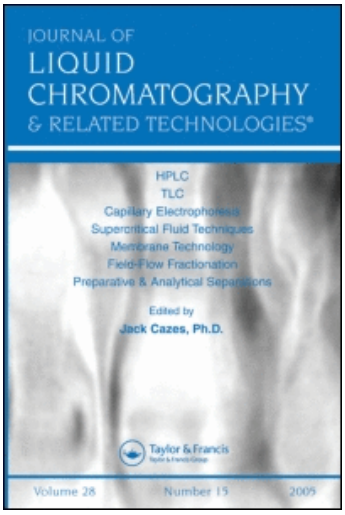

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DETERMINATION BY HPLC WITH ELECTROCHEMICAL DETECTION OF FORMOTEROL RR AND SS ENANTIOMERS IN URINE

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ABSTRACT

A method is described for the determination of the R,R- and S,S-enantiomer of the long-acting ß2-adrenoceptor agonist formoterol, which is marketed as a racemate for the treatment of asthma. The methodology is a modification of a previously published assay for formoterol. The sample clean-up from urine takes place by liquid liquid extraction followed by solid phase extraction. An AGP-column combined with electrochemical detection is used for the separation and detection of the enantiomers.

A diastereomer of formoterol (R,S- or S,R- configuration) is used as internal standard. The lower limits of detection of R,R-formoterol and S,S-formoterol were 60 and 75 pmol/L respectively. The method was validated by a cross-check of spiked urine samples with a GC/MS method for "racemic" formoterol (correlation of the sum of the enantiomers measured by HPLC with GC/MS results: r=0.999, n=17). The method appears to be well suited for pharmacokinetic studies of formoterol enantiomers in human subjects after inhalation of therapeutic doses of formoterol.

INTRODUCTION

Formoterol fumarate, 2-Hydroxy-5-[1RS)-1-hydroxy-2-{[(1RS)-(p-methoxyphenyl)-1-methylethyl]-amino}-ethyl]-formanilide fumarate dihydrate, is a new long-acting, $ß_2$-adrenoceptor agonist, with a high potency. Formoterol fumarate is a mixture of the two isomers with the configurations R,R and S,S; the RR-enantiomer being the active compound.[1,2] Pharmacokinetic data on the formoterol enantiomers are still missing due to the lack of a sensitive enantioselective assay.

Recently van den Berg et al. developed a high performance liquid chromatography (HPLC) assay with electrochemical detection to determine racemic formoterol in plasma.[3] This method was used to investigate the pharmacokinetics of racemic formoterol in plasma of human subjects after an oral dose of 168 μg of formoterol fumarate.[4] Despite the high sensitivity of this assay, it is still not sensitive enough to determine the plasma concentrations after inhaled therapeutic doses of formoterol fumarate and it is not suitable for the determination of the separate enantiomers. Formoterol is partly excreted into the urine, both after inhalation and oral administration.[5] Using a GC/MS method[7] it was shown that after inhalation of 12 μg formoterol, 24 ± 14 % of the dose was excreted as unchanged and conjugated formoterol into the urine within 12 hours, giving urinary concentrations which in pricipal could be determined using HPLC with electrochemical detection. Thus measuring the urinary excretion of formoterol is, at the moment, the only available option to get more information about the pharmacokinetics of formoterol and its enantiomers after inhalation of low therapeutic doses.

The four possible diastereomers of formoterol can be separated on a chiral column.[1] Based on this observation and our experience with electrochemical detection of formoterol, we have developed an enantioselective HPLC method

for the determination of R,R- and S,S-formoterol in human urine. Finding a good clean-up procedure for the urine samples, together with the selection of a suitable internal standard, was critical in the development of the assay.

MATERIALS

Apparatus

The HPLC system consisted of a Spectraflow 400 (Separations, Rotterdam, The Netherlands) with a Rheodyne 7125 injector with a PEEK 200 μL loop. An electrochemical detector Waters model EC 460 (Millipore-Waters Etten-Leur The Netherlands) was employed. The electrochemical detector was used with the original electrochemical cell or with an Antec electrochemical cell (Antec Leiden, The Netherlands), both with a glassy carbon working electrode, a reference Ag/AgCl and an auxiliary 316 stainlesss steel electrode. For appropiate functioning of the detector with the Antec electrochemical cell temperature fluctations must be minimized. For that reason the analytical column and the guard column were built into the cell-house of the detector, and the teflon tubing between column and cel was isolated with silicone tubing. The voltage of the working electrode was set on + 0.63 volt.

The enantioselective separation was achieved using a chiral α_1-acidglycoprotein (AGP) column 100x4.0 mm with an AGP guard column 10x4.0 mm (Baker, Deventer, The Netherlands) at ambient temperature (22°C).

