Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (6)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1999

R. BERGÉS, J. SEGURA, R. VENTURA, X. DE LA TORRE, M. MAS, M. FARRÉ, K.D. FITCH, A.R. MORTON, M. UEKI:

Oral *versus* Inhaled Salbutamol: Is Distinction Possible by Urine Analysis?

In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (6). Sport und Buch Strauß, Köln, (1999) 405-419

R. Bergés [1], J. Segura [1], R. Ventura [1], X. de la Torre [1], M. Mas [1], M. Farré [1], K.D. Fitch [2], A.R. Morton [2] and M. Ueki [3]

Oral *versus* Inhaled Salbutamol: Is Distinction Possible by Urine Analysis?

[1] Drug Research Unit, Institut Municipal d'Investigació Mèdica (IMIM). Doctor Aiguader 80, E-08003 Barcelona, Spain.

[2] Department of Human Movement & Exercise Science, University of Western Australia. Parkway Entrance No.3, Nedlands, Perth, Western Australia 6907.

[3] Mitsubishi Kagaku Bio-clinical Laboratories, Inc. 3-30-1 Shimura, Itabashi-ku, Tokyo, 174 Japan.

Introduction

The use of salbutamol in sports is only permitted by inhalatory route in the treatment of asthma. Administration by the oral or parenteral route is forbidden due to a strong adrenergic stimulation and an anabolic-like effect [1]. Owing to the necessity in doping control to distinguish between an authorized and a prohibited use of this drug, it is important to develop a urine test with adequate discriminatory capacity.

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 [2,3]. Metabolism, mainly sulfate conjugation, occurs in the intestine and the liver when the drug is given orally and is highly stereoselective in favor of the R(-)-enantiomer [4,5]. On the other hand, salbutamol does not appear to be extensively metabolized in the lungs, therefore its metabolic behavior following inhalation depends mainly upon the proportion of inhaled salbutamol relative to the proportion swallowed. The proportion of conjugated salbutamol present in urine is higher after oral than after inhaled administration due to the first pass metabolic reaction and, because of the stereoselective

sulfation, the excretion of S(+)-salbutamol is also greater than the excretion of R(-)-salbutamol [6].

In doping control, the screening procedure for determining urinary salbutamol is usually performed by means of gas chromatography/mass spectrometry (GC/MS) analysis of the enzymatically partially hydrolyzed urine [7]. However, following this procedure mainly free urinary salbutamol is measured because a limited hydrolysis of sulfated compound is achieved. ELISA tests are also used to measure total free plus conjugated salbutamol [8] but data do not afford clear cut results to conclude about the dose and the route of administration. Using the GC/MS methodology a criteria to distinguish between oral and inhaled salbutamol was proposed by measurement of the peak height ratio between androsterone and salbutamol [9].

Recently, a method for separation and quantitation of salbutamol enantiomers in urine samples has been developed [10]. Separation of salbutamol enantiomers is achieved by an enantioselective liquid chromatographic procedure involving solid-phase clean-up and fluorimetric detection that provides the sensitivity required and offers great reliability for assay of large numbers of urine samples.

The establishment of a criteria to distinguish between the correct and the incorrect use of salbutamol in sports seems possible using the simultaneous evaluation of different variables such as the absolute concentration of salbutamol and metabolite, the proportion of conjugated salbutamol as compared with the unchanged drug in the urine (percentage of conjugation), and ratios between S(+)- and R(-)- enantiomers of the unchanged drug. The aim of this work is to compare the discriminatory power of these variables to differentiate between oral and inhaled ingestion of salbutamol.

Experimental

Chemicals and reagents

ELISA test Generic Bronchodilators (Elisa Technologies Division, Neogen Corporation, Lexington, KY) was supplied by Labsystems (Sant Just Desvern, Barcelona, Spain).

Methanol, 2-propanol (both HPLC grade), *tert*-butylmethyl ether, 25% ammonia, di-sodium hydrogenphosphate anhydrous, sodium dihydrogenphosphate monohydrate, potassium carbonate, ammonium iodide, 2-mercaptoethanol (all analytical grade) and trifluoroacetic acid (TFA) (for spectroscopy) were purchased from Merck (Darmstadt, Germany). Dichloromethane (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).

N-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) for gas chromatography was obtained from Macherey-Nagel (Düren, Germany) and β-glucuronidase from *Escherichia Coli* was purchased by Boehringer Mannheim (GmbH, Germany).

Racemic atenolol and racemic salbutamol were purchased by Laboratorios ICI-Farma S.A. (Porriño, Pontevedra, Spain) and Laboratorios Glaxo S.A. (Madrid, Spain), respectively. Pure enantiomers of salbutamol were donated by Glaxo (Uxbridge, Middelsex, UK). Stock standard solutions (1 mg/ml, in free base form) of racemic salbutamol, S(+)-salbutamol, R(-)-salbutamol and racemic atenolol were prepared in methanol. Working solutions of 100 and 10 μg/ml were prepared by dilution of stock solutions in methanol. These solutions were checked by UV spectrophotometry and stored at −20° C.

