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Analytical methodology for enantiomers of salbutamol in human urine for application in doping control[1]

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Abstract

Liquid chromatographic procedure with fluorimetric detection for chiral separation and quantification of salbutamol enantiomers in urine samples has been developed. The extraction of free salbutamol from urine has been considered using liquid–liquid and solid-phase procedures. The effect of pH, salting-out effect and organic solvent has been studied in liquid–liquid extraction from aqueous and urine samples. For solid-phase extraction, different mechanisms (polar, non-polar, cation-exchange and interactions with a polymeric phase) have been tested and the effect of the urine matrix on the extraction recoveries has been considered. Bond-Elut Certify™ extraction cartridges provided the best specificity and good recoveries for salbutamol in urine. The sample is acidified, applied to the preconditioned cartridges and, after a washing step, salbutamol enantiomers are eluted with a mixture of chloroform and 2-propanol (80:20, v/v) containing 2% ammonia. Atenolol is used as external standard. Enantioselective separation is accomplished with a Chirex™ 3022 stationary phase (urea type silica-bonded chiral phase) using a mobile phase containing hexane–dichloromethane–methanol–trifluoroacetic acid (250:218:31:1, v/v) and fluorimetric detection with excitation and emission wavelengths set at 230 and 309 nm, respectively. The method proposed is rapid, selective and sensitive, and seems to be useful to differentiate between an authorized and a prohibited use of the drug in doping control. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Salbutamol

1. Introduction

Salbutamol (2-(*tert*.-butylamino)-1-(4-hydroxy-3-(hydroxymethyl)phenyl)ethanol), also known as albuterol, is a selective β_2-adrenoceptor agonist widely used in sport as a bronchodilator to prevent exercise induced asthma [1]. Moreover, β_2-agonist are drugs that potentially produce a certain amount of anabolic-like effect, depending on the dose and the route of administration [2]. For that reason, the use of salbutamol is restricted by the International Olympic Committee (IOC) and it is only permitted by inhalation and, even then, must be declared in writing to the relevant medical authority prior to the competition [3]. The usual detection of salbutamol carried out by many antidoping laboratories by means of GC–MS analysis of enzymatically partially hydrolyzed urine [4], does not afford clear cut results to

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conclude about the dose and the route of administration and to distinguish between an authorized and a prohibited use of the drug.

Salbutamol, which has an asymmetric carbon atom, is administered clinically as a racemic mixture even though the *R*(−) enantiomer is carrying most of the therapeutically bronchodilating effects [5–7]. Metabolism is mainly by sulphate conjugation of the phenolic hydroxyl group [8,9]. This metabolic reaction occurs in the intestine and the liver [10] when the drug is given orally and is highly stereoselective in favour of the *R*(−) enantiomer [11,12]. On the other hand, salbutamol does not appear to be extensively metabolized in the lungs, therefore its metabolic behaviour following inhalation depends mainly upon the proportion of inhaled salbutamol relative to the proportion swallowed [13].

Salbutamol is excreted in the urine as a mixture of the enantiomers of the unchanged drug and of the enantiomers of the 4′-*O*-sulphate ester (Fig. 1). The ratios in urine of unchanged drugs to metabolites and unchanged *S*(+) to *R*(−) enantiomers depend on the route of administration: after oral administration excretion of metabolite dominates and, because of the stereoselective sulphation, the excretion of unchanged *S*(+)-salbutamol is also greater than the excretion of unchanged *R*(−)-salbutamol [13–15].

Based on the above arguments, it is possible that the simultaneous evaluation of different variables measured in urine can be useful to establish the correct (inhaled) or incorrect (oral) use of salbutamol

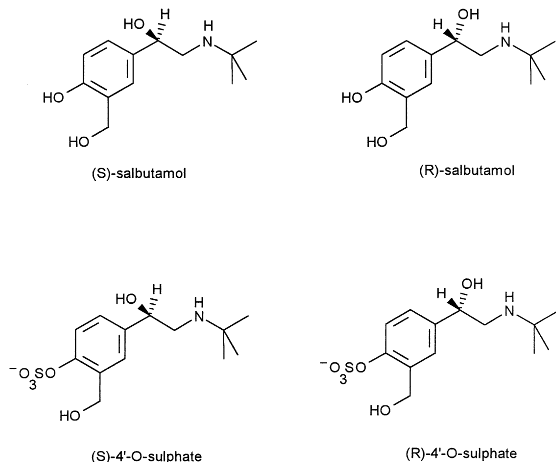

Fig. 1. Chemical structure of salbutamol enantiomers and its main metabolites in urine, the 4′-*O*-sulphate esters.

in sport. These variables could be the absolute concentration of salbutamol and metabolite, the proportion of conjugated salbutamol as compared with the unchanged drug in the urine, and ratios between *S*(+) and *R*(−) enantiomers of unchanged drug as well as between *S*(+) and *R*(−) enantiomers of metabolite. Development of analytical methods for the determination and quantitation of the metabolite have been limited by the lack of pure substance. Moreover, reliable hydrolysis procedures of salbutamol metabolite to the parent drug are not described in the literature. For these reasons, the parameters that could easily be used to differentiate between oral and inhaled ingestion of salbutamol are the absolute concentration of unchanged salbutamol and the ratio between its *S*(+) and *R*(−) enantiomers excreted in urine. Accordingly, a method for enantioseparation and quantitation of salbutamol enantiomers in urine is needed.

