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Short communication

HPLC–electrospray mass spectrometric assay for the determination of (R,R)-fenoterol in rat plasma

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ABSTRACT

A fast and specific liquid chromatography–mass spectrometry method for the determination of (R,R)-fenoterol ((R,R)-Fen) in rat plasma has been developed and validated. (R,R)-Fen was extracted from 125 μl of plasma using solid phase extraction and analyzed on Atlantis HILIC Silica 3 μm column. The mobile phase was composed of acetonitrile:ammonium acetate (pH 4.1; 20 mM) (85:15, v/v), at a flow rate of 0.2 ml/min. The lower limit of detection (LLOD) was 2 ng/ml . The procedure was validated and applied to the analysis of plasma samples from rats previously administered (R,R)-Fen in an intravenous bolus.

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1. Introduction

Fenoterol (Fen, Fig. 1) is a β_2-adrenoceptor agonist that may have clinical value in the treatment of congestive heart failure [1,2]. Fen possesses two chiral centers and the drug is supplied as a racemic mixture of (R,R)-Fen and (S,S)-Fen. Previous studies using cardiomyocytes contractility have demonstrated that (R,R)-Fen is the active component and this compound is currently under development as a therapeutic agent [1,2].

As part of the preclinical studies, the pharmacokinetics of (R,R)-Fen in rats was investigated, and an assay was developed to quantify (R,R)-Fen concentrations in rat plasma. The determination of Fen in plasma requires a sensitive assay as the plasma concentrations of the drug are commonly <10 ng/ml due to the compound's poor bioavailability and extensive metabolism via phase II pathways [3]. Plasma concentrations of Fen have been measured using a radioimmunoassay [4] and enzyme immunoassay [5] techniques as well as gas chromatography–MS [6,7], LC–APCI–MS [8] and HPLC with fluorescence detection [9]. However, these methods require either expensive antibodies, derivatization procedures or are not sensitive enough for the determination of low Fen plasma concentrations that are required for pharmacokinetic studies. Here, we present a fast, sensitive and specific assay for the determination of (R,R)-Fen in rat plasma which can be also adapted to other matrixes.

2. Experimental

2.1. Materials

(R,R)-Fenoterol ((R,R)-Fen) was prepared as previously described [2]. Ritodrine (Rit) hydrochloride, metaproterenol hemisulfate, ractopamine hydrochloride and formic acid were purchased from Sigma–Aldrich (St. Louis, MO). HPLC-grade acetonitrile, methanol and ethyl acetate were supplied by Fisher Scientific (Pittsburgh, PA). Purified water was prepared using a Milli-Q system (Millipore, Milford, MA). Control rat plasma collected from whole blood onto sodium EDTA was purchased from Innovative Research (Novi, MI).

2.2. Chromatographic conditions

The chromatography was carried out using an Agilent Technologies (Palo Alto, CA) 1100 LC/MSD Series (liquid chromatography-mass selective detector) composed of a vacuum degasser (G1379 A), a quarternary pump (1311A) a thermostated autosampler (G1329 A) and a thermostated column compartment (G1316A). The mass selective detector (MSD Quad SL, G1956B) was used with

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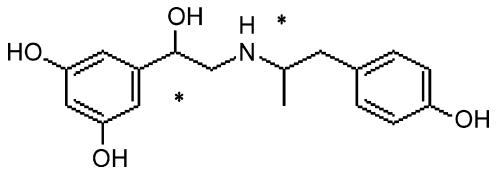

Fig. 1. Molecular structure of fenoterol. Asterisk "*" indicates position of a chiral carbon.

electrospray ionization interface (ESI) and on-line nitrogen generation system (Parker, Haverhill, MA, USA). The data was acquired by ChemStation software, Rev.A.10.02 [1757] (Agilent Technologies, Palo Alto, CA). The analysis was achieved with the use of an Atlantis HILIC Silica 3 μm (2.1 mm × 150 mm) column connected to an Atlantis HILIC Silica 3 μm guard column (2.1 mm × 10 mm) (Waters, Milford, MA).

The mobile phase was composed of acetonitrile:ammonium acetate (pH 4.1; 20 mM) (85:15, v/v) and was used at a flow rate ranging from 0.2 ml/min at time 0–10.0 min and from 15.1 to 17.0 min while flow rate of 0.3 ml/min was used between 10.01 and 15.0 min. To avoid excessive entry of complex plasma components into MS detector the flow was directed into the instrument only during the window time for the target compounds analysis, between 4.5 and 10 min of each chromatographic run.