Bond Elut Certify™ (130 mg/10 ml) columns, which contain a mixed phase with both ion exchange and lipophilic properties, were provided by Varian (Harbor City, CA, USA) and Detectabuse™ cartridges containing a non-ionic copolymer were supplied by Biochemical Diagnostics, Inc. (Edgewood, NY, USA).

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

ELISA analyses

Sample aliquots of 20 μl were added to each microplate well along with 180 μl of a diluted solution of the conjugate terbutaline-horseradish peroxidase. Wells were incubated by shaking (Heidolph mixer, Labsystems) for 60 min at room temperature and then were washed three

times with 200 µl of diluted washing buffer (Autowash I, Labsystems). After washing, 150 µl of peroxidase substrate solution (K-Blue, proprietary composition) were added to each well, and incubation for 30 min by shaking was performed for color development. The optical density was determined at 620 nm with an automated microplate reader (Anthos Reader 2001, Bercu Instruments S.L., Sant Adrià del Besós, Barcelona, Spain).

A calibration curve of salbutamol was analyzed in duplicate with each batch of samples. The following calibration levels were used: 0, 0.1, 1, 10 and 100 ng/ml of racemic salbutamol. Calibration curves of salbutamol were calculated using a sigmoidal equation. Samples were diluted 1:10, 1:100 or 1:1000 with dilution buffer to obtain a response in the range of the calibration curve. A blank urine and a positive control of 10 ng/ml of salbutamol were analyzed in each strip of wells.

Gas chromatography-mass spectrometry analyses

Salbutamol and androsterone were analyzed by GC/MS using the conventional screening procedure for anabolic agents [7]. Sample (2.5 ml) loaded with 25 µl of a mixture containing 50 µg/ml methyltestosterone, 5 µg/ml testosterone-d_3 and 50 µg/ml etiocholanolone-d_4 (internal standard solution) were added to Detectabuse™ columns preconditioned with 2 ml of methanol and 2 ml of deionized water. The columns were washed with 2 ml of deionized water, and the analytes eluted with 2 ml of methanol. The methanolic extract was evaporated to dryness under nitrogen stream in a 50° C water bath, and reconstituted with 1 ml of 0.2 mol/l sodium phosphate buffer, pH 7. Then 30 µl of β-glucuronidase from *Escherichia coli* were added and hydrolysis was performed in a water bath set at 55° C for one hour. The samples were cooled at room temperature, alkalinized with 250 µl of a 5% K_2CO_3 solution and extracted with 5 ml of *tert*-butylmethyl ether. After rocking at 40 movements/minute for 20 minutes and centrifugation (5 minutes at 3500 rpm), the organic phase was separated and evaporated to dryness under nitrogen stream in a water bath at 50° C. Samples were kept in a dessicator for 30 min, derivatized with a mixture of MSTFA:NH_4I:2-mercaptoethanol (1000:2:6, v/w/v) and heated in a dry bath at 60° C for 20 minutes.

GC/MS analysis was performed in a 5890A Series II gas chromatograph coupled to a 5970 electron impact mass-selective detector equipped with an HP 7673 automatic sampler (all

from Hewlett-Packard, Palo Alto, California, USA). The column was a 17 m x 0.2 mm I.D. fused silica cross linked methylsilicone with a 0.11 μm film thickness (Hewlett-Packard). Helium was used as a carrier was at a flow-rate of 0.8 ml/min. The injection port and detector temperatures were 280° C. The oven temperature was increased from 181° C to 230° C at 3° C/min, and then to 310° C at 40° C/min, with a final hold time of 3 minutes. The injection volume was 2 μl with a split ratio of 10:1. The MS acquisition was performed in selected ion monitoring (SIM) mode, by monitoring m/z 86, 369 and 440 for the TMS derivative of salbutamol and m/z 434 for androsterone. Salbutamol concentrations were estimated by response factors calculated from a calibration point containing 400 ng/ml of salbutamol.

Enantioselective HPLC analyses

Separation of salbutamol enantiomers was achieved using a solid-phase clean-up procedure followed by enantioselective HPLC separation and fluorescence detection [10]. Urine samples (2 ml) were acidified with 1 ml of 1 mol/l acetic acid solution, vortex mixed and applied to the Bond-Elut Certify™ cartridges preconditioned with 1 ml of methanol and 1 ml of deionized water. The columns were washed with 1 ml of water, 500 μl 1 mol/l acetic acid, 1 ml of methanol, and dried for five minutes 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. The combined eluates were added to 10 μl of a 100 μg/ml atenolol solution (external standard), vortex mixed and evaporated to dryness under a stream of nitrogen in a 40° C water bath. The dried extracts were finally reconstituted in 100 μl of a mixture dichloromethane/TFA in the same proportion as in mobile phase, vortex mixed for 1 minute and 25 μl were injected into the HPLC system.

Chromatographic analysis was carried out using a Series II 1090L liquid chromatograph (Hewlett Packard) linked to a HP ChemStation (Hewlett Packard) to acquire and analyze data. The effluent was monitored with a LS-5 fluorescence detector (Perkin-Elmer, Norwalk, CO, USA) equipped with a flow-through cell of 2 x 2 mm at an excitation and emission wavelength of 230 and 309 nm, respectively, with both excitation and emission slit widths set at 10 nm.

