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Automated online dual-column extraction coupled with teicoplanin stationary phase for simultaneous determination of (*R*)- and (*S*)-propranolol in rat plasma using liquid chromatography–tandem mass spectrometry

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Abstract

An automated online sample extraction method for rat plasma was developed and validated for the quantification of (*R*)- and (*S*)-propranolol following the intravenous administration of either the racemate or the individual enantiomers at 5 mg/kg. A dual-column extraction system coupled to a chiral stationary phase (CSP) was used in conjunction with liquid chromatography–tandem mass spectrometry. In this method, two Oasis HLB extraction columns (50×1.0 mm) in parallel were used for online plasma sample purification and teicoplanin CSP (Chirobiotic T) was used for the enantiomeric separation. This method allowed the use of one of the extraction columns for purification while the other was being equilibrated. Hence, the time required for re-conditioning the extraction columns did not contribute to the total analysis time per sample, which resulted in a relatively shorter run time and higher throughput. The lower limit of detection was 0.5 ng/ml and the lower limit of quantification was 2 ng/ml for each enantiomer using 25 μl of rat plasma. The method was validated with a linear calibration curve between 2 and 2000 ng/ml for (*R*)- and (*S*)-propranolol, respectively. The intra- and inter-day precision (C.V.) was no more than 7.6% and the accuracy of the assay was between 92 and 103%. The teicoplanin CSP proved to be rugged with excellent reproducibility of chromatographic parameters.

Keywords: Stationary phases, LC; Enantiomer separation; Teicoplanin; Propranolol

1. Introduction

Chirality has long been viewed as one of the critical issues in the drug design and discovery process. During the development stages, the use of a chiral drug requires an understanding of the pharmacokinetic attributes of each of the enantiomers since possible differences could arise in the absorption, distribution, metabolism, and excretion of each enantiomer [1–3].

In recent years, samples generated from large-scale clinical trials, along with the ambitious development timelines, have prompted the use of higher throughput bioanalytical alternatives. In this regard, substantial progress has been made in order

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to streamline sample purification steps in liquid chromatography–tandem mass spectrometry (LC–MS/MS) bioassays [4]. Numerous improvements in speed, sensitivity, and accuracy, augmented with innovations in automation, have placed MS among one of the versatile and multi-faceted analytical techniques available today.

Recently, we reported the application of an online purification system in conjunction with chiral LC–MS/MS to achieve high-throughput quantification of terbutaline enantiomers via direct-injection of human plasma samples [5]. Briefly, the biological samples were injected directly onto an extraction column packed with large size particles (25–60 μm), which allowed proteins to be washed away, while retaining the analyte inside the pores of the packing material. Using this high-flow online extraction approach, an accelerated purification (less than 30 s per sample) was achieved without compromising the extraction efficiency. The enantiomeric separation was achieved using a teicoplanin (Chirobiotic T) chiral stationary phase (CSP) under isocratic reversed-phase LC conditions [5]. However, the lack of reference standards for either terbutaline enantiomer, together with the fact that plasma samples from dosed subjects were not available, left room for improvement in the rigorous validation of the assay.

In order to extend the application of our automated online chiral LC–MS/MS approach, we have chosen to utilize the β-receptor blocking drug, propranolol, since racemic compound, as well as both (*R*)- and (*S*)-enantiomers, were available commercially. In addition, the stable isotope internal standard, D_7-racemic-propranolol, provided an ideal compound to minimize intra- and inter-day variations. A number of racemic LC assays, using MS [6–8] or fluorometric detection [9], for the screening of propranolol have been reported. Furthermore, several laboratories have demonstrated the feasibility of chiral LC in conjunction with fluorescence-based detection for the analysis of propranolol enantiomers [10–16]. The reported methods required derivatization procedures [10–12], or long chromatographic run time [14,15], and used off-line sample clean up steps [10–15]. However, to our knowledge, automated online-column purification, in conjunction with chiral LC–MS for the analysis of propranolol enantiomers in biological samples, has not been reported.

This paper presents the systematic evaluation of online extraction recoveries, matrix effect, ex vivo stability and analysis of plasma samples obtained from male Sprague–Dawley rats dosed intravenously with (*R*)-, (*S*)-, and racemic-propranolol utilizing an automated online chiral LC–MS/MS procedure. As will be seen, the pivotal chiral separation was achieved using the Chirobiotic T CSP recently introduced by Armstrong and co-workers [17–20].

2. Experimental

2.1. Chemicals and reagents

Racemic propranolol hydrochloride was purchased from Aldrich (Milwaukee, WI, USA). The enantiomers, (*S*)- and (*R*)-propranolol, as their hydrochloride salts, were obtained from Tocris Cookson (Ellisville, MO, USA). The internal standard, D_7-racemic-propranolol, was purchased from CND Isotopes (Quebec, Canada). Analytical-grade ammonium trifluoroacetate, ammonium acetate, and formic acid were purchased from Sigma (St. Louis, MO, USA). Methanol and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Drug-free rat plasma containing disodium–EDTA as the anticoagulant was obtained from Bioreclamation (Hicksville, NY, USA).