2.3. Optimization of the mass selective detector (MSD) parameters

An ESI was used with the MSD operating in the positive ion mode. The optimized conditions for (R,R)-Fen and Rit were as follows: fragmentor voltage 70 and 100 V, respectively, gain 10, drying gas flow 10 l/min, nebulizer pressure 20 psig, drying gas temperature at 350 °C, capillary voltage 4000 V. Target compounds were quantified in a single ion-monitoring (SIM) mode. (R,R)-Fen was monitored at m/z 304.2 while Rit at m/z 288.2.

2.4. Extraction procedure

Rat plasma samples, ~0.150 ml, were thawed and centrifuged at 2643 × g for 5 min, a 0.125-ml aliquot was transferred to a polypropylene Eppendorf tube, acidified with 0.250 ml of 2% formic acid and vortex-mixed for 30 s. An aliquot of 20 μl of internal standard (Rit hydrochloride, working solution concentration 0.2 μg/ml) was added and the solution was vortex-mixed again for 30 s. The analytes were extracted using solid-phase extraction (SPE) with Bond Elut Plexa 1 ml cartridges (Varian, Palo Alto, CA). Cartridges were conditioned with 1 ml of MeOH and equilibrated with 1 ml of 2% formic acid. The cartridges were then loaded with samples and washed with 1 ml of water. The analytes were eluted with 2 ml of a mixture of ethyl acetate:methanol (1:1, v/v). The solution was evaporated to dryness in a Speed-Vac (Thermo Savant, NY) for a 1.5 h at a temperature of 80 °C. The residue was reconstituted in 20 μl of methanol, vortex-mixed for 20 s followed by the addition of 80 μl of acetonitrile and vortex-mixed for 10 s. The reconstituted samples were centrifuged for 6 min at 2643 × g and a 15-μl aliquot of the supernatant was injected onto HPLC system.

2.5. Preparation of standard solutions

A stock solution of (R,R)-Fen was prepared in methanol at a concentration of 1 mg/ml. Working solutions were prepared in methanol in amounts corresponding to concentrations of standard curve points, 2.0, 4.0, 8.0, 40.0, 160.0, 400.0, 1600.0 ng/ml (final concentrations). All solutions were kept at −20 °C.

2.6. Calibration curves, quality controls

Calibration and quality controls were prepared daily by spiking 115 μl of plasma with 10 μl of a corresponding working solution. (R,R)-Fen calibration curves were prepared in the following concentrations: 2.0, 4.0, 8.0, 40.0, 160.0, 400.0, 1600.0 ng/ml using Rit (28 ng/ml) as internal standard (final concentrations). One standard curve along with two sets of quality control samples were prepared each day of analysis. The quality control concentrations were as follows: 4.0, 40.0 and 400.0 ng/ml for low quality control (LQC), middle quality control (MQC) and high quality control (HQC), respectively.

2.7. Matrix effect (ME), recovery (RE) and process efficiency (PE)

Matrix effect studies were performed according to Matuszewski et al. [10]. They were studied at three quality control levels, 4.0, 40.0, and 400.0 ng/ml. The detailed description of quantification has been previously published [11].

2.8. Precision and accuracy

Both precision and accuracy were studied on three different days by analyzing QC samples with $n = 5$. Precision and accuracy were also determined for lower limit of quantification (LLOQ) with $n = 5$. The acceptance criteria were taken from the FDA Guidance [12] where the mean accuracy value for QCs should be within 15% of the actual value except for LLOQ, 20%. Precision determined for each QC level also should not exceed 15% of CVs and 20% for LLOQ.

2.9. Stability studies

(R,R)-Fen stability tests investigated were freeze–thaw and post-preparative studies for plasma extracts kept in the auto-sampler up to 12 h. Freeze–thaw tests were performed for three levels of QCs over 3 days while post-preparative tests were done for low and high QC.

2.10. Animal study

The pharmacokinetic study of (R,R)-Fen was performed by SRI International (Menlo Park, CA). The study was approved by Institutional Animal Care and Use Committee (study nb. B246-07). The drug was administered to male Sprague–Dawley rats at a dose of 5 mg/kg by IV administration. The blood samples (0.250 ml) were collected and processed to plasma at nine timepoints; predose, 5, 15, and 30 min; 1, 2, 4, 5 and 6 h post-dose. Samples were shipped on dry ice and stored at −80 to −75 °C for 5 months before analysis.

