( $\mu\text{L}$ )	Sample preparation	Internal standard	Linearity (ng/mL)	Run time (min)	Application; incurred sample reanalysis	Ref.
Direct methods—using chiral columns	HPLC-fluorescence (excitation-229 nm, emission-298 nm); Chiralpak AD (250 mm $\times$ 4.5 mm, 10.0 $\mu\text{m}$ )	Human plasma/urine; 1000/100	LC-18 SPE for plasma and LLE for urine with dichloromethane-diisopropyl ether in the presence of 1.0 M NaOH	NR	10–100 in plasma, 250–25000 in urine	N.R.	Steady-state pharmacokinetics of metoprolol in 10 hypertensive patients; NR	[7]
	HPLC-UV <sup>a</sup> (225 nm); CelluCoat (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 5000	SPE on Sep-Pak C18 Cartridges	NR	NR	30.0	Study of plasma-protein binding along with 3 other $\beta$ -blockers; NR	[10]
	HPLC-fluorescence (excitation-228/272 nm, emission-306 nm); Chiral-AGP (100 mm $\times$ 4.0 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	LLE with dichloromethane-diethyl ether in the presence of 1.0 M NaOH	2-dehydroxy metoprolol	~0.5–200	20.0	Pharmacokinetic study with 200 mg metoprolol in healthy subjects; NR	[11]
	HPLC-fluorescence (excitation-275 nm, emission-315 nm); Chiralcel OD (250 mm $\times$ 4.6 mm, 10.0 $\mu\text{m}$ )	Human serum; 1000	LLE with diethyl ether in the presence of 2.0 M NaOH	<i>Rac</i> -verapamil	12.5–400	15.0	Pharmacokinetic study with 200 mg metoprolol in healthy subjects; NR	[12]
	HPLC-fluorescence <sup>b</sup> (excitation-272 nm, emission-306 nm); Chiralcel OD (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human/dog plasma or urine; 1000	LLE with diethyl ether in the presence of 1.0 M NaOH	( <i>S</i> )-alprenolol	2.5–250	35.0	Pharmacokinetic study with 10 mg/kg metoprolol in dogs; NR	[13]
	HPLC-fluorescence (excitation-220 nm, emission-320 nm); Chiralcel OD (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	SPE on octadecylsilane columns	<i>d</i> -propranolol	20–400	40.0	Pharmacokinetic study with 200 mg metoprolol in a healthy subject; NR	[14]
	HPLC-fluorescence <sup>c</sup> (excitation-276 nm, emission-309 nm); Chiralcel OD (250 mm $\times$ 4.6 mm, 10.0 $\mu\text{m}$ )	Human urine; 400	LLE with dichloromethane in the presence of 1.0 M Na <sub>2</sub> CO <sub>3</sub>	NR	NR	12.0	Pharmacokinetic study with 100 mg metoprolol in healthy subjects; NR	[17]
	HPLC-fluorescence (excitation-276 nm, emission-309 nm); (I) Phenomenex silica (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ ) and (II) Chiralcel OD (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human urine; 1000	LLE with dichloromethane in the presence of 1.0 M NaOH	Pindolol	100–2000	50.0	Pharmacokinetic study with 100 mg metoprolol in a healthy subject; NR	[18]
	HPLC-fluorescence (excitation-229 nm, emission-298 nm); (I) Chiralpak AD (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ ) and (II) Chiralcel OD-H (150 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	For column I: SPE on Supelclean LC-18; For column II: LLE with dichloromethane-diisopropyl ether in the presence of 1.0 M NaOH	NR	10–500 for the column I and II	10.0 for column I and 20.0 for column II	Kinetic disposition study with 200 mg metoprolol in a healthy subject; NR	[19]
	HPLC-fluorescence <sup>d</sup> (excitation-225 nm, emission-310 nm); Chirobiotic T bonded phase (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	SPE on silica bonded with ethyl group	<i>Rac</i> -atenolol	0.5–100	15.0	Bioavailability study with 100 mg metoprolol in healthy subjects; NR	[20]
	HPLC-fluorescence <sup>c</sup> (excitation-229 nm, emission-298 nm); Chiralpak AD (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Rat plasma; 1000	LLE with dichloromethane-diisopropyl ether in the presence of 1.0 M NaOH	NR	10–250	30.0	Pharmacokinetic study with 15 mg/kg of metoprolol in male Wistar rats; NR	[21]
	LC-MS/MS; Chiral ASTEC Chirobiotic T (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 900	LLE with ethyl acetate in the presence of 1.0 M NaOH	<i>Rac</i> -propranolol	0.5–50	11.0	Pharmacokinetic study with 190 mg and 47.5 mg metoprolol in patients; NR	[22]
	LC-MS/MS <sup>e</sup> ; Chiral-cellobiohydrolase (100 mm $\times$ 3.0 mm, 5.0 $\mu\text{m}$ )		Spotting on Whatman FTA DMPK-A cards followed by LLE		2.5–2500	3.0		[23]

Table 1 (continued)

Methods	Technique; column	Matrix; sample volume ( $\mu\text{L}$ )	Sample preparation	Internal standard	Linearity (ng/mL)	Run time (min)	Application; incurred sample reanalysis	Ref.
	LC-MS/MS; Lux Amylose-2 (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Dried blood spots (DBS); 20 Human plasma; 200	with ethyl acetate in the presence of 2% NaOH SPE on LiChrosep DVB HL cartridges	Rac-metoprolol-d7 Rac-metoprolol-d6	0.5–500	7.0	Comparison of developed DBS assay with a plasma assay; NR Bioequivalence study with 200 mg metoprolol in 14 healthy subjects; % change varied from -13.0 % to 13.0 %	PW
Indirect methods— via derivatization on achiral columns	GC-MS; Capillary column HP-5 MS (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu\text{m}$ film thickness)	Human urine; 1000	LLE with hexane-butanol in the presence of 0.05 M HCl followed by derivatization with (–)-MTPA-Cl, using MSTFA	Bisoprolol	100–4000	~25.0	Pharmacokinetic study with 100 mg metoprolol in a healthy subject; NR	[9]
	HPLC-fluorescence (excitation-220 nm, no emission filter); Stainless steel (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	LLE with chloroform in the presence of 0.1 M NaOH followed by derivatization with NEIC	<i>rac</i> -propranolol	5.0–500	30.0	Pharmacokinetic study with 100 mg metoprolol in a healthy subject; NR	[15]
	HPLC-fluorescence <sup>f</sup> (excitation-223 nm, emission-340 nm); Hypersil 5 C18 (250 mm $\times$ 4.6 mm, 5.0 $\mu\text{m}$ )	Human urine; 2000	LLE with ethyl acetate in the presence of 2.0 M $\text{K}_2\text{CO}_3$ followed by derivatization with <i>S</i> -(–)-MCF	<i>Rac</i> -toliprolol	375–11250	30.0	Excretion rate and stereoselective metabolism in young and old subjects; NR	[16]
	HPLC-fluorescence (excitation-223 nm, emission-340 nm); Lichrospher RP-8 (125 mm $\times$ 4.0 mm, 5.0 $\mu\text{m}$ )	Human plasma; 1000	LLE with dichloromethane-diisopropyl ether in presence of 1.0 M NaOH followed by derivatization with <i>S</i> -(–)-MCF	NR	10–500	15.0	Kinetic disposition study with 200 mg metoprolol in a healthy subject; NR	[19]

LLE: liquid-liquid extraction; SPE: solid-phase extraction; NR: not reported; PW: present work; (–)-MTPA-Cl: (–)- $\alpha$ -methoxy- $\alpha$ -(trifluoromethyl)-phenylacetyl chloride; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; NEIC: (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate; *S*-(–)-MCF: *S*-(–)-menthyl chloro formate

<sup>a</sup>Along with carazolol, oxprenolol and alprenolol;

<sup>b</sup>Along with four enantiomeric forms of  $\alpha$ -hydroxy metoprolol;

<sup>c</sup>Along with the enantiomers of its metabolite *O*-desmethyl metoprolol and  $\alpha$ -hydroxy metoprolol;

<sup>d</sup>Along with the diastereoisomers of  $\alpha$ -hydroxy metoprolol;

<sup>e</sup>Along with enantiomers of oxazepam, bupivacaine and terbutaline;

<sup>f</sup>Along with its major acidic metabolite;

formate, ammonium acetate, ammonium trifluoroacetate, ammonia solution (30%), diethyl amine and LiChrosep DVB HL cartridges (30 mg, 1cc) were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at  $-20^{\circ}\text{C}$  until use.

