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Determination of salbutamol enantiomers in human plasma and urine by chiral high-performance liquid chromatography

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Abstract

Enantiomers of salbutamol were directly separated ($R_s = 1.16$) and quantitated at therapeutic concentrations after solid-phase extraction from human plasma and urine by normal-phase high-performance liquid chromatography on a chiral column with fluorescence detection. The assay was linear for each enantiomer between 1.25 and 500 ng ml^{-1} and had a minimum limit of detection of 250 pg ml^{-1}. A 3-ml plasma or 1-ml urine sample was required for quantitation at therapeutic doses. Inter-day variation was 5.0% for *S*-(+)- and 6.5% for *R*-(−)-salbutamol. The assay was used to compare enantioselective disposition after single doses of racemate by the intravenous, oral and rectal routes.

1. Introduction

Salbutamol [2-(*tert.*-butylamino)-1-(4-hydroxy-3-(hydroxymethyl)phenyl)-ethanol], also known as albuterol, is a sympathomimetic drug with potent β_2-adrenoceptor stimulating properties. It is administered clinically as a racemic mixture for the relief of bronchospasm in reversible airways disease and for the prevention of preterm labour (tocolysis) during pregnancy [1]. The therapeutic activity of salbutamol has been shown to be associated with the *R*-(−) enantiomer with little or no adrenoceptor stimulation attributed to the *S*-(+) enantiomer [2].

Several studies have determined pharmacokinetic parameters of racemic salbutamol in man [3–5]. However, due largely to assay limitations, only preliminary pharmacokinetic studies of enantiomer disposition have been carried out. The first evidence for enantioselective metabolism of salbutamol emerged from a urinary excretion study in two volunteers given oral radiolabelled pseudoracemate [6]. The inactive *S*-(+) enantiomer was found to have a higher urinary excretion. A second small study of urinary excretion in man also suggested that the metabolism of salbutamol is enantioselective with the *R*-(−) enantiomer undergoing faster metabolism to the 4′-O-sulfate conjugate [7]. A series of in vitro studies using pure enantiomers has supported these observations by showing that the *R*-(−) enantiomer undergoes a higher rate of sulfation (up to 10-fold) by a phenolsulfotransferase enzyme [8,9]. In order to provide a more comprehensive pharmacokinetic profile of this enantioselective disposition in man a sensitive chiral assay for salbutamol enantiomers in plasma is required.

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It appears that only two enantioselective assays for salbutamol have been published, both involving HPLC. The first, by Tan and Soldin [7], used an α_1-acid glycoprotein column maintained at 0°C to directly separate salbutamol enantiomers. Baseline resolution was not achieved (R_s = 1.06) and the limit of detection using an amperometric detector was high (250 ng ml^{-1}), due partly to the small injection volume employed (1–7 μl). While this level of sensitivity is adequate for analysis of salbutamol in urine since it undergoes active renal excretion, it is not adequate for determination of therapeutic plasma levels which are usually less than 30 ng ml^{-1} of racemate [4,5]. A more recent method, by He and Stewart [10], involved derivatisation of salbutamol after solid-phase extraction. The assay used fluorescence detection and was sensitive enough for analysis of enantiomers in human serum. However, the procedure was applied to spiked serum only and was not used to determine enantiomer concentrations in clinical samples after racemic salbutamol administration.

This paper presents a convenient, sensitive and reproducible chiral HPLC assay for salbutamol enantiomers in biological samples using a Pirkle-type column containing a napthyl urea chiral stationary phase. Samples are subjected to solid-phase extraction to remove endogenous interference and concentrate salbutamol enantiomers before HPLC with fluorescence detection. The suitability of the procedure was assessed in a single dose pharmacokinetic study of salbutamol given as the racemate by the intravenous, oral, and rectal routes to a single human volunteer.

2. Experimental

2.1. Materials

Racemic salbutamol sulphate was purchased from Sigma (St. Louis, MO, USA). HPLC grade hexane, 1,2-dichloromethane, acetonitrile and methanol (ChromAR) were obtained from Mallinckrodt (Paris, KY, USA). HPLC grade trifluoroacetic acid (HiPerSolv) was from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). Salbutamol sulfate for intravenous and oral administration was commercially available Ventolin injection (0.5 mg ml^{-1}) and elixir (2 mg ml^{-1}), both from Allen and Hanburys Ltd. (Greenford, UK). Salbutamol sulfate B.P. for rectal administration was a gift from Glaxo (Palmerston North, New Zealand).