2.2. Instrumentation

Mass spectrometric analysis was performed on a PE Sciex API 3000 triple quadrupole mass spectrometer (Toronto, Canada) equipped with an atmospheric pressure chemical ionization (APCI) interface. PE Sciex Analyst software (version 1.1) was used for data acquisition and peak integration. The LC system consisted of an HTS-PAL autosampler (LEAP Technologies, Carrboro, NC, USA), Shimadzu LC-AD^{VP} binary pumps (Columbia, MD, USA), and Perkin-Elmer (Norwalk, CT, USA) Series 200 micro pumps. A 10-port switching valve equipped with microelectric two-position valve actuators from Valco (Houston, TX, USA) was connected to one of the Shimadzu pumps. Oasis HLB extraction columns (1.0×50-mm I.D., 25-μm particle size) and precolumn filters were purchased from Waters (Milford,

MA, USA). A Chirobiotic T CSP (4.6×100-mm I.D., 5-μm particle size) column was obtained from Advanced Separation Technologies (Whippany, NJ, USA). A Zorbax RX-SIL guard column (2.1×12.5-mm I.D., 5-μm particle size) was purchased from Agilent Technologies (Chadds Ford, PA, USA) and replaced after approximately 200 injections. Deep 96-well collection plates were purchased from Microliter Analytical Supplies (Suwanee, GA, USA).

2.3. Preparation of standard calibration and quality control samples

Stock solutions of (*R*)-, (*S*)- and racemic-propranolol were prepared, in methanol, at a concentration of 1.0 mg/ml and stored at 4 °C. Standard calibration working solutions were prepared in methanol at concentrations of 20, 50, 100, 250, 1000, 5000, 10 000, and 20 000 ng/ml of either (*R*)- or (*S*)-propranolol, respectively. The standard calibration curve samples were prepared by spiking 20 μl of the working solution (vide supra) into 180 μl of control rat plasma to obtain concentrations of 2, 5, 10, 25, 100, 500, 1000, and 2000 ng/ml of (*R*)- and (*S*)-propranolol. The working solutions and standard calibration samples were prepared freshly and separately for each enantiomer in each run. Four sets of QC samples were prepared in control rat plasma in order to evaluate the precision and accuracy of the method. The first set contained (*R*)-propranolol, the second set contained (*S*)-propranolol, the third set contained racemic-propranolol, and the fourth set contained (*R*)-/(*S*)-propranolol in ratios of 100:10 and 10:100. The first three sets of QC samples were prepared by spiking (*R*)-, or (*S*)-, or racemic-propranolol, at concentrations of 2, 6, 100, 1000, 1600, and 5000 (the dilution QC) ng/ml into control rat plasma. The fourth one was prepared by spiking both (*R*)- and (*S*)-enantiomer in ratios of 100:10 and 10:100 ng/ml in control rat plasma. Five different sources (lot numbers) of control rat plasma were used to prepare the QC samples.

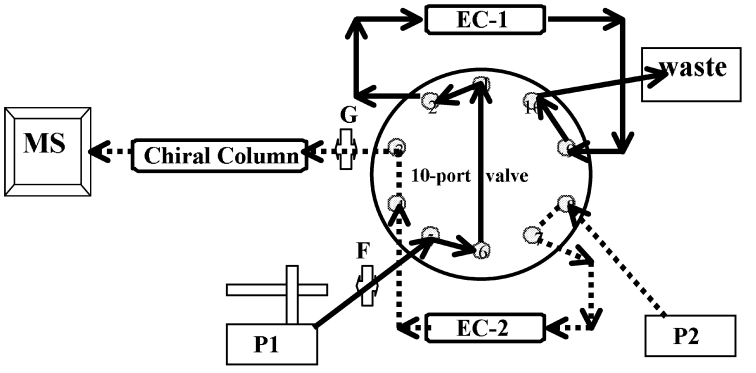
The internal standard solution, containing 250 ng/ml of D_7-racemic-propranolol, was prepared in a 0.5 *M* formic acid containing 20% methanol (v/v). The plasma samples and the dilution QC (5000 ng/ml), in which concentrations were above the upper limit of quantification, were diluted 5-fold with control rat plasma.

2.4. Online dual-column extraction and chiral LC–MS/MS process

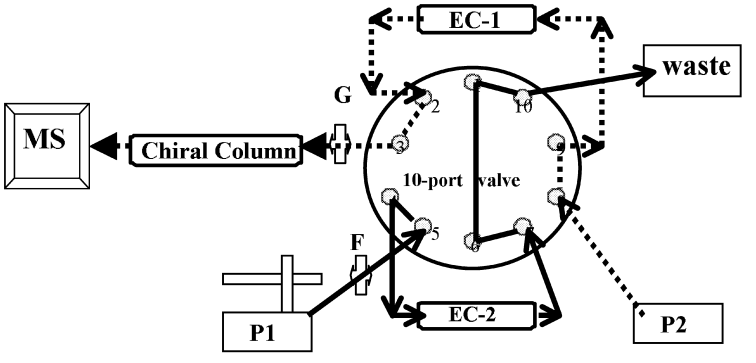
Prior to online analysis, a 25-μl aliquot of the internal standard working solution was added to 25 μl of drug-free (control) rat plasma (standard curve and QC samples) in a 96-well collection plate. The plate was then sealed and vortexed for 5 min and spun in a centrifuge for 10 min at 1650 *g* at 10 °C after which it was placed in the autosampler, at 10 °C, ready for analysis.

The schematic of our online dual-column extraction coupled with chiral LC–MS/MS system is shown in Fig. 1. The system consisted of a LEAP HTS-PAL autoinjector coupled with two Shimadzu LC-AD pumps (P1), two Perkin-Elmer Series 200 micro pumps (P2), a 10-port switching valve, a Chirobiotic T column, two Oasis HLB extraction columns (EC-1, EC-2), one in-line pre-column filter (F), one in-line guard column (G), and a PE Sciex API 3000 mass spectrometer. The loading mobile phase A was prepared by dissolving 0.77 g of ammonium acetate in 1.0 l of de-ionized water. The loading mobile phase B was 100% methanol. The eluting mobile phase C was prepared by dissolving 0.5 g of ammonium trifluoroacetate in 1.0 l of methanol. Lastly, the eluting mobile phase D was 100% methanol.

