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Rapid chiral high-performance liquid chromatographic assay for salmeterol and α-hydroxysalmeterol Application to in vitro metabolism studies

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Abstract

A rapid and sensitive chiral high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of salmeterol and its principal human metabolite α-hydroxysalmeterol is described. The two pairs of enantiomers were resolved on a chiral-cellobiohydrolase column and detected by electrochemical detection at +700 mV. Standard curves were linear over the concentration range 0.1 to 4.0 μ*M* for α-hydroxysalmeterol enantiomers and 2.5 to 40.0 μ*M* for salmeterol enantiomers. Intra- and inter-day coefficients of variation were <10%. The method was applied to a study of human hepatic metabolism in vitro which showed that microsomal metabolism of salmeterol to α-hydroxysalmeterol is not stereoselective.

Keywords: Chiral stationary phases, LC; Salmeterol; α-Hydroxysalmeterol

1. Introduction

β_2-Adrenoceptor agonists are used in the treatment of asthma and are generally administered via the lung [1]. The drugs are marketed as racemic mixtures although only the *R*-(−)enantiomers are pharmacologically active [2]. A number of studies have shown that the metabolism of β_2-adrenoceptor agonists is stereoselective [3–5]. Thus the sulphation of salbutamol in various human tissues occurs with an efficiency (V_{max}/K_m) about 10-fold higher for the *R*-(−)enantiomer than for its antipode. This has important consequences in the oral delivery of β_2-adrenoceptor agonists since they are subject to extensive first pass metabolism [6,7]. It may also be important in the action of inhaled β_2-adrenoceptor agonists since the vast majority of an inhaled dose is swallowed [8,9].

Salmeterol is a long-acting potent β_2-adrenoceptor agonist used via inhalation to improve lung function, reduce symptoms and provide a better quality of life for patients with asthma [10,11]. The β_2-agonist activity of the *R*-(−)enantiomer is 40 times greater than that of its antipode [12]. A non-chiral study has shown that salmeterol is predominantly metabolised by aliphatic oxidation to α-hydroxysalmeterol (Fig. 1) following oral administration to humans [13]. The reaction is mediated by cytochrome P450 3A [14]

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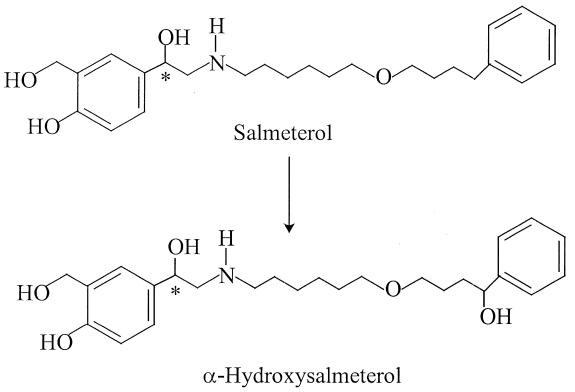

Fig. 1. The aliphatic oxidation of salmeterol to α-hydroxysalmeterol. Asterisk indicates the position of the asymmetric centre.

but nothing is known about the stereoselectivity of this reaction. We have developed a chiral HPLC assay for the simultaneous determination of the enantiomers of salmeterol and α-hydroxysalmeterol and applied it to a study of salmeterol metabolism by human liver microsomes.

2. Experimental

2.1. Reagents

Salmeterol xinafoate (*RS*-salmeterol) and α-hydroxysalmeterol xinafoate (GR127433) were kindly donated by Glaxo Wellcome Research and Development (Stevenage, UK). *R*-(−)- and *S*-(+)-salmeterol xinafoate were kindly supplied by Dr Thomas Walle, (Medical University of South Carolina, Charleston, SC, USA). NADPH was purchased from Sigma Chemical Company (St. Louis, MO, USA). HPLC grade methanol, 2-propanol and *tert*.-butyl methyl ether (MTBE) were from BDH (Poole, UK). AR grade toluene was from Ajax Chemicals (Auburn, Australia). EDTA–Na_2 was from May&Baker (Dagenham, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Chromatography