Several procedures for the determination of racemic salbutamol based on nonchiral high-pressure liquid chromatography (HPLC) on reversed-phase columns with electrochemical [16] or fluorescence [13,14,17–19] detection have been reported. A sample clean-up procedure either with solid-phase [13,19] or with liquid–liquid extraction [14,16,17], which are the most popular methods, is required. Hutchings et al. [17] and Morgan et al. [14] described an ion-pair liquid–liquid extraction procedure with chloroform as organic solvent and fluorescence detection for determination of salbutamol in plasma and urine. Sagar et al. [16] developed a method for salbutamol in human plasma that involved ethyl acetate in the extraction procedure and electrochemical detection. Solid-phase extraction clean up procedures described use silica phases for plasma [19] or Bond-Elut Certify™ (a mixed phase cartridge with both ion-exchange and lipophilic properties) for urine samples [13] combined with fluorescence detection. Alternative procedures of immunoaffinity chromatography for plasma sample clean up have been described [18] and offer the advantage of simultaneous extraction of salbutamol and its metabolite.

When enantioselective separations are considered, the methods most commonly used involve derivatization of the enantiomers to diastereoisomers followed by HPLC on a nonchiral stationary phase [20] or direct separation of enantiomers by HPLC on a chiral

stationary phase with fluorescence detection [15,21,22]. He and Stewart [20] reported an HPLC method for pharmacokinetic studies of salbutamol enantiomers in spiked plasma samples involving solid-phase extraction on octadecylsilane columns and fluorescence detection of the diastereoisomeric thioureas formed between *R*(−) and *S*(+)-salbutamol and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl isothiocyanate. Adams and Stewart [21] developed an enantioselective HPLC assay under normal-phase conditions on a Sumichiral OA4700 column for salbutamol enantiomers in human serum with fluorescence detection following a silica solid-phase extraction procedure for clean up. A normal-phase HPLC assay for salbutamol enantiomers on a silica-bonded chiral stationary phase of (*S*)-indoline-2-carboxylic acid linked to (*R*)-1-(α-naphthyl)ethylamine urea and to *N*-propylamine (known as Chirex™ 3022) was described by Boulton and Fawcett [15,22]. The assay allowed for efficient separation of the enantiomers and quantitation at therapeutic concentrations in plasma and urine by fluorescence detection following solid-phase extraction on silica cartridges. The method was applied to a pharmacokinetic study of salbutamol enantiomers following oral and intravenous administration of racemic formulation [15].

An enantioselective HPLC technique with an urea type enantioselective stationary phase using a ternary mobile phase containing hexane, dichloromethane and an alcohol seems to be the most effective in the direct separation of salbutamol enantiomers [23] and the fluorescence detection appears to offer the best specificity and selectivity for application to urine samples. In this work, the extraction procedure to remove endogenous interferences from urine samples and concentrate salbutamol enantiomers before enantioselective HPLC with fluorescence detection was optimized in order to develop a precise, sensitive and reproducible method for application to doping control.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile, ethyl acetate, 2-propanol (all HPLC grade), *tert*.-butylmethyl ether, 25% ammonia, ammonium chloride, sodium chloride, sodium acetate, sodium hydroxide (all analytical grade) and trifluoroacetic acid (TFA) (for spectroscopy) were purchased from Merck (Darmstadt, Germany). Dichloromethane and *tert*.-butyl alcohol (both analytical grade), chloroform, hexane and glacial acetic acid (all HPLC grade) were supplied by Scharlau (Barcelona, Spain). Deionized water was obtained by a Milli-Q system (Millipore Ibérica, Barcelona, Spain).

Ammonium chloride buffer was prepared by dissolution of 28 g of ammonium chloride in 100 ml of deionized water and adjusting the pH to 10 with concentrated ammonia solution. Acetate buffer was prepared adjusting the pH of a 1.1 mol/l sodium acetate solution to 5.2 with glacial acetic acid.

S(+)-Salbutamol and *R*(−)-salbutamol were donated by Glaxo (Uxbridge, UK). Racemic atenolol was supplied by Laboratorios ICI-Farma S.A. (Porriño, Pontevedra, Spain). Stock standard solutions (1 mg/ml, in free base form) of *S*(+)-salbutamol, *R*(−)-salbutamol and racemic atenolol (external standard) were prepared in methanol. Working solutions of 10 μg/ml were prepared by dilution of stock solutions with methanol. All these solutions were stored at −20°C.

Oasis™ HLB (60 mg/3 ml) and Sep-Pak™ C_{18} (200 mg/3 ml) columns were purchased from Waters (Milford, MA, USA); Bond-Elut™ Si (100 mg/10 ml) and Bond-Elut Certify™ (130 mg/10 ml) columns were provided by Varian (Harbor City, CA, USA).

Organic phases were evaporated to dryness under nitrogen stream with a Turbo-Vap LV evaporator from Zymark Corporation (Hopkinton, MA, USA).

2.2. Instrumental analysis

Chromatographic analysis was carried out using a Series II 1090L liquid chromatograph equipped with a diode array detector (Hewlett-Packard, Palo Alto, CA, USA). The instrument was linked to a HP ChemStation (Hewlett Packard). The effluent was monitored with a LS-5 fluorescence detector (Perkin-Elmer, Norwalk, CO, USA) equipped with a flow-through cell of 2×2 mm at an excitation and emission wavelengths of 230 and 309 nm, respectively, with both excitation and emission slit widths set at 10 nm. Before the conditions of the method

were optimized, the chromatograms obtained by measuring absorbance at 280 nm were used to check the specificity of the different extraction procedures tested.