Peak heights were recorded using a dual channel recordèr (Kipp & Zonen, Delft, The Netherlands), one channel, connected with the 1.0 V output, recorded the low range concentrations the other, connected with the 10 mV output, the high range concentrations.

The mobile phase consisted of isopropanol: phosphate buffer 50 mM pH 7.0 (1.5 : 100) with 1mM KCL and a small quantity of complexing agent (EDTA). The mobile phase was filtered using 0,22 μm filter (Millipore-Waters Etten-Leur, The Netherlands). Electrochemical detector and pump (flow 0.9 mL/min) were continuously running 24 hours a day.

Chemicals

Chemicals, water (18 MΩ) and the organic solvents (Merck, Darmstadt, Germany) were of analytical grade and used without further pretreatment. Racemic formoterol fumarate, its enantiomers R,R- and S,S-formoterol fumarate as well as the racemic mixture of their diastereomers (configuration R,S and S,R) were kindly provided by Ciba-Geigy Ltd., Basle, Switzerland. Solid-phase SiOH 100mg 1mL extraction columns were obtained from Baker (Baker, Deventer, The Netherlands).

Preparation of theInternal Standard

The internal standard working solution was prepared as follows: 50 μL of a stock solution of the diastereomers of formoterol fumarate in methanol (concentration: 2.5 mg/mL) was injected into the HPLC apparatus equipped with the chiral AGP column. An UV detector (210 nm) was used instead of the electrochemical detector, the mobile phase being 10 mM phosphate buffer pH 7.0 (flow 0.9 mL/min). The last eluting peak (retention time 120 min) was collected to a total volume of about 50 mL. This solution containing either the R,S- or S,R- enantiomer (the absolute configuration was not determined) was used directly as internal standard working solution.

METHODS

Clean-up

A stock solution of racemic formoterol fumarate in methanol was prepared at a concentration of 100 μg/mL and was stored at - 20°C. To prepare calibration samples, working solutions were made by appropriate dilutions of the stock solution in double distilled water. 100 μL of the respective working solution were added to 900 μL of drug free urine, collected from healthy volunteers during the day. The calibration samples covered a concentration range of 0.15 to 48 nmol/L of R,R- and S,S-formoterol.

A two step extraction procedure was needed for a satisfactory clean-up of the urine samples. In glass tubes 100 μL of the IS solution and 100 μL of 250 mM phosphate buffer pH 8 were added to each calibration sample and to 1 mL aliquots of urine samples with unknown concentrations. The mixture was briefly vortexed and 3 mL of ethyl acetate was added. Extraction was

performed by mechanical tumbling during 30 minutes at 25 rpm followed by centrifugation for 10 minutes at 4000 rpm.

The organic layer was carefuly removed with a Pasteur pipette and brought upon a preconditioned (see below) solid-phase silica extraction column. The columns were washed by successive rinsing with 1 mL of ethyl acetate and 10 mL of 5% isopropanol in water, and were then centrifuged for 10 minutes at 4000 rpm.

Formoterol and the internal standard were eluted from the columns with 3 mL of methanol, and the eluate was evaporated to dryness at 30°C under a continuous stream of nitrogen. The residue was redissolved in 100 μL of the mobile phase and injected onto the HPLC column.

The solid-phase extraction columns were conditioned by prewashing with 3 mL methanol followed by 3 mL ethylacetate. Between the conditioning and the concentration step, care was taken to ensure that the sorbent bed did not dry.

AnalyticalCross-check

The following samples were prepared at Ciba-Geigy, Basle, using blank urine obtained from a healthy subject: Six samples spiked with racemic formoterol, six samples spiked with either R,R- or S,S- formoterol and six samples spiked with both enantiomers. The concentration range was 0.4 to 40 nmmol/L for each enantiomer. Two blank urine samples completed the set of 20 samples. Each sample was split into two aliquots. One set of samples was sent deep-frozen to the Academic Medical Center, Amsterdam, where the samples were analysed by the described enantiospecific HPLC method. The second set of samples was stored below -18°C until analysis by a non-stereospecific gas chromatographic/mass-spectrometric (GC/MS) method at Ciba-Geigy, Basle.

The GC/MS method used was based on the method described by Kamimura et al.[1] The main modifications were as follows: Deuterium labelled $^2H_5$ to $^2H_7$) formoterol fumarate was used as internal standard. Gas chromatography was performed on a fused silica capillary column (12 m x 0.2 mm i.d.) coated with 5% diphenyl 95% dimethyl polysiloxane. The mass spectrometer was operated in the negative ion chemical ionisation mode (NCI) using methane as reagent gas and selected ion monitoring (SIM) was

performed on the fragment ions (M-HF) m/z 584 (derivative of formoterol) and m/z 589 (derivative of deuterated formoterol).

