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Development and validation of a stereoselective liquid chromatography–tandem mass spectrometry assay for quantification of *S*- and *R*-metoprolol in human plasma

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Abstract

A stereoselective liquid chromatography–tandem mass spectrometry assay was developed and validated for quantification of *S*- and *R*-metoprolol at concentrations of 0.5–50 μg/L in human plasma. Metoprolol was extracted from plasma by liquid–liquid extraction with ethyl acetate (82% recovery). Chromatographic separation of the enantiomers was achieved on a chiral Chirobiotic T column using an isocratic mobile phase consisting of methanol/acetic acid/ammonia (100/0.15/0.15, v/v/v). An ion trap mass spectrometer with an electrospray interface was used for detection in the positive mode, monitoring the *m/z* transition 268 → 191 for metoprolol. Standard curves for *S*- and *R*-metoprolol fitted quadratic functions ($r^2 \geq 0.9995$) over the range 0.5–50 μg/L in plasma, with 0.5 μg/L representing the limit of quantification. In this range, relative standard deviations were <6% for intra-day precision and <10% for inter-day precision. The accuracy was within the range of 92–105%.

Keywords: Metoprolol; Enantiomers; LC–MS/MS; Plasma

1. Introduction

Metoprolol, (±)1-(isopropylamino)-3-[*p*-(β-methoxyethyl) phenoxy]-2-propanol (Fig. 1), is a β1-selective adrenoreceptor blocking drug. It is widely used in the management of hypertension, ischaemic heart disease and heart failure [1,2].

Metoprolol is administered as the tartrate, succinate or fumarate salt in a racemic mixture. The β-blocking activity resides primarily in the *S*-enantiomer [2]. Metoprolol is extensively metabolised by phase I processes in the liver. The metabolic pathways are stereoselective, with the *S*-enantiomer being the predominant form in humans, reflected by a plasma ratio of *S*/*R*-metoprolol >1 in most people [3–5]. The metabolism of metoprolol is subject to genetic polymorphism since a large proportion involves the Cytochrome P450 2D6 (CYP2D6) enzyme [2,4]. CYP2D6 extensive metabolisers have been shown to have a greater *S*/*R*-metoprolol ratio in plasma than poor metabolisers [4]. For clinical studies of metoprolol, it is thus important to be able to selectively monitor the individual enantiomers.

Several analytical methods have been developed for the stereoselective analysis of metoprolol in biofluids. Direct methods of analysis, which require no derivatisation with chiral reagents to form diastereomers prior to separation, are preferred for clinical studies. Such methods have used chiral stationary phases that were based on either proteins [6–10] or polysaccharide derivatives [3,11–13] applied in the normal phase mode. In a single case, a stationary phase based on macrocyclic glycopeptides was used [14]. Following extraction of metoprolol from plasma or urine by solid-phase or liquid–liquid extraction, fluorescence detection was used in all methods, with limits of quantification in the ranges of 0.5–25 μg/L.

While fluorescence detection is generally a sensitive and relatively selective detection technique for HPLC, a higher degree of selectivity can be achieved using tandem mass spectrometry (MS). Metabolites, other drugs, or endogenous compounds will usually have a different mass to charge ratio (*m/z*) from the ana-

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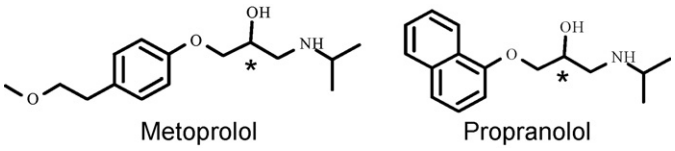

Fig. 1. Chemical structures of metoprolol and propranolol (internal standard). Chiral centres are indicated by (*).

lyte of interest. This reduces the risk of interferences and may also improve sensitivity.

The use of chiral HPLC with MS detection has recently been reviewed [15]. Many chiral HPLC methods require the use of normal phase modes for enantiomeric separation. Normal phase solvents, such as hexane, are generally incompatible with both electrospray ionisation (ESI) and atmospheric pressure ionisation (APCI) of mass spectrometers as they impose an explosion hazard in the presence of the high voltage of the electrospray needle used in ESI or in the presence of the corona discharge used in APCI. These solvents are thus best avoided unless safety measures are taken, such as extensive post-column addition of MS-compatible mobile phases [15,16]. Hence, simpler and safer methods such as chiral stationary phases that allow reversed phase or polar organic modes (100% organic phase with added acid or base) are preferred for MS detection. For this reason, the chiral stationary phases based on macrocyclic glycopeptides, such as the antibiotics teicoplanin, vancomycin and ristocetin, have become popular for LC–MS [17].