A Chirex™ 3022 30×4.0-mm guard column and a Chirex 3022™ 250×4.0-mm analytical column (Phenomenex, Torrance, CA, USA) were used. The mobile phase, initially consisting of hexane–dichloromethane–methanol–TFA (240:140:20:1, v/v), was degassed under a stream of helium before use. The flow-rate was 1.0 ml/min and the system was operated at ambient temperature. Once the best extraction procedure was chosen the composition of the eluent was changed and a mixture containing 250:218:31:1 (v/v) of hexane–dichloromethane–methanol–TFA was used as mobile phase in order to reduce the time of analysis. Reproducibility on retention times was calculated on different days ($n=10$) using fresh mobile phase preparation.

2.3. Liquid–liquid extraction procedures

Liquid–liquid extraction procedures with different solvents (chloroform, ethyl acetate, *tert*.-butylmethyl ether and a mixture *tert*.-butylmethyl ether–*tert*.-butyl alcohol 6:1, v/v) were tested. Sample (1 ml) was made alkaline with 100 μl of ammonium chloride buffer (pH 10), 0.5 g of sodium chloride were added to promote the salting-out effect and the mixture was vortex mixed and extracted with 5 ml of organic solvent. After mixing (rocking at 40 movements per min) for 20 min and centrifugation (1500 *g*, 5 min), the organic layer was separated, added to 50 μl of the external standard working solution, vortex mixed and taken to dryness under a nitrogen stream in a 40°C water bath. The residue was reconstituted either with 100 μl of mobile phase or with 100 μl of dichloromethane–TFA in the same proportion as in the mobile phase, vortex mixed for 1 min and 25 μl were injected into the HPLC system.

2.4. Solid–liquid extraction procedures

Different solid-phase extraction cartridges (Oasis™, Bond-Elut Certify™, Sep-Pak™ C_{18} and Bond-Elut™ Si) were tested.

2.4.1. Oasis™

Columns were conditioned by washing with 1 ml of methanol and 1 ml of deionized water. Samples (1 ml) were applied, the cartridges were washed with 1 ml of a methanol–water mixture (5:95, v/v) and salbutamol enantiomers were eluted with 1 ml methanol.

2.4.2. Bond-Elut Certify™

Cartridges were conditioned by washing with 1 ml of methanol and 1 ml of deionized water and prevented from drying before applying specimens. One ml of 1.1 mol/l acetate buffer (pH 5.2) was added to 1 ml of sample, vortex mixed and applied to the preconditioned cartridges. The columns were washed with 1 ml of water, 500 μl 1 mol/l acetic acid, 1 ml of methanol, and dried for 5 min under full vacuum. Two consecutive elutions (1 ml each) were carried out with a mixture of chloroform and 2-propanol (80:20, v/v) containing 2% ammonia.

2.4.3. Sep-Pak™ C_{18}

Cartridges were conditioned by washing with 1 ml of methanol and 1 ml of water. Sample (1 ml) was made alkaline with 100 μl ammonium chloride buffer (pH 10) and was applied to the preconditioned columns. The cartridges were washed with 1 ml of water and 1 ml of a methanol–water mixture (25:75, v/v), and two consecutive elutions (1 ml each) with methanol were carried out.

2.4.4. Bond-Elut™ Si (silica)

Columns were conditioned with 1 ml of acetonitrile and 1 ml of deionized water. Samples (1 ml) were applied to the preconditioned cartridges and eluted to waste. After drying for 2 min the cartridges were washed with 1 ml of water followed by 1 ml of acetonitrile. The columns were again dried (2 min) before salbutamol enantiomers were eluted with 2 ml of methanol.

In all solid-phase extraction procedures, the eluates were added to 50 μl of the external standard working solution, vortex mixed and evaporated to dryness under a stream of nitrogen in a 40°C water bath. The dried extracts were reconstituted in 100 μl of a mixture dichloromethane–TFA in the same proportion as in the mobile phase, vortex mixed for 1 min and 25 μl were injected into the HPLC system.

2.5. Optimization of the extraction procedure

The efficiency of the reconstitution step after liquid–liquid and solid–liquid extraction procedures was evaluated. Water and urine samples (1 ml) spiked with 200 ng/ml of each enantiomer were placed in 15-ml screw-capped disposable glass tubes, extracted ($n=3$) using the different procedures tested and reconstituted in 100 μl of either mobile phase or a mixture dichloromethane–TFA in the same proportion as in the mobile phase by vortex mixing for 1 min.

In all liquid–liquid extraction procedures the effect of sodium chloride addition and the effect of the organic solvent on the extraction recoveries were studied. For this purpose, water samples (1 ml) spiked with 200 ng/ml of each salbutamol enantiomer were subjected to the liquid–liquid extraction procedure with different organic solvents, and with or without the addition of sodium chloride.

The effect of the urine matrix on the extraction recoveries was also studied. Water and urine samples (1 ml) spiked with 200 ng/ml of each salbutamol enantiomer were subjected to extraction procedures tested using different organic solvents, all with salting-out effect, and different solid-phase cartridges.

