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Enantioselective disposition of (R,R)-formoterol, (S,S)-formoterol and their respective glucuronides in urine following single inhaled dosing and application to doping control

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Keywords

arformoterol, enantiomer, ADME, metabolism, SABA, beta2-agonist

Running title: (R,R)-formoterol and (S,S)-formoterol in urine

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Abstract (249 words)

Formoterol is a long-acting beta2-adrenoceptor agonist (LABA) used for treatment of asthma and exercise-induced bronchoconstriction. Formoterol is usually administered as a racemic (*rac*-) mixture of *(R,R)*- and *(S,S)*-enantiomers. While formoterol is restricted by the World Anti-Doping Agency (WADA), inhalation of formoterol is permitted to a predetermined dose (54 µg/24 hours) and a urine threshold of 40 ng/mL. However, chiral switch enantiopure *(R,R)*-formoterol is available, effectively doubling the therapeutic advantage for the same threshold. The aim of this study was to investigate whether formoterol exhibits enantioselective urinary pharmacokinetics following inhalation. Six healthy volunteers were administered a 12 µg inhaled dose of *rac*-formoterol. Urine was collected over 24-hours and analysed by enantioselective UPLC-MS/MS assay. Total (free drug plus conjugated metabolite) median(min-max) *rac*-formoterol urine levels following inhalation were 1.96(1.05-13.4) ng/mL, 1.67(0.16-9.67) ng/mL, 0.45(0.16-1.51) ng/mL, 0.61(0.33-0.78) ng/mL, and 0.17(0.08-1.06) ng/mL at 2, 4, 8, 12 and 24 hours, respectively, well below the 2019 urine threshold. The proportion of conjugation differed between enantiomers with glucuronide conjugation much greater for *(R,R)*-formoterol (around 30-60% of total) compared to (S,S)-formoterol (0-30%). There was clear evidence of inter-individual

enantioselectivity observed in the ratios of *(R,R):(S,S)*-formoterol, where *(S,S)*- was predominant in free formoterol, and *(R,R)*- predominant in the conjugated metabolite. In conclusion, *rac*-formoterol delivered by inhalation exhibits enantioselective elimination in urine following single dose administration. Enantioselective assays should be employed in doping control to screen for banned beta2-agonist chiral switch products such as *(R,R)*-formoterol, and total hydrolysed *rac*-formoterol is warranted to account for inter-individual differences in enantioselective glucuronidation.

Introduction

Formoterol (USAN, INN, BAN) is a long-acting beta2-adrenoceptor agonist (LABA) widely used for the treatment of airways diseases, particularly asthma and exercised-induced bronchoconstriction (EIB). Formoterol is a chiral compound consisting of *(R,R)*- and *(S,S)*-enantiomers, most commonly administered as 50:50 racemic (*rac*-) mixture with clinical use almost always preferred via inhalation. The *(R,R)*-formoterol enantiomer elicits the pharmacological bronchodilator response, while *(S,S)*-formoterol is considered pharmacologically inert (around 1000x less potent than the R-enantiomer) [1].

There is considerable debate surrounding beta2-agonists with regard to performance enhancing effects, with the need for balancing treatment of asthma and EIB while minimising the potential for doping [2]. Use of formoterol is restricted by the World Anti-Doping Agency (WADA) when taken orally or by other routes such as injection, but is permitted to be administered via inhalation (dry powder or metered dose inhaler) within a predetermined maximum dose of 54 µg over 24 hours. A corresponding urine threshold and decision limit of 40 and 50 ng/mL, respectively [3], have been introduced to minimise supratherapeutic

inhaled or oral dosing for doping purposes. However, these limits are based on the racemic drug [3, 4]. Formoterol is now available in some markets as an enantiopure chiral switch product consisting of (R,R)-formoterol (arformoterol). Optical isomer chiral switch products such as arfomoterol are prohibited by WADA [3], but enantioselective assays are not used for doping control, with only total formoterol reported. There is currently no information on the proportion of total drug in urine present as the *(R,R)*-formoterol enantiomer after an inhaled dose.

