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Pharmacokinetics and Metabolism of (*R,R*)-Methoxyfenoterol in Rat

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Abstract

1. (*R,R*)-Fenoterol (Fen), a β_2 -adrenoceptor agonist, is under clinical investigation in the treatment of congestive heart disease. The pharmacokinetics and metabolism of the 4-methoxyphenyl derivative of (*R,R*)-Fen, (*R,R*)-MFen, have been determined following intravenous and oral administration to the rat and compared with corresponding results obtained with (*R,R*)-Fen. Results of the study suggest that (*R,R*)-MFen can offer pharmacokinetic and metabolic advantages in comparison to an earlier (*R,R*)-Fen.
2. The oral administration revealed that the net exposure of (*R,R*)-MFen was about three-fold higher than that of (*R,R*)-Fen (7.2 versus 2.3 min $\times$ nmol ml⁻¹), while intravenous administration proved that the clearance was significantly reduced, 48 versus 146 ml min⁻¹ kg⁻¹, the $T_{1/2}$ was significantly longer, 152.9 versus 108.9 min and the area under the curve (AUC) was significantly increased, 300 versus 119 min $\times$ nmol ml⁻¹.
3. (*R,R*)-MFen was primarily cleared by glucuronidation associated with significant presystemic glucuronidation of the compound. After intravenous and oral administration of (*R,R*)-MFen, (*R,R*)-Fen and (*R,R*)-Fen-G were detected in the urine samples indicating that (*R,R*)-MFen was *O*-demethylated and subsequently conjugated to (*R,R*)-Fen-G. The total (*R,R*)-Fen and (*R,R*)-Fen-G as a percentage of the dose after intravenous administration was 3.6% while after oral administration was 0.3%, indicating that only a small fraction of the drug escaped presystemic glucuronidation and was available for *O*-demethylation.
4. The glucuronidation pattern was confirmed by the results from *in vitro* studies where incubation of (*R,R*)-MFen with rat hepatocytes produced (*R,R*)-MFen-G, (*R,R*)-Fen and (*R,R*)-Fen-G, while incubation with rat intestinal microsomes only resulted in the formation of (*R,R*)-MFen-G.

Keywords

Hepatocytes; liver microsomes; intestinal microsomes; high-performance liquid chromatography (HPLC); mass spectrometry; pharmacokinetics; phase II drug metabolism

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Introduction

Fenoterol, 5-[1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]-amino]-ethyl]-1,3-benzenediol (Fen, Figure 1), is a β_2 -adrenoceptor (β_2 -AR) agonist used in the prevention and treatment of asthma (Heel et al. 1978) and in obstetric practice as a tocolytic agent (Hochhaus and Mollmann 1992; Hildebrandt et al. 1993). Previous studies in cardiomyocytes from an animal model of congestive heart failure have suggested that Fen may also be effective in the treatment of this disease (Xiao et al. 2003).

Fen is a chiral compound with two asymmetric carbons and is marketed as a racemic mixture of (*R,R*)-Fen and (*S,S*)-Fen. In recent studies, (*R,R*)-Fen and (*S,S*)-Fen were independently tested for their activity in the cardiomyocyte model (Beigi et al. 2006; Jóźwiak et al. 2007). The data demonstrated that (*R,R*)-Fen produced a significant increase in the maximum contractile response and reduced the EC_{50} while (*S,S*)-Fen had no observed effect. In addition, (*R,R*)-Fen displayed submicromolar affinity for the β_2 -AR, $K_i = 0.35 \mu\text{M}$, and had a greater than 40-fold selectivity relative to its affinity to the β_1 -AR (Jóźwiak et al. 2007). Recent studies have also demonstrated that (*R,R*)-Fen selectively activates β_2 -AR coupled G_S signaling (Woo et al. 2009). Based upon these data, (*R,R*)-Fen is under clinical investigation as a therapeutic agent in the treatment of congestive heart disease.

Recently a series of fenoterol derivatives were synthesized and tested for β_2 -AR selectivity and activity (Jóźwiak et al. 2007; Woo et al. 2009). The results from this study indicate that (*R,R*)-methoxyfenoterol, (*R,R*)-MFen (Figure 1), had submicromolar affinity for the β_2 -AR, $K_i = 0.47 \mu\text{M}$, a greater than 40-fold selectivity relative to its affinity to the β_1 -AR and produced a significant increase in the maximum contractile response in the cardiomyocyte model. In addition, as with (*R,R*)-Fen, (*R,R*)-MFen selectively activates β_2 -AR coupled G_S signaling (Woo et al. 2009). The results of these studies suggest that (*R,R*)-MFen may also be useful in the treatment of congestive heart failure.

In the rat, Fen is rapidly eliminated from the body by glucuronidation (Koster et al. 1985, 1986) and the drug's extremely low bioavailability after oral administration, less than 1%, has been attributed to presystemic glucuronidation (Koster et al. 1985, 1986). The glucuronidation of Fen can occur at two sites, the 1,3-benzenediol and the 4-hydroxyphenyl moieties, which result in the previously designated *meta*- (Fen-MG) and *para*- (Fen-PG) metabolites, respectively (Figure 1) (Koster et al. 1986). In the rat, (*R,R*)-Fen is regioselectively metabolized to Fen-PG (Koster et al. 1986).

In the (*R,R*)-MFen molecule, the 4-hydroxyphenyl group has been converted into a 4-methoxyphenyl, thereby blocking the production of the (*R,R*)-MFen-PG (Figure 1). The reduction in the available sites for glucuronidation should result in an increased bioavailability of (*R,R*)-MFen relative to (*R,R*)-Fen as well as a more favorable pharmacokinetic profile, that is, decreased clearance and higher systemic exposure.