For Bond-Elut Certify™ extraction columns, the influence of sample amount, sample pH and ionic strength, as well as the influence of the elution volume and concentration of alcohol in the elution phase were considered. For this purpose, recoveries and detection limits were compared when 2 ml of urine were used, when the sample pH was adjusted to 3 and 10, when urine was diluted with water (1:1), when the elution volume was increased (2+2 ml) and when the elution step was performed with a mixture of chloroform and 2-propanol (50:50, v/v) containing 2% ammonia.

In all cases methanolic solutions of *S*(+)-salbutamol and *R*(-)-salbutamol in amounts equivalent to 100% recovery were dried and reconstituted to evaluate the extraction recoveries. The extraction recoveries ($n=3$) were calculated by comparison of peak area ratios between salbutamol enantiomers and the external standard obtained from the extracted samples with those of the pure standard equivalent to 100% recovery.

After optimization studies, the procedure involving Bond-Elut Certify™ solid-phase was chosen and the following procedure was finally used for the determination of salbutamol enantiomers in urine samples: 1 ml of 1 mol/l acetic acid were added to 2 ml of urine, vortex mixed and applied to the preconditioned cartridges. The columns were washed with 1 ml of water, 500 μl of 1 mol/l acetic acid, 1 ml of methanol and dried for 5 min under full vacuum. Two consecutive elutions, 2 ml each, were carried out with a mixture of chloroform and 2-propanol (80:20, v/v) containing 2% ammonia, and the combined eluates were added to 10 μl of a 100 μg/ml solution of external standard.

2.6. Assay validation

The final method was validated by determining its selectivity and specificity, salbutamol enantiomer recoveries from urine samples, linearity, limits of detection and quantification, and precision and accuracy.

The selectivity and the specificity of the method developed were studied analyzing a series of different blank urine (six male and six female) and checking for the presence of any interfering substances at the retention time of the compounds of interest, salbutamol enantiomers and atenolol.

Recoveries ($n=4$) of *S*(+)-salbutamol and *R*(−)-salbutamol from urine were calculated by comparison of peak area ratios between analytes and the external standard obtained from urine spiked samples (200 ng/ml) and the peak area ratios obtained when pure standards were added to an extracted blank urine.

For the study of linearity a calibration graph for both enantiomers of salbutamol was prepared covering the whole expected concentration range. Spiked urine samples with concentrations of 90, 250, 500, 750 and 1000 ng/ml of each salbutamol enantiomer were prepared daily by adding appropriate volumes of the stock solutions to 2-ml aliquots of drug-free urine. These samples were prepared and analyzed in duplicate and the ratio of the peak area of each enantiomer to the peak area of the external standard was plotted against analyte concentration.

Six replicates of spiked urine with concentrations of 100, 450 and 800 ng/ml for both enantiomers of

salbutamol were analyzed in the same day in order to determine the intra-assay precision and accuracy. The standard deviation of the estimated concentration values for the 100 ng/ml spiked sample was used as a measure of the noise for the calculation of the limits of detection (three times the standard deviation) and quantification (ten times the standard deviation). Moreover, the inter-assay precision and accuracy were determined using the calibration points of 90, 500 and 1000 ng/ml analyzed during different days. Precision is expressed as the relative standard deviation (RSD) of the spiked sample concentration calculated using the calibration curve, and accuracy is expressed as the relative error (%) of their estimated concentrations.

3. Results and discussion

3.1. Chromatographic analysis

The enantiomeric separation of salbutamol was achieved using a urea type chiral stationary phase of (*S*)-indoline-2-carboxylic acid linked to (*R*)-1-(α-naphthyl)ethylamine urea and to *N*-propylamine with an isocratic and normal-phase chromatographic method similar to the one previously described by Boulton and Fawcett [22]. The use of a ternary mobile phase comprising hexane, dichloromethane and methanol with the addition of TFA has been found to be effective in the separation of salbutamol enantiomers. The addition of dichloromethane increases the solubility of the solute molecules in the mobile phase and also inhibits the adsorption of the alcohol on the stationary phase. This permits better solute-stationary phase interactions resulting in a better enantiomeric resolution.

The elution order of salbutamol enantiomers was determined by chromatographic analysis of the individual enantiomers. Retention times with a mobile phase consisting of hexane–dichloromethane–methanol–TFA (250:218:31:1) were 7.78±0.12 and 9.06±0.16 (n=10) min for *S*(+)-salbutamol and *R*(−)-salbutamol, respectively. Atenolol was chosen as external reference substance for quantitation to avoid variability in results due to evaporation of solvents because of its chromatographic behaviour, eluting at a retention time close to those of salbutamol enantiomers and presenting good response in the fluorimetric detector at the selected wavelengths. The retention times of atenolol enantiomers were 12.29±0.28 and 13.33±0.33 (n=10) min, but the order of elution could not be determined due to the lack of pure standard for each enantiomer. Therefore, the peaks were designated as A_1 and A_2 based on the shortest and longest respective elution time. The quantitation of each salbutamol enantiomer was based on the ratio towards the peak area of the A_2 atenolol peak.

The fluorescence detection offers good specificity and selectivity for the determination of salbutamol in urine. As it is shown in Fig. 2, chromatograms of extracts of blank urine using fluorescence detection at an excitation and emission wavelengths of 230 and 309 nm, respectively, are free from interferences contrary to UV absorbance detection at 280 nm.