The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu Corporation, Kyoto, Japan), a manual injector fitted with a 50 μl loop (Rheodyne 7125, Cotati, CA, USA), a chiral-CBH (Cellobiohydrolase) 10×3.0 mm guard column and a chiral-CBH 100×4.0 mm analytical column (ChromTech AB, Hagersten, Sweden). Detection was via an ESA coulometric electrochemical detector with a Model 5020 guard cell operated at 1000 mV and a Model 5011 dual-electrode analytical cell (ESA, Inc., Bedford, MA, USA) with detectors 1 and 2 set at 300 and 700 mV, respectively. The signal from detector 2 was processed by a Hitachi D-2500 Chromato-Integrator (Hitachi Ltd., Tokyo, Japan) to obtain peak heights. The mobile phase consisting of 15% 2-propanol in 25 m*M* sodium phosphate buffer pH 6.0 with 50 μ*M* EDTA–Na_2 was filtered through a 0.45 m filter and degassed by sonication under vacuum before use. The flow-rate was 0.9 ml/min and the system was operated at ambient temperature. Under these conditions, the retention times were 8.0 and 9.2 min for the enantiomers of α-hydroxysalmeterol and 17.2 and 24.0 min for *R*-(−)- and *S*-(+)-salmeterol, respectively (Fig. 2). For metabolite enantiomers, the resolution factor (R_s) was 1.36 and the relative retention (α) was 1.67. Corresponding values for salmeterol enantiomers were 1.21 and 1.62. The four peaks were free of interference from compounds present in blank incubation mixture.

2.3. Human liver microsomes

Two human liver samples were obtained from registered organ donors who were healthy at the time of death. The use of the tissue was approved by the local Ethics Committee. The liver samples were stored at −84°C until used. Microsomes were prepared by differential centrifugation as previously described by Robson et al. [15]. The protein content of the microsomal preparation was determined by the Lowry method [16].

2.4. In vitro hepatic microsomal metabolism of salmeterol

The microsomal incubation mixture (final volume 250 l) consisted of 67 m*M* phosphate buffer pH 7.4, 500 μ*M* NADPH, an aliquot of microsomal preparation to a final protein concentration of 400 μg/ml and varying final concentrations of either *R*-(−)-salmeterol, *S*-(+)-salmeterol (2.5–40.0 μ*M*) or *RS*-

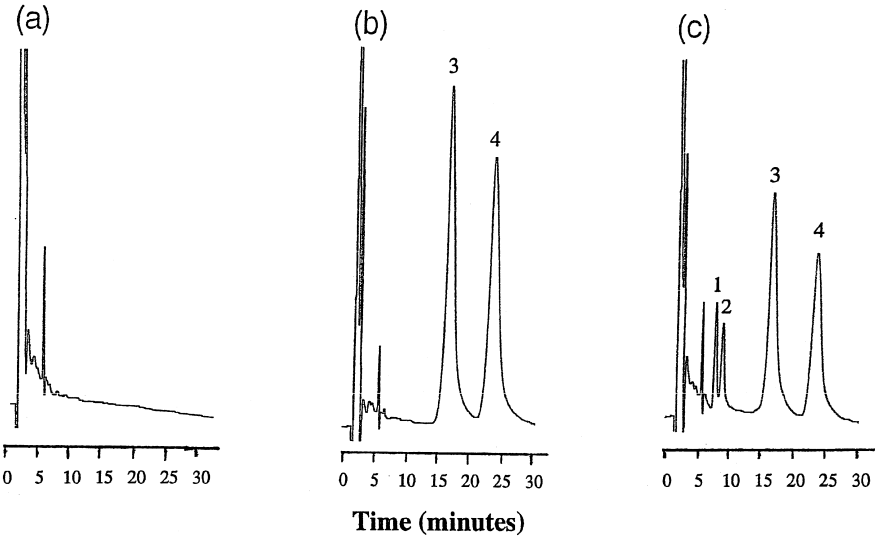

Fig. 2. HPLC chromatograms obtained for (a) blank incubation sample (without salmeterol), (b) incubation sample spiked with 10 μ*M* of *RS*-salmeterol (without NADPH) and (c) incubation sample spiked with 10 μ*M* of *RS*-salmeterol (with NADPH). Peaks: 1=*R*-α-hydroxysalmeterol; 2=*S*-α-hydroxysalmeterol; 3=*R*-(−)-salmeterol; 4=*S*-(+)-salmeterol.

