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ANALYSIS OF SALBUTAMOL ENANTIOMERS IN HUMAN URINE BY CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRELIMINARY STUDIES RELATED TO THE STEREOSELECTIVE DISPOSITION KINETICS IN MAN

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SUMMARY

Enantiomers of salbutamol, extracted from human urine, were successfully separated and quantitated by high-performance liquid chromatography with electrochemical detection. This direct resolution was accomplished using a chiral α_1-acid glycoprotein column (EnantioPac) maintained at 0°C and a mobile phase consisting of a 0.1% (v/v) triethylamine in 5.3 m*M* citrate buffer, pH 7.2. An amperometric detector incorporating a glassy carbon electrode was employed for detection. The between-day coefficients of variation for the determination of *R*(−)- and *S*(+)-salbutamol in human urine were 4.1 and 4.7%, respectively, at a drug level of 1.0 μg/ml. The urinary excretion ratio of the biologically active (−)-isomer to (+)-isomer in one healthy subject who received an intravenous dose of racemic salbutamol (1.0 mg) decreased continuously over a 12-h period. A similar excretion pattern exhibiting a far more extensive distortion in the enantiomeric ratio was found in three subjects dosed with a single 4-mg tablet of racemic salbutamol.

INTRODUCTION

Salbutamol, 2-*tert.*-butylamino-1-(4-hydroxy-3-hydroxymethyl)phenylethanol, is a relatively selective β_2-adrenoreceptor stimulant widely used as a therapeutic agent in bronchial asthma and other forms of reactive airways disease [1,2]. Like many β-adrenergic agents, it is used clinically as a racemic mixture of two optical isomers, *R*(−)- and *S*(+)-salbutamol. Even though both enantiomers show high selectivity of action as the racemate for β-adrenoreceptors in tracheobronchial muscle compared to cardiac muscle [3], the drug's agonistic activity resides mainly in the *R*(−) configuration [3–5], as is the case with other β-adrenoreceptor agonists.

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Stereoselective disposition of enantiomers can result in different pharmacological profiles owing to different rates of absorption or stereoselective presystemic metabolism, distribution or clearance [6–8]. To date there is no information on the disposition kinetics of individual salbutamol isomers in man or in animals after administration of the racemic drug. The present study was undertaken to investigate, using chiral high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED), if stereoselective disposition of $R(-)$- and $S(+)$-salbutamol exists in man after intravenous or oral dosing with the racemic drug.

The direct stereoisomeric separation and quantitation of $R(-)$- and $S(+)$-salbutamol was accomplished in this study using a chiral α_1-acid glycoprotein column which is commercially available as EnantioPac. A simple and specific urine analysis for the quantitative measurement of the enantiomers was developed based on the established separation system. The method requires sample clean-up procedures involving column extraction of the enantiomers and the internal standard followed by ion-pair extraction prior to HPLC analysis.

EXPERIMENTAL

Materials

Racemic salbutamol sulphate and terbutaline sulphate (the internal standard) were a generous gift from Glaxo Canada (Toronto, Canada) and Astra Pharmaceuticals (Mississauga, Canada), respectively. Glass-distilled ethyl acetate and HPLC-grade methanol were purchased from BDH (Toronto, Canada) and Caledon Labs. (Georgetown, Canada), respectively, and used without further purification. Di-(2-ethylhexyl) phosphate (DEHP) was a synthetic-grade reagent obtained from Sigma (St. Louis, MO, U.S.A.) and gold-label triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Disposable C_8 solid-phase columns were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). The remaining chemicals and solvents were of reagent grade and were used as purchased.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Beckman Model 100A pump (Berkeley, CA, U.S.A.), a Waters Model U6K injector (Milford, MA, U.S.A.), a 100×4.0 mm I.D. EnantioPac cartridge column (LKB-Produkter, Bromma, Sweden) with α_1-acid glycoprotein as the stationary phase, and an electrochemical cell containing a glassy carbon electrode controlled by a BAS Model LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) which was connected to a Perkin-Elmer Model 024 recorder (Norwalk, CT, U.S.A.). The detector was typically operated at a sensitivity of 50 nA full scale and at an applied potential of +0.75 V vs. Ag/AgCl reference electrode. The α_1-acid glycoprotein column was embedded in an ice–water mixture in order to maintain its temperature at about 0°C throughout the analysis.