3.2. Reconstitution experiments

To dissolve samples in the same solvent as the mobile phase is recommended for good chromatographic results, therefore the dried extracts resulting from the different extraction procedures were initially reconstituted in mobile phase. However, problems were noted because in some cases either the salbutamol enantiomers or atenolol were not fully redissolved in these conditions. Results indicated that the solvent used to redissolve the dried extracts does not influence the extraction recoveries of salbutamol enantiomers when extracted from aqueous samples, but its influence on recoveries from urine is relevant. Extraction yields in urine samples after reconstitution of the extracts with mobile phase or with the mixture dichloromethane–TFA are compared in Fig. 3. Results showed that salbutamol enantiomers were best recovered from urine samples using a mixture dichloromethane–TFA when solid-phase extraction or liquid–liquid extraction with ethyl acetate or a mixture *tert*.-butylmethyl ether-*tert*.-butyl alcohol (6:1, v/v) were used. For that reason, residues were reconstituted with 100 μl of mobile phase when the extraction was made in *tert*.-butylmethyl ether or chloroform, and reconstituted with 100 μl of dichloromethane–TFA in the same proportion as in mobile phase after liquid–liquid extraction with ethyl

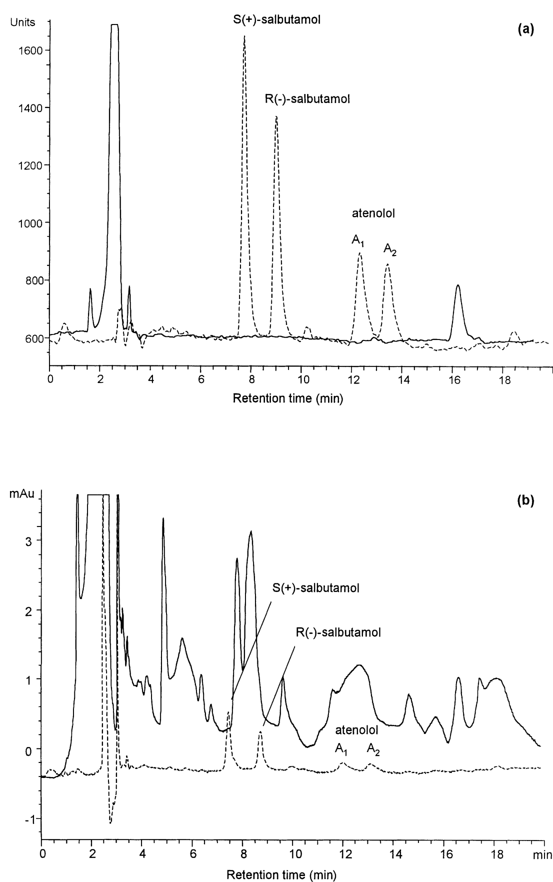

Fig. 2. Separation of pure standards (dotted lines) of salbutamol and atenolol (external standard) enantiomers compared with blank urines (solid-lines) after solid–liquid extraction with Bond-Elut Certify™ cartridges using (a) fluorimetric and (b) UV detection. A_1 and A_2 are assigned to the enantiomers of atenolol. Mobile phase: hexane–dichloromethane–methanol–TFA (250:218:31:1, v/v).

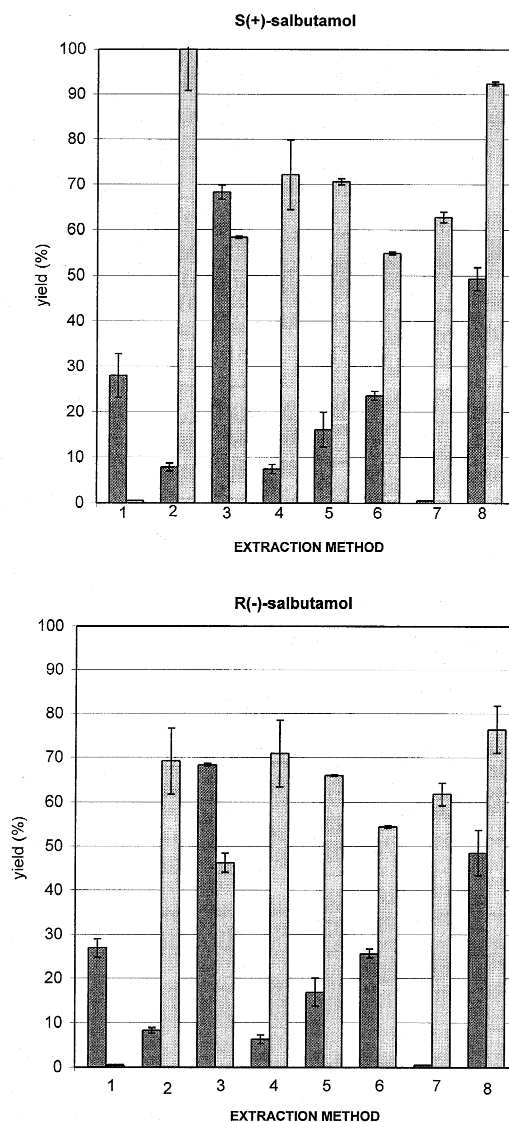

Fig. 3. Influence of the solvent used for reconstitution of the dried urine extracts after liquid–liquid (1 to 4, see below) and solid–liquid (5 to 8, see below) extraction with different organic solvents and cartridges [1=chloroform; 2=ethyl acetate; 3=*tert*.-butylmethyl ether; 4=*tert*.-butylmethyl ether–*tert*.-butyl alcohol (6:1, v/v); 5=Oasis™; 6=Bond-Elut Certify™; 7=Bond-Elut™ Si; 8=Sep-Pak™ C_{18}]. ▩, mobile phase; ▨, dichloromethane–TFA.

acetate or *tert*.-butylmethyl ether–*tert*.-butyl alcohol (6:1, v/v), and after solid–liquid extraction.

