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Development and Validation of a Sensitive LC-MS/MS Method for the Determination of Fenoterol in Human Plasma and Urine Samples

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Abstract

Due to the lack of sensitivity in current methods for the determination of fenoterol (Fen). A rapid, LC-MS/MS method was developed for the determination of (R,R)-Fen and (R,R;S,S)-Fen in plasma and urine. The method was fully validated and was linear from 50 pg/ml to 2000 pg/ml for plasma and from 2.500 ng/ml to 160 ng/ml for urine with a lower limit of quantitation of 52.8 pg/ml in plasma. The coefficient of variation was <15% for the high QC standards and <10% for the low QC standards in plasma and was <15% for the high and low QC standards in urine. The relative concentrations of (R,R)-Fen and (S,S)-Fen were determined using a chirobiotic T chiral stationary phase. The method was used to determine the concentration of (R,R)-Fen in plasma and urine samples obtained in an oral cross-over study of (R,R)-Fen and (R,R;S,S)-Fen formulations. The results demonstrated a potential pre-systemic enantioselective interaction in which the (S,S)-Fen reduces the sulfation of the active (R,R)-Fen. The data suggests that a non-racemic mixture of the Fen enantiomers may provide better bioavailability of the active (R,R)-Fen for use in the treatment of cardiovascular disease

Keywords

Congestive heart failure; bioavailability; chirobiotic T; β_2 -adrenergic receptor agonist

1. Introduction

Fenoterol (Fen), Fig. 1, is a β_2 -adrenergic receptor (β_2 -AR) agonist with 2 chiral centers resulting in 4 possible stereoisomers [1]. The racemic mixture of (R,R)-Fen and (S,S)-Fen, (R,R;S,S)-Fen, has been approved for the treatment of asthma [2] and this formulation is approved for clinical use in Europe. Recently, *in vitro* studies using a rat cardiomyocyte model has demonstrated that (R,R)-Fen is a potent and selective β_2 -AR agonist, which produces significant contractility in the model, while (S,S)-Fen was essentially

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pharmacologically inactive [3,4]. This observation has led to the development of (R,R)-Fen as a potential agent in the treatment of congestive heart failure. As part of the (R,R)-Fen development program, we have conducted an open label, dose escalation study in healthy volunteers to determine the safety, pharmacokinetics and bioavailability of (R,R)-Fen after oral administration and to compare the effects to equivalent doses of (R,R ;S,S)-Fen.

In humans, (R,R ;S,S)-Fen has a low bioavailability (~2%) after oral administration due to extensive presystemic sulfation by sulfotransferase (SULT) enzymes [5,6]. The SULT mediated metabolism of Fen is regioselective and enantioselective as SULT1A1 preferentially catalyzes sulfation at the 3,5-dihydroxyphenyl position and is selective for (S,S)-Fen, while SULT1A3 mediates sulfation at the 4-hydroxyphenyl position where it is highly selective for (R,R)-Fen and at the 3,5-dihydroxyphenyl position where it has a slight preference for (S,S)-Fen [7]. Because of the poor bioavailability, the study of the comparative pharmacokinetics (R,R)-Fen and (R,R ;S,S)-Fen after oral administration requires a sensitive and selective bioanalytical assay as pg/ml plasma levels of the drugs were expected. A number of methods have been reported for the determination of (R,R ;S,S)-Fen in plasma and urine of various species including absorptive voltammetry [8], radioimmunoassay [9], enzyme immunoassay [10], capillary electro-chromatography-mass spectrometry (MS) [11], LC-APCI-MS, capillary zone electrophoresis [12], gas chromatography-MS [13], HPLC with fluorescence detection [14,15] and LC-ESI-MS [16]. However, none of these methods had the sensitivity required for the current study. In addition, these methods involved extensive workup or derivatization. We have recently reported a sensitive LC-MS method for the determination of (R,R)-Fen in rat plasma after iv administration of the single enantiomer which had a lower limit of quantitation of 2 ng/ml [17]. This method was used as the starting point for the assay reported in this manuscript.