salmeterol (5.0–80.0 μ*M*). All concentrations of salmeterol and α-hydroxysalmeterol refer to the corresponding free bases. Incubation without the substrate served as blank and incubation without NADPH served as control. The reaction was initiated by the addition of NADPH and carried out in a shaking waterbath at 37°C for 15 min. All reactions were terminated by the addition of 0.5 ml methanol. Samples were vortex-mixed and centrifuged at 20 000 *g* for 5 min and 20 μl aliquots were injected into the HPLC system.

In the incubation samples, the concentrations of metabolite are much lower than those of the parent drug. To assay these low concentrations of α-hydroxysalmeterol (<0.8 μ*M*) without overloading the column with salmeterol, an extraction and concentration step was required. After incubation, 0.1 *M* sodium carbonate buffer pH 9.5 (0.5 ml) and toluene (4 ml) were added to incubation tubes. Samples were vortex-mixed and centrifuged at 1000 *g* for 10 min after which the organic phase was discarded. The toluene was shown to extract salmeterol but not α-hydroxysalmeterol. MTBE (5 ml) was then added and the tubes were vortex-mixed and centrifuged at 1000 *g* for 10 min. MTBE (3.5 ml) was transferred to clean plastic tubes and evaporated to dryness in a Speed Vac Concentrator with a −106°C trap (SVC 200H, Savant Instruments, Farmingdale, NY, USA) under reduced pressure and 400 *g*. Residues were dissolved in 100 μl of mobile phase and 20 μl aliquots were injected into the HPLC system.

2.5. Validation

All validation was based on analysis of spiked microsomal incubation mixtures (without NADPH, final volume 250 μl) as described in Section 2.4. For quantitation of salmeterol enantiomers, standard curves were determined using *RS*-salmeterol spiked standards of 5.0, 10.0, 20.0, 40.0 and 80.0 μ*M*. Intra- and inter-day variation of the assay at 10.0, 30.0 and 60.0 μ*M* were determined by the analysis of 10 samples of each concentration on the same day and analysis of two samples of each concentration on five different days [17]. Standard curves for assay of α-hydroxysalmeterol enantiomers were determined using spiked standards of 1.0, 2.0, 4.0 and 8.0 μ*M*. Intra- and inter-day coefficients of variation of the assay were determined at 1.5, 3.0 and 6.0 μ*M* as described above.

To validate the extraction and concentration step, α-hydroxysalmeterol spiked standards of 0.2, 0.4, 0.8 and 1.6 μ*M* were used. To determine recovery of the extraction procedure, an identical set of aqueous

standards was analysed. Intra- and inter-day coefficients of variation of the assay with extraction step were determined by the analysis of six samples of each concentration on the same day and of one sample on six different days, respectively.

2.6. Data analysis

Linear regression analysis was carried out using a validated computer program (Pharmaceutical Statistical Regression, School of Pharmacy, University of Otago, Dunedin, New Zealand). Enzyme kinetic parameters (maximum velocity of reaction, V_{max}, and the Michaelis–Menten constant, K_m) were determined using a least squares non-linear modelling program, MINIM (Dr R. Purves, Department of Pharmacology, University of Otago, Dunedin, New Zealand).

3. Results and discussion

Most β_2-adrenoceptor agonists in clinical use are relatively hydrophilic molecules which undergo phase II metabolism to sulphates (fenoterol, salbutamol and terbutaline) [18–20] or glucuronides (formoterol) [21]. In contrast, salmeterol is a lipophilic molecule which undergoes extensive oxidative metabolism to α-hydroxysalmeterol [13]. Under the in vitro conditions of our study, the amount of α-hydroxysalmeterol formed was equal to the amount of salmeterol lost, showing that this hydroxylated product is the only metabolite produced by human liver microsomes. Each enantiomer of salmeterol produced only one enantiomer of metabolite assigned to the corresponding enantiomer of α-hydroxysalmeterol. The order of elution of metabolite enantiomers was the same as that of parent drug.