The detailed description of the online-column sample extraction LC–MS/MS methodology has been reported elsewhere [4,5]. In summary, the extraction and analysis included loading, eluting, and equilibration steps. The total analysis time per sample was 10 min, which consisted of 0.5 min for loading, and 9.5 min for elution and equilibration. The online bioanalytical process for the odd-number injections is illustrated in Fig. 1 (configuration A). The online analysis began with the loading process, in which the prepared plasma sample (10 μl) was injected onto EC-1 by P1 using the loading mobile phase profile described in Table 1. During this 0.5-min loading process, propranolol was retained on EC-1 and proteins and salts were diverted to waste. Concurrently, P2 delivered the isocratic eluting mobile phase, consisting of the eluting mobile phases



Configuration A



Configuration B

P1 = Leap autosampler coupled with two Shimadzu LC-AD VP pumps
(⟶ loading mobile phase pathway)
P2 = two Perkin-Elmer 200 micro pumps
(⇢ eluting mobile phase pathway)
EC-1 & **EC-2** = the first and second extraction columns (Waters Oasis HLB, 1x50 mm ID)
Chiral Column = Chirobiotic T chiral column (4.6x100 mm ID, 5 μm)
10-port valve = a Valco ten-port switching valve
F = in-line filters
G = in-line guard column (Zorbax RX-Sil, 2.1x12.5 mm ID)
MS = PE Sciex API3000 mass spectrometer

Fig. 1. Schematic diagrams of the online dual-column extraction in conjunction with chiral LC–MS/MS system.

C and D (75/25, v/v) at a flow-rate of 1.5 ml/min through EC-2 to the Chirobiotic T followed by MS/MS detection. At t=0.5 min, the 10-port switching valve was switched from configuration A to B. The elution and equilibration steps took place simultaneously and lasted for 9.5 min. In configuration B, propranolol retained on EC-1 was back-eluted by P2 through the chiral column and into the MS for enantiomeric analysis. Meanwhile, EC-2 was being equilibrated by P1. The online sample analysis (odd-number injections) was completed in approximately 10 min.

Table 1
Loading LC gradient program used by P1

Time (min)	Flow-rate (ml/min)	A (%)	B (%)	10-Port valve	Event
0.0	4.0	100	0		Loading
0.5	4.0	100	0	Open	
0.6	4.0	0	100		Elution and
2.6	4.0	0	100		equilibration
2.7	0.1	100	0		
9.0	0.1	100	0		
9.1	4.0	100	0		
10	4.0	100	0		

A, 10 m*M* ammonium acetate in water; B, 100% methanol.

In configuration B (Fig. 1), the next plasma sample (the even-number injection) was injected onto EC-2 by P1 to initiate the loading process. Concurrently, P2 delivered the eluting mobile phase through EC-1 to the chiral column. At $t=0.5$ min, the 10-port valve was switched from configuration B to A, and the propranolol retained on EC-2 was back-eluted by P2 by way of the chiral column and into the MS for detection. Meanwhile, EC-1 was being equilibrated by P1. The timing for P1 was controlled by P2. The PE Sciex Analyst software (version 1.1) controlled the timing for P2. The switching valve was controlled by P1 using timed-event contact closures. All the columns were maintained at ambient temperature.

2.5. Mass spectrometric conditions

The PE Sciex API 3000 triple quadrupole tandem mass spectrometer, equipped with an APCI source, was operated in the positive ion mode using multiple reaction monitoring (MRM). The MRM transitions are m/z 260.2→m/z 116.2 for propranolol and m/z 267.3→m/z 116.2 for D_7-racemic-propranolol. The LC flow was directed into the APCI source without splitting. The current of the corona discharge needle was set at 3 μA. The auxiliary gas temperature was maintained at 450 °C. Nitrogen was used as the nebulizer, curtain, and collision gas. The mass resolution was set to a peak width of 0.7 mass units at half-height for both Q1 and Q3. The electron multiplier was set at 2000 V. The product ion spectra of racemic-propranolol and D_7-racemic-propranolol are shown in Fig. 2. The dwell time of each MRM transition was set at 1.5 and 0.5 s for propranolol and D_7-propranolol, respectively. Declustering potential (45 V), collision energy (25 eV, laboratory-frame), entrance potential (−10 V), focusing potential (130 V), and collision cell exit potential (9 V) were set as indicated.

2.6. Assay validation

The peak area ratios of (*R*)-propranolol to D_7-(*R*)-propranolol (the internal standard for (*R*)-propranolol), (*S*)-propranolol to D_7-(*S*)-propranolol (the internal standard for (*S*)-propranolol) were plotted as a function of the nominal concentrations of (*R*)- and (*S*)-propranolol, respectively. The standard calibration curves for each enantiomer were constructed using a weighted $1/x$ linear regression. The equations for the calibration curves of (*R*)- and (*S*)-propranolol were then used to calculate the concentrations of (*R*)- and (*S*)-enantiomer, respectively. The linearity of the assay was established using standard calibration curve samples containing individual (*R*)- and (*S*)-propranolol in duplicate on each day of validation. The precision and accuracy of the method were evaluated by determination of the intra- and inter-day assay coefficients of variation (C.V.) using the QC samples, in five replicates, on each day of validation, and percentage of bias from the nominal concentrations of the QC samples, respectively. Intra- and inter-day assay C.V. and accuracy for QC samples were calculated using Watson software (version 6.2.0.02).