In terms of mass balance, around half of administered formoterol is recovered in urine as both free drug and metabolites [5, 6]. Following inhaled delivery, formoterol recovered from the urine is mostly in the form of unchanged free drug (40-60%), as well as an O-demethylated metabolite (5-25%) and glucuronide conjugate (25-40%), the proportion of which may vary significantly between individuals [7]. Glucuronidation occurs at the phenolic position as well as the formation of a benzyl glucuronide [6]. This glucuronidation metabolism is enantioselective [8], and is generally susceptible to considerable pharmacogenetic variability [9]. Although *rac*-formoterol has been on the market for over 20 years, there is still little information regarding the pharmacokinetics of each enantiomer following inhaled delivery. It has been shown for unconjugated formoterol that urinary *(S,S)*-formoterol levels are consistently higher than *(R,R)*-formoterol indicating enantioselective pharmacokinetics both at high inhaled dose (120 µg) [10] and after oral dosing [5]. Unfortunately, there have been no reported studies looking at enantioselective pharmacokinetics of both formoterol and respective glucuronide metabolites in urine following inhaled dosing to date. This is highly relevant for doping control as formoterol is delivered by inhalation for therapeutic use and a threshold limit for total formoterol (free and glucuronide conjugate) is used [4].

Supratherapeutic dosing of beta2-agonists via the inhaled route has been shown to elicit performance-enhancing effects [11-15] as well as increase fat oxidation [16].

It is clear from the short acting beta2-agonist (SABA) salbutamol that there are enantioselective differences in metabolism between oral and inhaled routes of administration that have the potential to be exploited for doping control purposes. Data extracted from Schmekel et al [17] in a repeated dose pharmacokinetic study over 24 hours illustrates the significant potential of enantioselective chromatography to develop better approaches to salbutamol doping control with a mean 0-24 hour urine S:R ratio of 4.9 for oral versus 2.3 for inhaled delivery, presumably due to differences in first-pass metabolism in the liver following oral administration. This suggests there is potential to discriminate between permitted and prohibited routes of administration using enantiomer ratio and this is the focus of ongoing work in our laboratories.

The aim of this study was to investigate whether formoterol does exhibit enantioselective urinary pharmacokinetics following inhalation and the extent of inter-subject differences, and thereby improving our understanding of bioequivalent systemic levels of the active enantiopure *(R,R)*-formoterol. Secondly, if pharmacokinetic enantioselectivity is observed, this may lead to an application in sports doping control.

Method

Subjects

Six subjects (three females) were included in the study, all physically active in recreational sport and aged between 21-45 years of age and BMI within a healthy weight range 18.5-24.9. Subjects currently taking formoterol or salmeterol were excluded, along with those with moderate or severe persistent asthma according to the National Asthma Council Guidelines (Australia) or any contraindications to receiving a beta2-agonist. Subjects were instructed how to use a dry powder inhaler, before inhaling two 6 μg doses of formoterol as formoterol fumarate dihydrate (Symbicort ® Turbuhaler ®; AstraZeneca, NSW, Australia) one minute apart under supervision equivalent to a total dose of 12 μg. Subjects were provided with sample containers and self-collected urine at baseline, 2, 4, 8, 12 and 24 hours post dose, each ± 15 minutes, and stored frozen overnight until being returned to the laboratory the next day and stored at -30 °C until analysis. Subjects were asked to avoid dehydration but otherwise go about normal activities. All subjects gave informed consent and the study was approved by the Human Research Ethics Committee (Tasmania Network); H0014003.

Formoterol enantiomer determination in urine

Enantioselective formoterol analyses were undertaken using an UPLC-MS/MS (ultra performance liquid chromatograph-mass spectrometry) assay that was modified from previous work with chiral beta2-agonists in our laboratory [18-21]. An enzyme hydrolysis method was used to determine total formoterol enantiomers (free drug plus glucuronide) based on the previous method reported by Zhang et al [5], with formoterol glucuronide concentration determined by subtracting free (unconjugated parent drug) from total formoterol (after hydrolysis).

In brief, calibration samples were prepared consisting of concentrations of 0.1, 0.5, 2.0, 10, 40 ng/mL unlabeled formoterol in drug free human urine from *rac*-formoterol fumarate dihydrate (Carbosynth, Compton, UK). Internal standard *rac*-formoterol-D6 (Toronto Research Chemicals, Toronto, Canada) was first added to each study urine sample (400 µL) or calibration urine sample equivalent to 10 ng/mL in an Eppendorf® centrifuge tube.