The mobile phase consisted of a 0.1% (v/v) triethylamine in 5.3 m*M* citrate buffer, pH 7.2, containing no organic modifer. It was filtered through a 0.45-μm

membrane (Millipore, Bedford, MA, U.S.A.) before use. The flow-rate was set at 0.2 ml/min.

Determination of the order of elution

Those fractions of the column eluate containing the resolved enantiomers from the chromatography of racemic salbutamol were collected and evaporated to dryness by freeze-drying. Their direction of rotation (+ or −) was determined by using a Jasco Model J-41A spectropolarimeter (Jasco Spectroscopic, Tokyo, Japan).

Sample preparation

If necessary, the pH of a 1.0-ml urine sample was adjusted to 7.2–7.5 with 0.1 *M* phosphate buffer (pH 8.6). A 30-μl internal standard solution (3 μg racemic terbutaline sulphate) was then added. The C_8 minicolumn was pretreated by successively washing it with 2 ml methanol, water and 0.1 *M* phosphate buffer (pH 7.3). The urine sample was then slowly forced through the minicolumn until the meniscus was level with the top of the column packing. The column was then washed with 2 ml of 0.1 *M* phosphate buffer (pH 7.3) and 2 ml of water. Salbutamol and the internal standard were subsequently eluted with 500 μl methanol which was evaporated to dryness under a nitrogen stream. They were further extracted as the ion pairs with DEHP by vortexing vigorously for 1 min with 80 μl of 0.1 *M* phosphate buffer (pH 7.3) and 300 μl of 0.05% (v/v) DEHP in ethyl acetate. After centrifugation the organic phase was transferred to another microtube (50×6 mm) containing 70 μl of 15 m*M* hydrochloric acid into which salbutamol and terbutaline were back-extracted by vortexing for 1 min. The organic layer was discarded after centrifugation and a small volume (1–7 μl) of the aqueous phase was injected into the chromatograph.

Sample collection

Racemic salbutamol (4.0 mg) was given as a single tablet (commercial Ventolin, A & H) to three healthy volunteers (two males and one female) between the ages of 28 and 33. One male subject also received 1.0 mg of racemic salbutamol (Ventolin) administered by intravenous infusion over a period of 30 min. Urine samples were collected without addition of any preservative at frequent intervals through 12 h, stored at 4 °C and analysed in duplicate the following day.

Quantitation

Quantitation of salbutamol enantiomers was done by comparing the peak-height ratio of each enantiomer to terbutaline (T_1), the internal standard, in the unknown sample to those of control samples containing known quantities of salbutamol enantiomers, extracted and chromatographed in exactly the same way. Peak heights of salbutamol enantiomers were measured by the perpendicular distance from the peak apex to the projected baseline underneath the isomeric peaks. Readings were taken only when no shift in the zero baseline occurred, i.e. when the projected baseline was horizontal.

RESULTS AND DISCUSSION

Enantiomeric order of elution

The assignment of a + or − sign to the resolved enantiomers are in accordance with their Cotton effect at 276–280 nm as measured on the Jasco spectropolarimeter. Thus, under the established chromatographic conditions, $S(+)$-salbutamol was found to elute faster than $R(-)$-salbutamol from the chiral column. The order of elution of terbutaline enantiomers (the internal standard) was not determined. Therefore, T_1 and T_2 were assigned to the enantiomer with the shorter and longer elution time, respectively.