The enantiomers of salmeterol and α-hydroxysalmeterol were baseline resolved as shown in Fig. 2. Changes in pH value, buffer concentration and percentage of 2-propanol in the mobile phase were found to have a profound influence on the retention and enantioselectivity of the column. With higher buffer concentration and/or percentage of 2-propanol, enantioselectivity increased and retention decreased. At higher pH, both enantioselectivity and retention increased. With a mobile phase containing 15% 2-propanol (the maximum recommended by the manufacturer), optimum enantioselectivity and retention were achieved at pH 6.0 and 25 m*M* sodium phosphate buffer.

Three non-chiral assays for salmeterol in biological fluids have been published which employed gas chromatography–mass spectrometry (GC–MS) [22] and HPLC with fluorescence [23] and radiochemical detection [13]. The GC–MS and HPLC assays with fluorescence detection were applied to pharmacokinetic studies in human and animals, respectively. The HPLC assay with radiochemical detection determined salmeterol and α-hydroxysalmeterol simultaneously [13] and was applied in in vivo and in vitro metabolism and disposition studies in human [13,14]. The chiral assay reported here is the first to analyse the enantiomers of salmeterol and α-hydroxysalmeterol simultaneously. Electrochemical detection provides adequate sensitivity to determine α-hydroxysalmeterol enantiomers formed during in vitro incubation and at a signal-to-noise ratio of 3:1 gives a limit of detection of about 0.05 μ*M* (30 ng/ml) of enantiomer.

The standard curves for enantiomers of α-hydroxysalmeterol and salmeterol were linear ($r > 0.999$) over the concentration ranges 0.5 to 4.0 μ*M* and 2.5 to 40.0 μ*M*, respectively. Intercepts with the *y*-axis were not significantly different from zero. Intra- and inter-day coefficients of variation were $<10\%$. The standard curves for assay of α-hydroxysalmeterol enantiomers with extraction step were linear ($r > 0.99$) over the concentration range 0.1 to 0.8 μ*M*. The recovery for α-hydroxysalmeterol was $88.8 \pm 2.0\%$. Intra- and inter-day coefficients of variation for *R*- and *S*-α-hydroxysalmeterol were similar and varied from 9.4% at 0.1 μ*M* to 4.3% at 0.8 μ*M*.

On incubation of salmeterol with human liver microsomes, the rate of formation of α-hydroxysalmeterol was linear over 20 min at microsomal protein concentrations up to 600 μg/ml. Therefore, 400 μg/ml microsomal protein and an incubation time of 15 min were used in subsequent experiments. The formation of α-hydroxysalmeterol from pure *R*-(−)- and *S*-(+)-salmeterol as well as from *RS*-salmeterol is illustrated in Fig. 3. The formation of metabolite was described by Michaelis–Menten kinetics (Table 1). After incubation with pure en-