Since (*R,R*)-MFen and (*R,R*)-Fen appear to have equivalent pharmacological activities, (*R,R*)-MFen may represent a better therapeutic option for the chronic treatment of congestive heart failure. The objective of this study was to explore this possibility through the determination of the pharmacokinetics of (*R,R*)-MFen after intravenous and oral administration of the compound to the rat and to compare it with pharmacokinetics of (*R,R*)-Fen. The *in vivo* and *in vitro* metabolism of (*R,R*)-MFen was also investigated.

Materials and methods

Chemicals and reagents

(*R,R*)-Methoxyfenoterol fumarate ((*R,R*)-MFen) and (*R,R*)-fenoterol fumarate ((*R,R*)-Fen) were prepared as previously described (Jóźwiak et al. 2007). Ritodrine (Rit) hydrochloride, acetic acid, formic acid, uridine diphosphate glucuronic acid (UDPGA) and β -glucuronidase type HP-2S from *Helix pomatia* were purchased from Sigma-Aldrich, (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and ethyl acetate were supplied by Fisher Scientific (Pittsburgh, PA, USA). Purified water was prepared using a Milli-Q system (Millipore, Milford, MA, USA). Control rat plasma and urine were purchased from Innovative Research (Novi, MI, USA). Waymouth 752 culture medium was purchased from Gibco™ (Grand Island, NY, USA) and Biocoat® Collagen 1 Cellware twelve-well plates were purchased from Becton Dickinson Labware (Bedford, MA, USA). Pooled (male and female) rat intestinal microsomes (RIM) were purchased from XenoTech LLC (Lenexa, KS, USA).

Animal studies

(*R,R*)-MFen and (*R,R*)-Fen were administered intravenously via an in-dwelling catheter (5 mg/kg) and by oral gavage (25 mg/kg,) to male Sprague-Dawley rats (7-8 weeks of age, 277-314 g). Six rats were used in each group for a total of 24 rats. Blood samples (approximately 200 μ l each) were collected from all groups using a jugular vein catheter (separate from the catheter used to administer drug intravenously) and processed to plasma at pre-dose and 5, 15 and 30 min, and 1, 2, 4, 5 and 6 h post-dose. The urine was collected from 3 rats in each experimental group between 0-6 h and 6-24 h. The samples were stored at -80°C until analysis. General procedures for animal care and housing was in accordance with the National Research Council (NRC) *Guide for the Care and Use of Laboratory Animals* (1996) and the Animal Welfare Standards incorporated in 9 CFR Part 3, 1991.

In vitro metabolism assays

Incubation with rat hepatocytes—Hepatocytes were isolated from the whole liver of a male Sprague Dawley rat using previously described methods (Allen & Green 1993). The freshly isolated hepatocytes (viability of 81.4%) were immediately plated on Biocoat® plates at 1×10^6 cells per ml of culture medium (Waymouth 752 culture medium supplemented with hormones (Allen & Green 1993) with 10% heat-inactivated foetal bovine serum (FBS)). Cells were allowed to attach for 2 hours at 37°C, with 5% CO₂:95% air. The culture medium was replaced with 600 μ l of fresh media containing either 5, 10 or 50 μ M (*R,R*)-MFen. Incubations were performed for various time periods: 0, 0.5, 1, 2, 4, 8 and 24 hour. At the end of each time period the media were transferred to 2 ml microfuge tubes. All samples were frozen in dry ice and stored at approximately -70 °C.

Incubation with rat intestinal microsomes—(*R,R*)-MFen was incubated with rat intestinal microsomes (RIM) at three concentrations 5, 10 and 50 μ M for 0.5 and 1 h at 37°C. The incubation mixture consisted of 5 mM MgCl₂, 0.5 mg ml⁻¹ of RIM and Tris buffer (pH 7.4, 25 mM) in 200 μ l of total sample incubation volume. The reaction was started with uridine diphosphate glucuronic acid (UDPGA) (5mM) and stopped with cold acetonitrile (600 μ l). Samples were frozen in dry ice and stored at approximately -70 °C. No detergent was added to the incubation mixture due to the subsequent liquid chromatography-mass spectrometry (LC-MS) analysis.

Sample preparation for quantitative analysis

Rat plasma samples—The plasma samples obtained after intravenous administration were analysed for (*R,R*)-MFen and (*R,R*)-Fen concentrations using a previously described method

utilizing solid phase extraction coupled with LC-MS analysis (Siluk et al. 2008). The plasma samples obtained after oral administration were analysed for (*R,R*)-MFen and (*R,R*)-Fen concentrations using a previously described method employing on-line immunoextraction coupled with LC-MS (Kim et al. 2009).

Rat urine samples—(*R,R*)-MFen, (*R,R*)-Fen and their metabolites were extracted from rat urine using a modified version of the previously described solid phase extraction method (Siluk et al. 2008). In brief, a 25 or 5 μ l aliquot of urine was acidified with 2% formic acid (20 μ l), diluted with 500 μ l of ammonium acetate buffer (pH 5.0; 0.1 M) and 20 μ l of ritodrine (Rit) (0.2 μ g ml⁻¹ in acetonitrile) was added. The Bond Elut Plexa 1ml solid phase extraction cartridge (Varian, Palo Alto, CA) was conditioned with 1 ml of MeOH and equilibrated followed by 1 ml of water. The cartridge was then loaded with the sample, washed with 2 $\times$ 1 ml of water and the analytes eluted with 2 ml of a mixture of ethyl acetate:methanol (1:1, v/v). The elution mixture was evaporated to dryness in a Speed-Vac (Thermo Savant, NY, USA) for 1.5h at a temperature 80°C. The resulting residue was reconstituted in 400 μ l of a solution composed of MeOH (20 μ l), 2% formic acid (20 μ l) and acetonitrile (360 μ l), vortex-mixed and a 100 μ l aliquot was transferred to an HPLC vial and diluted with 900 μ l of acetonitrile. A 10 μ l aliquot of the resulting solution was injected onto LC-MS system.