VALIDATION AND RESULTS

Calibration

To construct calibration curves, urine samples with known concentrations were prepared as described in a previous section.

After the samples were processed, the entire extract was injected into the injection valve and released onto the analytical column via the 200 μL loop. The peak-heights of the compounds of interest were measured and ratios of R,R- and S,S- enantiomers to the internal standard were calculated by hand. The calibration curves for both the enantiomers were calculated with linear regression analysis. The following terms for the calibration curve (Y=A+Bx) in the range of 0.1 to 50 nmol/L were obtained: RR-calibration curve; intercept 0.0108, slope 0.2575, correlation coefficient 0.9998, SS-calibration curve; intercept 0.0018, slope 0.1952 , correlation coefficient 0.9999.

Table 1

Comparison of HPLC Results (Sum of Both Enantiomers: = y) vs. GC/MS Results (=x); Linear Least-Squares Regression Line y = a + bx; R = Coefficient of Correlation; N = Number of Samples

	a	b	R	N
First HPLC assay vs. GC/MS	-0.3340	0.9885	0.9987	17*
Second HPLC assay vs. GC/MS	-1.2666	1.0549	0.9953	18

*One sample could not be evaluated due to distrubances in the HPLC chromatogram.

Table 2

Comparison of Found (= y) vs. Given (= x) Concentrations; Linear Least-Squares Regression Line y = a + bx; R = Coefficient of Correlation; N = Number of Samples

	a	b	R	N
First HPLC assay: R, R-formoterol	-0.0485	0.9529	0.9979	14*
Second HPLC assay: R, R-formoterol	-0.3330	0.9939	0.9878	15
First HPLC assay: S,S-formoterol	-0.0684	0.9642	0.9995	14*
Second HPLC assay: S, S-formoterol	-0.5168	1.0234	0.9966	15
GC/MS: sum enantiomers	0.0184	0.9797	0.9999	18

*One sample could not be evaluated due to disturbances in the HPLC chromatogram.

The recoveries of R,R-formoterol and S,S-formoterol were 93,2% (CV% 2.6) and 92.3 (CV% 4.1) respectively.

Analytical Cross-check

The concentrations obtained with the HPLC and the GC/MS assay and also the given and found concentrations were compared and evaluated by means of linear regression ('zero' concentrations were not included). The results are summarized in Tables 1 and 2.

Table 3

Between-Day Precision and Accuracy of R,R-Formoterol

Expected (nmol/L)	Found (n=3) (nmol/L)	Inter-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	29.12	2.8	-2.1
11.89	11.41	2.9	-4.0
2.97	2.84	2.2	-4.4
1.49	1.46	5.2	-2.0
0.48	0.51	12.4	+6.3

Table 4

Between-Day Precision and Accuracy of S,S-Formoterol

Expected (nmol/L)	Found (n=3) (nmol/L)	Inter-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	28.85	2.7	-3.0
11.89	12.05	7.5	+1.3
2.97	3.01	2.7	+1.3
1.49	1.52	7.0	+2.0
0.48	0.56	12.7	16.7

Between-day Precision and Accuracy

Five spiked human urine samples with concentrations in the range of 0.68 - 29.73 nmol/L R,R-formoterol and S,S-formoterol were analyzed on three different days (Table 3 and 4).

The inter-assay coefficient of variation ranged from 2.2 to 12.4 % for R,R-formoterol and 2.7 to 12.7 % for S,S-formoterol. The deviation from the mean values ranged from -4.4 to + 16.7 %.

Table 5

Within-Day Precision and Accuracy of R,R-Formoterol

Expected (nmol/L)	Found (n=5) (nmol/L)	Intra-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	28.68	2.6	-3.5
2.97	2.99	1.6	+0.7
1.49	1.48	4.8	+0.7

Table 6

Within-Day Precision and Accuracy of S,S-Formoterol

Expected (nmol/L)	Found (n=5) (nmol/L)	Intra-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	29.02	2.1	-2.4
2.97	2.89	2.7	-2.7
1.49	1.43	4.0	-4.0

Within-Day Precision and Accuracy

Three spiked human urine samples with concentrations in the range of 1.49 to 29.73 nmol/L R,R-formoterol and S,S-formoterol were analyzed five times on the same day (Tables 5 and 6).