Since the study was designed to compare the bioavailability of (R,R)-Fen after administration of the racemic and single enantiomer formulations, the enantioselective separation of the (R,R)-Fen and (S,S)-Fen had to be included in the bioanalytical approach. Enantioselective separations of (R,R)-Fen and (S,S)-Fen have been reported using an amylose tris (3,5-dimethylphenylcarbamate) (Chiralpack AD) LC column [18], capillary zone electrophoresis [19], an CE separations using cyclodextrin derivatives [20–22] and -glycoprotein [23]. These methods all lacked sufficient sensitivity for our assays and in some cases also required extensive workup.

We now report the development and validation of a direct and sensitive LC-MS/MS method using an Atlantis HILIC stationary phase which can be used for the quantification of (R,R)-fenoterol after oral administration of the single enantiomer formulation and (R,R ;S,S)-Fen after the administration of the racemic formulation. In this approach, the relative concentrations of (R,R)-Fen and (S,S)-Fen were determined using a chirobiotic T chiral stationary phase. The method has been applied to the analysis of the plasma and urine samples from the cross-over study described above.

2. Materials and Methods

2.1. Materials

(R,R)-Fen, (R,R ;S,S)-Fen and (R,R)-4-methoxynaphthylfenoterol (MNF) were prepared as previously described [24]. Acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Purified water was prepared using a Milli-Q system (Novi, MI).

2.2. Chromatographic conditions

2.2.1. Instrumentation—The chromatographic experiments were carried out on a Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA). The samples were

introduced to the analytical column using Shimadzu SIL-20A autosampler and maintained at 4°C in the autosampler tray, and injections of 30 µl were made.

2.2.2. Achiral separations—The achiral separations of (R,R)-Fen and (R,R ;S,S)-Fen from their corresponding sulfate metabolites were accomplished using an Eclipse XDB-C18 guard column (4.6 mm × 12.5 mm) and an Atlantis HILIC Silica 3 µm analytical column (2.1 × 150 mm) purchased from Waters (Milford, MA). The internal standard used was MNF (5 µg/ml, for a final concentration of 20 ng/ml). The mobile phase consisted of water containing 0.1% formic acid as Component A and acetonitrile as component B. A linear gradient was run as follows: 0–2 min 50% B; 2min–5 min 80% B; 5–20min 50% B at a flow rate of 0.3 ml/min. The total run time was 20 min per sample.

2.2.3. Enantioselective separations—The enantioselective separation of (R,R)-Fen and (S,S)-Fen was accomplished using an Eclipse XDB-C18 guard column (4.6 mm × 12.5 mm) and an Astec Chirobiotic T 5 µm analytical column (4.6 mm × 25 cm) purchased from Sigma (St. Louis, MO). The mobile phase was methanol containing 0.2% acetic acid and 0.01% triethylamine. The flow rate was set at 0.8 ml/min. The total run time was 15 min per sample. Injection volume per sample was 60 µl.

2.2.4. Mass spectrometry conditions—MS/MS analysis was performed using a triple quadrupole mass spectrometer model API 4000 system from Applied Biosystems/MDS Sciex equipped with Turbo Ion Spray® (TIS) (Applied Biosystems, Foster City, CA, USA). The data was acquired and analyzed using Analyst version 1.4.2 (Applied Biosystems). Positive electrospray ionization data were acquired using multiple reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray), collision gas and ion spray voltage were 500°C, 10 psi, 60 psi, 60 psi, 8 psi and 5500 V, respectively. The TIS compound parameter settings for declustering potential, entrance potential, and collision cell exit potential were 70 V, 10 V, and 12 V, respectively. The collision energy setting was 20V for (R,R)-Fen and (R,R ;S,S)-Fen, and 30V for MNF and the sulfate metabolites of Fen. The standards were characterized using the following MRM ion transitions: (R,R)-Fen and (R,R ;S,S)-Fen (304.1/135.1); Fen sulfate metabolites (384.4/135.1); MNF (369.2/200.1).