2.7. Application of the assay

The method was used for the quantification of (*R*)- and (*S*)-propranolol in rat plasma after intravenous administrations of 5 mg/kg (free base) of (*R*)-, (*S*)-, or racemic-propranolol with the dosing vehicle of ethanol–water (10:90, v/v) at 1 ml/kg. Blood samples (400 μl) were withdrawn from cannulated, adult male Sprague–Dawley rats ($n=3$; approx. 300 g; Charles Rivers Laboratories) at time zero (pre-dose), 10, 20, 30, 45, 60, 120, and 180 min after dosing into tubes containing EDTA as the anti-coagulant. Plasma samples were obtained by centrifugation and stored at −70 °C pending analysis.

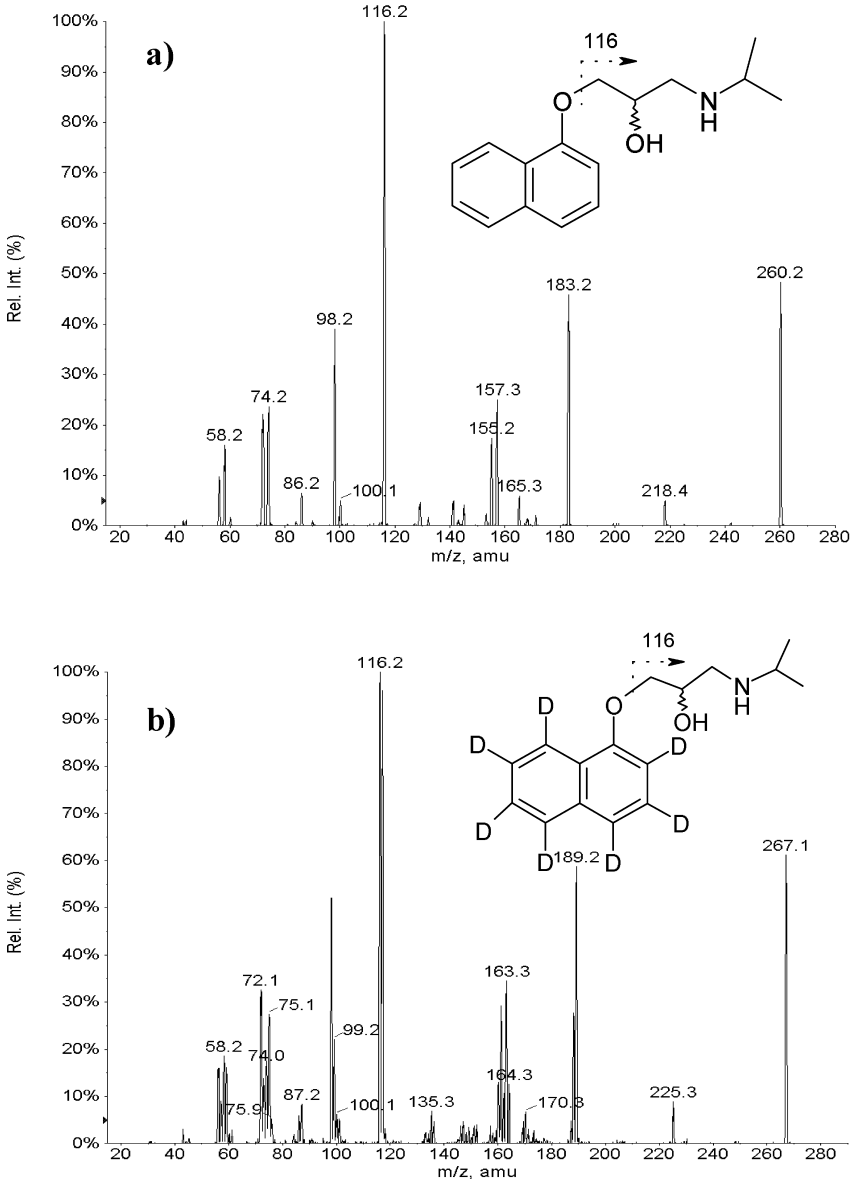


Fig. 2. Product ion spectra of (a) propranolol and (b) D_7-propranolol.

3. Results and discussion

3.1. Enantioseparation

Teicoplanin is a glycopeptide, which is produced by *Actinoplanes teichomyceticus*. Teicoplanin belongs to a class of novel macrocyclic antibiotics, which have several key characteristics including 23 stereogenic centers, seven aromatic rings, and three carbohydrate moieties that give rise to their relatively high surface activity. The teicoplanin CSP proved to be rugged and showed no deterioration of chromatographic separation after 600 injections (just one column was used for the entire validation and analysis of i.v. samples). The polar organic elution mobile phase, methanol containing 0.05% (by

weight) ammonium trifluoroacetate and methanol (75:25, v/v), was selected based on a previous report [5]. As shown in Fig. 3, the automated online column-extraction chiral LC–MS/MS method demonstrated excellent reproducibility and ruggedness. It illustrates the measured peak areas and retention times of D_7-(*R*)- and D_7-(*S*)-propranolol (from the internal standard) in rat plasma versus number of injections in a post-dosed analytical run. The relative standard deviation of the peak areas and retention times of the internal standard were within 8.3 and 1.6%, respectively, in more than 130 injections (equivalent to 23 h). Furthermore, under our assay conditions, no inter-conversion between (*R*)- and (*S*)-propranolol was detected (Fig. 4a,b). Fig. 4a shows the MRM chromatograms of (*S*)-propranolol