Enantioseparation studies

The most challenging task during the course of this study undoubtedly was to achieve a chromatographic separation of the stereoisomers of salbutamol. Our attempts to explore both the direct and indirect chromatographic resolution methods were partially limited by the requirements of an HPLC system compatible with ED. Our experiments to derivatise salbutamol with some commonly used chiral derivatising agents followed by diastereomeric resolution on a reversed-phase column were not successful. Additionally, direct resolution employing a reversed-phase mode of operation on several types of chiral stationary phase, such as D-phenylglycine or L-leucine (Pirkle covalent column), β-cyclodextrin (Cyclobond I column) and cellulose ester (Chiralcel Type OK column), also failed to resolve the enantiomers of salbutamol. Visibly discernible separation was first realised with the α_1-acid glycoprotein column (EnantioPac) at room temperature using a buffered aqueous mobile phase for elution. This separation was dramatically enhanced upon lowering the column temperature. The lack of a cooling device to maintain the column temperature at the recommended minimum of 4 °C prompted us to embed the column in an ice–water mixture. No serious deterioration in column performance was observed. Organic modifier was found to diminish both the retention and selectivity, and hence was excluded from the mobile phase. Higher ionic strength reduced selectivity and therefore the molarity of the buffer was kept low though high enough to maintain sufficient buffering capacity and current conduction. The pH of the buffer was also important as the capacity and resolution factor increased with an increase in pH, as expected for cationic substrates. A pH of 7.2 was chosen to avoid shortening column life. The type of buffer used was not critical but citrate buffer was chosen over acetate or phosphate buffer as it was observed to be significantly better in terms of selectivity. The addition of a tertiary amine, triethylamine, to the mobile phase followed by titration to the appropriate pH further improved the selectivity with optimal resolution being obtained at 0.1% (v/v) triethylamine. The final composition of the mobile phase was established through the extensive manipulations of these parameters until the best possible resolution was obtained, as shown in Fig. 1. The relatively low retention capacity ($1.0 < k' < 2.0$) of the column for salbutamol does not permit a total baseline separation but the resolution ($R_s = 1.06$) achieved here is believed to be adequate to allow good kinetic data to be obtained unless the concentration ratio of the enantiomers is $> 10:1$.

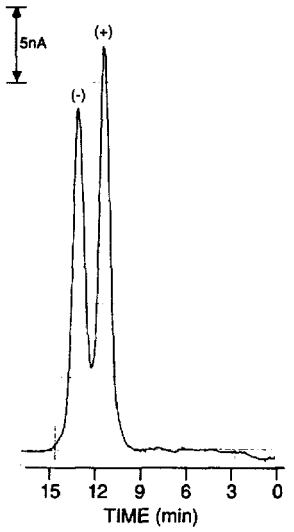

Fig. 1. Enantiomeric separation of 40 ng (−)- and (+)-salbutamol on the EnantioPac column. Chromatographic data: $k'_{(+)} = 1.22$; $k'_{(-)} = 1.55$; $\alpha = 1.27$; $R_s = 1.06$. Peaks: (+) = S(+)-salbutamol; (−) = R(−)-salbutamol.

Urinary assay

The enantiomeric separation system established above was then used for the quantitative determination of the enantiomeric composition of salbutamol in human urine. Fig. 2 represents typical chromatograms of extracted blank urine (A) and of a urine sample (B) to which 2 μg/ml racemic salbutamol had been added. No interfering peaks were observed in several different pools of blank urine when