Dilute ammonia solution (100 µL) was then added to each sample sufficient to give a final pH of 8.5 and vortex mixed, before the addition of 850 µL of HPLC grade ethyl acetate. This was vortex mixed for one minute and then centrifuged at 15 000 g for five minutes. The organic supernatant was then transferred to a glass autosampler vial, from which the solvent was evaporated under nitrogen at 40°C. Extraction of urine was repeated with a second 850 µL aliquot of ethyl acetate. Combined residue was reconstituted using 80 µL of methanol and vortex mixed prior to analysis via UPLC-MS/MS. To hydrolyse glucuronides of formoterol enantiomers, β-glucuronidase (1000 units from *Helix pomatia*; Sigma-Aldrich, Sydney Australia) in 250 µL of 0.1 M acetate buffer (pH 5) was added to each urine sample (250 µL) together with 10 ng of *rac*-formoterol-D6. These samples were incubated in a temperature controlled room at 37°C for 21 hours on a table mixer at 200 rpm. After incubation, dilute ammonia solution (100 µL) was added to each sample sufficient to give a final pH of 8.5 and vortex mixed, then samples were extracted with 2 mL of ethyl acetate and processed and analysed in the same manner as the unhydrolysed samples. A calibration curve was constructed from the ratio of analyte *(R,R)*-formoterol to internal standard *(R,R)*-formterol-D6 in each sample, and similarly *(S,S)*-formoterol to internal standard *(S,S)*-formterol-D6.

The UPLC instrument was a Waters Acquity® H-class UPLC system (Waters Corporation, Milford, MA). Chromatography was performed using an Astec® CHIROBIOTIC™ T2 chiral column (4.6 × 250 mm × 5 μm particles) (Sigma-Aldrich). The UPLC was coupled to a Waters Xevo® triple quadrupole mass spectrometer (Waters Corporation). Analyses were undertaken using multiple reaction monitoring (MRM) in positive electrospray ionisation mode. The UPLC was operated with a mobile phase consisting of 100% methanol with 0.2% acetic acid and 0.025% ammonium hydroxide. Elution was isocratic for 30 min. The flow rate was 0.8 mL/min and the column was held at room temperature. Injection volume was 50 μL. Electrospray ionisation was performed with a capillary voltage of 2.76 KV, a cone voltage of 30 V and individual collision energies for each MRM transition, as described below. The desolvation temperature was 450°C, nebulising gas was nitrogen at 950 L/h and cone gas was nitrogen at 50 L/h. MRM transition monitored for formoterol was (m/z) 345 to 149, (collision energy 19 V), and MRM transition monitored for formoterol-D6 was (m/z) 351 to 155, (collision energy 19 V). Dwell time per channel was 36 ms. Confirmation of enantiomer elution order was undertaken by analysis of *(R,R)*-formoterol standard (TLC Pharmaceutical Standards, Ontario, Canada). Basic assay performance measures (sensitivity, precision, accuracy, recovery, and linearity evaluated by r^2) were all determined as per standard laboratory protocols, with method detection limit defined as a signal-to-noise ratio of 3 and lower limit of quantification as a signal-to-noise ratio of 10 [22]. The "drop perpendicular" method of peak integration was used where a vertical line from the valley of the peaks is dropped to the horizontal baseline.

Statistical Analysis

Enantioselectivity in urine was determined using log (S,S):(R,R) ratio with one-sample t-test against a hypothetical value of 0 (no enantioselectivity). Results were log transformed to account for lack of symmetry with ratios less than 1. Analyses were performed using JMP 11.2.0 (SAS Institute Inc, NC, USA) and GraphPad Prism 6 for Mac OSX (GraphPad Software Inc, CA, USA) with results of $p<0.05$ considered statistically significant.

Results and Discussion

Formoterol enantiomers were satisfactorily resolved (Figure 1) (< 15% of peak height, peak resolution R_s=1.4) to allow accurate and reproducible quantitation with adequate assay sensitivity and performance required for determination of urine levels following inhaled dosing (Table 1). Figure 1 demonstrates that the background sample matrix baseline from a blank urine was low and flat, as expected from an MRM analysis. Of the four most common beta2-agonists (salbutamol, terbutaline, formoterol, salmeterol) in our laboratory, we have found that formoterol enantiomers are the most difficult to assay using UPLC-MS/MS detection. This is due to both the low doses used in inhaled delivery, and the need to optimise signal based on a trade-off between peak resolution and MS sensitivity, due to differences in electrospray ionisation suppression from the mobile phase additives required for enantiomer separation. Analytical performance is summarised in Table 1.

Rac-formoterol has a urinary threshold of 40 ng/mL with a decision limit of 50 ng/mL consisting of parent drug and glucuronide [4]. These limits are based on maximum doses permitted by WADA of 54 μg in a 24-hour period [3]. In the present study, we observed total (free drug plus conjugated) median(min-max) *rac*-formoterol urine levels of 1.96(1.05-13.4)

ng/mL, 1.67(0.16-9.67) ng/mL, 0.45(0.16-1.51) ng/mL, 0.61(0.33-0.78) ng/mL, and 0.17(0.08-1.06) ng/mL 2, 4, 8, 12 and 24 hours following inhalation, respectively. Median levels of formoterol enantiomers and their respective glucuronide conjugates are shown in Table 2. Individual subject enantiomer levels and glucuronide conjugation over time is shown in Figure 2, which depicts considerable variation between subjects with respect to overall levels and relative proportions of free and conjugated formoterol enantiomers.