2.2. Instrumentation and conditions

The HPLC system consisted of a Jasco 880-PU pump (Japan Spectroscopic Co., Tokyo, Japan), a manual injector fitted with a 200-μl loop (Rheodyne 7125, Cotati, CA, USA), a Chirex 3022 30 × 4.0 mm stainless-steel guard column, a Chirex 250 × 4.0 mm stainless-steel analytical column (Phenomonex, Torrance, CA, USA) and a fluorescence detector (Jasco FP-920). The excitation and emission wavelengths were set at 220 and 309 nm, respectively, and both excitation and emission slit widths were 18 nm. The mobile phase consisting of hexane–1,2-dichloromethane–methanol–trifluoroacetic acid (243:140:17:1) was filtered through a 0.45-μm filter and degassed by sonication under vacuum before use. The flow-rate was 1 ml min^{-1} and the system was operated at ambient temperature. Chromatographic data were acquired and analysed with a computerised integration system (Delta Chromatography Data System V4.02, Digital Solutions, Margate, Queensland, Australia).

2.3. Sample preparation

The sample clean-up procedure was modified from that of Bland et al. [11]. Solid-phase extraction cartridges (Extract-Clean) containing 100 mg (1 ml) and 500 mg (2.8 ml) silica packing and a vacuum manifold box were purchased from Alltech Assoc. (Deerfield, IL, USA). The vacuum pressure of the manifold box was maintained at −3 mm Hg for loading the cartridges and at −15 mm Hg for drying. The cartridges were preconditioned by washing with one volume of acetonitrile followed by one volume of deionised water. Urine (1 ml) or plasma (3 ml)

samples were applied to the preconditioned cartridges and eluted to waste. After drying, the cartridges were washed with one volume of deionised water followed by a further volume of acetonitrile. The cartridges were again dried before salbutamol was eluted into plastic tubes with two volumes of methanol. The tubes were placed in a Speed Vac concentrator with a −60°C trap (SVC 200H, Savant Instruments, Farmingdale, NY, USA) and the methanol evaporated to dryness under reduced pressure at 45°C and 1725 rpm. The dried extracts were finally reconstituted in 1 ml or 0.3 ml of mobile phase for urine and plasma, respectively, vortex-mixed for 1 min, and injected into the HPLC system.

2.4. Quantitation

To obtain standard curves for assay of plasma, three stock solutions of racemic salbutamol in distilled water were independently prepared and appropriate volumes added to 3 ml aliquots of drug-free plasma to give three replicate spiked standards of 2.5, 5, 10, 15, 30 and 60 ng ml^{-1}. Similarly, for assay of urine, three replicate spiked urine standards were prepared in 1-ml aliquots of drug-free urine with concentrations of 10, 100, 250, 500, 750 and 1000 ng ml^{-1} from three independently prepared stock solutions. The plasma and urine standards were subjected to solid-phase extraction and analysed as above. To determine recovery of the extraction procedure an identical set of aqueous standards was prepared with concentrations equal to those of spiked plasma and urine standards. These aqueous standards were rotary-evaporated, reconstituted in mobile phase and injected into the HPLC. Spiked standards were frozen at −80°C and used to check the standard curves on each assay day.

Intra-day variation of the assay at 1 and 10 ng ml^{-1} was assessed using aqueous standards by injecting five replicates of three independently prepared standards on the same day. Inter-day variation was determined at 10 ng ml^{-1} by injecting one of the standard solutions a further five times on two subsequent days [12].

2.5. Determination of the order of elution

Fractions containing single enantiomers from the chromatographic separation of racemic salbutamol sulfate standard solutions were collected and evaporated to dryness in a Speed Vac concentrator. The direction of rotation (+ or −) was determined using a Jasco DIP-370 digital polarimeter.

2.6. Single dose pharmacokinetic study

Racemic salbutamol sulfate (1.6 mg) was administered intravenously (400 μg loading dose followed by a 10 $\mu g\ min^{-1}$ infusion for 2 h), orally (4 mg) and rectally as a suppository (8 mg powder in dehydag base) to a 24-year-old healthy male volunteer on separate occasions at least one week apart. On each occasion, multiple venous blood samples (7 ml) were collected in heparinised tubes over 8 h following dosing. Plasma was obtained by centrifugation (1000 g) and stored at −80°C until analysed for salbutamol enantiomers as above. Urine was collected at hourly intervals for the 8 h of each study and samples frozen at −80°C until analysis. The study protocol was approved by the Southern Regional Health Authority Ethics Committee (Otago).