A Chirex^{TM} 3022 (30 x 4.0 mm) guard column and a Chirex 3022 (250 x 4.0 mm) analytical column (Phenomenex, Torrance, CA, USA) were used. The mobile phase was a mixture of hexane, dichloromethane, methanol and TFA (250:218:31:1, v/v) and it was degassed under a stream of helium before use. The flow-rate was 1 ml/min and the system was operated at ambient temperature.

Standard curves for both enantiomers were prepared every day by adding appropriate volumes of the working standard solutions to 2 ml aliquots of drug-free urine to give two replicate spiked samples of 90, 250, 500, 750 and 1000 ng/ml. Control samples with concentrations of 100, 450 and 800 ng/ml of each enantiomer were used to check the standard curves on each assay day. Quantitation was based on peak-area ratios salbutamol enantiomers to atenolol *versus* concentration of compound spiked.

Studies design

Field study. Study was designed to investigate the possibility to discriminate between 20000 μg of salbutamol taken orally over a 24 hours period (a common dosage practice) and 200 μg of inhaled salbutamol taken immediately before a training session. Fifteen asthmatic (9 male and 6 female) competitive swimmers and seventeen non-asthmatic (10 male and 7 female) recreational swimmers who could comfortably complete one-hour swim training were recruited. No recreational swimmer, who may have been required to undergo sports drug testing, was included in this study and all subjects were at least 18 years of age. Each subject was required to follow the normal routine training session after the administration of salbutamol (Ventolin®). This procedure simulates a sports drug test.

All subjects provided a urine sample prior to application of any treatment to allow the determination of baseline values. Urine samples were also collected during a 60-minute period after the training session. The urine was frozen, stored in dry ice and flown by courier to Barcelona (Spain) for analysis.

The asthmatic subjects received one inhaled treatment and the non-asthmatic group received two treatments, inhaled and oral, in random order and separated by at least 72 hours. For the inhaled phase, two inhalations (2 puffs) each of 100 μg salbutamol were administered 5

minutes prior to the start of the training session. For the oral treatment, five tablets each of 4 mg salbutamol were administered 6-hourly with the last tablet taken no longer than 2 hours prior to the swimming session. Asthmatic subjects were allowed to continue taking any prescribed medication and any necessary inhaled salbutamol even on the day of testing. The non-asthmatic subjects self-administered the salbutamol using a metered dose inhaler (MDI) while the asthmatic swimmers used a spacer with their MDI. The possible side effects from the administration of salbutamol were annotated.

Controlled study. A clinical trial involving six healthy volunteers (males) receiving salbutamol by inhalation and by the oral route under controlled conditions in absence of exercise was designed. The cross-over and non-blind study consisted of six drug conditions: single and multiple doses of oral salbutamol, and single and multiple doses of inhaled salbutamol with and without washing the mouth with water post-inhalation to reduce the amount swallowed. Drug conditions were applied in random order and separated by one week wash-out period.

All subjects provided a urine sample prior to application of treatments to allow the determination of baseline values. Urine samples were collected during a 48-hour period after the single dose administration, and during a 72-hour period after the multiple dose administration.

For the inhaled phase, subjects received a single dose consisting of four inhalations (4 puffs) each of 100 μg salbutamol or a multiple dose treatment consisting of four doses each of four inhalations (16 puffs) administered 8-hourly over 24 hours. These drug conditions, single and multiple dose, were reproduced adding a mouth-washing step post-inhalation. For the oral phase, subjects received a single dose of 4 mg salbutamol or a multiple dose consisting of four tablets each of 4 mg administered 8-hourly over 24 hours.

Results and discussion

All urine samples obtained from the field study were analyzed. The "free" urinary salbutamol levels and the peak height ratio between androsterone and salbutamol were determined by means of GC/MS analysis; the total salbutamol (free+conjugated) concentrations were determined using the ELISA test; and concentrations of S(+)- and R(-)-salbutamol

enantiomers excreted in urine were determined with the enantioselective HPLC procedure described in the experimental section.

Owing to the great differences between doses administered orally (mg) and by inhalation (μg), the total concentration of free salbutamol excreted in urine could be useful to differentiate the correct or incorrect use of salbutamol in sport. Concentrations of salbutamol, mainly free compound, and the peak height ratio between androsterone and salbutamol determined by GC/MS in urine samples obtained from all subjects considered in the field study are represented in Figure 1. Results obtained indicate that concentrations higher than 500 ng/ml of salbutamol are detected in urine after oral administration whereas after inhalation concentrations are in general lower than those values. However, some samples obtained after oral administration present concentrations of salbutamol lower than 500 ng/ml and to some extent similar to those obtained after inhaled administration.

The peak height ratio of androsterone to salbutamol found are lower for samples obtained after oral administration of the drug, but there is not a clear cut-off that allow to distinguish these samples from those obtained after inhalation. The detection and semi-quantitation of salbutamol by the usual anabolic steroids screening procedure involving enzymatic hydrolysis presents some analytical limitations: limited hydrolysis of conjugated salbutamol, irreproducibility of liquid-liquid extraction yield for salbutamol, and better extraction of androsterone than salbutamol. Moreover, semiquantitation of salbutamol and androsterone by one-point calibration is carried out, androsterone usually appears as a saturated peak and it is affected by treatments with other anabolic agents. Therefore, no validated criteria to establish the route of administration either by absolute concentration or by androsterone/salbutamol ratio exists.