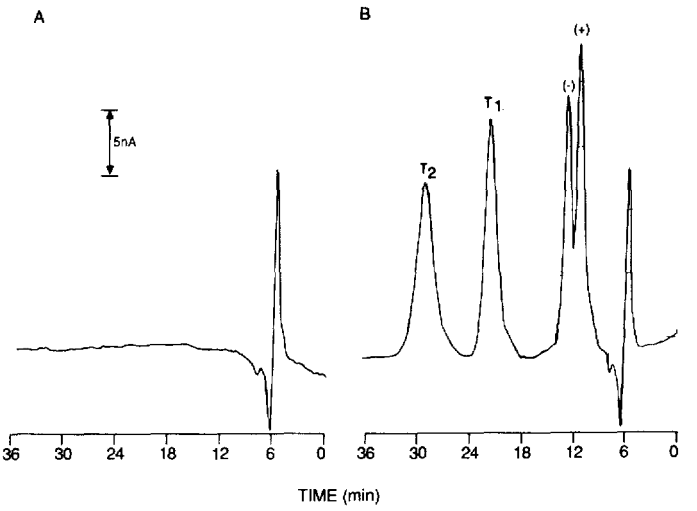

Fig. 2. Chromatograms obtained with (A) blank urine and (B) urine containing 2 μg/ml racemic salbutamol. Peaks: (+) = S(+)-salbutamol; (−) = R(−)-salbutamol; T_1, T_2 = (±)-terbutaline, the internal standard.

TABLE I

PRECISION AND RECOVERY DATA FOR ENANTIOMERIC SALBUTAMOL ASSAY

C.V. = coefficient of variation.

Racemic salbutamol concentration (μg/ml)	Precision ($n=6$)				Recovery (mean ± S.D., $n=7$) (%)	
	Within-day C.V. (%)		Between-day C.V. (%)			
	(+)	(−)	(+)	(−)	(+)	(−)
0.50	3.5	3.9	4.8	4.4		
2.00			4.7	4.1	57.4 ± 6.3	57.0 ± 6.5
5.00	3.0	3.8	4.2	4.0		

the sample injection volume was kept small. The resolution of salbutamol enantiomers in extracted urine samples appears to be significantly less than that of aqueous standards, presumably due to the medium in which salbutamol was injected into the chromatograph (hydrochloric acid versus water). Note that racemic terbutaline, a β_2-adrenoceptor agonist closely related to salbutamol and used as the internal standard in this analysis, is very well separated from both salbutamol enantiomers. The terbutaline enantiomer with the lower retention capacity, T_1, was used for calculation throughout the study.

Assay validation

The analytical recovery of salbutamol enantiomers in seven urine samples spiked with 2.0 μg/ml was essentially identical for both enantiomers, averaging 57% (Table I). The precision of the method was assessed by the repeated analysis of urine samples to which racemic salbutamol had been added at different concentrations ranging from 0.50 to 5.0 μg/ml. The coefficients of variation for both the within-day and between-day assays are summarized in Table I.

The standard curves of the two salbutamol isomers were constructed as the peak-height ratios of $R(-)$- or $S(+)$-salbutamol to terbutaline (T_1) versus concentrations of the salbutamol enantiomer. Concentration and peak-height ratio were verified to be linearly related throughout the concentration range examined (0.25–5.0 μg/ml), yielding a correlation coefficient of 0.9985 for each enantiomer.

Chromatographic analysis time for a urine sample requires 30–35 min. Specificity of the assay is good. In no instance did the pre-trial sample yield peaks with the same retention time as salbutamol or the internal standard. The sensitivity is limited by the small injection volume used (<10 μl).

Salbutamol enantiomers in human urine

Timed urine samples collected from the subject who received intravenous racemic salbutamol showed a continuously decreasing proportion of the active $R(-)$-isomer to the relatively inactive $S(+)$-isomer. The excretion ratio of (−)- to (+)-salbutamol obtained 1 h after drug infusion was 0.89 but declined to 0.58 for the 8–12 h collection period. On the other hand, urine specimens collected on