### 2.2. Liquid chromatography and mass spectrometric conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-20AD prominence pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-20 A<sub>3</sub> degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of analytes and IS was performed on a Phenomenex Lux Amylose-2 (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) column and was maintained at  $35^{\circ}\text{C}$  in a column oven. The mobile phase consisted of 15 mM ammonium acetate, pH 5.0 adjusted with acetic acid and 0.1% (v/v) diethyl amine in acetonitrile (50:50, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 1.0 mL/min with 80% flow splitting; flow directed to the ion spray interface was equivalent to 200  $\mu\text{L}/\text{min}$ . The autosampler temperature was maintained at  $5^{\circ}\text{C}$  and the pressure of the system was 1500 psi.

Ionization and detection of analytes and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with electrospray ionization and operating in positive ionization mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor  $\rightarrow$  product ion transitions of  $m/z$  268.3  $\rightarrow$  116.3 for *S*-(–)-metoprolol and *R*-(+)-metoprolol and  $m/z$  274.2  $\rightarrow$  122.2 for IS respectively. The source dependent parameters maintained for the analytes and IS were Gas 1 (Nebulizer gas): 30 psig; ion spray voltage (ISV): 5500 V, turbo heater temperature:  $400^{\circ}\text{C}$ ; entrance potential: 10 V; collisional activation dissociation (CAD): 5 psig and curtain gas, nitrogen: 10 psig. The optimum values for compound dependent parameters like declustering potential, collision energy and cell exit potential were set at 70, 26 and 10 eV respectively for both the analytes and IS. Analyst classic software version 1.4.2 was used to control all parameters of LC and MS.

### 2.3. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of 1.0 mg/mL for *S*-(–)-metoprolol and *R*-(+)-metoprolol respectively was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total volume of blank plasma) blank plasma with the stock solution. Calibration curve standards were made at 0.500, 1.00, 2.00, 4.00, 10.0, 20.0, 50.0, 100, 250 and 500 ng/mL concentrations respectively while QC samples were prepared at five levels, 400 ng/mL (HQC, high quality control), 200/30.0 ng/mL (MQC-1/2, medium quality control) and 1.50 ng/mL (LQC, low quality control) for both the analytes respectively. Stock solution (1.0 mg/mL) of the IS was prepared by dissolving requisite amount of *rac*-metoprolol-d<sub>6</sub> in methanol. An aliquot of 25  $\mu\text{L}$  of this solution was further diluted to 25.0 mL in

methanol to obtain a working solution of 1.0  $\mu\text{g}/\text{mL}$ . All the solutions (standard stock, calibration standards and quality control samples) were stored at  $2-8^{\circ}\text{C}$  until use.

### 2.4. Sample extraction protocols

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature for 30 min. To an aliquot of 200  $\mu\text{L}$  of spiked plasma sample, 50  $\mu\text{L}$  internal standard solution and 50  $\mu\text{L}$  of 0.1 M sodium hydroxide in water was added and vortex mixed for 20 s. The samples were then centrifuged at  $3200 \times g$  for 2 min at  $10^{\circ}\text{C}$  and loaded on LiChrosep<sup>®</sup> DVB-HL (1cc, 30 mg) extraction cartridges, which were preconditioned with 1.0 mL of methanol followed by 1.0 mL of water. Washing of the samples was done with 1.0 mL water, followed by 1 mL of methanol: 2% (v/v) ammonia solution in water (30:70, v/v). The analytes and ISs were eluted using 500  $\mu\text{L}$ , of 2% (v/v) acetic acid in methanol and collected in pre-labeled vials. The eluates were evaporated to dryness under gentle stream of nitrogen (20 psi) at  $40^{\circ}\text{C}$ . The dried samples were reconstituted with 100  $\mu\text{L}$  of the mobile phase, vortex-mixed for 30 s and 10  $\mu\text{L}$  was used for injection in the chromatographic system.

### 2.5. Method validation procedures

The method validation was performed as per the USFDA guidelines [24]. System suitability experiment was performed by injecting six consecutive injections of aqueous standard mixture of *S*-(–)-metoprolol and *R*-(+)-metoprolol (at MQC-1) and IS (1.0  $\mu\text{g}/\text{mL}$ ) at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without analyte and IS) and one ULOQ & LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. Carry over effect of autosampler was checked to verify any carryover of analyte at the start and at the end of each batch. The sequence of injection for this experiment was, extracted blank plasma  $\rightarrow$  ULOQ sample  $\rightarrow$  extracted blank plasma  $\rightarrow$  LLOQ sample  $\rightarrow$  extracted blank plasma.

The selectivity of the method towards endogenous plasma matrix components was assessed in ten different batches of plasma, of which, seven were normal K<sub>3</sub>EDTA plasma and one each of lipidemic, haemolysed and heparinized plasma. The selectivity of the method towards commonly used medications in human volunteers was done for acetaminophen, cetirizine, domperidone, ranitidine, diclofenac and ibuprofen in six different batches of plasma having K<sub>3</sub>EDTA as anticoagulant. Their stock solutions (1.00 mg/mL) were prepared by dissolving requisite amount in methanol. Further, their working solutions (500 ng/mL) were prepared in methanol:water (50:50, v/v) spiked in plasma and analyzed under the same SPE conditions at LQC and HQC levels in triplicate.

The linearity of the method was determined by analysis of five linearity curves containing nine non-zero concentrations. The area ratio responses (analyte/IS) were obtained from MRM for regression analysis. Each calibration curve was analyzed individually by least square weighted ( $1/x^2$ ) linear regression. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the



analyte response was at least ten times more than that of drug-free (blank) extracted plasma.

For determining the intra-batch accuracy and precision, plasma samples of *S*(-)-metoprolol and *R*(+)-metoprolol were analyzed in six replicates of LLOQ QC, LQC, MQC-2/1 and HQC samples along with a calibration curve in a single day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15%.

Qualitative ion suppression/enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by post-column analyte infusion experiment. A standard solution containing the analytes (at MQC level) and IS was infused post-column via a 'T' connector into the mobile phase at 10  $\mu$ L/min employing the infusion pump. Aliquots of 10  $\mu$ L of extracted control plasma were then injected into the column by the autosampler and MRM LC-MS/MS chromatograms were acquired for the analytes and IS.

The relative recovery, absolute matrix effect and process efficiency were evaluated at HQC, MQC1/2 and LQC levels in six replicates. Relative recovery for the analytes and IS was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. Absolute matrix effect was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with the mean area of neat standard solutions. The overall 'process efficiency' was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of neat standard solutions. The assessment of relative matrix effect was based on the calculation of precision (% CV) values for slopes of calibration lines from eight plasma lots (including heparinized, haemolysed and lipidemic). For a method to be practically free from relative matrix effect the % CV should not exceed 3–4% [25].

All stability results were examined by measuring the response (area ratio) of stability samples against freshly prepared comparison standards with identical concentration. Stock solution of the analytes and IS was checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from nominal value was within  $\pm 10.0\%$ . Autosampler stability (wet extract), bench top (at room temperature), dry extract (at  $-20$  °C), and freeze-thaw stability (at  $-20$  °C and  $-70$  °C) were performed at LQC and HQC using six replicates at each level. Similarly, the long term stability of spiked plasma samples stored at  $-20$  °C and  $-70$  °C was also studied at both these levels. Whole blood stability was also determined to ascertain any enzymatic degradation by spiking blood samples with analytes at both the QC levels at room temperature up to 2.0 h and in wet ice batch maintained below 10 °C.

To establish the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed on two different columns (same make but different batch no.), while the second batch was analyzed by two different analysts who were not part of method validation. Dilution reliability was evaluated by preparing a spiked standard at 900 ng/mL concentration of *S*(-)-metoprolol and *R*(+)-metoprolol respectively in the screened plasma. The precision and accuracy at 1/2 (450 ng/mL) and 1/10th

(90 ng/mL) dilution were determined by analyzing the samples against freshly prepared calibration curve standards.