The existing methods for separation of metoprolol enantiomers in human plasma have generally been based on normal phase modes [3,6–13] and are thus not transferable to MS detection. The only exception is the method by Mistry et al. [14] in which a teicoplanin column was used with a polar organic mobile phase consisting of acetonitrile/methanol/dichloromethane/glacial acetic acid/triethylamine. However, this mobile phase is not readily compatible with ion trap mass spectrometers as triethylamine contaminates the instrument leading to poor detection limits [18]. A novel method for stereoselective determination of *S*/*R*-metoprolol in human plasma by LC–MS was therefore required.

To our knowledge, there are only two studies in the literature involving separation of metoprolol enantiomers with MS detection. Bakhtiar and Tse [16] showed separation of the enantiomers, though not to baseline, on a teicoplanin (Chirobiotic T) column using a mobile phase consisting of methanol and ammonium trifluoroacetate and using APCI-MS in positive mode. A standard solution of 1 μg/L metoprolol in methanol was easily detected with a S/N > 10 but the method was not evaluated further. Nikolai et al. [19] developed an enantiomeric LC–MS method for metoprolol in wastewater effluents using a vancomycin column (Chirobiotic V) in the reversed phase mode. The method was used for quantification although the enantiomers were not completely resolved (resolution not reported). Positive mode ESI-MS was applied and a limit of detection of 4 μg/L in wastewater extract was obtained.

The aim of the current work was therefore to develop and validate a simple and stereoselective LC–MS/MS method for the determination of metoprolol enantiomers in human plasma. The assay is currently being applied in a clinical study of metoprolol in heart failure.

2. Experimental

2.1. Materials

S- and *R*-metoprolol were kindly donated by AstraZeneca (Mölndal, Sweden). Racemic metoprolol tartrate salt and racemic propranolol hydrochloride, both with a purity of ≥99%, were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC-grade methanol, 100% anhydrous acetic acid (glacial), ethyl acetate and analytical grade sodium hydroxide were purchased from Merck (Darmstadt, Germany) and ammonia solution sp.gr. ~0.88–0.89 was purchased from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System from Millipore (MA, USA). Human plasma used for assay blanks and preparation of standards was obtained from New Zealand Blood Services (Christchurch, New Zealand).

2.2. Instrumentation

LC–MS/MS analysis was performed with a Thermo Finnigan Surveyor HPLC system coupled to a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with an electrospray ionisation source (San Jose, CA, USA). Data acquisition was performed with XCalibur™ software ver. 1.3.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a chiral ASTEC Chirobiotic T column, 250 mm × 4.6 mm i.d., 5 μm from Supelco (Bellefonte, CA, USA) using an isocratic mobile phase consisting of methanol/acetic acid/ammonia (100/0.15/0.15, v/v/v) at a flow rate of 1 mL/min. A 4 mm × 3 mm i.d. C18 guard column was used (Phenomenex, Torrance, CA, USA). The column was maintained at ambient temperature whilst the autosampler was cooled to 5 °C. Injection volume was 50 μL. The first 4 min of the run was sent to waste and the total run time was 11 min.

2.4. Mass spectrometry

Positive mode electrospray ionisation with selective reaction monitoring (SRM) was used to acquire the mass spectra. The electrospray needle was held at +5000 V and the capillary was heated to 360 °C. Nitrogen was used as sheath and auxiliary gas at settings of 60 and 10 arbitrary units, respectively. Helium was used as collision gas. Collision energy was optimised at 36% for metoprolol at SRM 268.1 → 191.1 and 33% for propranolol at SRM 260.1 → 183.1. An isolation width of 2 Da was used.