In vitro medium sample preparation—A 25 μ l of hepatocyte incubation medium was acidified with 25 μ l of ammonium acetate buffer (pH 5.0; 0.1 M) and 25 μ l of 2% formic acid followed by the addition of 20 μ l of Rit (0.2 μ g ml⁻¹ in acetonitrile) and 355 μ l of acetonitrile. The resulting solution was vortex-mixed and centrifuged for 5 min at 2643g and a 15 μ l aliquot of the supernatant was injected onto LC-MS system.

In vitro rat intestine sample preparation—A 25 μ l aliquot of centrifuged RIM incubate was acidified with 25 μ l of 2% formic acid followed by the addition of 20 μ l of the Rit (0.2 μ g/ml in acetonitrile) and 380 μ l of acetonitrile. The resulting solution was vortex-mixed and a 15 μ l aliquot of the supernatant was injected onto LC-MS system.

Incubation of samples with beta-glucuronidase

Rat urine samples—Rat urine samples were deconjugated following the procedure described by Eap et al. (2004). In brief, a 25 μ l or 5 μ l aliquot of urine was acidified with 2% formic acid (20 μ l) followed by the addition of 500 μ l of ammonium acetate buffer (pH 5.0; 0.1 M) and 20 μ l of Rit (0.2 μ g ml⁻¹ in acetonitrile). A 30 μ l of β -glucuronidase (100 000 units ml⁻¹) was added, the resulting solution vortex-mixed for 10 sec and the sample was incubated for 20 h in 37°C with occasional vortex-mixing. The incubation sample was centrifuged for 5 min at 2643g followed by preparation for HPLC analysis as described above for rat urine samples.

In vitro medium samples— β -Glucuronidase (5 μ l of 20 000 units ml⁻¹) was added to a 25 μ l aliquot of the medium from the incubation reactions and acidified with 25 μ l of ammonium acetate buffer, (pH 5.0; 0.1 M). The resulting mixtures were incubated at 37°C for 20 h with occasional vortex-mixing. After incubation, 25 μ l of 2% formic acid, 20 μ l of Rit (0.2 μ g ml⁻¹ in acetonitrile) and 350 μ l of acetonitrile were added, the resulting solution centrifuged for 5 min at 2643g and a 15 μ l aliquot of the supernatant was injected onto the LC-MS system.

LC-MS analysis

The previously reported LC-MS method for analysis of (*R,R*)-Fen in rat plasma samples was adopted for the determination of (*R,R*)-MFen and metabolites in rat urine and for *in vitro* studies (Siluk et al. 2008). In this study, 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 quaternary pump (1311A) a thermostated autosampler (G1329 A) and a thermostated column compartment (G1316A). The mass selective detector (MSD Quad SL, G1956B) was used with 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 Agilent Technologies, Palo Alto, CA). The analysis was achieved with the use of an Atlantis HILIC Silica 3 μm (2.1×150 mm) column connected to an Atlantis HILIC Silica 3 μm guard column (2.1×10 mm) (Waters, Milford, MA, USA). (*R,R*)-MFen was monitored at m/z 318.0, (*R,R*)-MFen glucuronide at m/z 494.0, (*R,R*)-Fen at m/z 304.2, (*R,R*)-Fen glucuronide at m/z 480.2 and Rit at m/z 288.2.

MS/MS conditions—MS/MS analysis was performed using an Applied Biosystems API4000 triple quadrupole mass spectrometer (AB/MDS Sciex, Ontario, Canada) and positive mode electrospray ionizations (ESI) using a TurboIon Spray ion source were applied throughout the study. The typical MS/MS parameters were: ion source temperature (TEM) was set at 450°C , the ion spray voltage (IS) was set at 5500 V, curtain gas (CUR) flow was set 20 psi, gas 1 (GS1) was set at 55 psi, gas 2 (GS2) was 70 psi, collision gas (CAD) was set at 8, declustering potential (DP) was set at 95 V and collision energy was set at 26.5 V. Data analysis was performed in a scanning (m/z 100 – 650) and multiple reaction monitoring modes. Multiple reaction monitoring (MRM) transitions of (*R,R*)-Fen (m/z , 304.3 to 135), (*R,R*)-MFen (m/z , 318.3 to 149), (*R,R*)-Fen-G (m/z , 480.2 to 135) and (*R,R*)-MFen-G (m/z , 494.3 to 149) with a dwell time set to 200ms for each transition were used.

Validation of the analytical method

(*R,R*)-Fen and (*R,R*)-MFen standard curves ranged from 0.1 to $40 \mu\text{g ml}^{-1}$. Standard solutions were prepared in methanol in 6 concentrations (0.5; 2; 5; 20; 50 and $200 \mu\text{g ml}^{-1}$). 5 μl of each standard solution was added to vials and solvent was evaporated in a Speed-Vac (5 min, 80°C). After evaporation the residue was dissolved in a 20 μl of 2% formic acid and 500 μl of ammonium acetate buffer (pH 5.0; 0.1 M), followed by a 25 μl aliquot of urine and 20 μl of Rit ($0.2 \mu\text{g ml}^{-1}$ in acetonitrile). The sample preparation was identical as for rat urine samples. The LLOQ was determined at the lower end of the standard curve at $0.1 \mu\text{g/ml}$.

Standard curves and for low, medium and high quality control levels (LQC, MQC and HQC, respectively) for (*R,R*)-MFen and (*R,R*)-Fen were prepared each day of analysis following the procedures described above. Intraday precision was determined based on 5 determinations for LQC ($0.2 \mu\text{g ml}^{-1}$), MQC ($2.0 \mu\text{g ml}^{-1}$) and HQC ($20.0 \mu\text{g ml}^{-1}$). Inter-day precision of each assay was analysed based on determinations for LQC, MQC and HQC on three different days over a period of one week. Accuracy was determined using the same data set as for precision determination. Calibration curves were constructed using target compounds concentrations against peak area ratios (peak area of a target compound over a peak area of internal standard).