## 2.6. Bioequivalence study design and incurred sample reanalysis

The proposed method was applied to quantify plasma enantiomer concentration for a bioequivalence study conducted in 14 healthy Indian subjects after oral administration of test (200 mg metoprolol succinate extended release tablet, equivalent to 155.63 mg metoprolol from an Indian Pharmaceutical Company, India) and reference (Selo-zok<sup>®</sup>, 200 metoprolol succinate extended release tablet, equivalent to 155.63 mg metoprolol from AstraZeneca, Denmark) formulations under fasting conditions. The study was an open label, balanced, randomized single-dose, two-treatment, two-sequence, two-period crossover study. The primary target variables of the study were  $C_{max}$ ,  $AUC_{0-24}$ , and  $AUC_{0-inf}$ , which were analyzed using the confidence interval approach. The secondary end points of the study included  $AUC_{0-24}/AUC_{0-inf}$ ,  $T_{max}$ ,  $K_{el}$  and  $t_{1/2}$ . Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [26]. The subjects were fasted 10 h before administration of the drug formulation. They were orally administered a single dose of test and reference formulations after recommended wash out period of 7 days with 200 mL of water. The blood samples were collected in labeled K<sub>3</sub>EDTA-vacutainers at pre-dose time 0.00, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 12.0, 14.0, 16.0 and 24 h. Plasma was separated by centrifugation and kept frozen at  $-20$  °C till the completion of study and then at  $-70$  °C until analysis. During the study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of *S*(-)-metoprolol and *R*(+)-metoprolol were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). To determine whether the test and reference formulations were pharmacokinetically equivalent,  $C_{max}$ ,  $AUC_{0-24}$ , and  $AUC_{0-inf}$  and their ratios (test/reference) using log transformed data were assessed; their means and 90% CIs were analyzed by using SAS<sup>®</sup> software version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

The assay reproducibility test was also conducted by selection of 10% of total subject samples analyzed, which included samples near the  $C_{max}$  and the elimination phase in the pharmacokinetic profile of the enantiomers. The percent change in the values, which were calculated using the following formula, should not be more than  $\pm 20\%$  [27,28].

$$\% \text{ Change} = \frac{\text{Repeat value} - \text{Initial value}}{\text{Mean of repeat and initial values}} \times 100$$

## 3. Results and discussion

### 3.1. Method development

As evident from the available literature, bulk of the methods reported on enantiomeric analysis of metoprolol is based on

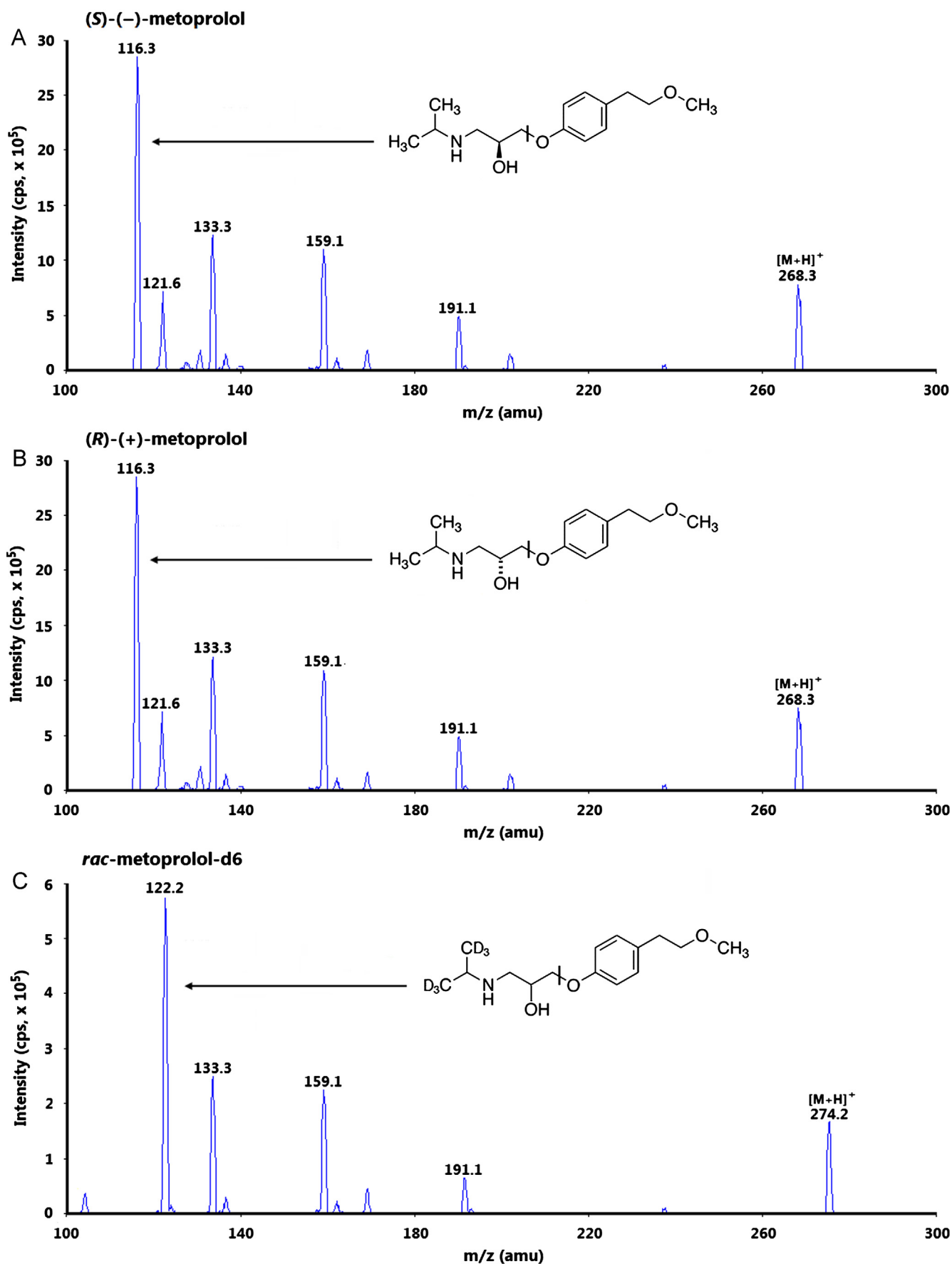


Fig. 1 Product ion mass spectra of: (A) *S*-(-)-metoprolol ( $m/z$  268.3 $\rightarrow$ 116.3, scan range 100–300 amu), (B) *R*-(+)-metoprolol ( $m/z$  268.3 $\rightarrow$ 116.3, scan range 100–300 amu), and (C) *Rac*-metoprolol-d<sub>6</sub>, internal standard ( $m/z$  274.2 $\rightarrow$ 122.2, scan range 100–300 amu) in positive ionization mode.

**Table 2** Mean relative recovery and absolute matrix effect of *S*-(-)-metoprolol and *R*-(+)-metoprolol by LLE and SPE at LQC level.

Extraction conditions	Relative recovery (%)		Absolute matrix effect (%)	
	<i>S</i> -isomer	<i>R</i> -isomer	<i>S</i> -isomer	<i>R</i> -isomer
Methyl <i>tert</i> -butyl ether:ethyl acetate (70:30, v/v) in the presence of 1.0 M NaOH	56.5	52.1	63.5	62.2
Methyl <i>tert</i> -butyl ether in the presence of 1.0 M NaOH	78.2	80.6	83.3	87.1
Ethyl acetate:dichloromethane (50:50, v/v) in the presence of 1.0 M NaOH	83.5	85.1	89.1	90.3
Dichloromethane in the presence of 1.0 M NaOH	79.5	82.3	85.3	86.1
Dichloromethane:diethyl ether (50:50, v/v) in the presence of 1.0 M NaOH	84.5	85.1	88.2	89.5
SPE using sample pretreated with 0.1 M NaOH	94.5	95.9	102.8	102.4

direct analysis on chiral columns [7,10–14,17–23]. This is apparently due to comparable or better assay sensitivity [19] without an additional derivatization step during sample processing than indirect methods. Moreover, easy availability of wide variety of stable and rugged chiral stationary phases has further propelled their use in enantioselective analysis. All five major classes of stationary phases including macrocyclic glycopeptide [20,22], polysaccharide [10,12–14,17–19,21], protein [11,17,23], cyclodextrin [17] and Pirkle-type [17] have been used in the separation and quantitation of metoprolol enantiomers from biological samples with varying degree of success. Moreover, these stationary phases can be used under normal and reversed-phase conditions and also with modified polar organic mobile phases, except the protein based stationary phase. Nevertheless, chiral columns coupled to mass spectrometric (MS) detection warrant careful optimization of mobile phase not only for enantiomeric resolution but also for compatibility with the MS interface and adequate MS response for assay sensitivity. In our effort to develop a sensitive, selective, high throughput and rugged method, this essential criterion was suitably optimized during method development along with the extraction procedure.

### 3.1.1. Mass spectrometry

The present study was conducted using ESI as the ionization source in the positive ionization mode as it gave high intensity for *S*-(-)-metoprolol, *R*-(+)-metoprolol and IS due to the presence of secondary amine which is easily protonated and a good linearity in regression curves. Initially, the precursor and product ions were optimized by infusing 500 ng/mL solutions in the mass spectrometer between 100 and 300 mass range. Q1 MS full scan spectra for the analytes and IS essentially contained protonated precursor  $[M+H]^+$  ions at  $m/z$  268.3 and 274.2 respectively. The most abundant and consistent product ions in Q3 MS spectra for the analytes and IS were observed at  $m/z$  116.3 and 122.2 respectively by applying 26 eV collision energy (Fig. 1). The product ion fragment at  $m/z$  116.3 for the analytes can be attributed to the substructure with an isopropyl amine group. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response for the analytes. A dwell time of 100 ms gave sufficient sampling points to obtain reproducible results for the analytes and IS and no cross talk was found between their MRMs.