3. Results and discussion

3.1. Chromatographic conditions

Since reversed-phase C_{18} columns are highly compatible with mass spectrometer, initial LC/MS studies of (R,R)-Fen were carried out by using a C_{18} column (Phenomenex Luna, 150 mm × 2.1 mm i.d.) with mobile phases containing varying mixtures of ammonium acetate (50 mM, pH 6.0) and methanol. Retention of (R,R)-Fen on the Phenomenex Luna C_{18} column was low ($k \leq 3$) even at high composition of aqueous mobile phase (95% of ammonium acetate). At a mobile phase composition of ammonium acetate (pH 6.0; 50 mM):methanol (95:5, v/v)) ion suppression was observed due

to the insufficient desolvation at MS interface and coelution of extraneous compounds with (R,R)-Fen.

An additional problem was that the reconstitution of plasma extracts with a high composition of ammonium acetate buffer produced hazy solutions that were not suitable for analysis by LC–MS. This problem was overcome by using methanol:acetonitrile (20:80, v/v) to reconstitute the sample. The high amount of organic solvent permitted the use of a hydrophilic interaction liquid chromatography (HILIC) column instead of the reversed phase column. The HILIC column gave several advantages. In HILIC mode, the weak mobile phase is organic solvent, thus, it was possible to use higher amount of acetonitrile in mobile phase which resulted in a more efficient desolvation of (R,R)-Fen in the MS interface and a higher ion signal.

The addition of modifiers (TFA and acetic acid) was also studied since these have substantial effects on selectivity and efficiency [13]. However, we found no significant improvements of the HPLC separation or peak shape of (R,R)-Fen. Thus, the optimized mobile phase condition was acetonitrile:ammonium acetate (pH 4.1; 20 mM) (85:15, v/v).

A variable mobile phase flow rate was used during the chromatographic run. The mobile phase was delivered at a flow rate ranging from 0.2 ml/min at time 0–10.0 min and from 15.1 to 17.0 min while flow rate of 0.3 ml/min was used between 10.01 and 15.0 min. The change of mobile phase flow rate to 0.3 ml/min within the run (10.01–15.0 min) was used to elute extraneous materials from HILIC column. A representative chromatogram of the standards is presented in Fig. 2A. Retentions of (R,R)-Fen and Rit on HILIC column under optimized conditions were the same with capacity factors equal 1.9 for both analytes.

3.2. Extraction procedure

The initial SPE studies used polymeric ion exchange columns, which had been previously suggested [14]. Mixed-mode cation exchange (MCX) and mixed mode anion (MAX) exchange cartridges were studied. However, recoveries of the target compounds from neat solutions were less than 50% in both cases. The losses were probably due to rapid oxidation of (R,R)-Fen in basic solutions [unpublished data] which were used in different steps of extraction procedures for both ion exchange sorbents. Losses of Fen during evaporation from basic solutions have been observed by Josefsson and Sabanovic [14]. Neutralization of the solution (ammonium hydroxide in methanol) used to elute (R,R)-Fen from MCX cartridges, with 100 μl of 20 mM tartaric acid or with 0.5% trifluoroacetic acid in volumes varying from 50 to 500 μl did not significantly increase the RE of (R,R)-Fen.

Therefore, cartridges with other packing materials were tested: C_{18} (Varian Bond Elut), aminopropyl (Varian SPEC NH2), phenyl (Varian SPEC PH) and polymeric (Varian Bond Elut Plexa). The most promising were polymeric Bond Elut Plexa cartridges partially due to the fact that it was not necessary to use basic solutions throughout the extraction procedure. During the optimization step it was found that the most efficient approach was to perform the intermediate wash with pure water and to elute with 2 ml of ethylene acetate:methanol (1:1, v/v). Chromatograms of extracted blank plasma and extracted spiked plasma are presented in Fig. 2B and C, respectively.