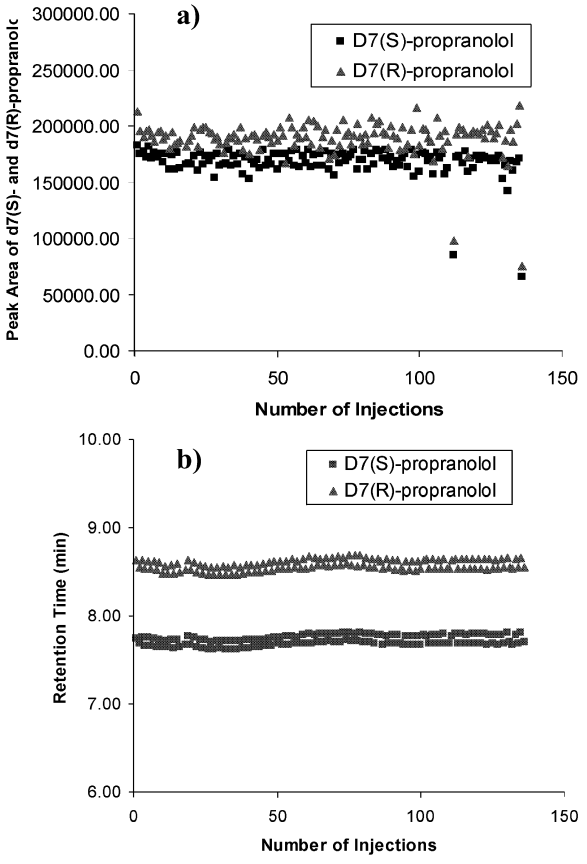


Fig. 3. (a) Peak areas; (b) retention times of D_7-propranolol measured from rat plasma samples versus number of injections during the analysis of propranolol in incurred samples.

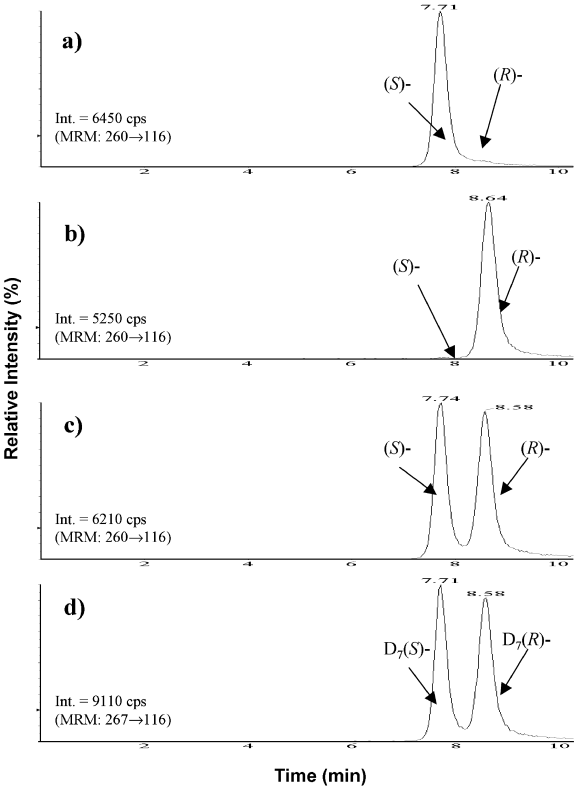


Fig. 4. MRM chromatograms of the quality control samples at 100 ng/ml in rat plasma obtained by using the online dual-column extraction coupled with Chirobiotic T and APCI-MS/MS system for (a) (*S*)-, (b) (*R*)-, (c) racemic-propranolol, (d) D_7-propranolol at 250 ng/ml in rat plasma.

QC sample at 100 ng/ml in rat plasma. There was no distinguishable (*R*)-propranolol signal at its respective retention time. Likewise, as indicated in Fig. 4b, there was no evidence for the ex vivo conversion of the (*R*)- to (*S*)-propranolol. The estimated resolution (R_s) and selectivity (α) factors were 1.83 and 1.16, and retention factors (k') were 2.57 and 2.97 for the (*S*)- and (*R*)-propranolol, respectively.

3.2. Online dual-column extraction

Recently, the use of high LC flow through an extraction column for online purification has been widely applied in assaying achiral drugs and metabolites in biological matrices. The detailed theory of the online-column extraction has been described previously [4,5,21,22]. An advantage for the use of

online-column extraction technique is that pretreatment of the biological sample has been eliminated, since it is injected directly onto extraction columns for the automated purification process. Although some online extraction equipment is commercially available, our assembled unit proved to be cost effective and simple to operate. We assembled the online dual-column LC–MS/MS system by simply installing a switching valve to the existing LC equipment, such as autoinjector, pump and mass spectrometer, already in our laboratory. The method development of the purification was relatively simple and the loading mobile phase was adapted from our previous work [5]. We have noticed that, in order to avoid the accumulation of endogenous material from plasma, adequate re-conditioning of the extraction column between each injection was essential to achieve good recovery and reproducibility. The use of single extraction column approach needed extra time for the re-conditioning process. The dual-column extraction approach eliminated the additional time required for re-conditioning of the extraction column because of the set-up of the system. This method allowed the use of one of the extraction columns for purification while the other was being equilibrated. Hence, the time required for re-conditioning the extraction columns did not contribute to the total analysis time per sample, which resulted in a relatively shorter run time and higher throughput. The Oasis HLB extraction column showed excellent ruggedness and reproducibility and at least 300 injections were made without loss of performance. The combination of the online extraction and chiral LC–MS/MS technique proved to be robust, simple and had a high throughput.

3.3. Mass spectrometry

Fig. 2a,b illustrates the protonated precursor ions of propranolol and D_7-propranolol at m/z 260 and 267, respectively. The most intensive product ion was at m/z 116 for both propranolol and D_7-propranolol. Although the MRM transitions had the common product ion of m/z 116, the precursor ions had different protonated molecular ions (m/z 267, 260). The prominence of this ion led us to propose that both propranolol and D_7-propranolol fragmented to yield the putative 1-methylethyl-amino-2-propanol ion. The MRM transitions at m/z 260.2→m/z 116.2 for propranolol and m/z 267.3→m/z 116.2 for D_7-racemic-propranolol were adopted in the current method since monitoring them resulted in good sensitivity and reproducibility of the method.