2.3. Sample preparation

2.3.1. Plasma—Plasma sample (475 µl) was combined with 500 µl of 2% formic acid, 15 µl methanol and 10 µl of the internal standard, MNF (5 µg/ml). The extractions were carried out as previously described [17]. Briefly, the 1 ml solid phase extraction (SPE) cartridges (Bond Elut Plexa, Varian, Palo Alto, CA) were preconditioned with 1 ml of methanol, followed by 1 ml of water containing 2% formic acid. The cartridge was then loaded with sample and washed with 1 ml of water and then the compounds were eluted with 1 ml of ethyl acetate: methanol (1:1), which was transferred to the autosampler vial for analysis.

2.3.2. Urine—Urine sample (100 µl) was combined with 390 µl methanol and 10 µl of the internal standard, MNF (5 µg/ml). The samples were then vortex mixed for 30 s and centrifuged at 20,800 × g for 10 minutes at 4°C and a 30 µl aliquot of the supernatant was injected into the chromatographic system and analyzed.

2.4. Validation of the achiral method- plasma and urine

2.4.1. Stock solution—A concentrated stock solution was prepared containing 1 mg/ml. The solution was prepared in ultra-pure water and stored in polypropylene tubes at –20°C. Serial dilutions of the stock solution were used to prepare the samples for the calibration curves and quality control standards (QC).

2.4.2. General procedures—The quantification of (R,R)-Fen or (R,R ; S,S)-Fen was accomplished using area ratios calculated using MNF as the internal standard. Quality control standards were prepared daily by adding 10 µl of the corresponding spiking standard solution to 475 µl of drug free plasma or 100 µl of drug free urine purchased from Bioreclamation (East Meadow, NY, USA).

2.4.3. Calibration curves and QC standards (plasma)—(R,R)-Fen calibration curves were prepared in the following concentrations: 52.79, 105.58, 211.17, 281.56, 375.42, 500.56, 667.41, 889.89, 1186.52, 1582.03, 2109.37 pg/ml using MNF (20 ng/ml) as internal standard (final concentrations). One standard curve along with at least three sets of quality control samples was prepared each day of analysis. The quality control concentrations were as follows: 105.58, 281.56, 667.41 and 1126.5 pg/ml for low quality control (QC₁), middle quality control (QC₂; QC₃) and high quality control (QC₄), respectively.

2.4.4. Calibration curves and QC standards (urine)—(R,R)-Fen calibration curves were prepared in the following concentrations: 2.5, 5, 10, 20, 40, 80, 160 ng/ml using MNF (20 ng/ml) as internal standard (final concentrations). One standard curve along with at least three sets of quality control samples was prepared each day of analysis. The quality control concentrations were as follows: 5, 20 and 80 pg/ml for low quality control (QC₁), middle quality control (QC₂) and high quality control (QC₃), respectively.

2.4.5. Extraction efficiency and matrix effects—The extraction efficiency was determined by injecting three replicates at the four QC concentrations for (R,R)-Fen in plasma and urine. Blank plasma was spiked with fenoterol and MNF before and after liquid–liquid extraction. The percent extraction efficiency from plasma and urine was calculated as the mean peak area of samples spiked before extraction divided by that of samples spiked after extraction.

Matrix effects were also evaluated using 5 lots. Blank plasma/urine was spiked with (R,R)-Fen and MNF post-extraction and the peak areas of the post-spiked sample was compared to the peak areas of the (R,R)-Fen and MNF spiked in standard solution. The matrix effect percentage was calculated as the mean peak area of the reconstituted solution over the mean peak area of the reference solution for each QC.

2.4.6. Stability—Analyte stability was evaluated under a variety of conditions. Prepared sample stability was determined by triplicate measurements of the QC's stored at 4 °C for 24 h after preparation and 1 h and 4 h at room temperature. Additionally, stability was demonstrated for spiked plasma/urine with three freeze–thaw cycles. Acceptable stability was achieved if the sample could be quantified within ±15% of the expected value.