2.5. Preparation of standards and quality control samples

Two separate stock solutions of racemic metoprolol were prepared by dissolving 10 mg of accurately weighed *Rac*-

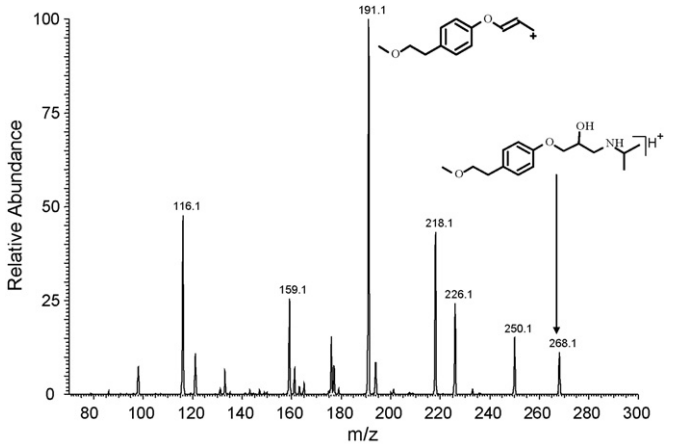

Fig. 2. Positive mode product ion spectrum (MS/MS) of metoprolol (*m/z* 268.1 → spectrum). The structure of metoprolol and a proposed structure of the most intense product ion at *m/z* 191.1 are shown.

metoprolol in 10 mL methanol (0.5 mg *S*- and *R*-metoprolol free base/mL). One solution was used for preparation of standard curves and the other for quality control (QC) samples. Likewise, a stock solution of the internal standard (IS) *Rac*-propranolol was prepared in methanol (0.5 mg *S*- and *R*-propranolol free base/mL). The stock solutions were stored at −30 °C.

A working solution of 2500 μg/L of *S*/*R*-metoprolol was prepared in human plasma. This solution was used to spike plasma samples to obtain calibration standards of 0.5, 1, 5, 10, 25 and 50 μg/L of *S*/*R*-metoprolol. Quality control samples were prepared by spiking plasma samples to 1, 10 and 50 μg/L of *S*/*R*-metoprolol. The spiked plasma standards and QC samples were stored in aliquots at −30 °C until analysis. A working solution of *S*/*R*-propranolol (IS) at 2000 μg/L was prepared in methanol and stored at −30 °C.

2.6. Sample preparation and extraction procedure

Plasma samples (0.9 mL) were alkalinised with 100 μL of a 1 M NaOH solution followed by addition of 50 μL of *S*/*R*-propranolol 2000 μg/L and 4 mL of ethyl acetate. The samples were shaken for 30 s and centrifuged at 2500 × *g* for 5 min. The organic phases were transferred to conical tubes and evaporated to dryness under vacuum. The residues obtained were redissolved in 90 μL methanol and 50 μL aliquots were injected into the LC–MS system. The peak area ratios of *S*-metoprolol/*S*-propranolol and *R*-metoprolol/*R*-propranolol were determined and used for calculation of metoprolol concentrations.

2.7. Assay validation

Sets of spiked standards and QC samples ($n = 5$ at each concentration) were prepared and analysed on three different days to evaluate linearity, precision and accuracy. Precision and accuracy was also assessed at the lowest concentration of the standards (0.5 μg/L), representing the limit of quantification (LOQ) for the assay ($n = 5$).

Extraction recovery for *S*- and *R*-metoprolol was determined by comparing the obtained peak areas of the QC samples to the peak areas of blank plasma extracts spiked with *S*/*R*-metoprolol (representing 100% recovery) at 1, 10 and 50 μg/L ($n = 5$). Matrix effects from the plasma extracts were assessed by comparing the peak areas of the spiked blank plasma extracts to the peak areas from samples spiked with *S*/*R*-metoprolol to methanol (representing 100%, i.e. no matrix effect) at 1, 10 and 50 μg/L ($n = 4$).

The stability of metoprolol (and propranolol) in plasma and stock solutions has already been demonstrated [8,9,12,14,20–22], thus stability evaluation was limited to two combined experiments in this work. For determination of long and short term stability as well as freeze–thaw stability, spiked QC samples at 10 and 50 μg/L of *S*/*R*-metoprolol ($n = 3$ at each conc.) were stored for 6 months at −80 °C, allowed to thaw at room temperature and refrozen at −80 °C for 12–24 h (three cycles) and finally left at room temperature for 4 h. The samples were analysed and the results for *S*- and *R*-metoprolol were compared to those of freshly prepared QC samples. For determination of in-process stability, a worst-case scenario of storage times was followed. Spiked QC samples at 1, 10 and 50 μg/L of *S*/*R*-metoprolol ($n = 3$ at each conc.) were thawed at room temperature and kept at this temperature for 3 h. Following extraction, the dry extracts were left at room temperature for 5 h before reconstitution and then kept at 5 °C in the autosampler for 30 h. The samples were analysed and the results for *S*- and *R*-metoprolol were compared to those of freshly prepared samples.