3.4. Calibration

Table 2 presents a summary of the standard curve data obtained for the 3-day validation of propranolol in rat plasma. The calibration curves for the (*R*)- and (*S*)-propranolol were linear from 2 to 2000 ng/ml with the coefficient of correlation better than 0.998. The accuracy of the back-calculated concentrations

Table 2
Precision and accuracy of calibration standard samples for propranolol enantiomers in rat plasma

Nominal conc.	(*S*)-Propranolol			(*R*)-Propranolol		
	Measured conc. (ng/ml)	RSD[a] (%)	Accuracy[b] (%)	Measured conc. (ng/ml)	RSD[a] (%)	Accuracy[b] (%)
2	2.03	8.9	102	1.93	8.3	97
5	4.96	7.9	99	5.01	8.8	100
10	9.78	6.8	98	9.83	6.1	98
25	25.81	5.5	103	25.64	4.4	103
100	98.56	4.8	99	99.98	6.0	100
500	487.49	5.8	98	508.84	6.2	102
1000	1026.81	4.3	103	1025.03	5.9	103
2000	1986.55	4.7	99	1965.75	5.6	98

[a] Expressed as coefficient of variation (C.V. %, $n=6$).

[b] [mean measured concentrations/nominal concentration]×100 ($n=6$). The mean slope ($n=3$) was 6.96×10^{-3} (SD=0.19×10^{-3}) for (*S*)- and 6.36×10^{-3} (SD=0.16×10^{-3}) for (*R*)-propranolol, respectively. The coefficient of correlation ($n=3$) was above 0.998 for both enantiomers.

from their nominal values was between 97 and 103%. The relative standard deviations (RSD) ranged from 4.3 to 8.9% for (*S*)- and 4.4–8.8% for (*R*)-propranolol, respectively.

3.5. Precision and accuracy

Table 3 summarizes the intra- and inter-day results obtained for the QC samples covering the anticipated dynamic concentration range of the method. The rat plasma QC samples were chosen to contain individual (*R*)-, (*S*)-, racemic- and (*R*)-/(*S*)-propranolol in ratios of 100:10 and 10:100. The intra- and inter-day precision values were within 7.6 and 3.6% for (*R*)-, and 6.9 and 4.5% for (*S*)-, respectively. The assay accuracy for the QC samples was between 97–103% for (*R*)-, and 92–100% for (*S*)-propranolol from their nominal values.

3.6. Recovery

The recovery studies were evaluated by a comparison between the response of propranolol spiked into plasma with one in which propranolol was spiked into a control plasma eluent using six different lots of control rat plasma at two concentrations (2 and 2000 ng/ml). To obtain the control plasma eluent, blank rat plasma samples were injected onto the extraction column for 0.5 min and the eluent was diverted to waste. The endogenous materials retained on the extraction column were back-eluted into a tube with the elution mobile phase for 5 min. The eluent was collected and evaporated under a stream of nitrogen at 30 °C. Subsequently, propranolol prepared in eluting mobile phase C, was spiked into the tube containing the dried eluent (Table 4). The recovery of propranolol was between 84.5 and 106% (see Table 4). The recovery for the internal standard D_7-racemic-propranolol was measured at concentration of 250 ng/ml and was between 103 and 105% (Table 4).

3.7. Specificity

The specificity of the method was examined by analysis of blank rat plasma. As shown in Fig. 5a,b, no interference was detected in the corresponding retention times of (*R*)-, (*S*)-propranolol, and D_7-propranolol, respectively. Fig. 5c,d shows the propranolol enantiomers in rat plasma at the lower limit of detection (0.5 ng/ml) and lower limit of quantification (2 ng/ml), respectively. The excellent sensitivity and selectivity were achieved by injection of only 10 μl of prepared rat plasma (Section 2.4), which contained 50% plasma and 50% the internal standard working solution.

The total reduction of the peak responses was evaluated by comparing the response of propranolol spiked into plasma with one in which propranolol was spiked into methanol. Table 4 shows that the propranolol response in plasma was reduced in about 9.5–17% in comparison to that in methanol. Since

Table 3
Precision and accuracy of QC samples for propranolol enantiomers in rat plasma

Nominal conc. (ng/ml)	(*S*)-Propranolol				(*R*)-Propranolol				*n*
	Mean measured conc. (ng/ml)	Precision[a] Intra-day (%)	Precision[a] Inter-day (%)	Accuracy[b] (%)	Mean measured conc. (ng/ml)	Precision[a] Intra-day	Precision[a] Inter-day (%)	Accuracy[b] (%)	
2	2.00	6.5	2.8	100	2.01	6.8	0.0	101	36
6	5.92	6.9	0.0	99	6.04	6.5	0.0	101	30
10	9.54	3.1	3.0	95	9.74	6.5	1.9	97	15
100	97.80	4.6	3.4	98	99.14	6.3	0.0	99	45
1000	959.94	2.4	4.5	96	995.86	7.6	0.0	99	30
1600	1479.98	3.3	3.1	92	1560.22	4.1	3.3	97	30
5000	4958.16	3.3	2.4	99	5145.03	2.9	3.6	103	25

[a] Expressed as coefficient of variation (C.V. %).
[b] [mean measured concentrations/nominal concentration]×100.