In the optimization step several compounds were tested for IS as deuterated Fen was not commercially available at the time of the study. Ractopamine, metaproterenol and Rit were chosen for

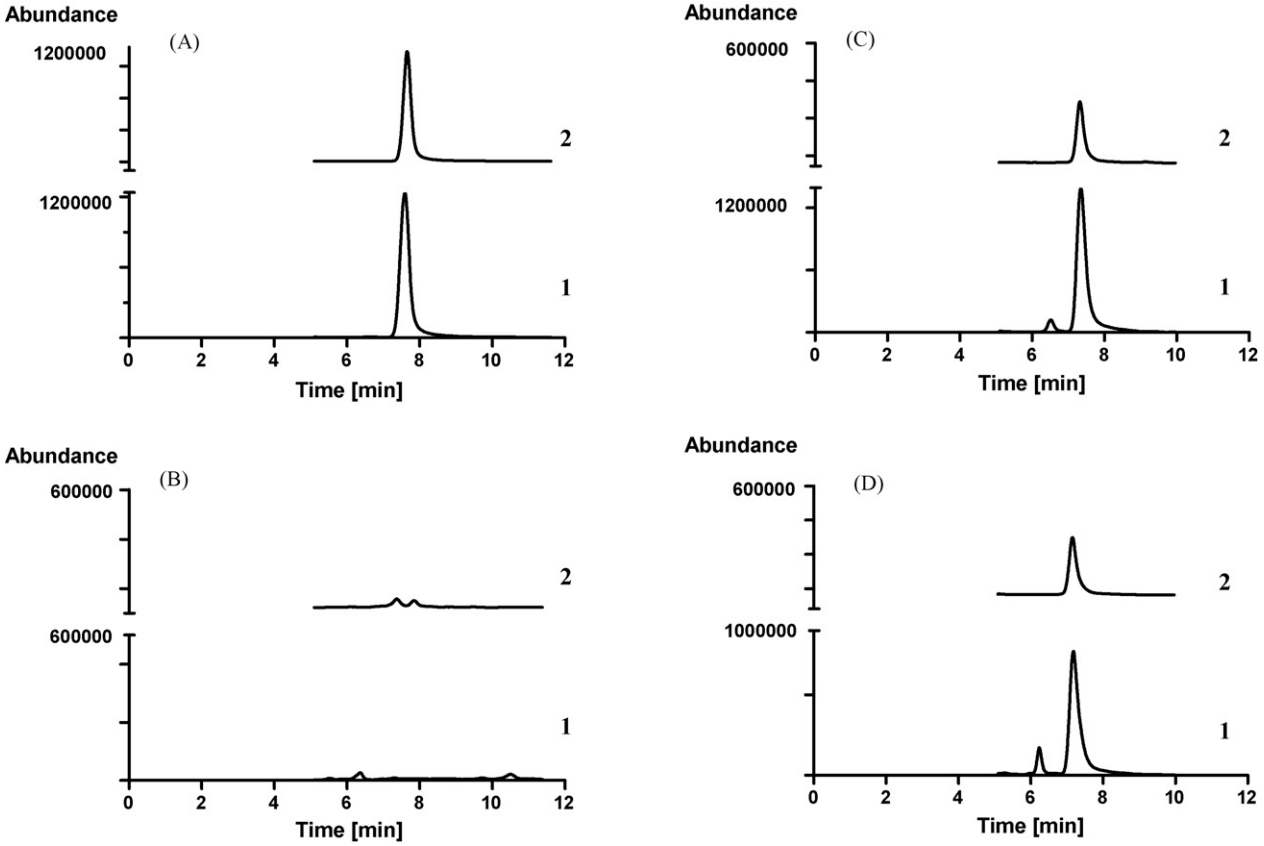

Fig. 2. HPLC chromatogram of 15 μl injection of an extract of (A) neat solution of (R,R)-Fen and Rit, (B) blank rat plasma extract, (C) control plasma spiked with (R,R)-Fen (HQC) and Rit and (D) rat plasma extract at 15 min after IV administration of (R,R)-Fen in a dose of 5 mg/kg. The quantified drug concentration was 245.5 ng/ml. Numbers 1 and 2 on the graphs correspond to *m*/*z* 304.2 ((R,R)-Fen) and *m*/*z* 288.2 (Rit), respectively.