3. Results and discussion

3.1. Method development

Antibiotic chiral stationary phases have been a popular choice for LC–MS as they allow for reversed phase or polar organic modes [17]. Andersson et al. [23] developed a generic chiral LC screen and found that four β-blockers (including metopro-

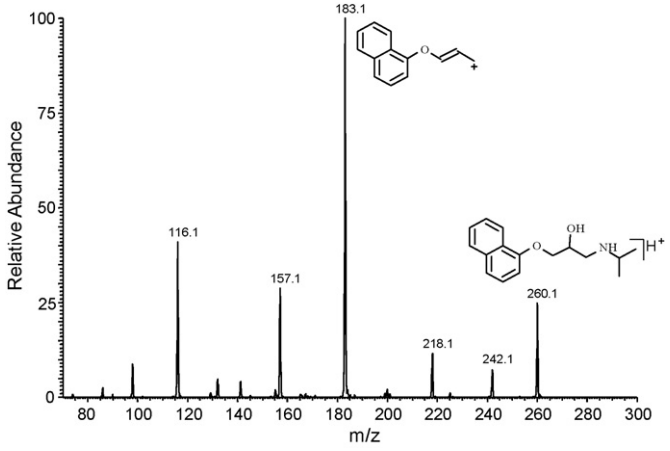

Fig. 3. Positive mode product ion spectrum (MS/MS) of the internal standard, propranolol (*m/z* 260.1 → spectrum). The structure of propranolol and a proposed structure of the most intense product ion at *m/z* 183.1 are shown.