2.7. Data analysis

Linear regression analysis for standard curves was carried out by a validated computer programme (Pharmaceutical Statistical Regression, School of Pharmacy, University of Otago). Data is quoted as mean ± standard deviation (S.D.) unless otherwise stated. Pharmacokinetic parameters were calculated by standard procedures [13].

3. Results

3.1. Assay validation

Salbutamol enantiomers chromatographed with typical retention times of 22 and 27 min for *S*-(+)- and *R*-(−)-salbutamol, respectively. The

resolution factor (R_s) for the enantiomers was 1.16, and the relative retention (α) was 1.24. The two peaks were free of interference from any peaks present in blank plasma or urine (Fig. 1).

The standard curves for both enantiomers based on peak height vs concentration in spiked plasma and urine and in aqueous standards gave correlation coefficients greater than 0.98 with y-intercepts not significantly different from zero. The solid-phase extraction procedure gave a high recovery of salbutamol (99.1 ± 3.7%, $n = 36$) in agreement with previously reported results [11]. Regression analysis of standard curves for spiked and aqueous standards gave parameters which were not significantly different, consistent with this high solid-phase extraction recovery.

The limit of detection was 750 pg ml^{-1} for each enantiomer (signal-to-noise, 3:1) from a 1-ml sample reconstituted in 0.3 ml of mobile phase. Intra-day variation for S-(+)- and R-(−)-salbutamol, respectively, ($n = 15$) was 3.4% and 2.6% at 10 ng ml^{-1} and 7.6% and 7.2% at 1 ng ml^{-1}. Inter-day variation ($n = 15$) at 10 ng ml^{-1} was 5.0% for S-(+)- and 6.5% for R-(−)-salbutamol.

3.2. Single dose pharmacokinetic study

Plasma concentration and cumulative urinary excretion vs time profiles following intravenous, rectal and oral administration of racemic salbutamol are shown in Fig. 2. The ratios of R-(−)/S-(+) were similar in plasma and urine over the same time period. Clear differences were observed in the concentrations of salbutamol enantiomers in plasma and urine with the S-(+) enantiomer usually present in larger concentration. This difference was most pronounced following oral administration with bioavailabilities (f) for S-(+) and R-(−) being 0.76 and 0.39, respectively. A smaller difference was evident following rectal administration with $f = 0.40$ and 0.33 for S-(+) and R-(−), respectively. Following intravenous administration, differences between pharmacokinetic parameters (S-(+) and R-(−)) were observed for half life (3.34 and 2.28 h),