Table 1
Precision and accuracy data in determination of (R,R)-Fen in rat plasma

Compound	Nominal concentration (ng/ml)	Precision		Accuracy	
		Intra-day CV (%) ($n = 5$)	Inter-day CV (%) ($17 \leq n \leq 19$)	Concentration calculated ($n = 5$)	Average (%) ($n = 5$)
(R,R)-Fenoterol	2.0 (LLOQ)	9.2	ND	1.7	82.7
	4.0 (LQC)	12.2	12.3	3.9	97.0
	40.0 (MQC)	14.8	10.8	38.6	96.6
	400.0 (UQC)	6.0	6.2	379.6	94.9

screening tests because of their structural similarity to analyte of interest. Ractopamine had to be eliminated due to cross-talk with (R,R)-Fen and metaproterenol due to a large difference in retention between R,R-Fen and metaproterenol ($k = 1.9$ for (R,R)-Fen and 3.0 for metaproterenol). Rit had the closest k ($k = 1.9$) to that of (R,R)-Fen and was chosen for further studies (Fig. 2B). Although the extracted blank plasma contained some interferences around the retention time of Rit, the validation process proved that the interferences were insignificant and that the selected compound was suitable for the assay (see below).

3.3. Calibration curves

The calibration curves were generated by a nonlinear regression with a use of a weighted ($1/X$) polynomial second-order equation. The standard curve was constructed daily for area ratios of (R,R)-Fen over Rit in the range from 2 to 1600 ng/ml. The average correlation coefficient was equal to 0.9996 ($\pm$0.0005) ($n = 10$).

3.4. Method validation

The intra-day and inter-day precision data investigated for QCs are presented in Table 1. Both parameters were in agreement with the FDA guidance [12]. ME data along with RE and PE are presented in Table 2. Results of the freeze and thaw study are presented in Table 3. Freeze–thaw assay reviled that (R,R)-Fen is unstable in low concentrations and that the analyte is stable only during two cycles. Stability of spiked plasma extracts in the autosampler was tested up to 12 h and they were only stable up to 6 h (Table 3).

Table 2
Matrix effect (ME), recovery (RE) and process efficiency (PE) data for (R,R)-Fen studied in rat plasma (quantified from peak area ratios of a compound/IS)

	Matrix effect (ME) %	Recovery (RE) %	Process efficiency (PE) %
4 (ng/ml)	61.5	103.2	63.5
40 (ng/ml)	64.0	105.2	67.3
400 (ng/ml)	75.6	95.6	72.3
Average	67.0	101.3	67.7
S.D.	7.5	5.1	4.4
CV	11.2	5.0	6.5

Table 3
Stability of compound in the freeze–thaw and post-preparative tests

Nominal concentration (ng/ml)	CV (%)	Accuracy (%)
Freeze–thaw studies ($n = 3$)		
4.0 (LQC)	22.9	82.0
40.0 (MQC)	14.2	99.4
400.0 (UQC)	9.2	87.8
Post-preparative stability studies (up to 6 h)		
4.0 (LQC)	2.4	102.8
400.0 (UQC)	4.0	88.5

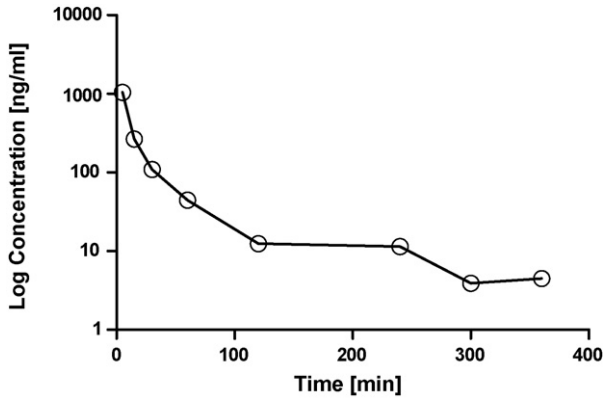

Fig. 3. Representative plasma–concentration time plot after IV administration of (R,R)-Fen in a dose of 5 mg/kg to a Sprague–Dawley rat.

3.5. Assay application

The validated method was applied in a pharmacokinetic study of (R,R)-Fen administered to male Sprague–Dawley rats as 5 mg/kg intravenous bolus. A representative chromatogram of rat plasma obtained 15 min after administration is presented in Fig. 2D and a representative plasma–concentration time plot is presented in Fig. 3.

4. Conclusions

The presented results prove that the assay is accurate, precise, reproducible and sensitive and can be applied to determination of (R,R)-Fen in plasma after IV administration of the drug to rats.

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