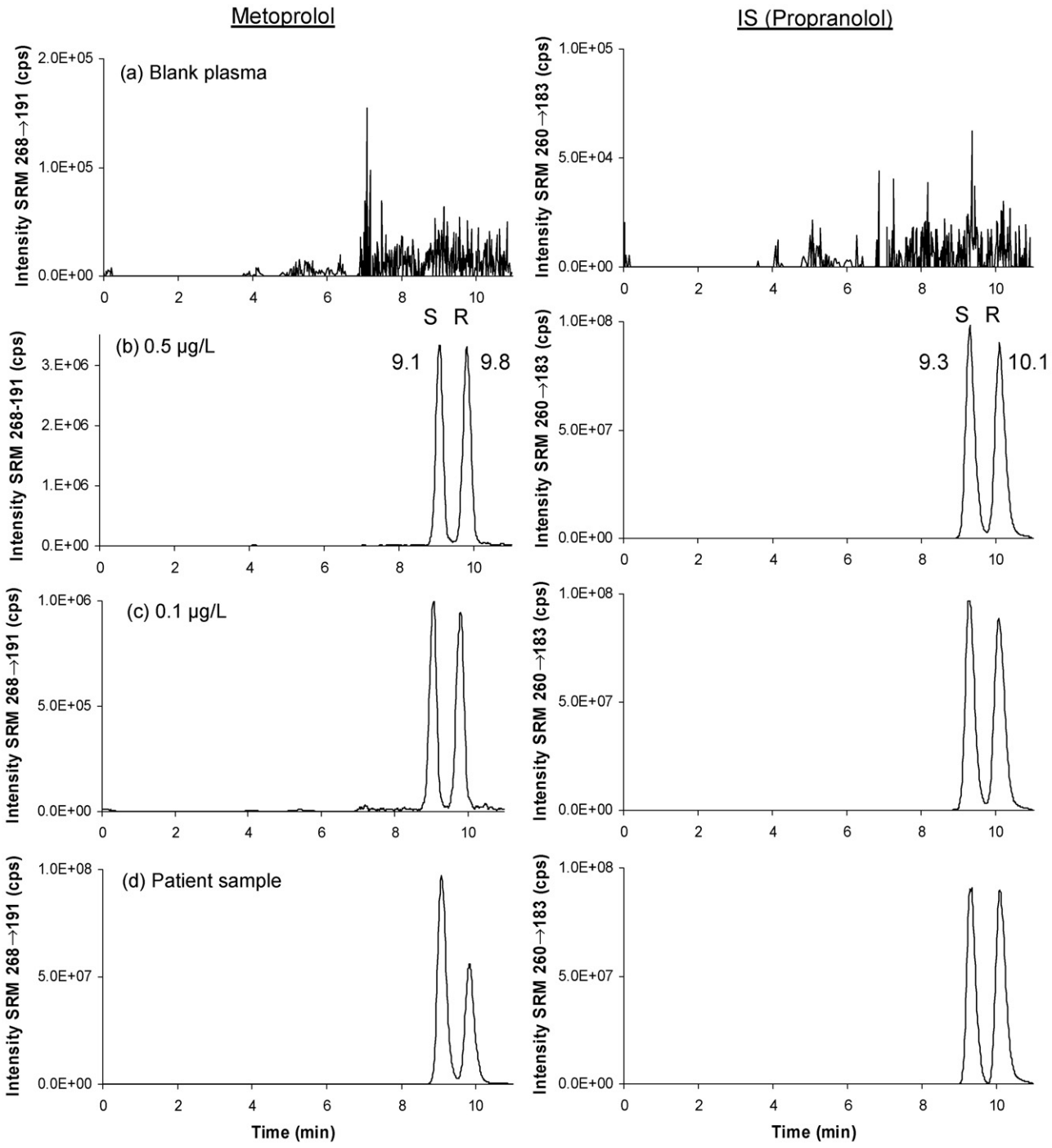

Fig. 4. Representative LC–MS/MS chromatograms of (a) blank plasma, (b) plasma sample spiked with *S*/*R*-metoprolol at 0.5 μg/L, (c) plasma sample spiked with *S*/*R*-metoprolol at 0.1 μg/L and (d) patient sample (*S*-metoprolol 28.4 μg/L, *R*-metoprolol 16.4 μg/L). Metoprolol is shown in the left pane and the internal standard (*S*/*R*-propranolol 50 μg/L) in the right pane. Fifty microlitres plasma extracts were injected.

lol) were well separated on either teicoplanin or vancomycin columns in polar organic mode. It was generally found that the polar organic mode resulted in better enantio-resolution of basic compounds than reversed phase mode [23]. Likewise, polar organic mobile phases were found to give better enantio-resolution in a shorter time than reversed phase mobile phases on vancomycin columns in a study of six β-blockers [24]. Based on these reports, and the result of a metoprolol standard solution by Bakhtiar and Tse [16], a teicoplanin (Chirobiotic T) column was chosen. No other columns were tried as satisfactory separation was obtained on this column.

With respect to the mobile phase, best enantiomer separation has been reported using polar organic phases consisting of methanol modified with triethylamine and glacial acetic acid [19,23,24] or ammonium trifluoroacetate [16]. However, we wished to avoid triethylamine due to the risk of instrument contamination [18] and found that ammonia and glacial acetic acid in equal volumes were superior to ammonium trifluoroacetate.

Table 1
Intra- and inter-day precision (RSD) and accuracy for *S*- and *R*- metoprolol in human plasma

Analyte	Spiked conc. (μg/L)	Found conc. (μg/L) (mean ± S.D., $n = 5$)	RSD (%)	Accuracy (%)
Intra-day				
S-Metoprolol	0.5 (LOQ)	0.521 ± 0.015	2.9	104
	1 (LQC)	1.04 ± 0.054	5.2	104
	10 (MQC)	10.0 ± 0.17	1.7	100
	50 (HQC)	45.9 ± 0.99	2.2	92
R-Metoprolol	0.5 (LOQ)	0.523 ± 0.029	5.5	105
	1 (LQC)	1.03 ± 0.058	5.6	103
	10 (MQC)	10.1 ± 0.37	3.6	101
	50 (HQC)	46.1 ± 0.39	0.9	92
Analyte	Spiked conc. (μg/L)	Found conc. (μg/L) (mean ± S.D., 3 runs of $n = 5$)	RSD (%)	Accuracy (%)
Inter-day				
S-Metoprolol	0.5 (LOQ)	0.486 ± 0.043	8.9	97
	1 (LQC)	0.930 ± 0.093	10.0	93
	10 (MQC)	10.2 ± 0.33	3.2	102
	50 (HQC)	50.8 ± 4.68	9.2	102
R-Metoprolol	0.5 (LOQ)	0.517 ± 0.036	6.9	103
	1 (LQC)	0.944 ± 0.078	8.3	94
	10 (MQC)	10.0 ± 0.45	4.5	100
	50 (HQC)	48.9 ± 2.46	5.0	98

Acceptable resolution could also be obtained in the reversed phase mode using the same modifiers, but this required longer run times, a finding similar to previously reported [23,24].