2.5. Crossover pharmacokinetic study of (R,R')-Fen and (R,R';S,S')-Fen after oral administration

The bioavailability, clearance and excretion of (R,R)-Fen and (R,R ;S,S)-Fen after oral administration of (R,R)-Fen or (R,R ;S,S)-Fen was determined in healthy volunteers using an open label dose escalation and crossover clinical design (Clinical Trials.gov identifier - NCT01440335). After obtaining informed consent, the volunteers received an oral dose of each formulation, i.e. (R,R)-Fen and (R,R ;S,S)-Fen, in a randomized order with a one-week washout between dosing. The test compounds were administered in the following escalating dose levels: 2.5 mg (R,R)-Fen, 5 mg (R,R ;S,S)-Fen; 5.0 mg of (R,R)-Fen, 10 mg (R,R ;S,S)-Fen; 10 mg (R,R)-Fen, 20 mg (R,R ;S,S)-Fen. The drug substances and formulations used in this study were manufactured and formulated by SRI International

under the NIA contracts N01-AG-3-1009 and N01-AG-3-1010. Blood samples were drawn for the determination of plasma drug concentrations before administration and at 0, 30, 60 and 90 min, and at 2, 4, 6 and 8 hours after administration. A total 24-hour urine sample was also collected.

3. Results and Discussion

3.1. Quantitation of (R,R')-Fenoterol and (S,S')-Fenoterol using an achiral-chiral method

It was necessary to improve the sensitivity of the LC-MS/MS method over existing methods, as they lacked the sensitivity needed for our protocol. (R,R)-Fen and (R,R ;S,S)-Fen concentrations were initially determined using a validated achiral LC-MS/MS method in plasma or urine with an LOQ = 52.8 pg/ml, which was a significant improvement over existing methods. The retention factors for (R,R)-Fen and (R,R ;S,S)-Fen in the analysis of plasma and urine samples were 1.63 and 1.61, respectively, and the retention factors for the corresponding sulfate metabolites (Fen-Sulf) were 0.25 and 0.40, respectively. The optimized chromatographic conditions were applied to the analysis of plasma (Figure 1a) and urine (Figure 1b) samples obtained from a patient receiving 10 mg (R,R ;S,S)-Fen or 5 mg of (R,R)-Fen.

To compare the bioavailability of (R,R)-Fen after administration of the racemic and single enantiomer formulations, the enantioselective separation of (R,R ;S,S)-Fen (Figure 2b,c) of the extracted samples was achieved on an Astec Chirobiotic T 5 μ m analytical column. The (R,R)- and (S,S)-Fen enantiomers were resolved with an α = 1.09 and R_s = 1.4, similar to or slightly improved from previously reported methods [3,19], where α = 1.21 and R_s = 1.06 [3] and α = 1.19 and R_s = 2.0 [19] were obtained. The current method differed from previously reported methods having (R,R)-Fen eluting first (figure 2a).

3.2. Validation of the method

In plasma, the calibration curves and the standards for quality control were prepared using (R,R)-Fen and ranged from 52.7–2109.3 pg/ml. The calibration curves were linear ($y = 3 \times 10^{-5}x - 0.0006$) with r^2 values ranging from 0.98–0.99. The method was then validated for intra-day and inter-day accuracy and precision using the QC samples listed in Table 1a. The four quality control (QC) standards were 105.5, 281.5, 667.4 and 1126.5 pg/ml. The coefficient of variation was <15% for the high QC standards and <10 % for the low QC standards. The LLOQ for (R,R)-Fen was 52.8 pg/ml. In urine, a seven point calibration curve used to measure (R,R)-Fen ranged from 500–32000 pg/ml. The calibration curves were linear ($y = 0.0003x + 0.0136$) with r^2 values of 0.99. The method was then validated for intra-day and inter-day accuracy and reproducibility using the QC samples listed in Tables 1b. The three quality control (QC) standards for (R,R ;S,S)-Fenoterol were 5, 20 and 80 ng/ml. The coefficient of variation was <15% for the high QC and low QC standards.