The coefficient of variation ranged from 1.6 to 4.8 % for R,R- enantiomer and 2.1 to 4.0 % for S,S-formoterol. The deviation from theory of the mean values ranged from -3.5 to +0.7 % for R,R-enantiomer and -4.0 to -2.4 % for S,S-formoterol.

DISCUSSION

Isolation of Formoterol from Urine

When developing the assay for urine samples with electrochemical detection and enantiomers separation with a AGP-column, we had to face problems concerning variable matrix composition of the urine samples, instability of the electrochemical detector and low resolution of the AGP column. The finding of an internal standard which has a close chemical resamblance to the components of interest, allowed a thorougly cleanup. We assume that both formoterol and the internal standard are trapped on silica by means of their formanilide and amine functional groups.

Chromatography and Detection

R,R-formoterol, S,S-formoterol and diastereomer R,S- or S,R-formoterol retention times were 8.7, 11.3 and 15.5 min., respectively (Figure 1). No endogenous substances with similar retention times were seen.

The use of an ANTEC electrochemical detector cell made it possible to detect the components of interest in the pmol/L range. According to a signal-to-noise ratio of 3:1, the lower limits of detection of R,R- formoterol and S,S-formoterol were 60 and 75 pmol/L respectively.

As was discussed before[3] it is especially the ortho position of the phenolic hydroxygroup of the molecule which makes electrochemical detection a viable choice for highly sensitive determination.

Tuning the mobile phase composition did not effect the enantioselectivity. Variable organic modifiers were used, such as acetonitril,methanol, aceton and isopropanol, and also the phosphate buffer molarity. These did not change the selectivity but only effect the capacity.

The method appeared to be well suited for pharmacokinetic studies of formoterol enantiomers in human subjects after inhalation of therapeutic doses of formoterol.

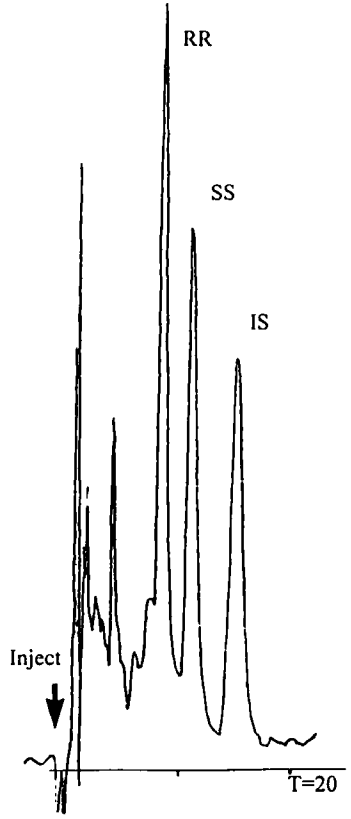

Figure 1. Separation of the enantiomers of formoterol (RR and SS) and its diastereomer used as internal standard (IS).

Application

The described method was applied to the determination of both enantiomers in urine of healthy volunteers after single inhaled doses of 12, 24, 48, 96 μg formoterol fumarate. The profiles of the urinary excretion rate of the enantiomers as obtained in a representative subject after inhalation of the 48 μg dose are shown in Figure 2.

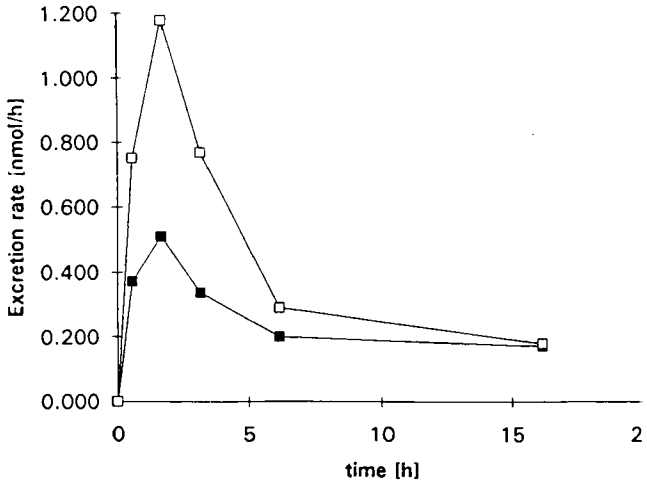

Figure 2. Urinary excretion rate of R,R- (9) and S,S-formoterol (;) in a healthy volunteer after a single inhaled dose of 48 μg of formoterol fumarate.

CONCLUSIONS

The two step clean-up procedure appears to be very well suited for determination of the enenatiomers of formoterol in urine using a AGP-column for the chiral separation with oxidative electrochemical detection. This method can be used in pharmacokinetic- pharmacodynamic studies of formoterol enantiomers in urine of healthy volunteers and patients.

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