The total salbutamol concentrations determined by the immunological screening method (ELISA) as well as the proportion of unchanged salbutamol excreted in urine for samples obtained in the field study are shown in Figure 2. After oral administration, we found extremely high concentrations (>2000 ng/ml) of salbutamol and a percentage of free salbutamol between 20 and 40%. On the other hand, after inhalation concentrations in general lower than 1000 ng/ml were found but percentages of the unchanged compound varying from 20 to 90% were detected. Therefore, no confirmatory criteria can be developed from

quantitation of total (free+conjugated) salbutamol and differential percentage of conjugation between oral and inhaled administration of the drug.

Concentration of salbutamol estimated by conventional screening procedures can be used as a preliminary measure for selecting suspicious cases of oral salbutamol administration. Suspicious cases should be further analyzed for free content of S(+)- and R(-)- enantiomers of salbutamol by solid-phase extraction, enantioselective HPLC separation and fluorimetric detection. Using the chromatographic procedure described in the experimental section [10], salbutamol enantiomers can be easily separated and their determination is not interfered by any peak present in blank urine (see Figure 3). Retention times of salbutamol enantiomers are (mean±standard deviation) 7.78±0.12 and 9.06±0.16 (n=10) minutes for S(+)- and R(-)-salbutamol, respectively. Atenolol enantiomers are used as external reference for quantitation to avoid variability due to evaporation of solvents, and their retention times are (mean±standard deviation) 12.29±0.28 and 13.33±0.33 (n=10) minutes. Fluorescence detector provides the sensitivity required, with limits of detection of 10.8 and 10.4 ng/ml for S(+) and R(-)-salbutamol, and offers great reliability for assay of large numbers of urine samples.

The simultaneous evaluation of concentration of unchanged salbutamol and ratio between S(+)- and R(-)- enantiomers measured in urine seem to be useful to establish the correct or incorrect use of the drug. Plots of total free salbutamol concentration excreted in urine *versus* S(+)/R(-) ratio for samples considered are shown in Figure 4. From results obtained, it can be observed that all urine samples collected after oral administration of racemic salbutamol present simultaneously concentrations of free salbutamol higher than 500 ng/ml and S(+)/R(-) ratios higher than 2.5, and that this distribution of values can be clearly separated from that obtained for samples after inhalation. Dotted line in Figure 4 could be useful to separate samples obtained after inhalation of salbutamol from those samples obtained after oral ingestion of the drug.

Samples (n=23) collected after competition at the Nagano Winter Olympic Games from athletes having declared the ingestion of inhaled salbutamol and showing salbutamol by the gas chromatography/mass spectrometry (GC/MS) method normally used for screening were analyzed with the enantioselective HPLC procedure proposed. As can be observed in Figure 5, all these samples can be clearly distinguised from those samples obtained after oral ingestion of salbutamol according to the two variables proposed.

Total concentration of free salbutamol *versus* the ratio between its enantiomers for urine samples collected after inhaled (single and multiple dose) and after oral (single and multiple dose) ingestion of the drug from one of the volunteers involved in the clinical trial are shown in Figure 6. Distribution of data for urine samples is in accordance with that obtained in the field study showing a clear separation between those samples obtained after inhalation and after oral administration of racemic salbutamol. Data obtained for all the volunteers involved in the clinical trial will be useful for pharmacokinetic studies.

This study demonstrate that a clear distinction between prohibited oral and authorized inhaled salbutamol in doses that are adequate for all asthmatic athletes to compete can be achieved by urinalysis.

Conclusion

Concentration of salbutamol estimated by conventional procedures for antidoping analysis can be used as a preliminary measure for selecting suspicious cases of oral salbutamol administration: common anabolic steroid screening, wich measures a value slightly higher than free salbutamol; and immunological screening method for β-adrenergic drugs (ELISA) which measures approximately "total" salbutamol. Suspicious cases should be further analyzed for free content of S(+)- and R(-)- enantiomers of salbutamol by solid-phase extraction, HPLC with enantioselective separation and fluorimetric detection.

Differential confirmation of oral suspicious samples as compared with therapeutic use of inhaled salbutamol should be related to criteria based simultaneously on the sum of the concentration of both enantiomers and the ratio between them.

Acknowledgments

The authors would like to thank Dr. Pleasance from Glaxo Wellcome (Uxbridge, Middelsex, UK) for kindly donation of pure salbutamol enantiomer standards. The technical assistance of Silvia Berruezo and Cintia Jiménez is gratefully appreciated. Financial support from the International Olympic Committee (IOC) is also acknowledged.

References

[1] L. Martineau, M.A. Horan, N.J. Rothwell, R.A. Little, *Clin. Sci.*, 83 (1992) 615.

[2] S. Sanjar, A. Kristersson, L. Mazzoni, J. Morley and E. Schaeublin, *J. Physiol.*, 425 (1990) 43.