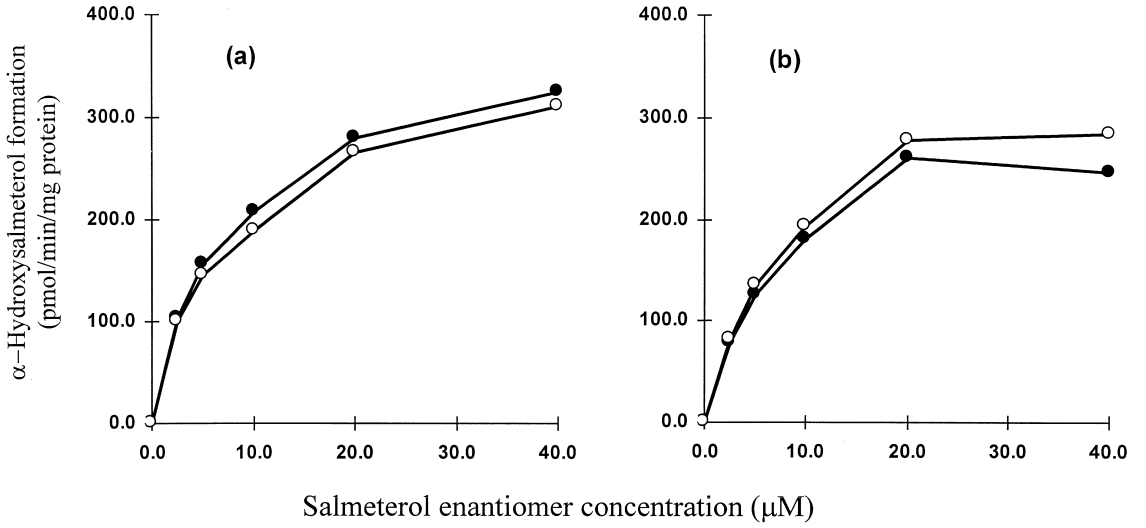

Fig. 3. Aliphatic oxidation of *R*-(−)- and *S*-(+)-salmeterol during incubation of (a) pure enantiomers and (b) *RS*-salmeterol with human liver microsomes. The microsomal protein concentration was 400 μg/ml and the incubation time was 15 min. ●, (*R*)-α-Hydroxysalmeterol; ○, (*S*)-α-hydroxysalmeterol. Data points represent the means of duplicate incubations with microsomes from one human liver (subject 1).

antiomers, the V_{max} and K_m values of the enzyme for the formation of *R*-α-hydroxysalmeterol were almost identical to those for the formation of *S*-α-hydroxysalmeterol. After incubation with racemate, the enantiomeric ratio *R*/*S* for salmeterol or α-hydroxysalmeterol remained at about 1 and again the V_{max} and K_m values of the enzyme for the formation of the two α-hydroxysalmeterol enantiomers were similar. Values of V_{max} and K_m determined in incubations of pure enantiomers were larger than corresponding values determined in incubations of racemate but the efficiencies (V_{max}/K_m) for the *R*-enantiomer were similar to those for the *S*-enantiomer for the two livers examined in the study.

Because salmeterol is a highly potent drug used by inhalation only, doses are small (25 μg/puff) and plasma concentrations in human are very low (pg/ml). This makes it difficult to study the metabolism and disposition of salmeterol enantiomers after therapeutic dosing. The assay described here was developed to determine enantiomers of α-hydroxysalmeterol and salmeterol at the concentrations involved in the metabolism of salmeterol enantiomers by human liver microsomes. The cytochrome P450 3A involved in salmeterol metabolism has K_m values in the micromolar range which is far higher than the levels encountered at therapeutic doses. Thus, in the clinical setting there is unlikely to be any interaction

Table 1
Enzyme kinetic parameters for the formation of α-hydroxysalmeterol enantiomers by human liver microsomes

Drug	Metabolite	Subject	K_m [a]	V_{max} [a]
R-(−)-Salmeterol	*R*-α-Hydroxysalmeterol	1	7.4	381.8
		2	13.2	450.6
S-(+)-Salmeterol	*S*-α-Hydroxysalmeterol	1	9.0	378.8
		2	12.4	431.5
	R-α-Hydroxysalmeterol	1	7.1	320.0
RS-Salmeterol		2	10.8	344.4
	S-α-Hydroxysalmeterol	1	8.5	354.6
		2	9.0	353.4

[a] $K_m = \mu M$, V_{max} = pmol/min/mg protein.

of salmeterol with other cytochrome P450 3A inducers or inhibitors used simultaneously.

In conclusion, a rapid and sensitive chiral HPLC assay for the simultaneous determination of the enantiomers of α-hydroxysalmeterol and salmeterol has been developed and applied to in vitro studies of human hepatic metabolism. The metabolism of salmeterol to α-hydroxysalmeterol in human is not stereoselective.

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