3.3. Liquid–liquid extraction procedures

Chemical structure of salbutamol is shown in Fig. 1. Two dissociation constant values have been described for this compound [24]: 9.3, which corresponds to the deprotonation equilibrium of the secondary amine group, and 10.3, which probably corresponds to the ionization equilibrium of the phenolic group. In order to optimize the extraction pH, recoveries at sample pH of 9.5, 10, 10.5 and 11.5, using chloroform as solvent, were calculated. Results obtained indicate that extraction of salbutamol is maximum when the pH of the sample is adjusted to 10, in agreement with the fact that in that

situation the amine group is not protonated and the phenolic group is not ionized.

The effect of sodium chloride addition on the extraction recoveries from aqueous samples, and the effect of the urine matrix on the liquid–liquid extraction procedures with the different solvent tested are shown in Fig. 4a and 4b, respectively. The salting-out effect helps in enhancing the extraction yields for all the organic solvents used but differences were greater in case of the less polar solvents, chloroform and *tert*.-butylmethyl ether. Recoveries of salbutamol enantiomers from aqueous samples using ethyl acetate, *tert*.-butylmethyl ether and a mixture *tert*.-butylmethyl ether-*tert*.-butyl alcohol (6:1, v/v) with the addition of sodium chloride were between 60 and 80%, whereas with chloroform were only about 25%. The effect of the urine matrix was not very important and recoveries were practically identical to those obtained in aqueous samples except when ethyl acetate was used. In this case, an interference co-eluting with *S*(+)-salbutamol resulted in an overestimation of its concentration. The higher recoveries of *S*(+)- and *R*(−)-salbutamol in urine samples were obtained with *tert*.-butylmethyl ether or a mixture *tert*.-butylmethyl ether–*tert*.-butyl alcohol (6:1, v/v) and the addition of sodium chloride. The mixture *tert*.-butylmethyl ether–*tert*.-butyl alcohol (6:1, v/v) had been suggested for the extraction of salbutamol from horse urine [25], but extracts presented less endogenous peaks when *tert*.-butylmethyl ether alone was used as organic solvent in the extraction.

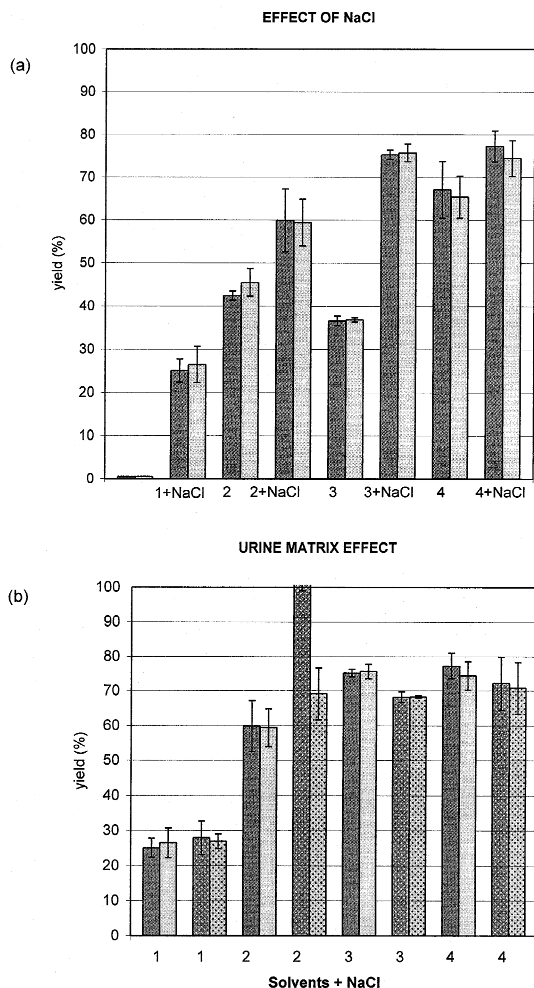

Fig. 4. Liquid–liquid extraction recoveries for salbutamol enantiomers using different organic solvents. (a) Effect of the addition of sodium chloride in aqueous samples and (b) effect of urine matrix [1=chloroform; 2=ethyl acetate; 3=*tert*.-butylmethyl ether; 4=*tert*.-butylmethyl ether–*tert*.-butyl alcohol (6:1, v/v)]. ▩, *S*(+) water; ▢, *R*(−) water; ▩, *S*(+) urine; ▩, *R*(−) urine.

3.4. Solid-phase extraction procedures

Salbutamol molecule contains polar and non-polar groups as well as an ionizable amine function in its chemical structure able to interact with different solid-phases. Extraction mechanisms involving polar (silica), non-polar (C_{18}), interactions with a polymeric sorbent with dual retention capability to retain polar and non-polar compounds (Oasis™) and interactions with a combined phase involving hydrophobic and cation-exchange bonding (Bond-Elut Certify™) have been tested.

The effect of the urine matrix after the solid-phase extraction procedures proposed with the different cartridges tested is shown on Fig. 5. Recoveries of salbutamol enantiomers from urine were similar to those obtained in aqueous samples in all cases except for the silica cartridges because polar interactions between salbutamol enantiomers and silica are favoured when the biological sample is passed through the sorbent.