Table 4
Recoveries of propranolol in rat plasma following on-line LC–MS/MS analysis

		Peak area (mean±SD); $n=6$					
		2 ng/ml		2000 ng/ml		250 ng/ml	
		(*R*)-	(*S*)-	(*R*)-	(*S*)-	D_7-(*R*)-	D_7-(*S*)-
(a) Plasma sample		2.29±0.18e3	2.24±0.12e3	2.60±0.03e6	2.09±0.03e6	1.85±0.04e5	1.58±0.06e5
(b) Solvent sample		2.76±0.19e3	2.53±0.13e3	2.85±0.06e6	2.31±0.03e6	2.08±0.07e5	1.78±0.03e5
(c) Eluent sample		2.71±0.36e3	2.49±0.25e3	2.46±0.06e6	2.00±0.03e6	1.79±0.05e5	1.51±0.03e5
Total reduction	[(a)/(b)]×100%	83.0	88.5	91.2	90.5	88.9	88.8
Recovery	[(a)/(c)]×100%	84.5	90.0	106	105	103	105
Ion suppression effect[a]		1.5	1.5	14.8	14.5	14.1	15.2

[a] Ion suppression effect was determined by subtracting recovery from total reduction of peak response.

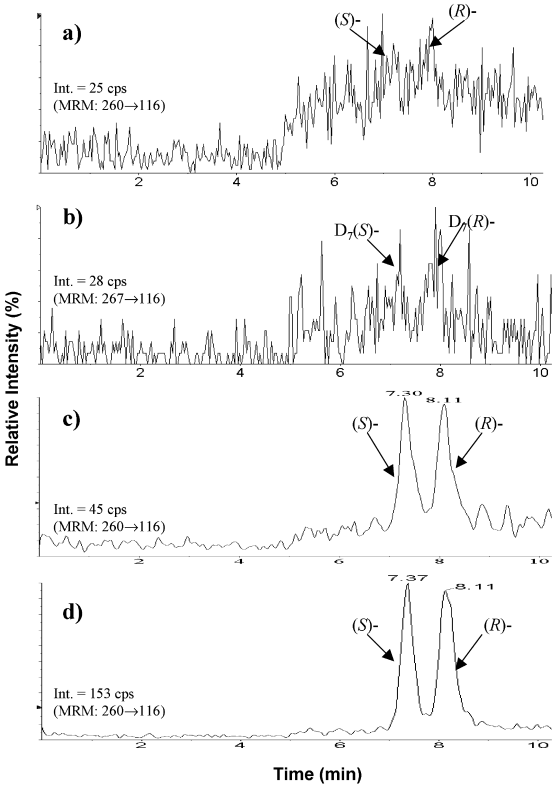


Fig. 5. MRM chromatograms obtained by using the online dual-column extraction coupled with Chirobiotic T and LC–APCI-MS/MS system for (a) MRM channel for a double blank rat plasma; (b) MRM channel for D_7-racemic-propranolol in a single blank of rat plasma; (c) racemic-propranolol at 0.5 ng/ml in rat plasma; (d) racemic-propranolol at 2 ng/ml in rat plasma.

the total reduction of the MRM chromatographic peak responses between plasma and methanol samples was the sum of reduction of recovery and the potential co-eluting endogenous materials [23–25], the matrix effect can be obtained by subtracting the recovery from the total reduction of peak response. Thus, the ion suppression of the matrix effect from six different sources of rat plasma on the analyte response was found to be between 1.5 and 15.2%. The underlying reason for this variability is still unclear and requires additional investigation.

3.8. Stability

The stability of propranolol in rat plasma was evaluated with QC samples at concentrations of 6, 100, 1000, 1600 ng/ml for (*R*)-, (*S*)-, and racemic-propranolol, respectively. These samples were analyzed in triplicate following different storage conditions. Propranolol was found to be stable in rat plasma stored in ambient temperature for at least 4 h, in −70 °C for at least 30 days. Propranolol was also stable for at least 48 h in the autosampler at 10 °C after addition of the internal standard working solution. Propranolol in methanol was stable for at least 30 days at 4 °C.

3.9. Assay application

The pharmacokinetics of propranolol in rats have been well-established [26–29] thereby providing a good measure of the success of our current assay

methodology. Our validated method was applied to the analysis of plasma samples from male Sprague–Dawley rats administered (*R*)-, or (*S*)-, or racemic-propranolol at 5 mg/kg (free base), by the intravenous route. Table 5 summarizes the pharmacokinetic parameters for the current i.v. study. Our data are in qualitative agreement with the study reported by Bode et al. [29]. The extrapolated area under the curve ($AUC_{0-\infty}$) was approximately 2-fold lower for the rats dosed with the racemate as compared to the individual enantiomers (Table 5). This observation was expected since the racemate consists of a 1:1 ratio of each enantiomer (i.e., 2.5 mg/kg of (*S*)-propranolol in the racemate versus 5 mg/kg in the pure (*S*)-enantiomer dose). The plasma elimination half-life ($t_{1/2}$) was not altered in animals dosed with the individual enantiomers compared to the animals that were dosed with racemic propranolol. Fig. 6 shows typical MRM chromatograms obtained from post dosing at 2 and 120 min. As shown in Fig. 6a, (*R*)- and (*S*)-propranolol were detected at 2 and 120 min after dosing of racemic-propranolol. In Fig. 6b, only the (*S*)-enantiomer was detected at 2 and 120 min subsequent to dosing of (*S*)-propranolol enantiomer to the rats. Likewise, Fig. 6c shows that only the (*R*)-enantiomer was detected at 2 and 120 min after dosing (*R*)-propranolol enantiomer. Individual pharmacokinetic parameters are shown in Table 5. Thus, there was no indication of an in vivo interconversion between the two enantiomers. The mean plasma concentration–time profiles for each dose group are also depicted in Fig. 7. The mean plasma con-