The maximum recorded individual urine level (Table 2) of *rac*-formoterol (free plus glucuronide) was 13.4 ng/mL which is in broad agreement with previous work that has been undertaken at higher doses [23-25]. Previous reports have demonstrated a maximum concentration of 19.6 ng/mL total drug (free plus glucuronide) following a dose of 18 μg over 8 hours (one third of daily limit) but most routine samples were below 10 ng/mL [25]. Similar results were observed by Deventer et al [23], where maximum concentration of free drug was 8.5 ng/mL following a 18 μg dose, and by Eibye et al [24] with a maximum concentration of 25.6 ng/mL corrected for specific gravity following inhalation of repetitive doses up to 72 μg in 6 hours. This has led to criticisms that the current threshold is too low and that further validation is required [23]. While our work did not correct for urine specific gravity, other single dose pharmacokinetic studies with high dose inhaled salbutamol has demonstrated that exercise and dehydration do affect urine concentrations compared to rest, resulting in a greater risk of exceeding the WADA decision limit, but correcting for specific gravity results in only modest improvements [26].

Although free formoterol enantiomers have been previously measured following inhalation (albeit at high dose; [10]), to our knowledge, this is the first report of total urine formoterol

enantiomer levels (free plus glucuronide) following inhaled dosing, and only the second study to report total urine formoterol enantiomer levels in urine (the previous report following oral dosing) [5]. Enantioselectivity can be clearly seen in Figure 3 and 4. It is evident that the proportion of conjugation differs between enantiomers (Figure 3) where the extent of glucuronide conjugation is much greater with *(R,R)*-formoterol (around 30-60% of total) compared to *(S,S)*-formoterol (0-30%). There is also clear evidence of enantioselectivity observed in the ratio of *(R,R):(S,S)*-formoterol, both for free formoterol, and formoterol glucuronide (Figure 4), where *(S,S)*- is predominant in free formoterol, and *(R,R)*- is predominant in the conjugated metabolite. These ratios are consistent with that observed up to eight hours after oral dosing by Zhang et al [5] and as reported by Lecaillon et al [10] which measured free drug only. We did not observe any reversed enantioselectivity, which was observed in the sole female participant in the study by Zhang et al [5].

The pharmacokinetics of each formoterol enantiomer following inhaled delivery are clearly different, but from our work here, it is unclear what effect repeated or cumulative dosing has on the ratio. From this data alone, it would seem reasonable to propose that the *(S,S):(R,R)* ratio for conjugated formoterol would be higher with cumulative dosing, and may offer discriminatory capability between permitted and prohibited dosing that warrants further exploration for doping control applications. Furthermore, from our data, we can see that given a *rac*-formoterol urine level in the first four hours after a dose, based on an *(S,S):(R,R)* of 1.6, only 38% of the drug would be present as pharmacologically active *(R,R)*-formoterol. So not only are there criticisms the current urine threshold is too low for *rac*-formoterol, if an athlete uses a bioequivalent dose chiral switch *(R,R)*-formoterol product, their levels are going to be almost one-third lower for the same therapeutic advantage. Presence of (R,R)-

formoterol only in urine would be indicative of prohibited arformoterol administration but further studies are needed to rule out chiral conversion in an individual.

Conclusion

Rac-formoterol delivered by inhalation exhibits enantioselectivity in urine following single dose administration that changes during the elimination phase. Enantioselective pharmacokinetics differs between parent drug and metabolite, and may offer the potential of improved discriminatory detection capability for doping applications with formoterol. Enantioselective assays should be employed in doping control to screen for banned beta2-agonist chiral switch products such as *(R,R)*-formoterol. Quantitative analysis using total hydrolysed *rac*-formoterol is warranted to account for inter-individual differences in enantioselective glucuronidation.

Conflicts of Interest

Glenn Jacobson has received funding from the World Anti-Doping Agency (WADA) to investigate the enantioeselctive pharmacokinetics of salbutamol (13D24GJ) and formoterol (14A32GJ) in urine and their application to doping control. Morten Hostrup, Haydn Walters, Christian Narkowicz and David Nichols have no conflicts of interest relevant to the content of this article.