Three sets of the four quality controls and a blank with internal standard were prepared for evaluation of sample recovery and matrix effects (i.e. suppression or enhancement of ionization). While no significant matrix was observed for urine, there was a significant enhancement of ionization observed for plasma samples (Table 2). However, this enhancement was constant for all QCs, therefore, the matrix effect of the assay was not a concern for the sensitivity of the assay. Similarly, while the recovery was almost 100% for urine, the recovery averaged about (64.9 $\pm$ 9.1)% for plasma, however, as it was consistent for all the QCs and had low variability, therefore the method was shown to be reliable. Also, as no significant matrix effect was observed for five different lots of plasma, it was concluded that the matrix did not affect the accuracy and precision of fenoterol determination.

Freeze–thaw stability was determined as the recovery (%) compared with the nominal value for all QCs in plasma and urine (Table 3a and 3b, respectively). Deviation and precision were less than 15% for all QCs in both plasma and urine for three freeze thaw cycles. Autosampler stability (stability of fenoterol in the eluate) was estimated by analysis of QC samples after storage for 24 h at 4°C in the autosampler. For both plasma and urine, the results differed less than 15% for plasma and 10% for urine from nominal value, therefore fenoterol was stable during storage in the autosampler. Finally, room-temperature stability was assessed by leaving the QCs at room temperature for 1 h or 4h before sample processing. There was no significant differences (<15%) from nominal value for fenoterol. All the results of the stability tests presented in table 3 imply the high stability of fenoterol for the developed method

The results of these studies indicate that the achiral analytical method is accurate and reproducible within accepted limits, and can be used to analyze plasma and urine samples obtained from human patients participating in the Fenoterol study.

3.3. Application

Timed plasma samples and a 24h cumulative urine sample were obtained from a healthy volunteer who received an oral dose of 10 mg (R,R ;S,S)-Fen and 5 mg (R,R)-Fen in a two-way crossover study with a one week washout between dosing. The plasma samples were collected at 30m, 60m, 90m, 120m, 240, 360m, 480m post administration of the drug. The total plasma concentration of (R,R ;S,S)-Fen was determined using the achiral method and the plasma concentration-time curve is presented in Fig. 3a. The peak plasma concentration was observed in the 60 min sample and significant (>400 pg/ml) concentrations were present in the sample obtained 360 min after administration of the racemate. The enantioselective separation achieved on the chirobiotic T column was used to determine the relative (R,R)-Fen and (S,S)-Fen concentrations. In this subject, the initial plasma concentrations of (R,R)-Fen and (S,S)-Fen were essentially equivalent 30 min after administration, were significantly different in the 60 min and 90 min samples with (R,R)-Fen/(S,S)-Fen ratios of 1.5 and 2.6, respectively, and were equivalent in the 120 min to 360 min samples, Fig. 3a. Although, this is a limited data set, the preliminary data suggest that the rate of the pre-systemic sulfation of (R,R)-Fen is slower than (S,S)-Fen allowing for a higher bioavailability of the (R,R) enantiomer or that (S,S)-Fen competitively inhibits the sulfation of (R,R)-Fen. The enantioselective bioavailability was consistent with the higher urinary concentrations of (S,S)-Fen where the relative urine concentration of (R,R) and (S,S)-Fen was 0.89 (Table 4). To our knowledge this represents the first report of the relative enantiomeric concentrations of these compounds in human plasma and urine after the administration of 10mg (R,R ;S,S)-Fen.