Pharmacokinetic analysis

Non-compartmental analysis—Individual animal plasma concentration versus time data were subjected to a non-compartmental analysis (WinNonlin 5.0.1, Pharsight, Mountain View, CA, USA). The observed and calculated pharmacokinetic parameters included the maximum plasma concentration (C_{max}), the time at which C_{max} was achieved (T_{max}), the terminal elimination half-life ($T_{1/2}$), area under the plasma concentration versus time curve ($\text{AUC}_{0-\text{inf}}$), clearance (Cl), volume of distribution at steady-state (V_{SS}) and concentration at time zero (C_0 - intravenous administration).

Pharmacokinetic modelling—A pooled analysis was conducted to estimate a single set of pharmacokinetic parameters for each compound after oral and intravenous administration.

Plasma concentration versus time profiles were described using a standard linear two-compartment mammillary model defined by the following equations:

$$dA_p/dt = \text{Input} - (k_{12} + k_{10}) \cdot A_p + k_{21} \cdot A_t \quad (1)$$

$$\begin{aligned} dA_t/dt &= k_{12} \cdot A_p \\ &- k_{21} \cdot A_t; \quad A_t(0) = 0 \end{aligned} \quad (2)$$

where A_p and A_t are the amounts of drug in the central and peripheral compartments, k_{10} is the first-order elimination rate constant, k_{12} and k_{21} are first-order distribution rate constants, and plasma concentrations are defined as the ratio of A_p and the volume of the central compartment:

$$V_p: C_p = A_p / V_p$$

The input function and initial condition for equation (1) after intravenous administration is:

$$A_p(0) = \text{Dose}; \text{Input} = 0$$

After oral administration, these terms are defined as:

$$\begin{aligned} A_p(0) &= 0; \\ \text{Input} &= F \cdot \text{Dose} \cdot \frac{k_{a1} \cdot k_{a2}}{(k_{a2} - k_{a1})} \cdot [e(-k_{a1} \cdot t) - e(-k_{a2} \cdot t)] \end{aligned} \quad (3)$$

where the input function consists of two sequential first-order absorption processes defined by k_{a1} and k_{a2} and F is the systemic bioavailability.

Pooled plasma concentration versus time profiles for each drug after oral and intravenous administration were modelled simultaneously (equations 1-3) using the ADAPT II computer program (Biomedical Simulation Resource, University of Southern California, Los Angeles, CA, USA). Model parameters were estimated by maximum likelihood, and a standard variance model was defined as:

$$\text{Var}_j = \sigma^2 \cdot M(\theta, t_j)^2 \quad (4)$$

where the function, M , represents model predicted values at the j^{th} time point based on model parameters (θ), and σ is an estimated variance model parameter. Estimated parameters were used to calculate total systemic clearance (CL) and the steady-state volume of distribution (V_{ss}) according to:

$$CL = k_{10} \cdot V_p \quad (5)$$

$$V_{ss}=V_p \cdot (1+k_{12}/k_{21}) \quad (6)$$

The terminal elimination half-life was also calculated as:

$$T_{1/2,\lambda z}=\ln(2)/\beta \quad (7)$$

with β defined as:

$$\beta=0.5 \cdot (k_{12}+k_{21}+k_{10}) - \sqrt{(k_{12}+k_{21}+k_{10})^2+4 \cdot k_{21} \cdot k_{10}} \quad (8)$$

Model development was guided by visual inspection of fitted profiles, distribution of residuals, Akaike Information and Schwarz criteria, and precision of parameter estimates (CV%).

Statistical analysis

Statistical differences in pharmacokinetic parameters were tested using Student's *t*-test while normal distribution was tested with Shapiro-Wilk's *W* test (STATISTICA 8.0, StatSoft, Inc., Tulsa, OK, USA). In a case of lacking normal distribution the Mann-Whitney *U*-test test was performed.

Results

Analysis of plasma samples

The plasma concentrations of (*R,R*)-MFen and (*R,R*)-Fen following oral and intravenous administration were determined using previously described and validated HPLC-MS methods (Siluk, et al. 2008; Kim, et al. 2009). Under the chromatographic conditions, the retention times of (*R,R*)-MFen and (*R,R*)-Fen were 6.8 and 7.3 min, respectively. (*R,R*)-MFen and (*R,R*)-Fen were monitored at *m/z* 318.0 and 304.2, respectively, and no interferences were detected in the control or experimental chromatograms. The plasma concentration versus time curves obtained after intravenous and oral administrations of (*R,R*)-MFen and (*R,R*)-Fen are presented in Figure 2.

Pharmacokinetic modelling

The pharmacokinetic model (equations 1-3) provides a reasonable description of the time-courses of (*R,R*)-Fen and (*R,R*)-MFen plasma concentrations after intravenous and oral administration in rats (Figure 3). Pharmacokinetic profiles after intravenous administration for both compounds and the (*R,R*)-MFen concentrations after oral dosing were well fitted, whereas the predicted curve shows a slight systematic deviation for the oral dosing profile of (*R,R*)-Fen. The final model represents a linear two-compartment model (equations 1 and 2) with two sequential first-order input processes (equation 3). Model building was initiated with simpler input functions, such as a standard linear first-order absorption rate constant with and without a time-lag; however, the sequential model was the simplest function that provided a reasonable fit to oral pharmacokinetic profiles of both compounds, as well as for a related (*R,R*)-Fen analogue (data not shown). The Akaike Information and Schwarz criteria decreased by 14 and 10 points for MFen, respectively, and lower sums of squared residuals for both compounds further supported the use of the more complex absorption model. It is possible that the first

two time-points for the oral (*R,R*)-Fen data are outliers or contain experimental error, as removing these values resulted in a significant improvement in the goodness of fit criteria (data not shown). Of importance is that changing the input function did not affect the estimates of the other pharmacokinetic parameters describing the disposition of the study compounds, and so this minimal model was determined to be acceptable. Model parameters were estimated with good precision (relatively low CV%), with the exception of k_{a2} , and the global pharmacokinetic parameters are in good agreement with the non-compartmental analysis (Table 1).