Acknowledgements

This project has been carried out with the support of the World Anti-Doping Agency (WADA; reference 14A32GJ), and the Discipline of Pharmacy, School of Medicine University of Tasmania.

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Table 1. Analytical method performance data of formoterol enantiomers

	(R,R)-formoterol	(S,S)-formoterol
Correlation coefficient r^2	0.9998	0.9997
Intra-day accuracy (%, n=5)		
0.1 ng/mL	5.9	-3.4
2.0 ng/mL	-1.5	-5.3
Intra-day precision (%RSD, n=5)		
0.1 ng/mL	4.1	5.5
2.0 ng/mL	5.6	1.3
Method detection limit (MDL)[¶] ng/mL	0.007	0.007
Lower limit of quantification (LLoQ) [¶] ng/mL	0.022	0.023
Recovery (%)	98	98
Freeze-thaw robustness (% loss)		
20 ng/mL	4.1	2.7

[¶] method detection limit and lower limit of quantification was determined from the signal-to-noise ratio (S/N) at the 0.1 ng/mL level with MDL and LLoQ defined as S/N=3 and S/N=10 respectively [1].

Table 2. Formoterol enantiomers and their respective glucuronides in urine following a single inhaled dose of 12 µg.

Time	(R,R)-formoterol	(S,S)-formoterol	(R,R)-formoterol glucuronide*	(S,S)-formoterol glucuronide*	*rac*-total	Fraction of total present as glucuronide*
	Median (min-max) ng/mL	Median (min-max) ng/mL	Median (min-max) ng/mL	Median (min-max) ng/mL	Median (min-max) ng/mL	Median (min-max) %
0	0.00	0.00	0.00	0.00		-
2	0.61 (0.38-0.96)	1.01 (0.50-1.72)	0.35 (0.11-7.10)	0.11 (0-3.64)	1.96 (1.05-13.4)	73 (20-90)
4	0.35 (0.07-1.00)	0.60 (0.09-1.75)	0.56 (0.01-4.76_	0.19 (0.00-2.16)	1.67 (0.16-9.67)	50 (28-94)
8	0.12 (0.03-0.38)	0.19 (0.03-0.58)	0.12 (0.00-0.47)	0.06 (0.00-0.17)	0.45 (0.16-1.51)	62 (36-99)
12	0.14 (0.07-0.26)	0.17 (0.09-0.38)	0.11 (0.00-0.16)	0.05 (0.00-0.12)	0.61 (0.33-0.78)	55 (49-82)
24	0.06 (0.03-0.27)	0.08 (0.03-0.32)	0.03 (0.00-0.48)	0.00 (0.00-0.16)	0.17 (0.08-1.06)	87 (40-100)