When the plasma concentrations of (R,R)-Fen were determined after the administration of the single isomer formulation, the plasma concentration peaked at 60 min after dosing and was measurable at the 360 min time point. It is of interest to note that at the 60 min and 90 min sampling points, the plasma concentrations of (R,R)-Fen after the administration of just the (R,R)-enantiomer were lower than the corresponding levels of (R,R)-Fen observed after the administration of (R,R ;S,S)-Fen. The calculated AUCs were also different as the AUC_{0–360} of (R,R)-Fen after administration of the single enantiomer was 91,763 h-pg/ml and 120,929 h-pg/ml after administration of the racemate. Since equivalent doses of (R,R)-Fen were administered in each arm of the study, the results suggest that the presence of (S,S)-Fen does indeed increase the bioavailability of the (R,R)-enantiomer. A more detailed comparison of the bioavailability of (R,R)-Fen after single isomer or racemic administration as well as the associated pharmacodynamics data will be presented elsewhere.

Conclusion

A highly sensitive LC-MS/MS method for the determination of Fen in plasma and urine was developed and validated. The analysis of the plasma data from the oral cross-over study of (R,R)-Fen and (R,R ;S,S)-Fen formulations demonstrate a potential pre-systemic enantioselective interaction in which the (S,S)-Fen reduces the sulfation of the active (R,R)-Fen. The data suggests that a non-racemic mixture of the Fen enantiomers may prove to be a more effective clinical formulation of the drug in the treatment of cardiovascular disease.

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- developed an LC-MS/MS method for the determination of Fenoterol enantiomers
- improved quantitation limit from 2 ng/ml to 50 pg/ml over existing methods.
- enantioselective competitive interaction affects fenoterol presystemic sulfation.

Figure 1a.

Figure 1b.

Figure 1.

Figure 1a. The chromatographic trace from the achiral analysis of a plasma sample (A) and urine sample (B) obtained 60 min after administration of a 10 mg (R,R ;S,S)-Fen to a healthy volunteer.

Figure 2a.

Figure 2b

Figure 2c.

Figure 2.

Figure 2a. The chromatographic trace from the chiral analysis of a plasma sample spiked with racemic fenoterol and (R,R)-Fen (A) and a plasma sample (B) and urine sample (C) obtained 60 min after administration of a 10 mg (R,R ;S,S)-Fen to a healthy volunteer.

Figure 3a.

3B)

Figure 3.

Figure 3a. Circulating plasma levels of a healthy volunteer receiving 10mg (R,R ;S,S)-Fen () and levels of (R,R)-Fen () and (S,S)-Fen () resolved using an Astec Chirobiotic-T column

3B) Circulating plasma levels of a healthy volunteer receiving 5mg (R,R)-Fen () and circulating plasma levels of (R,R)-Fen () in the volunteer receiving 10mg (R,R ;S,S)-Fen.

(*R,R'*)-fenoterol

(*S,S'*)-fenoterol

4-methoxy-1-naphthylfenoterol

SCHEME I.

Table 1a

Summary of the intra-day and inter-day accuracy and precision of (R,R)-Fen in human plasma (n = 9)

		Fen			Fen
QC _{1intra}	Mean	99.3	QC _{1inter}	Mean	114.9
	SD	7.53		SD	12.3
	RSD(%)	7.6		RSD(%)	10.72
	Accuracy(%)	95.05		Accuracy(%)	108.7
QC _{2intra}	Mean	284.3	QC _{2inter}	Mean	243.6
	SD	18.6		SD	18.9
	RSD(%)	6.55		RSD(%)	7.77
	Accuracy(%)	100.95		Accuracy(%)	86.5
QC _{3intra}	Mean	654.5	QC _{3inter}	Mean	606.0
	SD	49.4		SD	28.31
	RSD(%)	7.5		RSD(%)	4.67
	Accuracy(%)	98.06		Accuracy(%)	90.8
QC _{4intra}	Mean	1209.7	QC _{4inter}	Mean	1076.5
	SD	44.5		SD	153.7
	RSD(%)	3.68		RSD(%)	14.3
	Accuracy(%)	101.95		Accuracy(%)	90.7

Table 1b

Summary of the intra-day and inter-day accuracy and precision of (R,R)-Fen in human urine (n = 9) in ng/ml