[3] I.D. Chapman, K.H. Buchheit, P. Manley and J. Morley, *Trends Pharmacol. Sci.*, 13 (1992) 231.

[4] U.K. Walle, G.R. Pesola and T. Walle, *Br. J. Clin. Pharmacol.*, 35 (1993) 413.

[5] T. Walle, U.K. Walle, K.R. Thornburg and K.L. Schey, *Drug Metab. Dispos.*, 21 (1993) 76.

[6] D.W. Boulton and J.P. Fawcett, *Br. J. Clin. Pharmacol.*, 41 (1996) 35.

[7] A. Solans, M. Carnicero, R. de la Torre and J. Segura, *J. Anal. Toxicol.*, 19 (1995) 104.

[8] R. Ventura, G. González, M.T. Smeyers, R. de la Torre and J. Segura, *J. Anal. Toxicol.*, 22 (1998) 127.

[9] M. Donike, personal communication, 1994.

[10] R. Bergés, J. Segura, X. de la Torre and R. Ventura, *J. Chromatogr.*, accepted for publication.

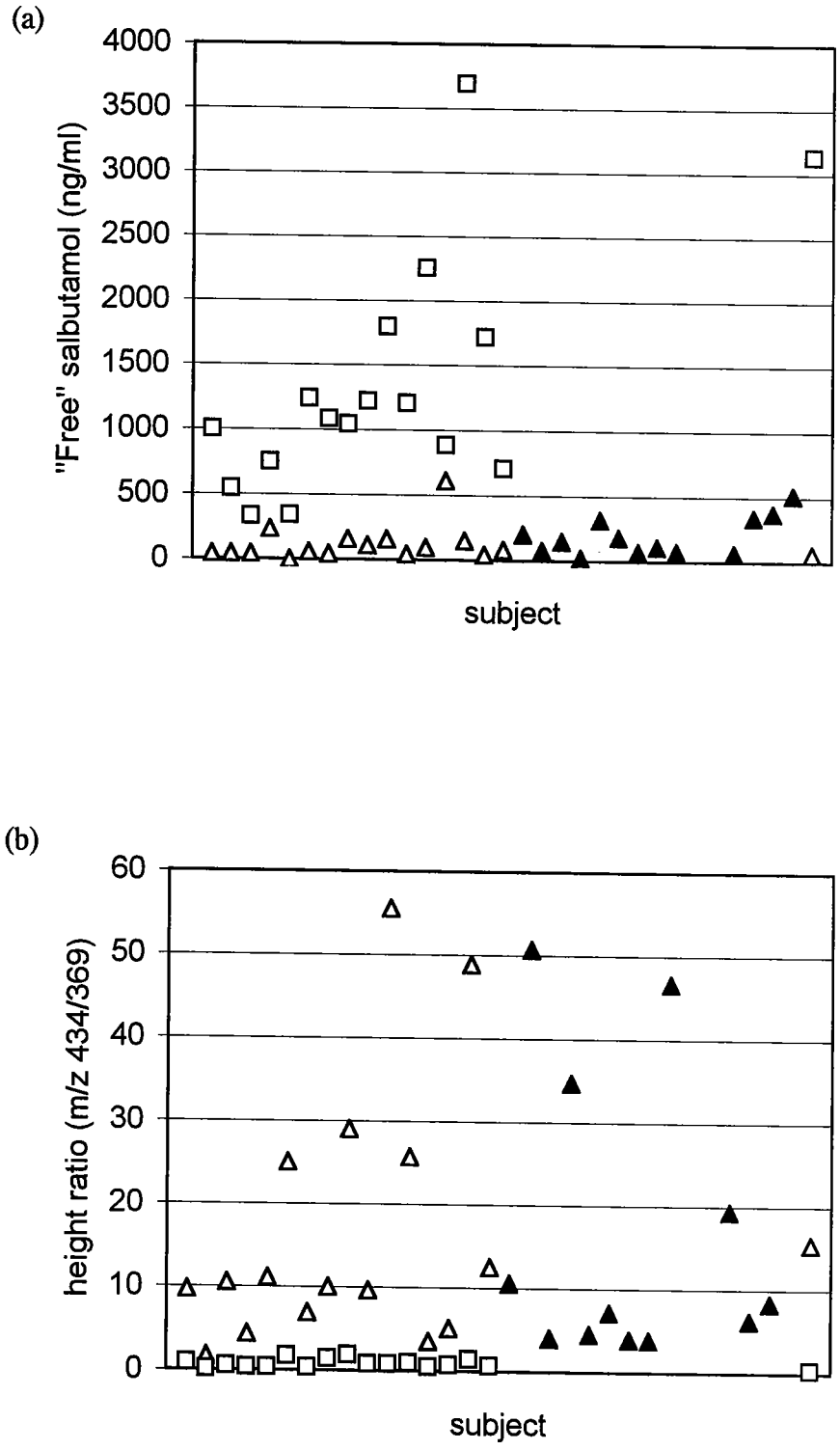
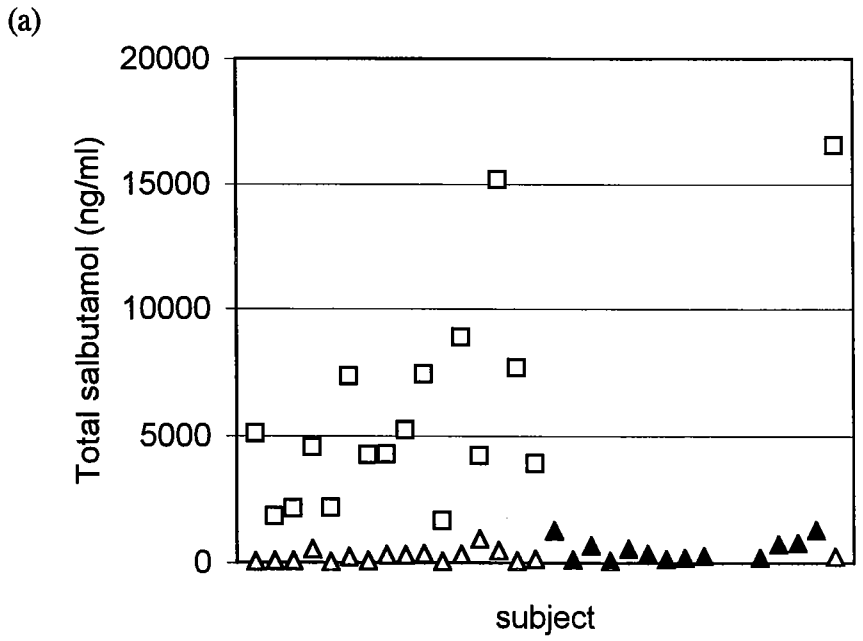