* Free formoterol equivalents

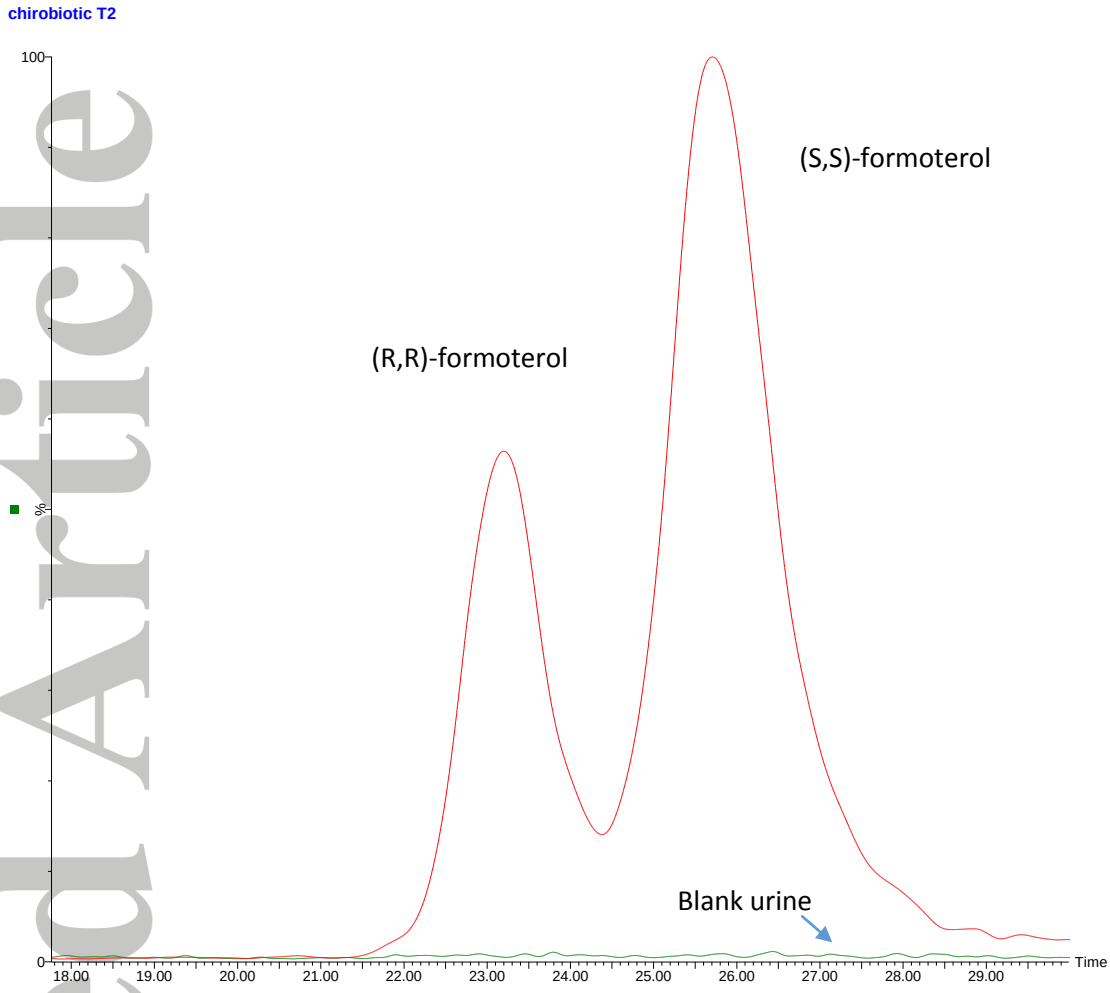

Figure 1. Example UPLC-MS/MS chromatogram of formoterol enantiomers in a subject's urine following a 12 µg inhaled dose of *rac*-formoterol overlaying a blank urine sample; *(R,R)*-formoterol 0.65 ng/mL and *(S,S)*-formoterol 1.09 ng/mL.