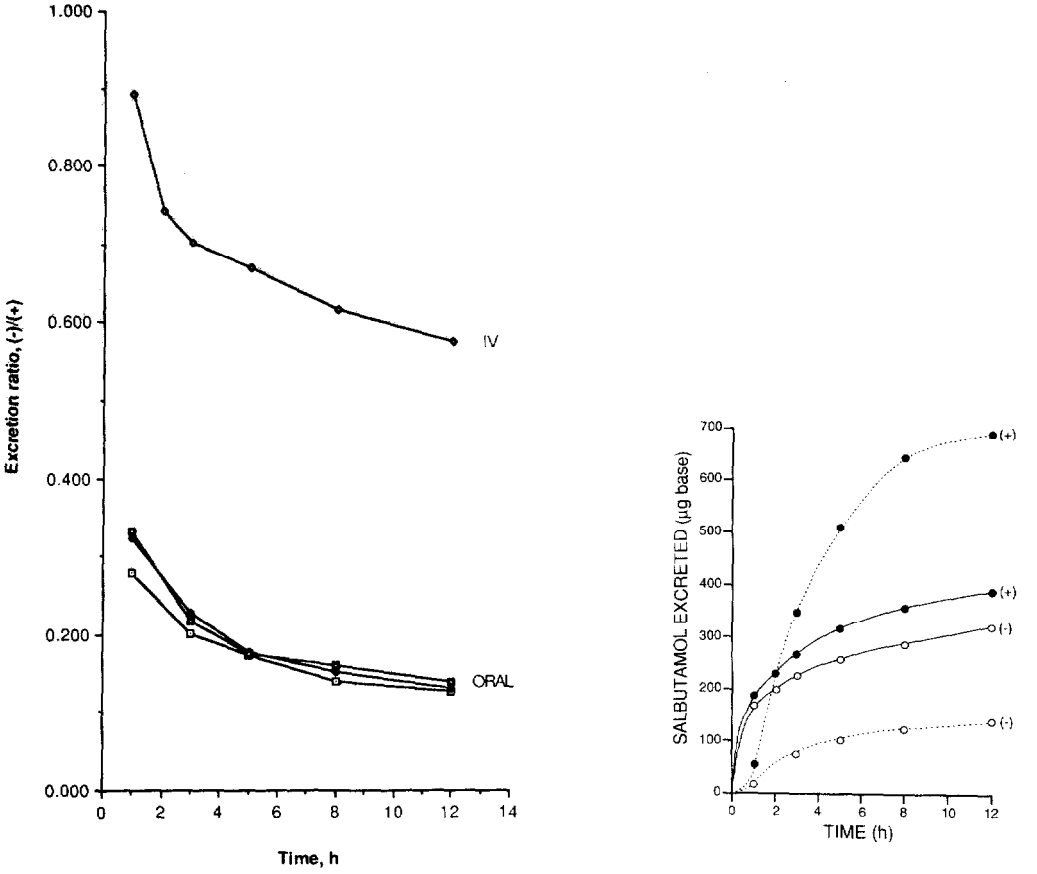

Fig. 3. Decreasing excretion ratio of the active (−)-salbutamol in urine collected up to 12 h after the oral ingestion and intravenous infusion of 4.0 and 1.0 mg of (±)-salbutamol, respectively, in a total of four experiments.

Fig. 4. Cumulative urinary excretion of *S*(+)-salbutamol (●) and *R*(−)-salbutamol (○) through 12 h from one male subject (age 28 years, weight 50 kg) who received oral (---) and intravenous (——) administration of 4.0 and 1.0 mg of racemic salbutamol, respectively, on two separate occasions.

the same subject plus two others after oral ingestion revealed a similar pattern of excretion but an even more extensive distortion in the enantiomeric ratio was observed, as shown in Fig. 3. The extent of such distortion remained relatively constant over a 12-h period for the three subjects studied. Fig. 4 shows the 12-h cumulative amount of the isomers excreted in the one subject who was dosed with both intravenous and oral racemic salbutamol on two separate occasions.