Figure 1. (a) Concentrations of salbutamol and (b) peak height ratio androsterone/salbutamol determined by GC/MS in urine samples collected from asthmatic (A) and non-asthmatic (NA) subjects involved in the field study. Symbols: Δ, inhaled NA; ▲, inhaled A; □, oral NA.



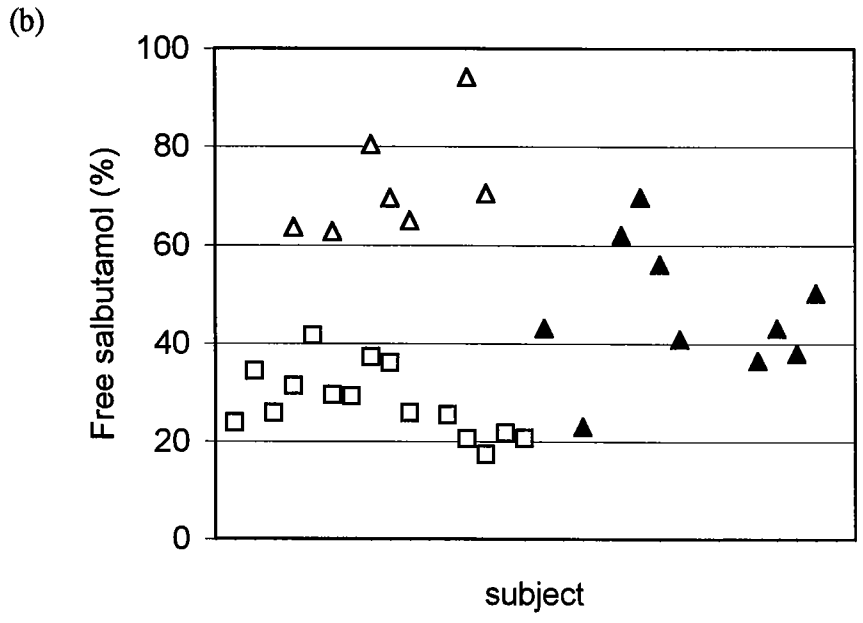


Figure 2. (a) Total salbutamol concentrations determined by ELISA and (b) proportion of free salbutamol in urine samples collected from asthmatic (A) and non-asthmatic (NA) subjects involved in the field study. Symbols: △, inhaled NA; ▲, inhaled A; □, oral NA.