Pharmacokinetic data analysis

In these studies, (*R,R*)-MFen exhibited slower elimination in comparison to (*R,R*)-Fen indicated by a decreased systemic clearance of 47.7 versus 145.7 ml min⁻¹ kg⁻¹ and longer terminal half-life (152.9 versus 108.9 min for (*R,R*)-Fen) (Table 1). The steady-state volume of distribution suggested extensive distribution of both agents in the body (6.6 and 8.2 l kg⁻¹ for (*R,R*)-MFen and (*R,R*)-Fen, respectively. After oral administration of a 25 mg kg⁻¹ dose the average maximum plasma concentration (C_{max}) of (*R,R*)-MFen was about 2.5 times higher than that observed after administration of (*R,R*)-Fen, 17.7±0.9 versus 6.9±0.3 nM (5.6±0.3 versus 2.1±0.1 ng ml⁻¹), while the T_{max} values were comparable (120 min). The absolute bioavailabilities of both compounds did not exceed 0.5% indicating an extensive first-pass metabolism. However, the net exposure of (*R,R*)-MFen was about three times higher than (*R,R*)-Fen, AUC_{MFen} = 7.2 min×nmol ml⁻¹ (2297 min×ng ml⁻¹) versus AUC_{Fen} = 2.3 min×nmol ml⁻¹ (692 min×ng ml⁻¹), (Table 1 and Figure 2), which is consistent with the reduced clearance of (*R,R*)-MFen relative to (*R,R*)-Fen.

Analytical method for the analysis of urine samples

The previously described method for the analysis of (*R,R*)-Fen in plasma samples after intravenous administration (Siluk et al. 2008) was adapted for the analysis of (*R,R*)-MFen, (*R,R*)-Fen and their metabolites in urine and *in vitro* samples. The assays were validated for use with the analysis of urine samples, Table 2. In these studies, the standard curves ranging from 0.1 to 40 µg ml⁻¹ for (*R,R*)-MFen and (*R,R*)-Fen were fitted with polynomial second order equations. The standard curves correlation coefficients for (*R,R*)-MFen and (*R,R*)-Fen were greater than 0.99. The intra-day and inter-day precision CVs for (*R,R*)-Fen for three QC levels (LQC=0.2 µg ml⁻¹; MQC=2.0 µg ml⁻¹ and HQC=20 µg ml⁻¹) were less than 13.4% (n = 5) and 12.9, respectively (n = 4 days). For (*R,R*)-MFen, the intra-day and inter-day precision CVs were less than 7.5 (n = 5) and 13.6% (n = 4 days). The corresponding average accuracies for (*R,R*)-Fen and (*R,R*)-MFen were 97.5 and 104.4%, respectively.

Analysis of urine samples

When the assay was applied to the urine samples obtained after the intravenous and oral administration of (*R,R*)-MFen, the internal standard eluted at 7.0 min, Fig. 4A, and the chromatograms contained peaks at 6.8 min corresponding to (*R,R*)-MFen and 7.3 min, which was identified as (*R,R*)-Fen (Figure 4A and B). The chromatograms also contained two additional peaks at 9.8 and 11.1 min. The peaks were analysed by mass spectrometry and the resulting mass spectra contained parent ions at m/z 494 and 480 which corresponded to the [M + H]⁺ ion from the glucuronidated forms of (*R,R*)-MFen and (*R,R*)-Fen, at m/z 494 [(*R,R*)-MFen-G] and 480 [(*R,R*)-Fen-G]. The presence of (*R,R*)-MFen-G and (*R,R*)-Fen-G was confirmed by treatment of the urine samples with beta-glucuronidase. After incubation, the peaks at 9.8 and 11.1 min were no longer detected in the chromatograms and there were corresponding increases in the areas of the (*R,R*)-MFen and (*R,R*)-Fen peaks (Figure 4C). The chromatograms obtained from the analysis of urine samples analysed after the administration of (*R,R*)-Fen only contained peaks corresponding to (*R,R*)-Fen and (*R,R*)-Fen-G (data not shown).

The Fen molecule contains two potential sites of conjugation, the 1,3-benzenediol and the 4-hydroxyphenyl moieties which result in the previously designated *meta*- (Fen-MG) and *para*- (Fen-PG) metabolites (Figure 1) (Koster et al. 1986). However, while MS/MS analysis was able to confirm the presence of (*R,R*)-Fen-G, it was unable to identify whether the ion peak arose from (*R,R*)-Fen-PG, (*R,R*)-Fen-MG or both. When (*R,R*)-MFen was studied, MS/MS analysis confirmed the presence of the mono-glucuronide, (*R,R*)-Fen-MG.

Cumulative urinary excretion

Not all animals urinated during the first period 0-6 h of urine collection. In the (*R,R*)-Fen group only one out of three animals urinated in the group of oral administration and two out of three in the intravenous administration group. In the case of (*R,R*)-MFen given orally urine was collected from two out of three animals in the group.

After intravenous administration of (*R,R*)-Fen, about 7% of the initial dose was excreted in the cumulative 24-h urine collection whereas about 5% of the dose was excreted as (*R,R*)-Fen-G (Table 3). After oral administration of (*R,R*)-Fen, less than 1% of the dose was excreted as the unchanged drug and about 8% of the dose was excreted as (*R,R*)-Fen-G (Table 3). These results are consistent with previously reported data which indicate that in the rat (*R,R*)-Fen is primarily cleared by presystemic and systemic glucuronidation (Koster et al. 1985,1986).