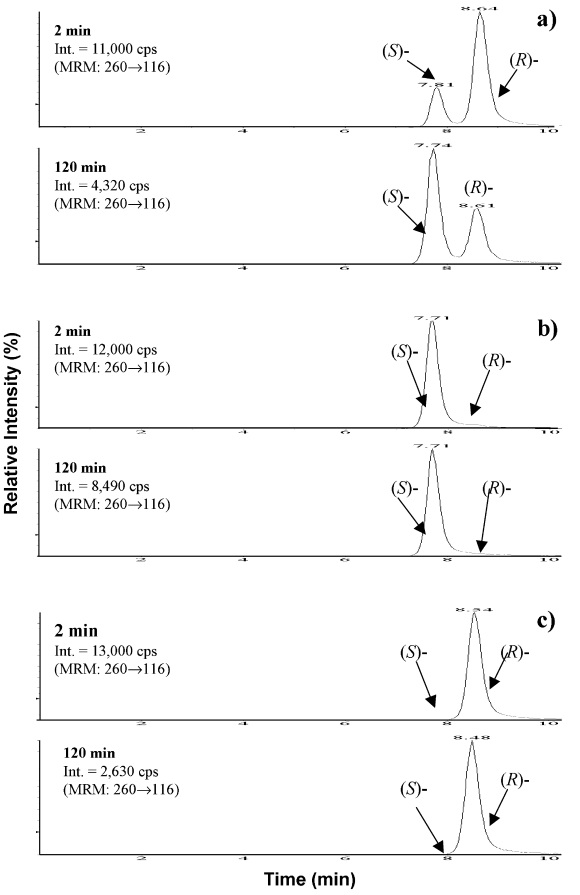


Fig. 6. Representative MRM chromatograms of rat plasma samples at 2 and 120 min after i.v. administration to rats: (a) racemic-propranolol; (b) (*S*)-propranolol; (c) (*R*)-propranolol.

Table 5
Mean pharmacokinetic parameters of propranolol enantiomers following intravenous administration of racemic-propranolol, (*R*)-propranolol, or (*S*)-propranolol to male Sprague–Dawley rats at 5 mg/kg (free base)

Dosed intravenously	*Racemic*	*Racemic*	(*S*)-	(*R*)-
Enantiomer measured	(*S*)-	(*R*)-	(*S*)-	(*R*)-
Body weight (g)	374±19	374	390±14	377±23
Propranolol dose (mg/kg)	4.59	4.59	4.47	4.07
Number of rats	3	2	3	3
$AUC_{0-\infty}$ [(μg·min/ml]	27.4±6.6	45.3	62.7±13.7	71.3±8.2
AUC_{norm} [(μg·min/ml]	59.7±14.4	98.8	70.2±15.3	87.7±10.1
$t_{1/2}$ (min)	60±19	26	66±5	25±5
Cl_p (ml/min)	28.8±6.0	17.4	24.8±4.2	17.5±2.0
V_d (l)	2.50±0.17	0.66	1.02±0.03	0.66±0.07

Cl_p, plasma clearance; V_d, volume of distribution at steady state; AUC, area under the plasma concentration–time profile. Values are mean±SD for $n=3$ rats except for (*R*)-propranolol in racemic-propranolol dose ($n=2$ rats, one outlier was rejected). AUC_{norm} [(μg·min/ml]: normalized to dose of 5 mg/kg.

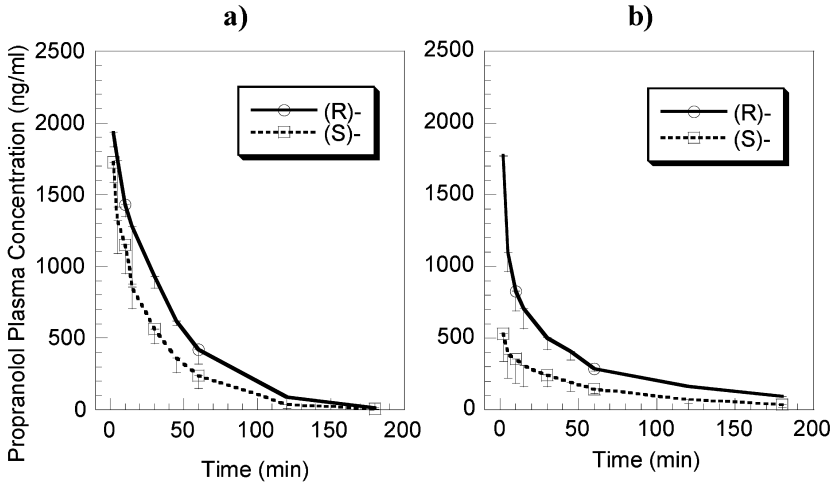


Fig. 7. Plasma concentration–time profile of (*R*)- and (*S*)-propranolol after intravenous (5 mg/kg, free base) administration of (*R*)-, (*S*)-, and racemic-propranolol to rats. Results are expressed as mean±SD ($n=3$ per group): (a) administration of (*R*)- or (*S*)-enantiomer; (b) administration of racemate.

centrations of the (*S*)-propranolol (the pharmacologically more potent isomer) were consistently lower than the (*R*)-enantiomer [27].

4. Conclusions

An automated, simple and selective online dual-column extraction in conjunction with chiral LC–APCI-MS/MS assay for the quantitative determination of propranolol enantiomers in rat plasma was developed and validated. High-flow online extraction, followed by enantiomeric separation provided higher throughput for the analysis of propranolol. Importantly, this approach has expanded the feasibility of the high-flow online column extraction LC–MS methodology from achiral to enantiomeric bioanalysis. Plasma samples from rats dosed intravenously with propranolol were analyzed successfully. The method proved to be rugged, accurate, and reproducible with no indication of any ex vivo interconversion of propranolol enantiomers. We envision the applicability of this assay to other biomatrices such as cerebral spinal fluid, brain extracts, and whole blood.

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