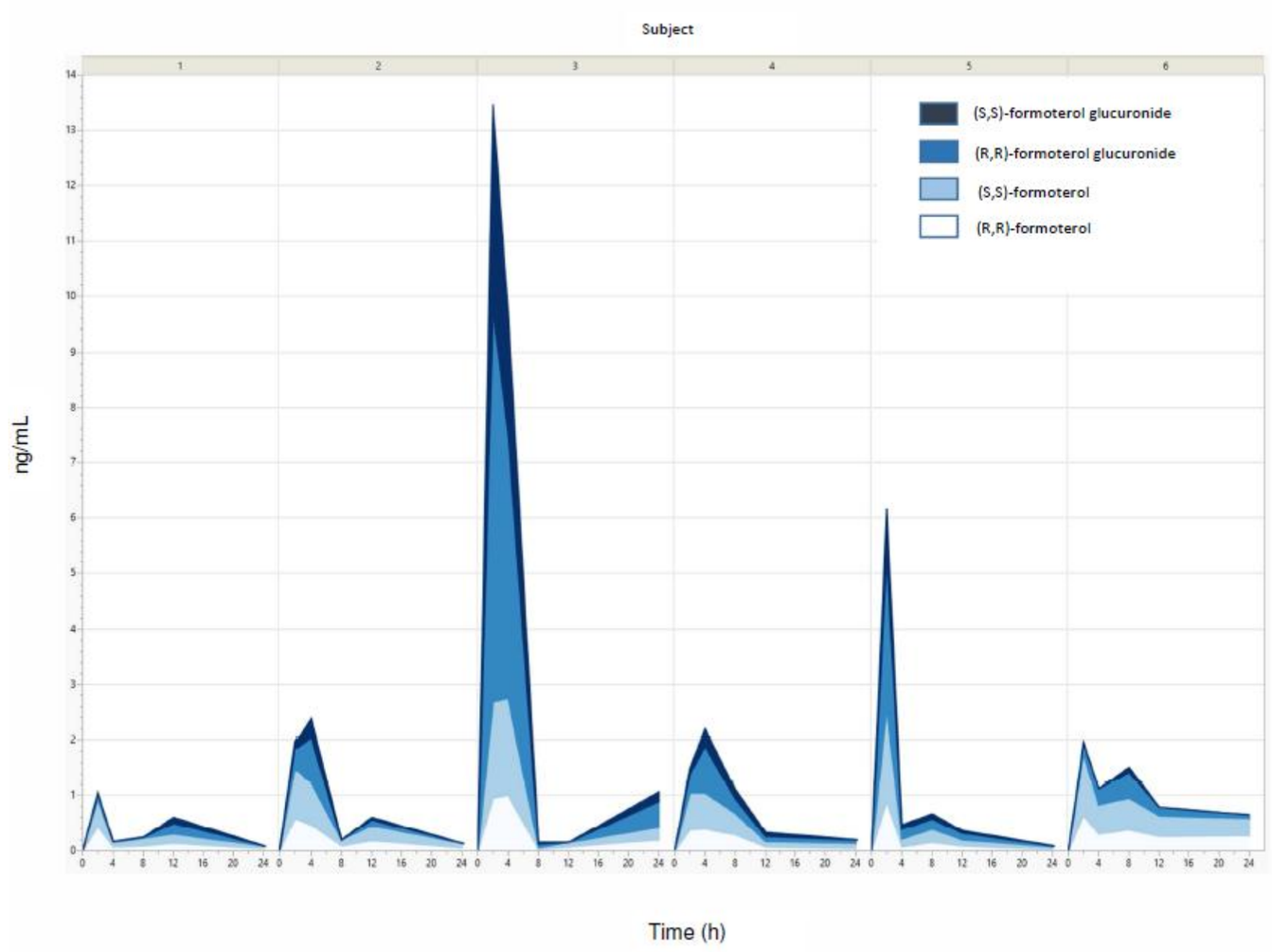

Figure 2. Urine levels (mean±SEM) of formoterol enantiomers (A) and formoterol enantiomer glucuronides (B) following a 12 µg inhaled dose of *rac*-formoterol demonstrating enantioselective pharmacokinetics.