High recoveries from urine, about 80%, were obtained with the Sep-Pak™ C_{18} cartridges. With this phase, pH must be adjusted in order to have sal-

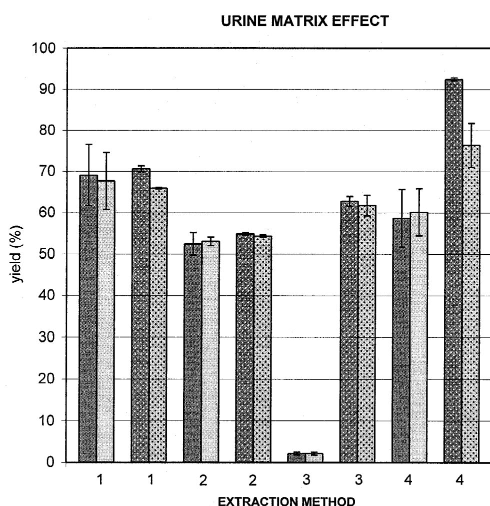

Fig. 5. Solid-phase extraction recoveries for salbutamol enantiomers using different cartridges: effect of urine matrix (1=Oasis™; 2=Bond-Elut Certify™; 3=Bond-Elut™ Si; 4=Sep-Pak™ C_{18}). ▩, S(+) water; ▩, R(-) water; ▩, S(+) urine; ▩, R(−) urine.

butamol molecules in their neutral form and allow hydrophobic interactions with the sorbent. However, an interference co-eluting with the S(+) enantiomer of salbutamol resulted in an overestimation of its concentration.

Oasis™ extraction cartridges contain a macroporous copolymer [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] designed to have a hydrophilic–lipophilic balance, that is supposed to give high and reproducible recoveries for acidic, basic and neutral compounds. The retention properties of this sorbent and its capability to retain substances with different polarities resulted in a poor selectivity of the extraction procedure. Some endogenous compounds of urine sample are extracted and chromatograms with interfering peaks at retention times similar to those of salbutamol enantiomers were obtained.

Bond-Elut Certify™ cartridges contain a mixed phase with both ion-exchange and lipophilic properties, depending on the pH conditions. Working at an acidic pH allows hydrophobic interactions and cationic exchange simultaneously between the sorbent and the substances. The extraction procedure involving this phase provided an effective and selective extraction of salbutamol enantiomers but recoveries were about 55% and the limit of detection was not higher than 100 ng/ml. Therefore, some adjustments were made in the extraction procedure for sample clean up and quantitation of salbutamol enantiomers in urine with Bond-Elut Certify™ in order to obtain better results. Recoveries for salbutamol increased when the elution volume was increased and when samples were acidified with 1mol/l acetic acid (pH 3), probably because interactions with anionic groups of the extraction columns are favoured. Moreover, the method sensitivity was improved by increasing the amount of urine sample extracted.

The optimized solid-phase extraction procedure with Bond-Elut Certify™ cartridges was chosen because is rapid and selective, and thus validated for the determination and quantitation of salbutamol enantiomers in urine. The proposed method provided recoveries similar to those obtained with liquid–liquid extraction using *tert.*-butylmethyl ether but, as can be observed in Fig. 6, provided extracts with less interfering compounds.

3.5. Assay validation

After analyzing a series of different blank urines by the optimized Bond-Elut Certify™ solid-phase extraction method, no interferences were found at the retention times of the S(+)- and R(−)-salbutamol enantiomers. However, an interfering peak co-eluting with the A_1 peak of atenolol (external standard) was observed in some samples and for that reason the second peak (A_2) was used for quantitation. Extraction recoveries for S(+)-salbutamol and R(−)-salbutamol were 69.5±5.2% and 70.2±5.8% (n=4), respectively.

Quantitation was based on peak-area ratios of compounds to external standard versus concentration of compound spiked. The reported method was found to be linear (r>0.99) over the standard curve range studied from 90 to 1000 ng/ml for both enantiomers of salbutamol; the slopes ranged from 2.5×10^{-3} to 3×10^{-3}, and the estimated intercepts did not differ significantly from the origin.

The limits of detection calculated as three times the standard deviation of the estimated concentration values obtained for a spiked sample containing 100 ng/ml of S(+)-salbutamol and R(−)-salbutamol were 10.8 and 10.4 ng/ml, respectively. The limits of quantification calculated as ten times that standard

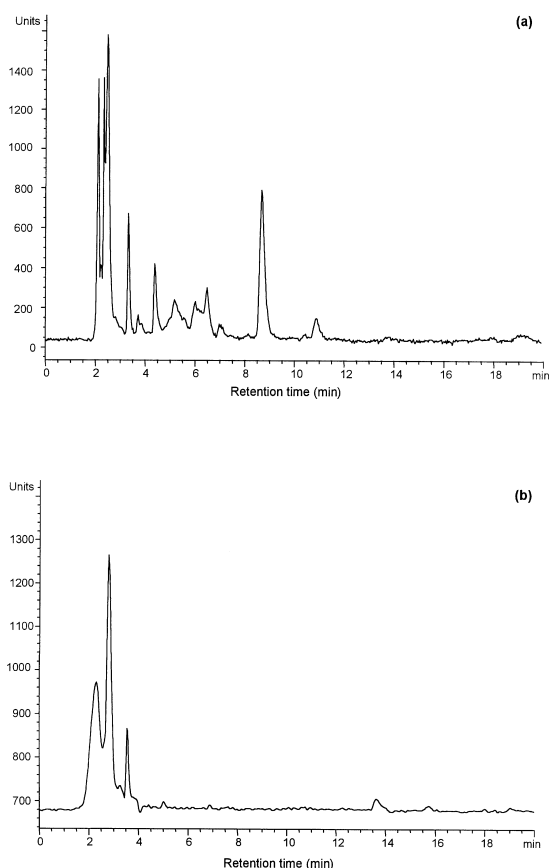

Fig. 6. Chromatograms from blank urine after (a) liquid–liquid extraction with tert.-butylmethyl ether using sodium chloride to promote the salting-out effect, and after (b) solid-phase extraction with Bond-Elut Certify™ cartridges. Mobile phase: hexane–dichloromethane–methanol–TFA (240:140:20:1, v/v).