### 3.1.2. Optimization of extraction procedure

All reported methodologies based on direct approach have used either SPE [10,14,19,20] or liquid–liquid extraction (LLE) in the presence of a base [7,11–13,17–19,21,22] for quantitative recovery of analytes. As metoprolol is a basic drug (pKa 9.7), it can be readily converted to an unionized state with a base for better extraction efficiency during LLE. Several solvent systems such as dichloromethane [17,18], dichloromethane-diisopropyl ether [7,19,21], diethyl ether [12,13], dichloromethane-diethyl ether [11] and ethyl acetate [22] have been used previously for LLE. Thus, both these conventional techniques were tested for optimum recovery with minimum matrix interference during method development trials. LLE was tried in some of these solvents as well methyl *tert*-butyl ether, while SPE was done on LiChrosep DVB HL cartridges. The results obtained for different extraction trials are summarized in Table 2. As evident, the results with SPE were superior compared to LLE with minimum matrix interference and thus was accepted in the present study. Further, use of 2% (v/v) acetic acid in methanol during SPE elution ensured good assay reproducibility and analyte recovery.

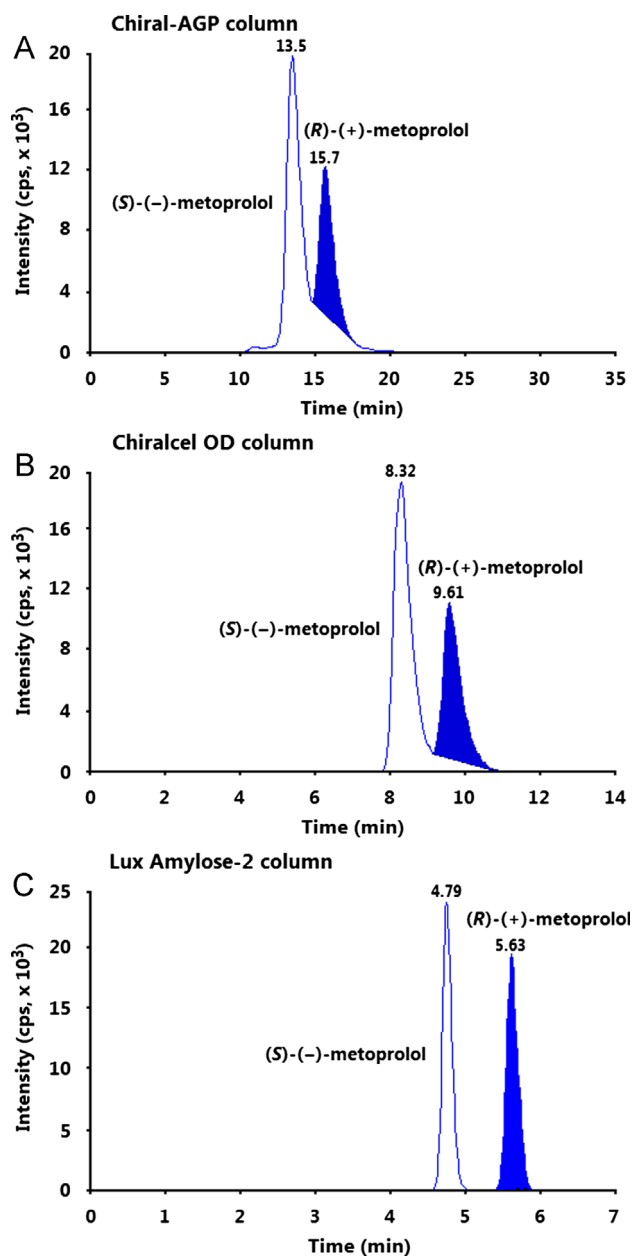
### 3.1.3. Optimization of chromatographic conditions

Chromatographic resolution of *S*-(-)-metoprolol and *R*-(+)-metoprolol was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short analysis time on two polysaccharide based stationary phases namely Lux Amylose-2 (250 mm × 4.6 mm, 5 μm) and Chiralcel<sup>®</sup> OD (250 mm × 4.6 mm, 5 μm) and one protein based Chiral-AGP (250 mm × 4.6 mm, 5 μm) columns. Different mobile phase compositions (methanol/acetonitrile and aqueous), buffer solutions (ammonium acetate/acetic acid, ammonium formate/formic acid) and modifier (diethyl amine) for basic analytes were investigated to evaluate the impact on retention and resolution of analyte enantiomers on the columns. Protein based columns showed enantioselectivity for broad spectrum of analytes; however, can be used only under reversed-phase conditions with limited proportion of organic solvent (generally less than 50%) in the mobile phase. Moreover, ionic strength, pH, type and concentration of organic modifiers play a significant role in the retention and resolution on protein-based phases [1]. Chiral-AGP column has been used previously by Persson et al. [11] with a mobile phase consisting of phosphate buffer (pH 7.0) and acetonitrile under gradient HPLC conditions. Similarly, Kim et al. [17] used a 20 mM



phosphate buffer as the mobile phase to obtain a resolution ( $R_s$ ) of 1.79 for metoprolol enantiomers under isocratic conditions. However, due to incompatibility of phosphate buffer with MS detection, a combination of ammonium acetate-acetic acid buffer (pH 5.0)-methanol/acetonitrile (70:30, v/v) was tried initially under isocratic conditions. Due to low organic solvent content the retention time for the analytes was too long ( $\sim 16.0$  min), with poor resolution ( $\sim R_s$  0.2) between the enantiomers. Addition of 0.1% diethyl amine in the mobile phase afforded a marginal increase in the resolution ( $\sim R_s$  0.5) as shown in Fig. 2A. Thus, polysaccharide based stationary phases, Chiralcel OD [cellulose tris(3,5-dimethylphenylcarbamate)] and Lux Amylose-2 [amylose tris(5-chloro-2-methylphenylcarbamate)] were tested which can withstand polar organic mobile phase and thus assist in shortening the retention times for analytes. Polysaccharide based stationary phases can be used with normal mobile phases consisting of alkane-alcohol mixture for HPLC applications. This combination along with modifiers such as diethyl amine or octylamine has been well established for enantioselective analysis of metoprolol on Chiralcel OD column [12–14,17,18]. Nonetheless, use of an alkane in the mobile phase is not worthwhile when coupling the column to APCI or ESI source for MS detection due to its inflammable nature. Thus, different volatile salts like ammonium acetate, ammonium formate and ammonium trifluoroacetate along with 0.1% diethyl amine in acetonitrile/methanol were tested on Chiralcel OD and Lux Amylose-2 columns. These salts facilitate ionization and provide enhanced MS detection for assay sensitivity. Both the columns offered adequate retention and resolution of metoprolol enantiomers compared to Chiral-AGP column using 15 mM ammonium acetate, pH 5.0 adjusted with acetic acid-0.1% diethyl amine in acetonitrile (50:50, v/v) as the mobile phase (Fig. 2B and C). Nevertheless, based on higher resolution, peak shape, shorter chromatographic run time and superior response, Lux Amylose-2 was selected for further study. Baseline separation ( $R_s$ , 2.24) of the analytes was possible within 7.0 min, with a retention time of 4.79 and 5.63 min for *S*-(-)-metoprolol and *R*-(+)-metoprolol respectively. The efficiency of the Lux Amylose-2 column expressed as the theoretical plate number was 2997 and 3170 for *S*-(-)-metoprolol and *R*-(+)-metoprolol respectively with a separation factor of ( $\alpha$ )=1.4. This helps in maintaining column efficiency and extending its life time for more number of injections. Further, the reproducibility of retention time for both the analytes, expressed as % CV was  $\leq 0.6\%$  for more than 100 injections on the same column. The deuterated internal standard (*rac*-metoprolol-*d*6) used in the study had similar chromatographic behavior and was quantitatively extracted with the proposed extraction procedure. Also, there was no effect of IS on analyte recovery, sensitivity or ion suppression.