After the intravenous administration of (*R,R*)-MFen, the 24-h cumulative urinary excretion contained the expected parent drug and glucuronide and peaks corresponding to (*R,R*)-Fen and (*R,R*)-Fen-G (Figure 4A). About 13% of the administered dose was excreted as the unchanged drug, 8% as (*R,R*)-MFen-G, 2% as (*R,R*)-Fen and 2% as (*R,R*)-Fen-G (Table 3 and Figure 5). The identity of the (*R,R*)-Fen and (*R,R*)-Fen-G metabolites were established using MS/MS analysis and the total excretion of the glucuronidated metabolites was estimated by indirect analysis using beta-glucuronidase deconjugation. As with (*R,R*)-Fen, after the oral administration of (*R,R*)-MFen, the urine samples contained the parent drug, (*R,R*)-MFen-G, (*R,R*)-Fen and (*R,R*)-Fen-G (Figure 4B). Only about 2% of the administered dose was excreted as (*R,R*)-MFen and the drug was predominately excreted as (*R,R*)-MFen-G, about 14% of the administered dose (Table 3 and Figure 5). Trace amounts of (*R,R*)-Fen and (*R,R*)-Fen-G were also detected in the cumulative urine samples (Table 3 and Figure 5).

Basing on the total amount of unchanged drug excreted into urine and on total clearance data obtained from noncompartmental analysis, renal and nonrenal clearances were calculated. The obtained parameters are presented in Table 4.

In vitro incubations and metabolism analysis

In chromatograms of rat hepatocyte incubation medium an unidentified peak with retention time 9.1 min was detected which was also present in the chromatogram of blank medium (Figure 6A, solid line, blank sample chromatogram insert). In extracts of solutions after RIM incubation with (*R,R*)-MFen no extra peaks were detected (data not shown).

To confirm the metabolic transformations of (*R,R*)-MFen, the compound was incubated with rat hepatocytes and with rat intestinal microsomes (RIM). The medium collected after the incubation of (*R,R*)-MFen with rat hepatocytes contained three metabolites, (*R,R*)-MFen-G, (*R,R*)-Fen, and (*R,R*)-Fen-G (Figure 6A). The experiments were performed with three different (*R,R*)-MFen concentrations (5, 10 and 50 μ M) and the recognized patterns of metabolites formation were similar for all concentration levels (data not shown). When (*R,R*)-MFen was incubated with RIM the metabolite observed in the incubate was (*R,R*)-MFen-G (Figure 6B). The metabolites were identified by chromatographic retention times and by MS/MS analysis.

Discussion

Non-compartmental or statistical moment analysis provides reliable estimates of typical pharmacokinetic parameters of interest with a minimal number of assumptions (that is, system linearity) in individual animals. In this study, the calculated $T_{1/2}$ of (*R,R*)-Fen was 108.9 min (Table 1), which was 2-fold greater than the previously reported $T_{1/2}$ of 46.8 min (Koster et al. 1985). A similar trend was observed for the calculated clearance, 146 ml min⁻¹ kg⁻¹ (this study) versus 72.8 ml min⁻¹ kg⁻¹, V_{ss} = 8.2 versus 1.3 l kg⁻¹ and k_{10} = 0.043 versus 0.197 min⁻¹. The differences between the two studies most probably reflects the fact that this study utilized (*R,R*)-Fen at the intravenous dose of 5 mg kg⁻¹ while the earlier study employed racemic-Fen, that is, (*R,R*; *S,S*)-Fen at 2 mg kg⁻¹. In addition, this study utilized male Sprague-Dawley rats, whereas the previous study was conducted using male Wistar rats. A final pharmacokinetic model was successfully developed to simultaneously characterize intravenous and oral profiles with a single set of system parameters. Table 1 shows that the global pharmacokinetic parameters were in good agreement and the model may prove useful for simulating system behavior under different conditions (for example, various dosing regimens).

Although there were differences in the calculated pharmacokinetic parameters, the observations that the predominant route of elimination of Fen is glucuronidation and that after oral administration a significant proportion of this transformation takes place in the intestinal mucosa are consistent with the previous studies (Rominger & Pollmann 1972; Koster et al. 1985). Due to the presystemic metabolism, the reported bioavailability of Fen after oral administration to the rat is extremely low, less than 1% (Koster et al. 1985, 1986; Hildebrandt et al. 1994), which was also observed in this study. The estimated bioavailability is an apparent value, as the methods used in this paper assume linear stationary kinetics, and the clearance of Fen has been shown to be dose-dependent (Koster et al. 1985).

The Fen molecule contains two potential sites of conjugation, the 1,3-benzenediol and the 4-hydroxyphenyl moieties which result in the previously designated *meta*- (Fen-MG, Fen-MS) and *para*- (Fen-PG, Fen-PS) metabolites, respectively (Figure 1) (Koster et al. 1986; Wilson et al. 1997). Using isolated rat hepatocytes and enterocytes, Koster and co-workers demonstrated that (*R,R*)-Fen is converted to (*R,R*)-Fen-PG with Cl_{int} values of 1.48 ± 0.32 and 0.79 ± 0.20 μ l min⁻¹ kg⁻¹, respectively. The data also demonstrated that (*R,R*)-Fen was converted into (*R,R*)-Fen-MG. Since the Fen molecule has three equivalent hydroxyl moieties, the authors concluded that based upon probability, a fraction of 0.33 for (*R,R*)-Fen-PG formation should be expected. However, the observed fractions were 0.28 ± 0.01 (hepatocytes) and 0.52 ± 0.01 (enterocytes), suggesting that the glucuronidation of the 4-hydroxyphenyl moiety is preferred in the enterocytes.

In the (*R,R*)-MFen molecule the 4-hydroxyphenyl moiety is blocked and it was assumed that this would affect the glucuronidation of the compound and thereby its bioavailability and clearance. The data from this study demonstrates that this alteration did indeed change the pharmacokinetic properties of (*R,R*)-MFen relative to (*R,R*)-Fen.