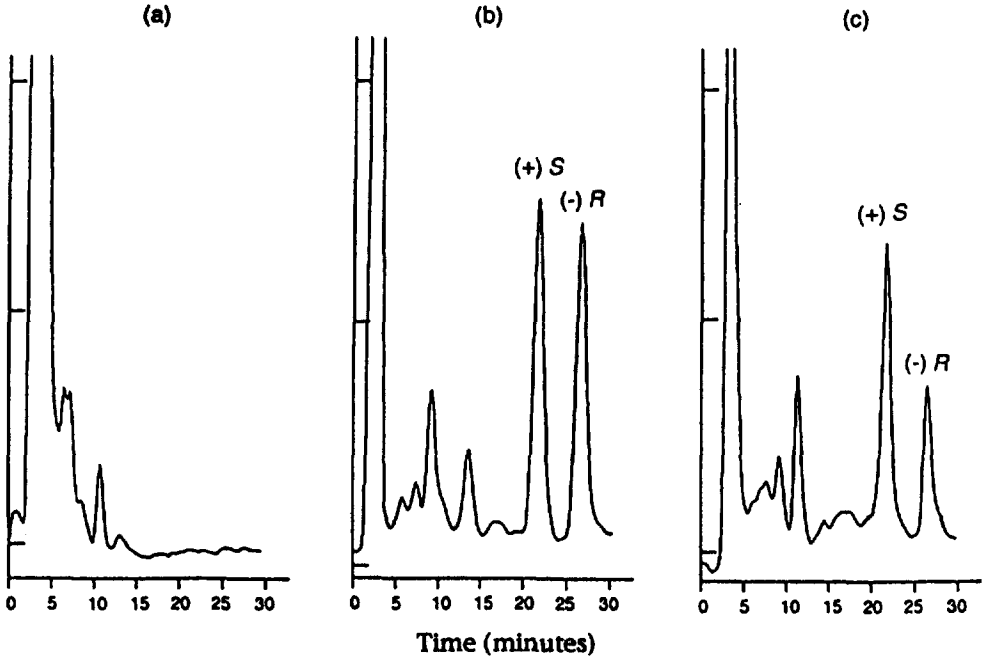

Fig. 1. HPLC chromatograms obtained for (a) blank plasma, (b) plasma spiked with 40 ng ml^{-1} of racemic salbutamol and (c) plasma taken from a healthy volunteer 60 min after the rectal administration of 8 mg racemic salbutamol.

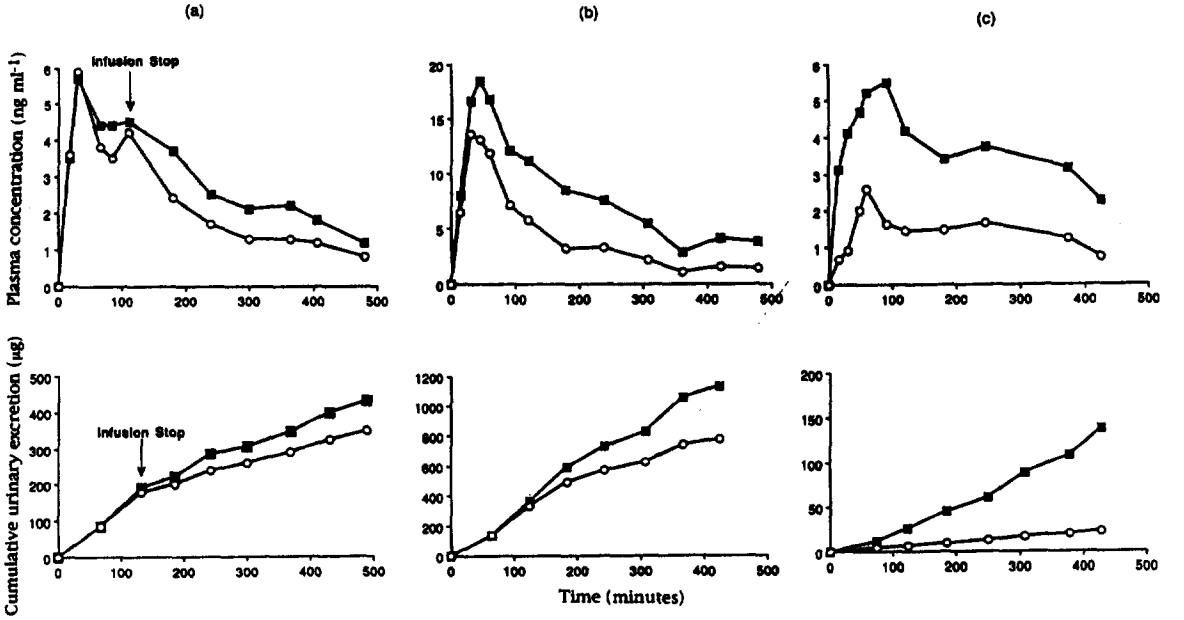

Fig. 2. Plasma concentration (ng ml^{-1}) vs time (top) and cumulative urinary excretion (μg) vs time (bottom) profiles for *S*-(+)- (■) and *R*-(−)- (○) salbutamol following (a) intravenous, (b) rectal and (c) oral dosing of 1.6, 8.0 and 4.0 mg of racemic salbutamol, respectively, to a healthy male volunteer.

clearance (424 and 640 ml min^{-1}) and the amount excreted unchanged in the urine (75% and 59%). Other parameters such as steady-state volume of distribution (134 and 125 l) and renal clearance (428 and 434 ml min^{-1}) were very similar.

4. Discussion

The low plasma concentration of salbutamol following therapeutic doses requires an assay with high sensitivity. Non-enantioselective assays for salbutamol in biological fluids have employed gas chromatography [14], gas chromatography–mass spectrometry [15] and high-performance liquid chromatography with electrochemical [16–19] or fluorescence detection [4,5,11,20,21]. For HPLC analysis, amperometric detection has the necessary sensitivity and has been widely employed in salbutamol assays despite requiring frequent maintenance in terms of electrode replacement. Fluorescence detection has the sensitivity required and offers greater reliability for assay of large numbers of samples.