deviation were 36.1 and 34.6 ng/ml for *S*(+) and *R*(−)-salbutamol, respectively.

Results obtained for intra-assay and inter-assay precision and accuracy are given in Table 1 and 2. The intra-assay precisions were between 1.5 and 3.5% for both salbutamol enantiomers, and the inter-assay precision varied from 5.3 to 10.3% for *S*(+)-salbutamol and from 6.2 to 13.5% for *R*(−)-salbutamol over the whole concentrations range studied. The accuracy was always better than 10% for *S*(+)-salbutamol and better than 5% for *R*(−)-salbutamol. Apart from this small difference in accuracy, the overall method proposed does not discriminate any of the two enantiomers and, therefore, appears useful for salbutamol metabolic studies.

The analytical methodology developed has been applied to urine samples obtained after administration of racemic salbutamol and, as an example, chromatograms for urine obtained after oral and inhaled administration are shown in Fig. 7. Results obtained suggest that after oral administration absolute concentrations of unchanged salbutamol are higher than after inhaled administration and that the ratio of *S*(+) to *R*(−) enantiomer is favoured. Therefore, simultaneous evaluation of absolute concentration in urine of salbutamol and the ratio between *S*(+) and *R*(−) enantiomer may be a useful tool to differentiate oral and inhaled administrations. Works are in progress in that direction with a large number of subjects.

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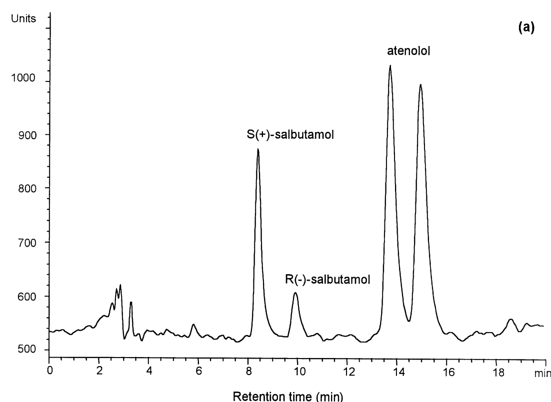
Table 1
Intra-assay precision and accuracy obtained for quantification of *S*(+)-salbutamol and *R*(−)-salbutamol added to urine

Compound	Concentration (ng/ml)	*n*	Estimated concentration (mean±SD) (ng/ml)	Precision (RSD, %)	Accuracy (relative error, %)
S(+)-Salbutamol	100	5	104.8±3.6	3.44	4.82
	450	5	447.7±11.4	2.55	−0.50
	800	6	796.8±12.4	1.56	−0.39
R(−)-Salbutamol	100	5	102.1±3.5	3.39	2.09
	450	6	449.8±7.7	1.71	−0.03
	800	6	794.6±16.4	2.07	−0.68

Table 2
Inter-assay precision and accuracy obtained for quantification of $S(+)$-salbutamol and $R(-)$-salbutamol added to urine

Compound	Concentration (ng/ml)	n	Estimated concentration (mean±SD) (ng/ml)	Precision (RSD, %)	Accuracy (relative error, %)
$S(+)$-Salbutamol	90	10	97.4±10.0	10.28	8.21
	500	10	506.3±38.5	7.61	1.27
	1000	10	999.2±53.1	5.31	−0.08
$R(-)$-Salbutamol	90	10	94.4±12.8	13.54	4.93
	500	10	509.2±41.6	8.17	1.85
	1000	10	996.5±61.5	6.18	−0.35



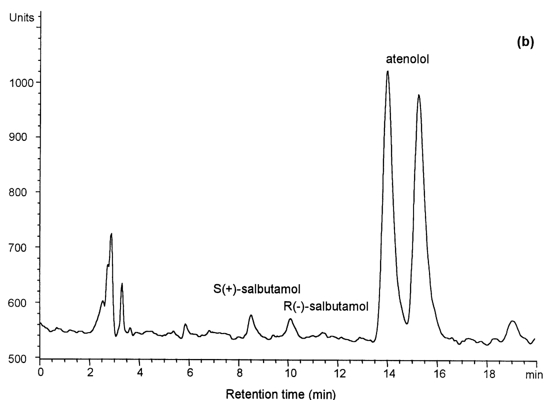


Fig. 7. Chromatograms from urine samples obtained after (a) oral administration and (b) inhaled administration of racemic salbutamol. Concentrations for $S(+)$-salbutamol and $R(-)$-salbutamol after oral administration were 948.2 ng/ml and 292.7 ng/ml; and after inhalation concentrations were 133.1 ng/ml and 117.3 ng/ml for $S(+)$ and $R(-)$-salbutamol, respectively.

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