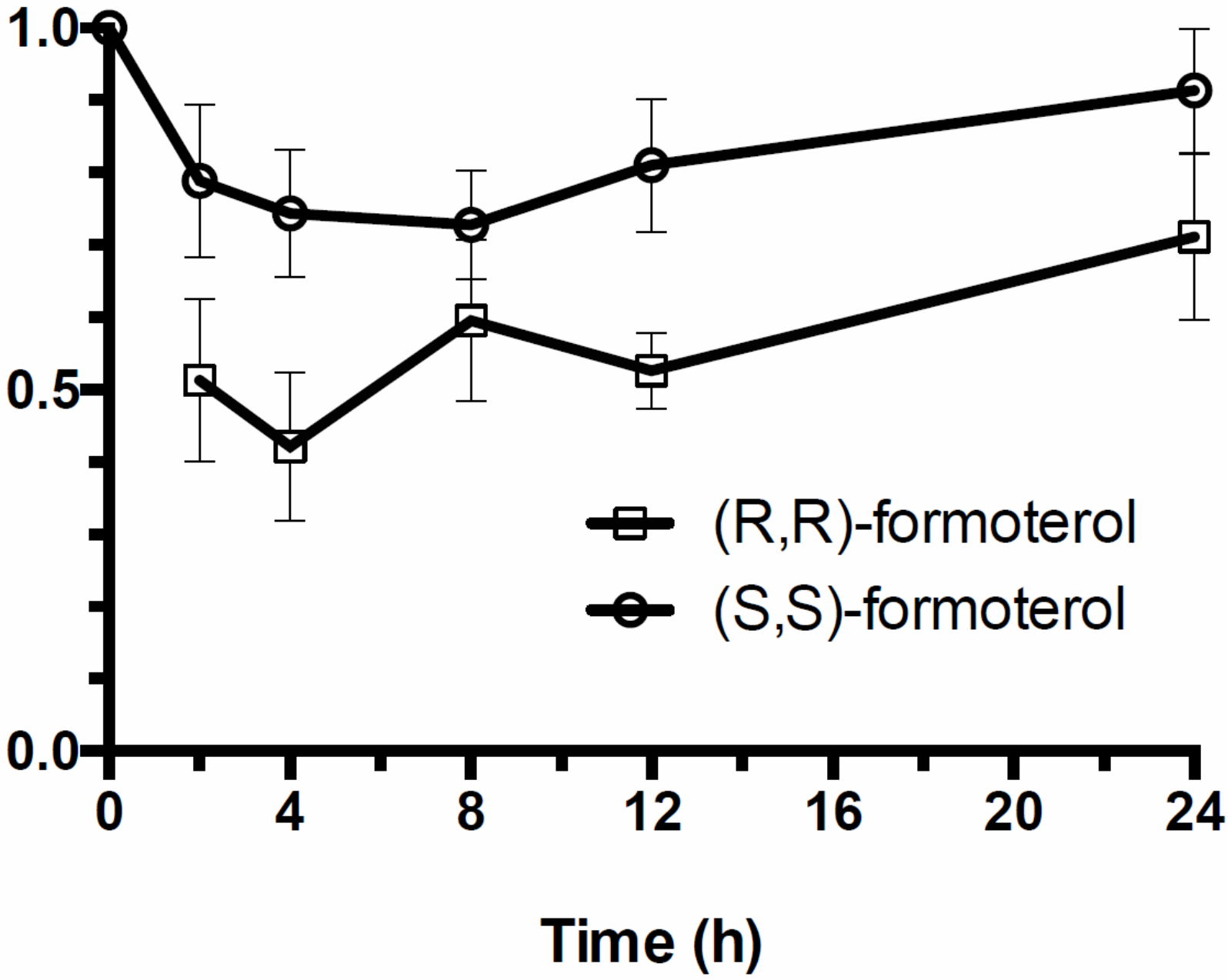

Figure 3. Extent of conjugation (free formoterol as a proportion of total) for each enantiomer.

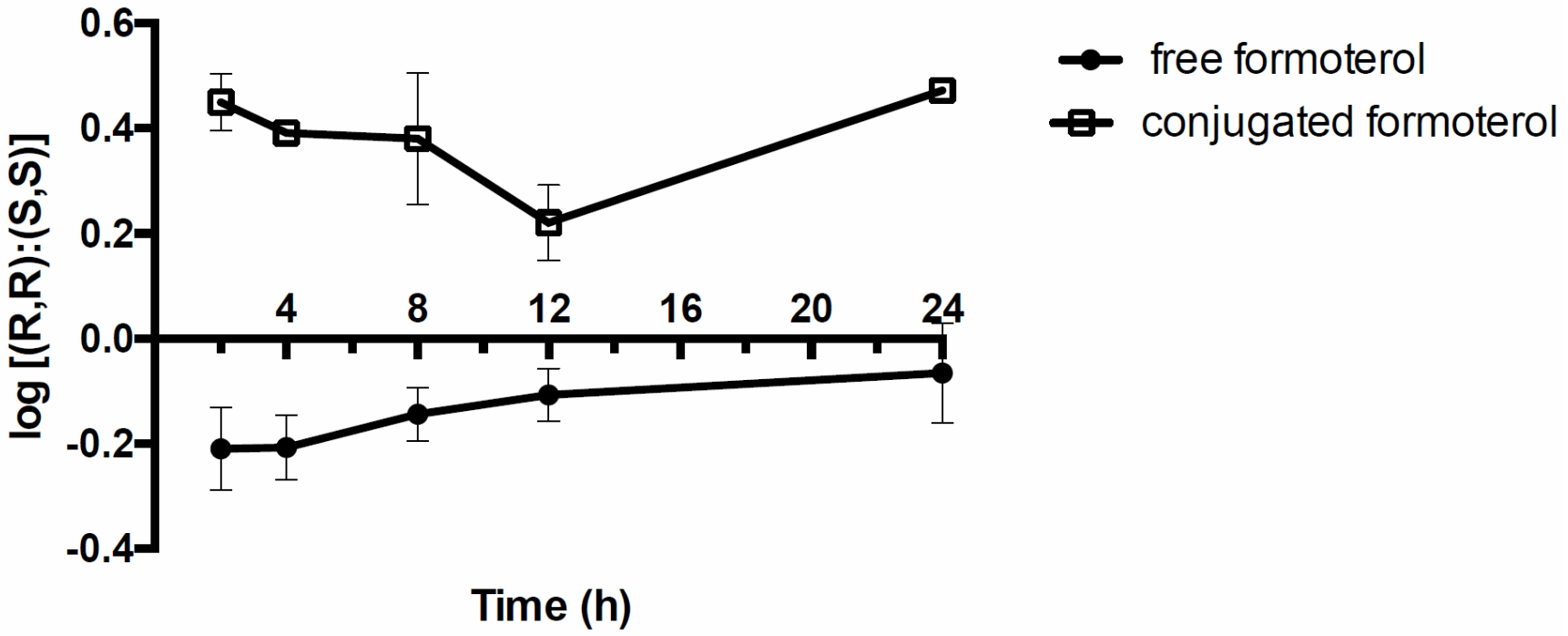

Figure 4. Enantioselectivity of formoterol (free) and formoterol glucuronide (conjugated) in urine following a 12 µg inhaled dose of *rac*-formoterol demonstrating enantioselective pharmacokinetics, $p<0.05$.