The findings above clearly indicate that the renal excretion rates of the two parent (unmetabolized) enantiomers are not the same and are dependent on the route of administration. As salbutamol is known to undergo extensive presystemic extraction in man following oral administration [9,10], the present work suggests strongly the existence of a stereoselective first-pass metabolism with the more potent *R*(−)-isomer being preferentially metabolised, resulting in a re-

TABLE II

EFFECTS OF ARYLSULFATASE HYDROLYSIS OF EXTRACTED URINE SAMPLES (0–5 h) ON SALBUTAMOL ENANTIOMERIC RATIO

Measured in terms of chromatographic peak heights. Unless otherwise stated, the hydrolytic conditions were: incubation volume, 0.5 ml; incubation time, 15 h; incubation medium, 0.1 *M* acetate buffer at pH 5.0; amount of enzyme, 2200 U.

Enzyme quantity		Incubation time		Incubation medium	
Unit (U)	(−)/(+) ratio	Time (h)	(−)/(+) ratio	Medium, pH 5.0	(−)/(+) ratio
0	0.21	2	0.48	Urine	0.35
80	0.26	15	1.00	Phosphate buffer	0.32
2200	1.00	20	1.05	Acetate buffer	1.00
4400	1.07	40	1.08		

duced systemic availability of the active drug. Such preferential elimination of the more potent isomer during first-pass metabolism is well documented with the calcium antagonist verapamil, and explains the apparent paradox that following oral ingestion, two to three times higher plasma verapamil concentrations in comparison to intravenous administration were required to produce an equivalent dromotropic effect on atrioventricular conduction in man [11]. The major metabolite of salbutamol in human urine has been isolated and identified to be the 4′-O-sulphate ester [12,13]. Morgan et al. [14] have recently suggested that the first-pass elimination of salbutamol is due entirely to sulphate conjugation. In our hands, enzymatic hydrolysis at pH 5.0 with arylsulfatase of *Helix pomatia* of this pharmacologically inactive conjugate, which was extracted from the 0–5 h urine samples collected after oral ingestion using C_{18} minicolumns (Water Assoc., Milford, MA, U.S.A.), increased the (−)- to (+)-salbutamol ratio to near or above unit, thus confirming that the former isomer is preferentially conjugated with sulphate. As phosphate ion is known to inhibit the arylsulfatase activity of *Helix pomatia* [12], neither incubation of the sulfatase directly in urine sample (which contains phosphate ions) nor incubation with the extracted urine sample in phosphate buffer produced any significant increase in the amount of the free (−)-salbutamol (Table II). It thus appears that stereoselective sulphate conjugation is the most predominant mechanism responsible for the differential disposition kinetics of (+)- and (−)-salbutamol seen in man, particularly after oral administration.

CONCLUSIONS

This paper describes a chiral HPLC separation method for the optical isomers of salbutamol, a bronchodilatory drug widely used in asthma therapy around the world. The established separation technique was employed to quantitatively determine the two salbutamol enantiomers extracted from human urine after the oral or intravenous administration of the racemic drug. The resolution achieved

with the chiral α_1-acid glycoprotein column has allowed sufficiently good pharmacokinetic data to be derived until the concentration difference between the isomers becomes substantial. Preliminary results strongly favour the existence of an enantioselective mechanism(s) that results in the preferential removal of the active $R(-)$-salbutamol from the body particularly after oral ingestion of the racemate. Enzymatic hydrolysis studies point to the first-pass biotransformation, specifically the 4′-O-sulphate conjugation, as the dominant mechanism responsible for the differential disposition of the enantiomers seen in man after oral ingestion. In light of the present findings, it appears important to study concentration–effect relationships of salbutamol, and possibly of other β-adrenoceptor agonists, based on the measurement of the active isomers. To quote Ariëns [15], the neglect of the stereoselectivity in drug action degrades many otherwise good pharmacokinetic studies to expensive "highly sophisticated pseudoscientific nonsense".

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