The representative MRM ion chromatograms in Fig. 3 of extracted blank human plasma (double blank), blank plasma fortified with IS, analytes at LLOQ and an actual subject sample at 5.0 h demonstrate the selectivity of the method. Based on the selectivity achieved in plasma samples and a high signal to noise ratio ( $S/N \geq 75$ ), it was possible to lower the LLOQ down to 0.070 ng/mL; however, it was not required based on the concentration of enantiomers found in subject samples. None of the commonly used medications by human volunteers (acetaminophen, cetirizine,



**Fig. 2** Chromatograms of metoprolol enantiomers ( $m/z$  268.3  $\rightarrow$  116.3) obtained on: (A) protein based Chiral-AGP (250 mm  $\times$  4.6 mm, 5  $\mu$ m), (S), (B) Chiralcel<sup>®</sup> OD (250 mm  $\times$  4.6 mm, 5  $\mu$ m) and (C) Lux Amylose-2 (250 mm  $\times$  4.6 mm, 5  $\mu$ m). Mobile phase for column (A) 15 mM ammonium acetate, pH 5.0 adjusted with acetic acid-0.1% diethyl amine in acetonitrile (70:30, v/v), for (B) and (C) 15 mM ammonium acetate, pH 5.0 adjusted with acetic acid-0.1% diethyl amine in acetonitrile (50:50, v/v).

domperidone, ranitidine, diclofenac and ibuprofen) interfered in the determination of analytes. Moreover, due to difference in their MRM transitions there was no interference in the quantification. The accuracy results were within 95.9–102.7% for *S*-(-)-metoprolol and *R*-(+)-metoprolol at both the QC levels. The average matrix factor value calculated as the response of post spiked sample/response of neat solution in reconstitution solution at LLOQ level was between 1.02 and 1.04 for both the analytes, which indicates negligible

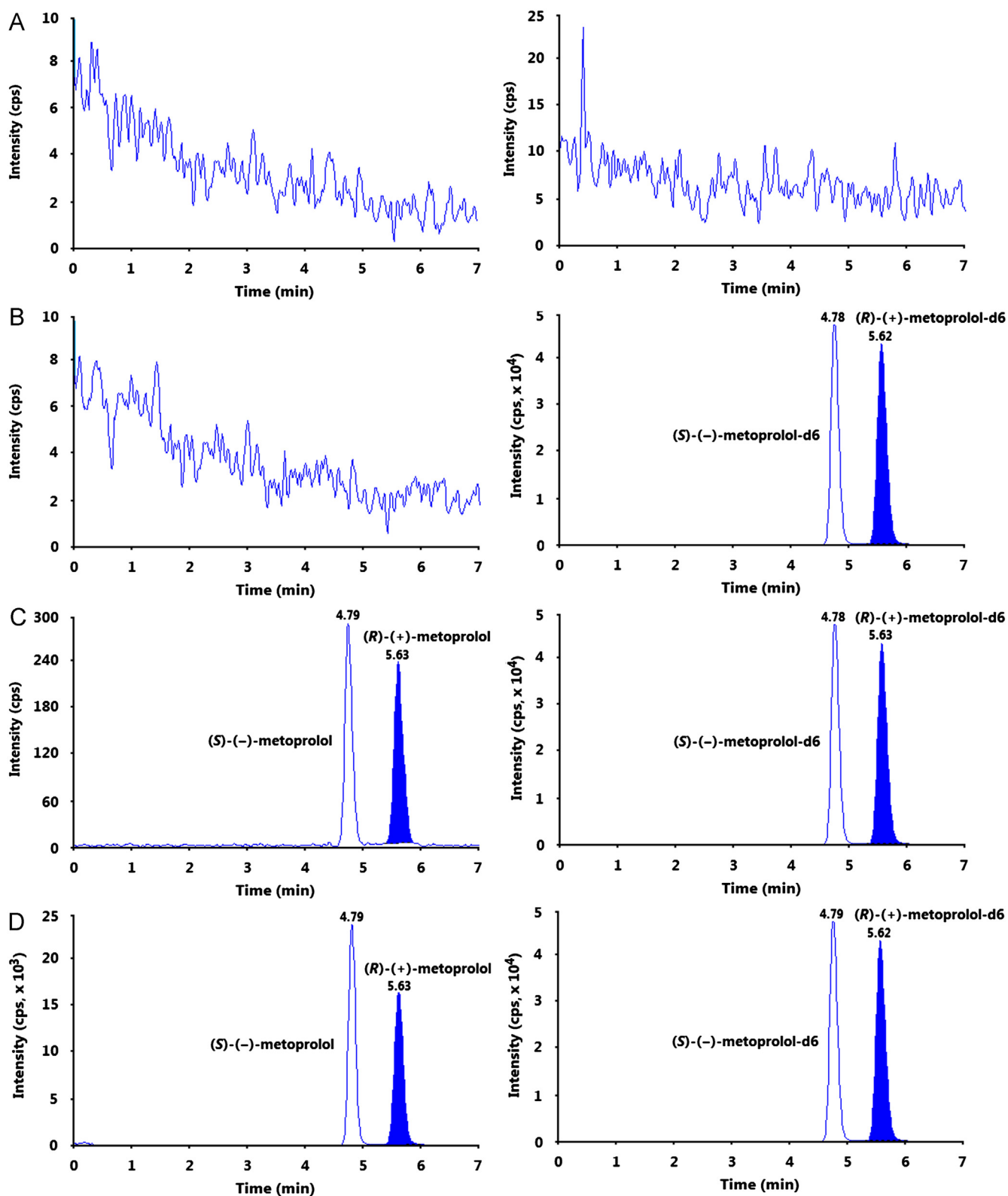
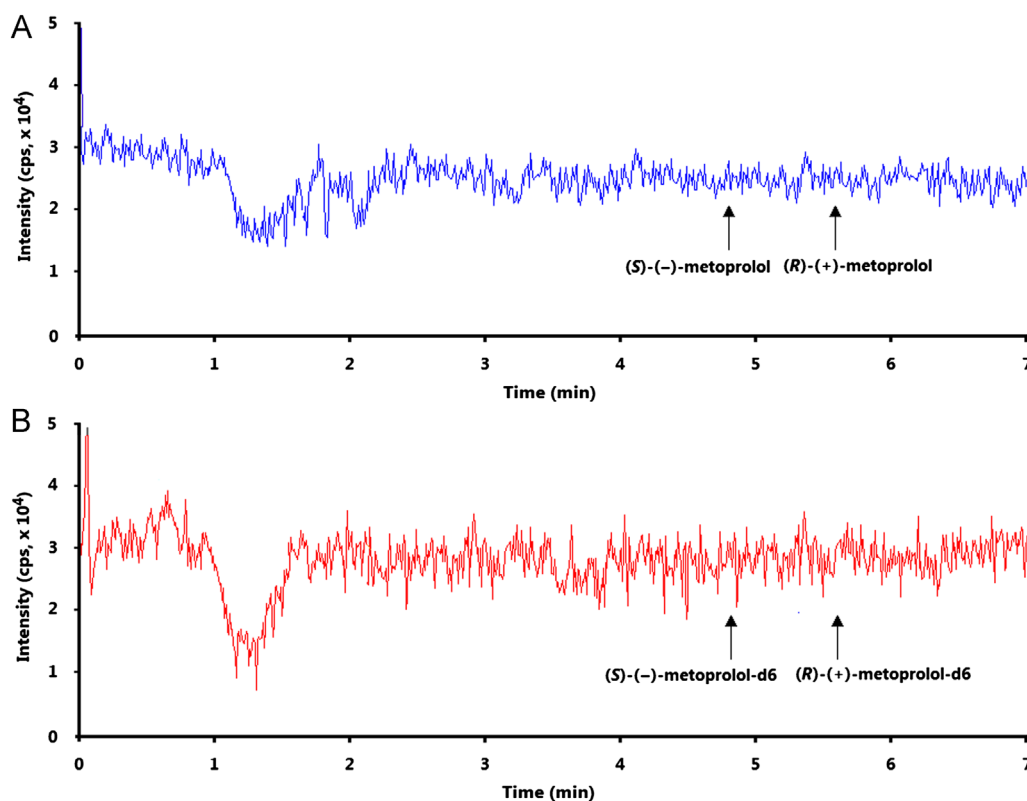


Fig. 3 MRM ion-chromatograms of: (A) double blank plasma (without IS), (B) blank plasma with *rac*-metoprolol-d6 (IS), (C) *S*-(-)-metoprolol and *R*-(+)-metoprolol at LLOQ level ( $m/z$  268.3  $\rightarrow$  116.3) and IS ( $m/z$  274.2  $\rightarrow$  122.2), and (D) real subject sample at 5.0 h after administration of 200 mg extended release metoprolol tablet.

enhancement. The chromatograms for post column infusion experiment show no ion suppression or enhancement at the retention time of *S*-(-)-metoprolol and *R*-(+)-metoprolol

and IS (Fig. 4). Though significant ion suppression was observed around 1.0–1.5 min, nevertheless it did not interfere in the quantitation of analytes.



**Fig. 4** Post-column analyte infusion MRM LC–MS/MS chromatograms for: (A) *S*(–)-metoprolol and *R*(+)-metoprolol ( $m/z$  268.3  $\rightarrow$  116.3), and (B) *Rac*-metoprolol- $d_6$  ( $m/z$  274.2  $\rightarrow$  122.2).