While the replacement of the 4-hydroxyphenyl moiety with a 4-methoxyphenyl group did not affect the absolute bioavailabilities of (*R,R*)-MFen and (*R,R*)-Fen, both were less than 0.5%, the net exposure of (*R,R*)-MFen was about 3 fold higher than that of (*R,R*)-Fen (Table 3). The CL of (*R,R*)-MFen was less than that of (*R,R*)-Fen, 47.7 versus 145.7 ml min⁻¹ kg⁻¹, the $T_{1/2}$ longer, 152.9 versus 108.9 min and the V_{ss} was reduced, 6.6 versus 8.2 l kg⁻¹. The urinary excretion data also indicates that after the oral administration of (*R,R*)-MFen there is a significant presystemic glucuronidation of the compound, which is consistent with the extensive presystemic metabolism of (*R,R*)-Fen. The detection of (*R,R*)-MFen-G after

intravenous administration also indicates that the drug is conjugated by hepatic glucuronosyltransferases, which has also been previously observed for Fen (Koster et al. 1986). The incubations with rat liver hepatocytes and RIM confirmed the *in vivo* observations. In both cases (*R,R*)-MFen and (*R,R*)-Fen along with their metabolites were eliminated predominantly within the first six hours of urine collection especially after intravenous administration.

After intravenous and oral administration of (*R,R*)-MFen, (*R,R*)-Fen and (*R,R*)-Fen-G were detected in the urine samples (Figure 5). The data suggests that (*R,R*)-MFen is *O*-demethylated to (*R,R*)-Fen and the (*R,R*)-Fen is subsequently conjugated to (*R,R*)-Fen-G (Figure 1). The extent of transformation through *O*-demethylation appears to depend on the route of administration and is markedly higher when (*R,R*)-MFen was given intravenously (Figure 5 and Table 3). The total (*R,R*)-Fen and (*R,R*)-Fen-G as percent of the a dose after intravenous administration was about 3.6% while after oral administration it was about 0.3%, indicating that only the small fraction of the drug that escaped presystemic glucuronidation was available for *O*-demethylation. The *in vitro* studies confirmed that (*R,R*)-MFen-G, (*R,R*)-Fen and (*R,R*)-Fen-G (Figure 6A), were produced during incubation of (*R,R*)-MFen with rat hepatocytes. Incubation of the substrate with RIM confirmed that formation of (*R,R*)-MFen-G takes place in a significant amount already in the intestinal part of the gastrointestinal tract (Figure 6B). Thus it is unlikely that *O*-demethylation will affect the therapeutic index of orally administered (*R,R*)-MFen.

In the literature it was documented that in rat fenoterol is almost exclusively metabolized by glucuronidation (Rominger & Pollmann 1972; Koster et al. 1986). In humans, however, previous studies of the metabolic clearance of Fen has indicated that Fen is conjugated to sulphate (Fen-S) and glucuronidate (Fen-G) and that the Fen-S is the predominate presystemic metabolite (Hildebrandt et al. 1994; Wilson et al. 1997). *In vitro* studies with recombinant human phenolsulphotransferase isoforms, M-PST and P-PST demonstrated that the sulphation of Fen was regioselective (Wilson et al. 1997). In these studies, the conjugation of the 4-hydroxyphenyl moiety of (*R,R*)-Fen, that is, the production of (*R,R*)-Fen-PS, by M-PST was the preferred pathway. Sulphation at the 1,3-benzenediol moiety by P-PST was also observed. However, the authors conclude that while P-PST may contribute to the sulphation of Fen after oral dosing, this isoform may play a reduced role in Fen clearance as its K_m was about ten-fold higher than for M-PST.

In rats, as indicated above, the regioselective preference of the conjugation site during the second phase of metabolism process follows the same pattern as in humans. The results from the current study confirmed the assumption about a slower elimination of (*R,R*)-MFen in comparison to (*R,R*)-Fen in rats. (*R,R*)-MFen, an (*R,R*)-fenoterol derivative with conjugation site blocked at the 4-hydroxyphenyl moiety was proved to exhibit more beneficial pharmacokinetic profile, especially in the light of its potential use in congestion heart failure. Therefore the data from the current study indicating the enhanced exposure of (*R,R*)-MFen to (*R,R*)-Fen is encouraging and further data in human systems is required. Moreover, the determination of the metabolism of (*R,R*)-Fen in human hepatocytes and enterocytes is in progress and the data will be reported elsewhere.

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Figure 1.
Molecular structures of compounds and a scheme of (*R,R*)-MFen metabolism

Figure 2. Time versus concentration curves for *(R,R)*-Fen and *(R,R)*-MFen. (A) Intravenous administration (5 mg kg⁻¹); (B) oral administration (25 mg kg⁻¹) to rats.

Figure 3. Concentration versus time profiles of (R,R)-MFen and (R,R)-Fen after intravenous and oral administration to Sprague-Dawley rats. Filled circles represent observed concentration values, while lines depict model-fitted profiles.

A **B**

C **Figure 4.**

HPLC chromatograms of extracted rat urine after intravenous administration of (*R,R*)-MFen in a dose 5 mg kg⁻¹ (6-24 hr collection time) (A); after oral administration of (*R,R*)-MFen in a dose 25 mg kg⁻¹ (6-24 hr collection time) (B) and after intravenous administration of (*R,R*)-MFen in a dose 5 mg kg⁻¹ after incubation with β -glucuronidase (6-24 hr collection time) (C). For a better transparency in figures A, B and C the IS chromatogram is not shown.

Figure 5. Total urine excretion (percentage of dose) of (*R,R*)-MFen and metabolites after intravenous administration of 5 mg kg⁻¹ (A) and after oral administration of 25 mg kg⁻¹ (B) to rats.

A

B

Figure 6. HPLC chromatograms of in vitro samples. Representative chromatograms of an extracted medium after rat hepatocytes incubation with 50 μM (*R,R*)-MFen (chromatogram of medium blank sample in right top corner) (A) and after incubation with rat intestinal microsomes at concentration 10 μM (B). (IS signal not shown).

Table 1

Estimated pharmacokinetic parameters of (R,R)-methoxyfenoterol ((R,R)-MFen) and (R,R)-fenoterol ((R,R)-Fen) after intravenous and oral administration to rats at doses 5 and 25 mg kg⁻¹, respectively.