In the development of this assay, a fluorescence detector capable of providing a low excitation wavelength of 220 nm with a variable emission slit width was required for plasma analysis of salbutamol enantiomers at therapeutic levels. The mobile phase had high native fluorescence at this low excitation wavelength and gave emission peaks at 290, 335 and 450 nm which were traced to impurities in the hexane. This background fluorescence could be suppressed using the adjustable slit width and gain settings while still maintaining adequate sensitivity. The emission maximum of salbutamol at 309 nm was close to the minimum observed between the 290 and 335 nm mobile-phase peaks.

The low plasma levels of salbutamol required concentration of samples to enhance the sensitivity of the HPLC assay. The solid-phase extraction procedure allows at least a 10-fold concentration of salbutamol from a 3-ml plasma sample resulting in an effective limit of detection

of 250 ng ml^{-1} for each enantiomer. Similar concentration of urine samples is not required since higher concentrations are present due to active renal excretion. A suitable internal standard for the assay could not be found since other β_2-agonists such as *R*-(−)- and *S*-(+)-terbutaline, *R*-(−)- and *S*-(+)-isoprenaline, (±)-bamethan, (±)-fenoterol, and (±)-orciprenaline were either too unstable, coeluted on the HPLC column or were not retained on the solid-phase extraction column under the conditions that were optimal for salbutamol extraction.

The HPLC assay of salbutamol in human urine using fluorescence detection has been found by some authors to be subject to interference at low excitation wavelengths [4,23]. The problem was resolved by exciting at the second absorption maximum of salbutamol at 278 nm and monitoring the emission at 609 nm. Unfortunately this results in a loss of sensitivity with limits of detection of 25–50 ng ml^{-1} being found for a 1-ml urine sample. The assay described here is free from interference presumably because the solid-phase extraction procedure removes it and because the normal-phase HPLC system elutes interfering compounds before the enantiomers of salbutamol. This reduction of interference allows the use of a low excitation wavelength resulting in a significant gain in sensitivity compared to earlier reversed-phase HPLC assays. The potential application of our assay to studies of enantiomeric disposition of salbutamol following lung administration is currently under investigation.

To demonstrate the suitability of the assay procedure for investigating enantioselective disposition of salbutamol in man, plasma and urine samples from a single volunteer taking salbutamol by three different routes were analysed. The assay clearly demonstrates a difference in the rate of metabolism of the two enantiomers of salbutamol over the 8 h following a single dose. The extent of this difference appears to be route-dependent since the bioavailability of the two enantiomers differs more after oral dosing than after rectal dosing. Salbutamol has been shown to undergo significant first-pass metabolism in the gut wall and liver [4] and the difference in bioavailability of the two enantiomers is most likely due to a difference in the extent of their first-pass metabolism. The lower overall bioavailability of the rectal dose is probably due to incomplete absorption from the rectum. It should be noted, however, that the bioavailability of the active enantiomer by the rectal route is similar to that observed by the oral route.

Irrespective of the route of administration, metabolism of salbutamol in man is enantioselective and appears to be faster for the *R*-(−) enantiomer. The similar enantiomeric ratios in plasma and urine at equivalent times suggest the active urinary excretion is not enantioselective confirming the interpretation of results from the two previous studies of urinary excretion [6,7].

This assay represents a significant improvement over the earlier direct method of Tan and Soldin [7] using reversed-phase HPLC on a chiral column maintained at 0°C. In our assay the column can be maintained at ambient temperature and can be loaded with significantly larger amounts of sample. The assay can detect therapeutic levels of salbutamol enantiomers in plasma from 7-ml blood samples after an efficient solid-phase extraction and concentration procedure. The assay is simple, accurate and reproducible and does not involve a derivatisation step. The run time is similar to those previously reported for separation of β_2-agonist enantiomers or their derivatives [7,10,22]. The assay can be applied to clinical trials, in vitro metabolism studies and analysis of racemic or enantiomerically "pure" pharmaceutical dosage forms. In addition, it can be applied to metabolite analysis after hydrolysis of the 4′-O-sulfate ester to free salbutamol using previously published methods [4,23].

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