### 3.2. Assay performance and validation

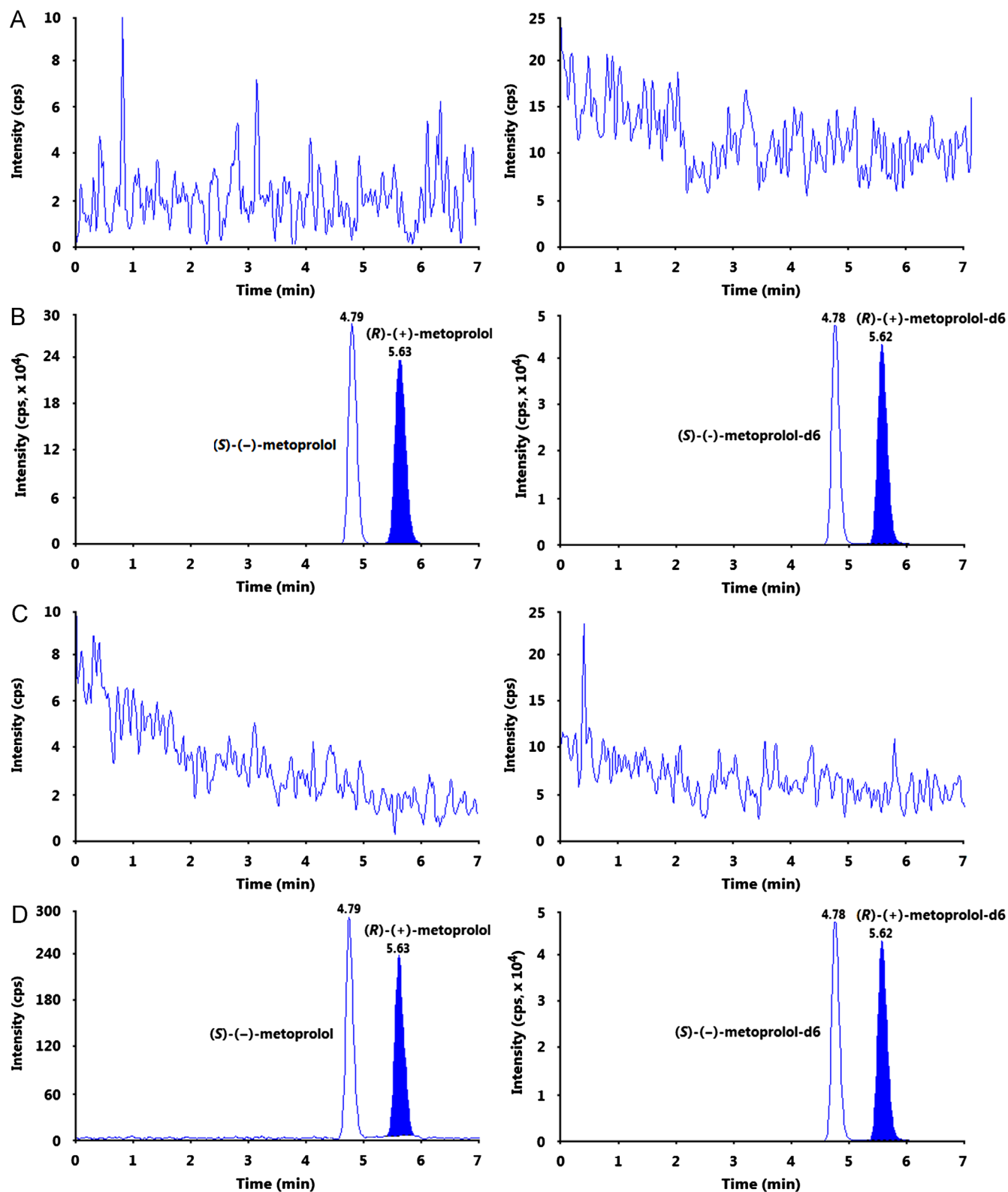
The precision (% CV) in the measurement of retention time and the response (area ratio) for system suitability test was observed in the range of 0.05–0.34% and 0.32–1.54% respectively, while the signal to noise ratio for system performance was  $\geq 75$  for both the enantiomers. Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. As evident from Fig. 5A–D, there was practically negligible carry-over ( $\leq 0.82\%$ ) during auto-sampler carryover experiment. All five calibration lines for *S*(–)-metoprolol and *R*(+)-metoprolol were linear over the concentration range of 0.500–500 ng/mL. The mean linear equation was  $y = (0.0058 \pm 0.0001)x + (0.0011 \pm 0.0001)$  and  $y = (0.0059 \pm 0.0001)x + (0.0011 \pm 0.0000)$  for *S*(–)-metoprolol and *R*(+)-metoprolol respectively, where  $y$  is the peak area ratio of the analyte/IS and  $x$  the concentration of the analyte. The mean correlation coefficient ( $r^2$ ) was  $\geq 0.9987$  for both the analytes.

The intra-batch and inter-batch precision and accuracy values at five QC levels are shown in Table 3. The intra-batch precision (% CV) and accuracy ranged from 1.2% to 3.6% and 94.7% to 105.3% respectively for both the enantiomers. Similarly, for the inter-batch experiment, the precision varied from 0.3% to 1.3% and the accuracy was within 98.2–103.5% for both the analytes. The relative recovery, absolute matrix effect and process efficiency data for *S*(–)-metoprolol and *R*(+)-metoprolol and IS are presented in Table 4. The mean relative recovery for SPE of the analytes was  $> 96\%$  across four QC levels. For IS,

the mean extraction recovery was greater than 93%. The absolute matrix effect values ranged from 100.6 to 103.2 for both the enantiomers. The coefficient of variation (% CV) of the slopes of calibration lines for relative matrix effect in eight different plasma lots was  $\leq 2.9$  for both the analytes (Table 5).

The stability of *S*(–)-metoprolol and *R*(+)-metoprolol was systematically evaluated in stock solutions and in plasma samples under different storage conditions. Stock solutions for short-term stability remained stable at room temperature up to 7 h, and for minimum of 7 days at refrigerated temperature of 5 °C for long term stability of both the analytes and IS. *S*(–)-metoprolol and *R*(+)-metoprolol in control human plasma (bench top) at room temperature were stable for at least 10 h at 25 °C and for minimum of five freeze-thaw cycles. Spiked plasma samples stored at –20 °C and –70 °C for long term stability experiment were found stable for a minimum period of 326 days. For blood sample stability the percentage change from comparison samples was less than 1.2% at two QC levels. Autosampler stability and dry extract stability of the analytes were determined up to 70 h without significant drug loss. Different stability experiments in plasma at two QC levels; with the values for percent change are shown in Table 6.

For method ruggedness, the precision (% CV) and accuracy values for two different columns ranged from 0.6% to 3.6% and 96.5% to 104.8% respectively for both the analytes at all QC levels. Similarly, for the experiment with different analysts, the results for precision and accuracy were within 1.0%–3.6% and 96.4%–101.8% respectively. The dilution



**Fig. 5** MRM ion-chromatograms for carry-over test of: (A) double blank plasma (without analyte and IS), (B) *S*-(-)-metoprolol and *R*-(+)-metoprolol at ULOQ level and IS, (C) double blank plasma (without analyte and IS) and (D) *S*-(-)-metoprolol and *R*-(+)-metoprolol at LLOQ level and IS.

reliability experiment was performed to validate the dilution test to be carried out on higher analyte concentration above ULOQ, which may be encountered during real subject

sample analysis. The precision for dilution integrity of 1/2 and 1/10th dilution was 1.2% and 0.8%, while the accuracy results were 93.2% and 96.8% respectively.

**Table 3** Intra-batch & inter-batch accuracy and precision for *S*(–)-metoprolol and *R*(+)-metoprolol.

Nominal concentration (ng/mL)	Intra-batch ( <i>n</i> =5; single batch)			Inter-batch ( <i>n</i> =25; 5 from each batch)		
	Mean concentration found (ng/mL)	Accuracy (%)	Precision (%)	Mean concentration found (ng/mL)	Accuracy (%)	Precision (%)
<i>S</i> (–)-metoprolol						
400	421	105.3	1.9	411	102.8	0.5
200	209	104.5	1.3	207	103.5	0.5
30.0	30.8	102.7	1.2	30.2	100.7	0.5
1.50	1.53	102.0	3.6	1.49	99.3	1.3
0.500	0.491	98.2	3.3	0.493	98.6	1.1
<i>R</i> (+)-metoprolol						
400	415	103.8	1.2	409	102.3	1.1
200	204	102.0	1.5	202	101.0	1.2
30.0	28.4	94.7	1.3	30.1	100.3	0.3
1.50	1.48	98.7	2.3	1.51	100.7	1.2
0.500	0.489	97.8	3.1	0.491	98.2	0.5

**Table 4** Absolute matrix effect, relative recovery and process efficiency for *S*(–)-metoprolol and *R*(+)-metoprolol.