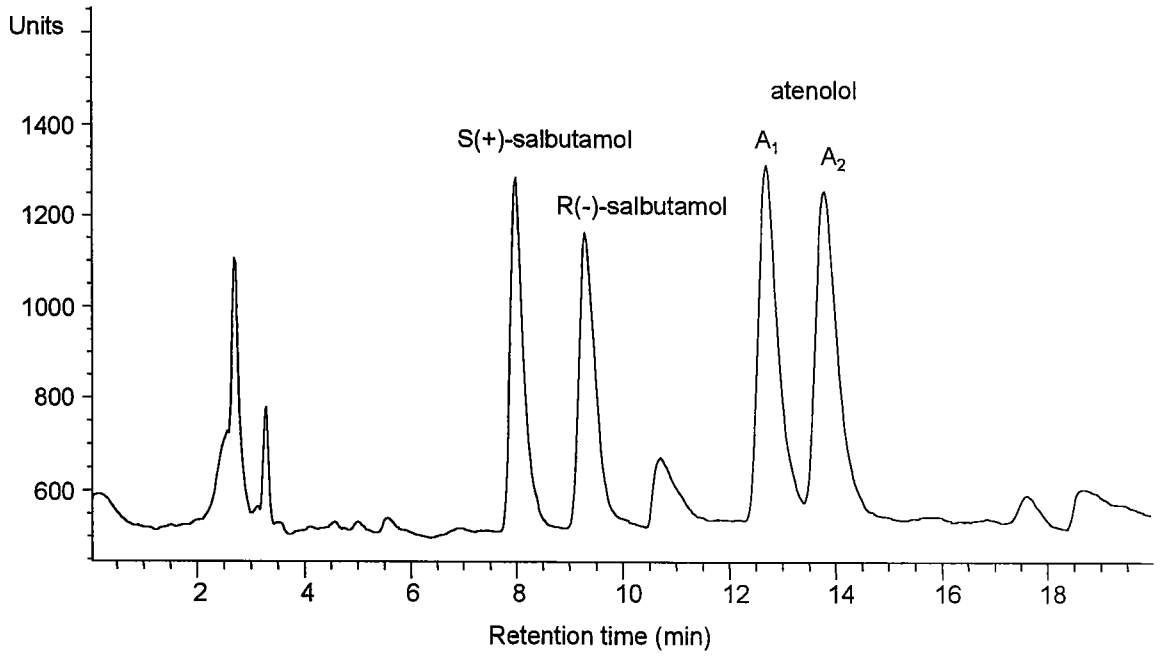


Figure 3. Chromatogram of a urine sample spiked with 500 ng/ml of each salbutamol enantiomer. First eluting peak of atenolol (A_1) is used as external standard for quantitation.

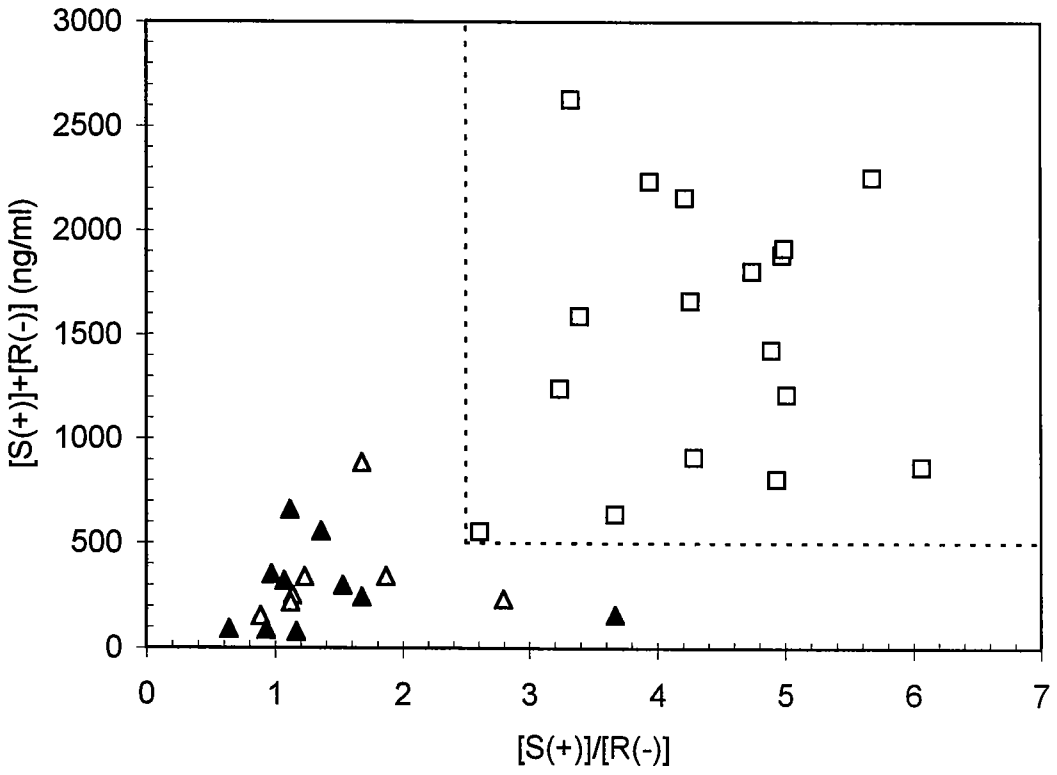


Figure 4. Plot of total free salbutamol concentration *versus* the ratio between S(+) and R(-) salbutamol enantiomers for urine samples collected from asthmatic (A) and non-asthmatic (NA) subjects involved in the field study. Symbols: Δ, inhaled NA; ▲, inhaled A; □, oral NA.