A mobile phase consisting of methanol/acetic acid/ammonia (100/0.1/0.1, v/v/v) at a flow rate of 2 mL/min resulted in adequate resolution of the metoprolol enantiomers ($R_s > 1.4$) within a run time of 7.5 min. Ionisation by APCI was tried as this was the source of ionisation used by Bakhtiar and Tse [16]. By changing to ESI, reducing the flow rate to 1 mL/min (to avoid splitting the flow), and changing the mobile phase to methanol/acetic acid/ammonia (100/0.15/0.15, v/v/v) an improvement in sensitivity of a factor of ~10 was gained. Under these conditions, a resolution of $R_s > 1.4$ could be obtained within a runtime of 11 min and this method was thus chosen.

Figs. 2 and 3 represent the mass spectra of metoprolol and propranolol and their respective product ion mass spectra. The most intense product ions were obtained from the *m*/*z* transitions of 268 → 191 for metoprolol and 260 → 183 for propranolol, and these were used for quantification. Representative chromatograms of plasma samples spiked with metoprolol and propranolol are shown along with a blank sample in Fig. 4.

Propranolol was found to be suitable as an internal standard. Ideally, isotopically labelled *S*/*R*-metoprolol should be used. However, non-labelled internal standards can be used if they match the chromatographic parameters, recovery and ionisation properties of the analyte and are not present in the sample. *S*/*R*-propranolol was found to have similar retention times to metoprolol (less than 0.3 min difference in retention time as seen in Fig. 4), similar recovery (see Section 3.2) and similar ionisation properties as seen in Figs. 2 and 3. Propranolol is not expected to be found in plasma samples from patients as it is highly unlikely that patients would be on propranolol and metoprolol concurrently. Certainly, in the study for which this assay was developed, it was controlled that patients were only given metoprolol. *S*/*R*-alprenolol was also tried as an internal standard but was found to have chromatographic properties that varied more widely.

Metabolites of metoprolol were not assessed in this assay as they are not commercially available. All the known metabolites of metoprolol [2] have different *m*/*z* values from metoprolol except one, which is a carboxylic acid, that would not be extrac-

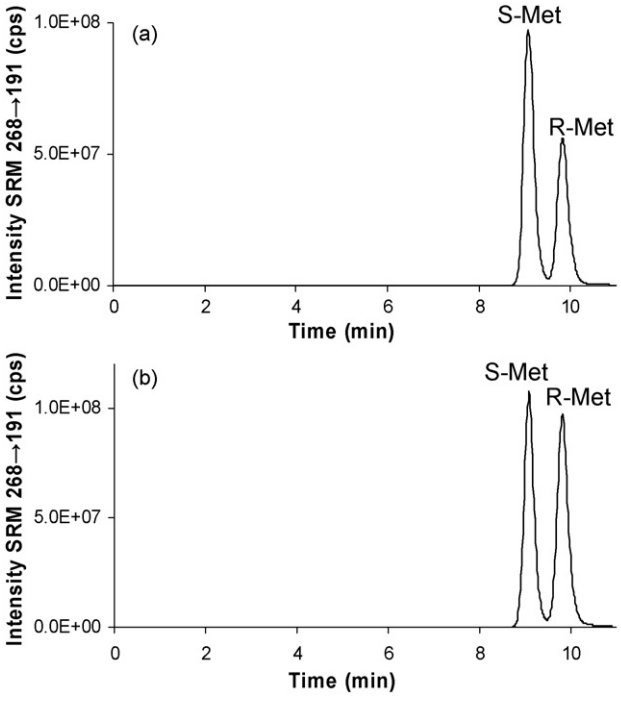

Fig. 5. Representative chromatograms of patient samples from (a) CYP2D6 extensive metaboliser on a 190 mg daily dose of metoprolol and (b) CYP2D6 poor metaboliser on a 47.5 mg daily dose of metoprolol.