Model parameters			Non-compartmental analysis		
Parameter (units)	(R,R)-MFen Mean (CV%) ^d	(R,R)-Fen Mean (CV%) ^d	(R,R)-MFen Mean (SD) ^e	(R,R)-Fen Mean (SD) ^e	
k ₁₂ (min ⁻¹)	0.142 (14%)	0.023 (26%)	<i>Intravenous administration</i>		
k ₂₁ (min ⁻¹)	0.022 (9%)	0.012 (18%)	C ₀ (ng ml ⁻¹)	4050	2237 (711.3)
k ₁₀ (min ⁻¹)	0.053 (16%)	0.043 (15%)	T _{max} (min)	5.0	5.0
V _c (l kg ⁻¹)	0.989 (18%)	3.94 (20%)	T _{1/2α} (min)	152.9	108.9 (40.0)
k _{a1} (min ⁻¹)	0.007 (10%)	0.004 (39%)	V _{ss} (l kg ⁻¹)	6.6	8.2 (1.7)
k _{a2} (min ⁻¹)	0.096 (23%)	0.167 (79%)	Cl (ml min ⁻¹ kg ⁻¹)	47.7	145.7 (39.3)
F (%)	0.436 (25%)	0.473 (25%)	AUC _{0-inf} (min×ng ml ⁻¹)	95.3×10 ³	36.2×10 ³ (8.9×10 ³)
CL (ml min ⁻¹ kg ⁻¹) ^a	52 (3%)	170 (8%)	<i>Oral administration</i>		
V _{ss} (l kg ⁻¹) ^b	7.3 (7%)	11.6 (15%)	C _{max} (ng ml ⁻¹)	5.6	2.1 (0.1)
T _{1/2α} (min) ^c	125 (5%)	96.4 (12%)	T _{max} (min)	120	120
			T _{1/2α} (min)	206.7	168.5 (5.9)
			AUC _{0-inf} (min×ng ml ⁻¹)	2296.8	691.7 (46.2)
			Bioavailability (%)	0.48	0.38

Notes:

^a Calculated parameter according to equation (5).

^b Calculated parameter according to equation (6).

^c Calculated parameter according to equations (7) and (8).

^d CV, coefficient of variation of a model estimate.

^e SD, standard deviation indicating population variability.

* p<0.05.

p<0.01,

p>0.001.

Table 2

Precision and accuracy of the measurement of (*R,R*)-fenoterol (*(R,R)*-Fen) and (*R,R*)-methoxyfenoterol (*(R,R)*-MFen) in rat urine.

		Nominal concentration ($\mu\text{g ml}^{-1}$)	Concentration determined ($\mu\text{g ml}^{-1}$) (n=5)	Intraday precision (%) (n=5)	Accuracy (%) (n=5)	Interday precision (%) (4 days)	Accuracy (%) (4 days)
<i>(R,R)</i> -Fen	LLOQ	0.1	0.11	14.4	111.4	nd	nd
	LQC	0.2	0.19	8.2	93.8	12.9	99.6
	MQC	2	1.82	3.8	90.8	10.3	90.2
	HQC	20	20.05	13.4	100.2	12.9	102.7
<i>(R,R)</i> -MFen	LLOQ	0.1	0.11	16.9	103.3	nd	nd
	LQC	0.2	0.20	3.2	101.3	13.6	105.7
	MQC	2	1.84	6.9	91.9	9.0	99.8
	HQC	20	21.66	7.5	108.3	8.3	107.7

Note: LLOQ, lower limit of quantification; LQC, low-quality control level; MQC, medium-quality control level; HQC, high-quality control level; n.d., not determined.

Table 3

Dose (%) excreted into urine after intravenous (5 mg kg^{-1}) and oral (25 mg kg^{-1}) administration of (*R,R*)-methoxyfenoterol ((*R,R*)-MFen) and (*R,R*)-fenoterol ((*R,R*)-Fen) to rats ($n \leq 3$).

Compound	Time period (h)	Route of administration	
		Intravenous	Oral
(<i>R,R</i>)-Fen	0-6	2.24	0.17
		7.75	
	6-24	1.70 ± 1.77	0.16 ± 0.12
(<i>R,R</i>)-Fen-G	0-6	1.71	4.43
		7.65	
	6-24	0.14 ± 0.23	3.72 ± 1.80
(<i>R,R</i>)-MFen	0-6	11.04 ± 5.13	1.86
			0.47
	6-24	1.75 ± 0.85	1.07 ± 0.57
(<i>R,R</i>)-MFen-G	0-6	4.37 ± 0.73	11.0
			5.26
	6-24	3.25 ± 0.98	5.62 ± 1.09
(<i>R,R</i>)-Fen	0-6	1.43 ± 0.21	0.05
			0.04
	6-24	0.17 ± 0.05	0.02 ± 0.01
(<i>R,R</i>)-Fen-G	0-6	1.45 ± 0.38	0.10
			0.02
	6-24	0.52 ± 0.12	0.13 ± 0.08

Table 4

Total, non-renal and renal clearances calculated for (*R,R*)-methoxyfenoterol ((*R,R*)-MFen) and (*R,R*)-fenoterol ((*R,R*)-Fen).

Compound	f_e^a (%)	Cl_{tot}^b (ml min ⁻¹ kg ⁻¹)	Cl_{ren}^c (ml min ⁻¹ kg ⁻¹)	Cl_{nonren}^d (ml min ⁻¹ kg ⁻¹)
(<i>R,R</i>)-MFen	12.8	48	6.1	41.9
(<i>R,R</i>)-Fen	6.7	146	9.8	136.2

Notes:

^a f_e , fraction excreted unchanged in urine (see Table 3).

^b Cl_{tot} , total clearance (see Table 1).

^c Cl_{ren} , renal clearance ($Cl_{ren} = f_e \times Cl_{tot}$).

^d Cl_{nonren} , non-renal clearance ($Cl_{nonren} = Cl_{tot} - Cl_{ren}$).