Analyte	Area response			Absolute matrix effect (B/A) × 100 (Internal standard)	Relative recovery (C/B) × 100 (Internal standard)	Process efficiency (C/A) × 100 (Internal standard)
	A, reconstitution solution (Precision, %)	B, spiked in extracted blank plasma (Precision, %)	C, spiked before extraction (Precision, %)			
<b>HQC</b>						
<i>S</i> (–)-metoprolol	1860324 (5.0)	1870973 (1.8)	1815582 (4.7)	100.6 (95.2)	97.0 (92.5)	97.6 (88.1)
<i>R</i> (+)-metoprolol	1913390 (5.4)	1941257 (2.4)	1911388 (7.1)	101.5 (101.4)	98.5 (91.8)	99.9 (93.1)
<b>MQC1</b>						
<i>S</i> (–)-metoprolol	997723 (4.1)	1012560 (5.0)	987886 (2.5)	101.5 (97.9)	97.6 (89.7)	99.0 (87.8)
<i>R</i> (+)-metoprolol	1031698 (3.5)	1055489 (6.2)	1017144 (3.4)	102.3 (101.2)	96.4 (91.7)	98.6 (92.8)
<b>MQC2</b>						
<i>S</i> (–)-metoprolol	168005 (5.5)	171960 (5.1)	162838 (3.8)	102.4 (96.9)	94.7 (97.1)	96.9 (94.1)
<i>R</i> (+)-metoprolol	167777 (5.6)	173124 (5.3)	168069 (4.0)	103.2 (102.4)	97.1 (91.2)	100.2 (93.4)
<b>LQC</b>						
<i>S</i> (–)-metoprolol	8570 (4.7)	8812 (6.3)	8327 (5.2)	102.8 (94.4)	94.5 (94.6)	97.2 (88.9)
<i>R</i> (+)-metoprolol	8620 (4.4)	8824 (7.5)	8462 (5.4)	102.4 (94.2)	95.9 (96.0)	98.2 (90.4)

### 3.3. Assay application and incurred sample reanalysis (ISR) results

To the best of our knowledge there are no reports on the pharmacokinetics/bioequivalence study of metoprolol enantiomers in Indian subjects. The validated method was successfully used to quantify *S*(–)-metoprolol and *R*(+)-metoprolol concentration in human plasma samples after administration of a 200 mg extended release metoprolol test and reference formulations to 14 healthy Indian subjects. Fig. 6 shows the plasma concentration vs. time profile of *S*(–)-metoprolol and

*R*(+)-metoprolol under fasting condition. Approximately 1000 samples including the calibration, QC, volunteer samples and ISR samples were run and analyzed during a period of 6 days and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulations are presented in Table 7. No significant difference was found in  $T_{\max}$  and  $t_{1/2}$  values between the enantiomers as observed previously [7]. However, the  $C_{\max}$  and AUC values of *S*(–)-metoprolol were slightly higher than those of *R*(+)-metoprolol. This observation is in line with several other reports [7,19,20]. The  $C_{\max}$



enantiomeric ratio ( $S/R$ ) of 1.19 was higher compared to the work of Lanchote et al. [19] with identical dose strength. However, AUC enantiomeric ratio ( $S/R$ ) of  $\sim 1.05$  was significantly lower compared to 1.37 and 1.39 for studies involving single dose of *rac*-metoprolol [6]. This indicates that both the isomers are metabolized almost to the same extent in Indian subjects and consequently have similar accumulation in plasma. The possible reason for this difference could be the race of subjects, gender type (body size and muscle mass), type of food etc. However, the 90% confidence interval of individual ratio geometric mean for test/reference was within 80%–125% for  $C_{max}$ ,  $AUC_{0-t}$  and  $AUC_{0-inf}$  as shown in Table 8. These observations confirm the bioequivalence of the test formulation with the reference product in terms of rate and extent of absorption. Further, there was no adverse event during the course of the study.

ISR study has now become essential to check the reproducibility and reliability of a validated bioanalytical method. The

assay reproducibility expressed as % change for selected 68 samples was within  $\pm 13\%$  for both the enantiomers (Fig. 7).

### 3.4. Comparison with reported methods

The validated method has the highest sensitivity compared to majority of the methods developed for stereoselective analysis of metoprolol enantiomers [7,10,12–19,21,23] and identical with few others [11,20,22] in biological matrices. The plasma volume for samples preparation is only 200  $\mu\text{L}$ , which is considerably less compared to all other procedures [7,10–14,17–23] using chiral stationary phase except the work of Liang et al. [23] on dried blood spots. Additionally, the total chromatographic analysis time is the shortest compared to all other methods except one report [23]. Also, the on-column loading of metoprolol enantiomers at ULOQ level was only 20 ng per sample injection volume, which is significantly lower compared to all reported procedures.

## 4. Conclusion

The proposed LC–MS/MS method is rapid, sensitive and rugged for the quantification of metoprolol enantiomers in human plasma. The assay was found to be reliable and reproducible to support a bioequivalence study in healthy volunteers. Absence of matrix interference is adequately demonstrated by post-column infusion technique and by the precision (% CV) values for the calculated slopes of calibration curves. The validated method is selective in the presence of some commonly used medications by healthy volunteers. The stability data have been extensively evaluated in plasma and also in whole blood samples. A wide linear dynamic concentration range can adequately support pharmacokinetic applications with higher dose formulations of *rac*-metoprolol.

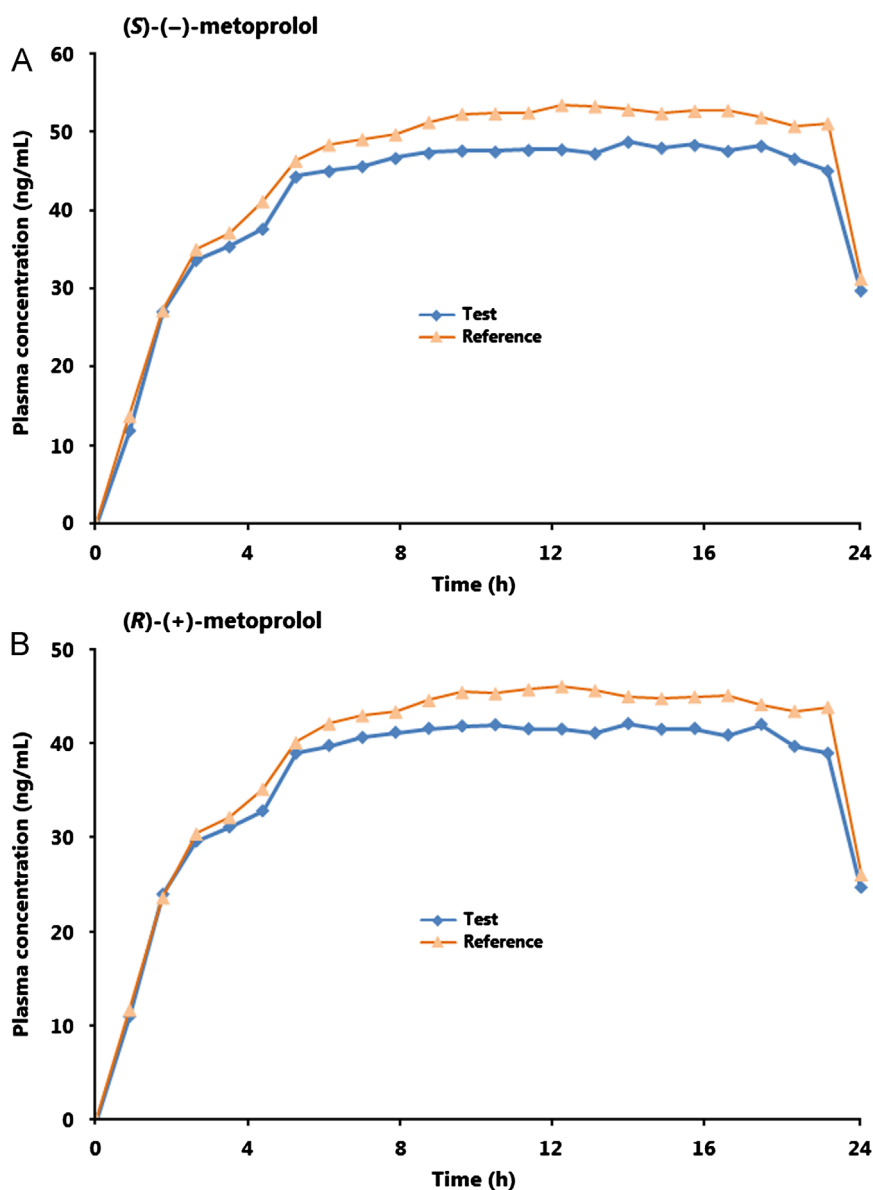
**Table 5** Relative matrix effect in different human plasma lots for *S*(–)-metoprolol and *R*(+)-metoprolol.