		Fen			Fen
QC _{1intra}	Mean	5.65	QC _{1inter}	Mean	4.98
	SD	59.96		SD	67.0
	RSD(%)	9.8		RSD(%)	9.4
	Accuracy(%)	112.97		Accuracy(%)	99.69
QC _{2intra}	Mean	21.01	QC _{2inter}	Mean	19.06
	SD	249.3		SD	410.0
	RSD(%)	11.02		RSD(%)	14.62
	Accuracy(%)	105.02		Accuracy(%)	95.31
QC _{3intra}	Mean	83.60	QC _{3inter}	Mean	75.45
	SD	519.2		SD	1357.2
	RSD(%)	6.09		RSD(%)	12.53
	Accuracy(%)	104.5		Accuracy(%)	94.31

Table 2
Extraction recovery and matrix effect of (R,R)-Fen in human plasma and urine ($n = 3$)

	Nominal Concentration (pg/ml)	Measured concentrations (pg/mL)	Extraction recovery (%)	Accuracy (%)	RSD(%)	Matrix effect (%)	RSD (%)
Plasma	105.5875	94.2	76.1	86.33	14.40	124.3	17.41
	281.56	235	58.4	84.81	4.46	141.7	5.35
	667.42	792	66.1	101.82	6.25	122.6	16.52
	1186.52	1310	55.6	97.38	9.14	120.3	18.25
Urine	5000	4540	98.3	90.8	13.09	94.8	15.61
	20000	21150	105	105.75	5.65	99.0	10.12
	80000	82000	105	102.50	8.48	96.5	9.34

Table 3aStability of (R,R)-Fen in human plasma ($n = 3$)

Sample condition	Nominal Concentration (pg/mL)	Measured concentrations (pg/mL)	Accuracy (%)	RSD (%)
Autosampler (24 h, 4°C)	105.59	104.27	98.75	9.90
	281.57	318.22	113.02	10.64
	667.42	626.37	93.85	3.07
	1186.52	1221.17	102.92	6.67
Three freeze/thaw cycles	105.59	115.15	109.05	8.96
	281.57	291.48	103.52	8.69
	667.42	651.59	97.63	8.46
	1186.52	1302.49	109.77	12.9
Room Temp 1 h	105.59	109.1	103.33	12.5
	281.57	244.7	86.91	8.9
	667.42	640.4	95.95	6.8
	1186.52	1048.2	88.35	7.3
Room Temp 4 h	105.59	102.75	97.32	6.1
	281.57	297.67	105.72	10.3
	667.42	655.84	98.26	8.3
	1186.52	1260.5	106.23	13.2

Table 3bStability of (R,R)-Fen in human urine ($n = 3$)

Sample condition	Nominal Concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy R.E. (%)	RSD (%)
Autosampler (24 h, 4 °C)	5	5.4	108.0	7.57
	20	21.78	108.9	7.83
	80	82.325	102.9	2.69
Three freeze/thaw cycles	5	4.955	99.1	12.2
	20	17.70	88.51	11.96
	80	90.47	113.1	12.6
Room Temp 1 h	5	5.95	119.0	7.21
	20	19.76	98.8	12.19
	80	69.59	87.0	7.35
Room Temp 4h	5	5.235	104.7	17.9
	20	20.375	101.9	8.13
	80	73.345	91.7	10.31

Table 4

Concentration of (R,R ;S,S)-Fen (pg/ml) in plasma and urine and the enantiomeric ratio of (R,R)-- and (S,S)-Fen in a healthy volunteer receiving 10 mg (R,R ;S,S)-Fen at 30, 60 and 90 min

Time (min.)	Total (R,R ;S,S)-Fen	(R,R)-Fen	(S,S)-Fen	R,R /S,S
30	643.05	330.26	312.79	1.06
60	1226.24	716.42	509.82	1.41
90	801.67	485.33	316.34	1.53
Urine	12218.5	5763.5	6455	0.89