Table 2
Extraction recovery and matrix effect (100% representing no effect) for *S*- and *R*-metoprolol in human plasma

Analyte	Spiked conc. (μg/L)	Extraction recovery (%) (mean ± S.D., $n = 5$)	Matrix effect (%) (mean ± S.D., $n = 4$)
S-Metoprolol	1	83.0 ± 5.9	94.3 ± 1.6
	10	84.7 ± 1.6	90.6 ± 2.6
	50	77.9 ± 1.9	85.8 ± 1.0
R-Metoprolol	1	81.5 ± 3.0	97.6 ± 1.8
	10	87.1 ± 3.4	86.9 ± 2.4
	50	78.9 ± 4.3	84.7 ± 1.1

ted under the alkaline conditions used (pH 11). Further, the chromatographic properties of the metabolites are likely to differ from metoprolol. Interference from metoprolol metabolites would thus not be expected in this assay.

3.2. Assay validation

Sets of spiked standard and quality control samples were prepared and analysed on three different days. The best fit for the standard curves for *S*- and *R*-metoprolol over the range 0.5–50 μg/L in plasma was obtained by using a quadratic function ($r^2 \geq 0.9995$). Linearity could be obtained by reducing the dynamic range and excluding the highest standard. However, as the assay was to be used for clinical samples in the range of 0.5–50 μg/L of *S*/*R*-metoprolol in plasma, the quadratic function was used. The limit of quantification was 0.5 μg/L and a representative chromatogram is shown in Fig. 4b.

If a lower LOQ is needed, the highest standard (50 μg/L) could be excluded and linear regression used. In this way, an LOQ of 0.1 μg/L could be obtained with a precision of <13% relative standard deviation (RSD) and accuracy within 85–108% ($n = 5$) using linear regression ($r^2 \geq 0.9995$). A representative chromatogram of 0.1 μg/L of *S*/*R*-metoprolol in plasma is shown in Fig. 4c.

Results for precision and accuracy of the assay over the range 0.5–50 μg/L metoprolol in human plasma are summarised in Table 1. Both precision and accuracy were within acceptable ranges for bioanalytical purposes. Relative standard deviations were <6% for intra-day precision and <10% for inter-day precision. The percent accuracy was within the range of 92–105%. Blank plasma samples from six donors were analysed and no interfering peaks were found.

Extraction recoveries for the liquid–liquid extraction of metoprolol from plasma are summarised in Table 2. The recovery was consistent and reproducible with an overall recovery of 82 ± 4% (mean ± S.D., $n = 15$). For propranolol, the extraction recovery was 85 ± 8% (mean ± S.D., $n = 3$, data not shown).

To investigate whether the matrix (plasma extract) influenced the ionisation, the response of plasma extracts spiked with metoprolol were compared to methanol spiked with metoprolol (representing 100%, i.e. no matrix effect). The results are summarised in Table 2. The plasma extract was found to have a small impact on the ionisation as the overall results were 90 ± 5% (mean ± S.D., $n = 12$) as compared to methanol.

Metoprolol was found to be stable in plasma during freezing at −80 °C for 6 months and for three freeze–thaw cycles. The mean values of stored samples at two concentrations ($n = 3$ at each conc.) deviated <4% from freshly prepared samples. To assess in-process stability, a worst-case scenario of storage time during the process was followed. The mean values of the slowly processed samples at three concentrations ($n = 3$ at each conc.) deviated <9% from freshly prepared samples.

3.3. Assay application

This LC–MS/MS assay was used in a clinical study involving >300 injections of samples. The assay was found to be robust and reliable with quality control samples showing a precision of <10% RSD and accuracy within the range of 93–104%. Part of the clinical study involved comparing *S*/*R*-metoprolol ratios between patients with various genotypes of the metabolic enzyme CYP2D6. Patients with high CYP2D6 activity were found to have greater *S*/*R*-metoprolol ratios in plasma than patients with lower activity. Representative chromatograms of patient samples from a CYP2D6 extensive metaboliser and poor metaboliser, respectively, are shown in Fig. 5. The outcomes of the clinical study will be reported elsewhere.

4. Conclusion

A stereoselective liquid chromatography–tandem mass spectrometry assay was developed and validated for quantification of *S*- and *R*-metoprolol at concentrations of 0.5–50 μg/L in human plasma. The assay was found to be reliable, accurate and precise and was applied to analysis of samples from a clinical study. Adequate sensitivity for clinical applications was obtained.

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