Plasma lot	Slope of calibration curve	
	<i>S</i> (–)-metoprolol	<i>R</i> (+)-metoprolol
Lot-1	0.0059	0.0059
Lot-2	0.0058	0.0058
Lot-3	0.0057	0.0058
Lot-4	0.0058	0.0060
Lot-5	0.0058	0.0060
Lot-6 (heparinized)	0.0062	0.0061
Lot-7 (haemolysed)	0.0059	0.0057
Lot-8 (lipidemic)	0.0061	0.0061
Mean	0.0059	0.0059
$\pm$ SD	0.00017	0.00015
Precision (%)	2.9	2.5

**Table 6** Stability results for *S*(–)-metoprolol and *R*(+)-metoprolol under different conditions ( $n=6$ ).

Storage condition	Level	<i>S</i> (–)-metoprolol		<i>R</i> (+)-metoprolol	
		Mean stability sample (ng/mL) $\pm$ SD	% Change	Mean stability sample (ng/mL) $\pm$ SD	% Change
Bench top stability, 10 h	HQC	394 $\pm$ 1.6	–1.5	391 $\pm$ 3.2	–2.3
	LQC	1.46 $\pm$ 2.3	–2.7	1.45 $\pm$ 4.7	–3.3
Wet extract stability; 70 h, 5 $\pm$ 3 $^{\circ}\text{C}$	HQC	430 $\pm$ 0.9	7.5	424 $\pm$ 0.9	6.0
	LQC	1.62 $\pm$ 1.2	8.0	1.57 $\pm$ 3.1	4.7
Dry extract stability; 69 h, –20 $^{\circ}\text{C}$	HQC	435 $\pm$ 1.2	8.7	429 $\pm$ 0.8	7.3
	LQC	1.61 $\pm$ 5.4	7.3	1.57 $\pm$ 1.9	4.7
Freeze-thaw stability; 5 cycles, –20 $^{\circ}\text{C}$	HQC	380 $\pm$ 0.7	–5.0	378 $\pm$ 0.8	–5.5
	LQC	1.37 $\pm$ 1.7	–8.7	1.39 $\pm$ 3.1	–7.3
Freeze-thaw stability; 5 cycles, –70 $^{\circ}\text{C}$	HQC	382 $\pm$ 0.7	–4.5	379 $\pm$ 0.9	–5.3
	LQC	1.39 $\pm$ 0.6	–7.3	1.41 $\pm$ 1.5	–6.0
Long term stability in plasma; 324 days, –20 $^{\circ}\text{C}$	HQC	374 $\pm$ 3.9	–6.5	372 $\pm$ 0.3	–7.0
	LQC	1.41 $\pm$ 2.8	–6.1	1.40 $\pm$ 4.5	–6.6
Long term stability in plasma; 324 days, –70 $^{\circ}\text{C}$	HQC	391 $\pm$ 1.2	–2.3	380 $\pm$ 1.0	–5.0
	LQC	1.45 $\pm$ 6.3	–3.3	1.42 $\pm$ 6.7	–5.3

$$\% \text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100.$$



**Fig. 6** Mean plasma concentration-time profile of: (A) *S*-(-)-metoprolol and (B) *R*-(+)-metoprolol after oral administration of test (200 mg metoprolol succinate extended release tablet from an Indian Pharmaceutical Company, India) and reference (Selo-zok<sup>®</sup>, 200 mg metoprolol succinate extended release tablet from AstraZeneca, Denmark) formulations to 14 healthy Indian subjects under fasting conditions.

**Table 7** Mean pharmacokinetic parameters of *S*-(-)-metoprolol and *R*-(+)-metoprolol (Mean  $\pm$  SD).

Parameter	<i>S</i> -(-)-metoprolol		<i>R</i> -(+)-metoprolol	
	Reference	Test	Reference	Test
$C_{max}$ (ng/mL)	52.41 $\pm$ 3.03	49.72 $\pm$ 5.35	43.76 $\pm$ 4.81	41.08 $\pm$ 3.76
$T_{max}$ (h)	9.36 $\pm$ 4.19	10.18 $\pm$ 4.23	9.57 $\pm$ 4.44	9.04 $\pm$ 4.40
$t_{1/2}$ (h)	2.50 $\pm$ 1.11	2.36 $\pm$ 0.71	2.05 $\pm$ 0.85	1.95 $\pm$ 0.67
AUC <sub>0-24</sub> (ng h/mL)	1554 $\pm$ 188	1499 $\pm$ 151	1421 $\pm$ 186	1392 $\pm$ 193
AUC <sub>0-inf</sub> (ng h/mL)	2106 $\pm$ 397	2046 $\pm$ 397	2086 $\pm$ 417	1996 $\pm$ 396
$K_{el}$ (1/h)	0.39 $\pm$ 0.07	0.38 $\pm$ 0.06	0.31 $\pm$ 0.11	0.28 $\pm$ 0.15

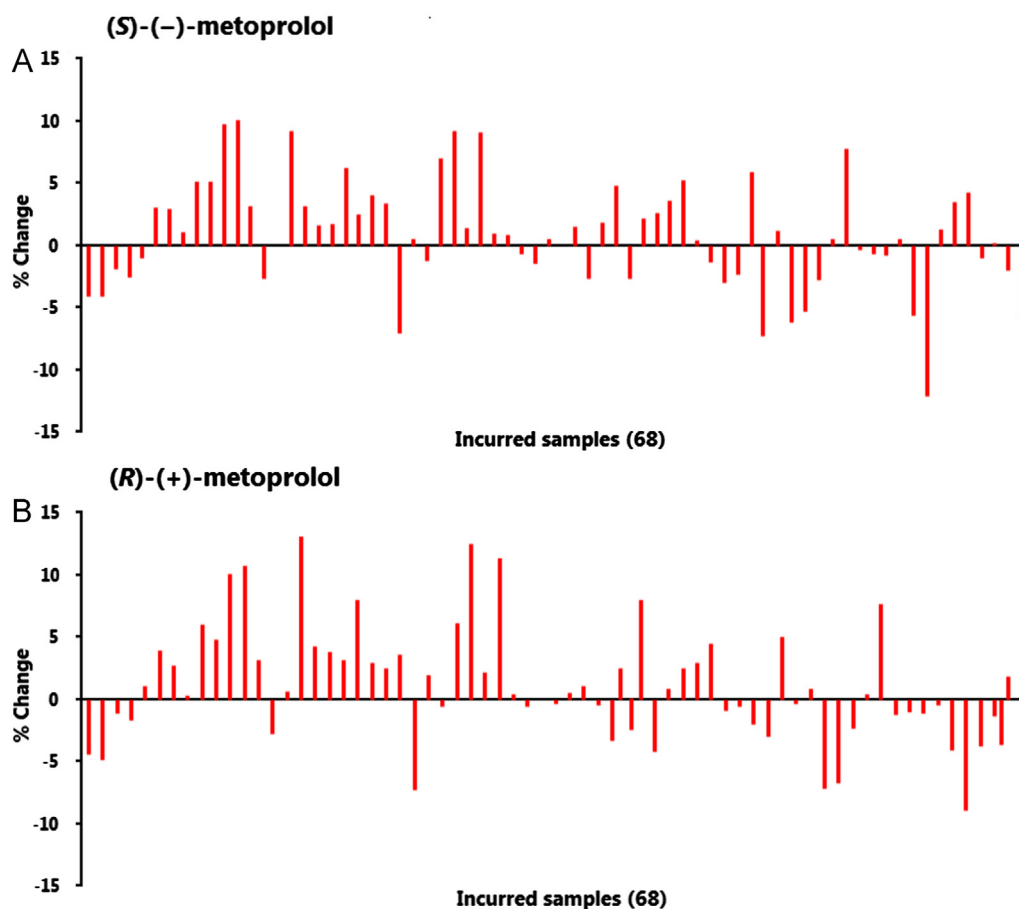


Fig. 7 Graphical representation of results for 68 incurred samples of *S*-(-)-metoprolol and *R*-(+)-metoprolol.

**Table 8** Comparison of treatment ratios and 90% CIs of natural log (Ln)-transformed parameters for *S*-(-)-metoprolol and *R*-(+)-metoprolol under fasting condition.

Parameter	Ratio (test/reference)		90% confidence interval (lower-upper)		Power		Intra-subject variation, precision (%)	
	<i>S</i> -isomer	<i>R</i> -isomer	<i>S</i> -isomer	<i>R</i> -isomer	<i>S</i> -isomer	<i>R</i> -isomer	<i>S</i> -isomer	<i>R</i> -isomer
$C_{max}$	94.8	93.9	90.8–97.2	89.1–97.2	1.000	1.000	4.3	6.1
$AUC_{0-24}$	96.4	97.9	94.8–98.5	93.3–100.5	1.000	1.000	2.6	5.3
$AUC_{0-inf}$	97.2	95.7	95.1–99.9	91.9–99.4	1.000	1.000	2.5	4.6

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