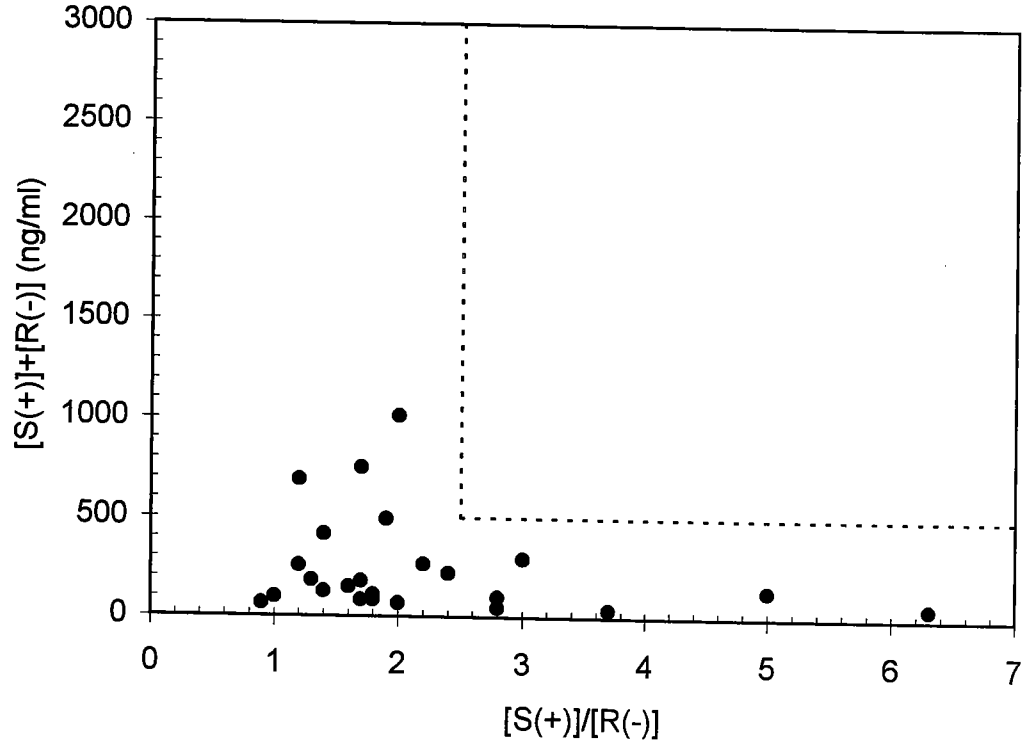


Figure 5. Plot of total free salbutamol concentration *versus* the ratio between S(+) and R(-) enantiomers for urine samples collected at the Nagano Winter Olympic Games from athletes having declared the ingestion of inhaled salbutamol.

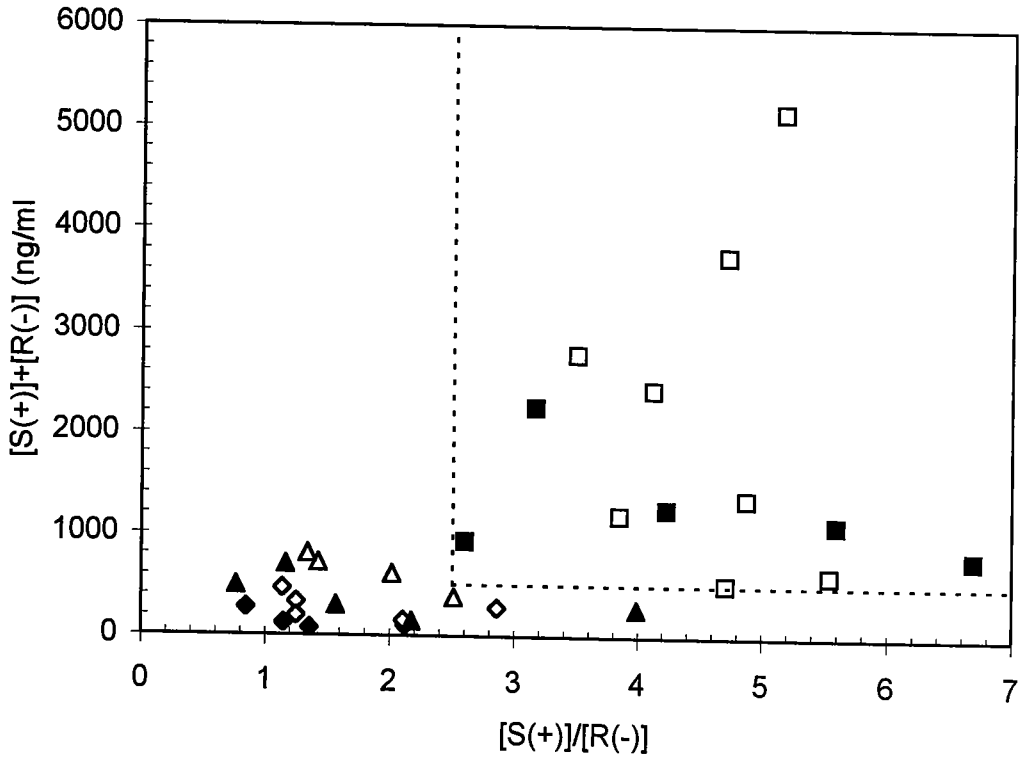


Figure 6. Plot of total free salbutamol concentration *versus* the ratio between S(+) and R(-) salbutamol enantiomers for urine samples collected from one volunteer involved in the clinical trial. Symbols: ◆, inhaled + washing (single dose); ◇, inhaled + washing (multiple dose); ▲, inhaled (single dose); △, inhaled (multiple dose); ■, oral (single dose); □, oral (multiple dose).