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Discrimination of prohibited oral use of salbutamol in sport from authorized inhaled asthma treatment: update

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Introduction

The use of salbutamol in sports is forbidden by the oral route due to a strong adrenergic stimulation and an anabolic-like effect [1]. Therefore, it is important to have an urine test for doping control with adequate discriminatory capacity to distinguish between an inhaled (authorized) and an oral (prohibited) use of this β_2-agonist. This distinction must extend to the maximum dosage of inhaled salbutamol compatible with treatment of asthma for competing athletes as well as for providing protection from exercise-induced asthma (EIA) during prolonged exercise.

Salbutamol is excreted in urine as a mixture of the unchanged drug and its sulfate conjugate metabolite. The proportion of conjugated salbutamol present in urine is higher after oral than after inhaled administration due to first pass metabolism. In the lungs, salbutamol is not extensively metabolized, and the proportion of metabolite after inhalation depends mainly on the percentage of the dose that is inadvertently swallowed. In addition, salbutamol is administered as a racemic mixture of two enantiomers, S(+) and R(-), and it has been demonstrated that the enantiomers are conjugated at a different rate by the body tissues [2,3]. The establishment of criteria to distinguish between the IOC authorized use (inhaled) and the IOC prohibited use (oral) of salbutamol is possible using the simultaneous evaluation of

different variables such as the concentration of non-conjugated enantiomers of salbutamol excreted in urine and the ratio between them. The method should be useful to confirm suspicious samples identified after application of conventional screening procedures in doping control.

Screening of urinary salbutamol is usually performed by the conventional procedure for anabolic agents by means of gas chromatography/mass spectrometry (GC/MS) analysis of the enzymatically partially hydrolyzed urine [4] or using ELISA procedures [5]. ELISA test allows the analysis of total free plus conjugated compound but provides low selectivity and specificity. In the GC/MS procedure mainly non-conjugated urinary salbutamol is measured because of a limited hydrolysis of the sulfated compound. Therefore, these usual screening procedures do not afford clear-cut results to conclude about the dose and the route of administration and to distinguish between an authorized and a prohibited use of the drug. The aim of this study was to develop a discriminatory procedure to differentiate between oral and inhaled ingestion of salbutamol. The method should be useful to confirm suspicious cases of oral salbutamol intake identified after application of conventional screening procedures in doping control.

Procedures

Studies design. This work included three different studies in which urine samples were collected after oral and inhaled administration of salbutamol. Urine samples from non-asthmatic and asthmatic competitive swimmers were obtained after a normal training session in Studies 1 and 2. Swimming was selected as the mode of exercise as historically asthmatics have been heavily involved in swimming and the majority of asthmatics in Olympic Games teams have been in the swimming events [6]. Study 1 was designed to investigate the possibility to discriminate between 20,000 μg (20 mg) of salbutamol taken orally over a 24-hour period and 200 μg of inhaled salbutamol taken immediately before a training session [7]. Study 2 was to determine whether it is possible to distinguish between oral and inhaled salbutamol if the inhaled medication is increased to the maximum advisable dosage for competing asthmatics. Subjects were administered sixteen inhalations each of 100 μg of salbutamol (1,600 μg) in 24 hours including eight inhalations (800 μg) in the last four hours. A 25 ml sample of urine was collected 60 minutes after the last inhalation. This high dosage is

within the manufacturer's recommendations and allows several doses during a competition lasting two or three hours [8].

Urine samples from a crossover clinical trial (Study 3) involving the administration of single and repeated doses of inhaled and oral salbutamol in random order were also obtained [7]. These samples were used to check the final discriminatory procedure proposed.

Urine analysis. All urine samples obtained from the studies were analyzed. The total salbutamol (free plus conjugated) concentrations were determined using the ELISA test [7], the non-sulfated compound was detected and quantified with the GC/MS screening procedure [7] and S(+)- and R(-)-salbutamol enantiomers concentrations excreted in urine were determined using a previously described methodology involving solid-phase clean-up followed by an enantioselective HPLC separation with fluorescence detection [9].

Stability experiments. Some experiments were designed in order to evaluate the stability of salbutamol and salbutamol sulfate in human urine samples after long-term storage. For this purpose, several urine samples collected after oral and after inhaled administration of the drug were stored during 45 days at different temperatures: -20°C, 4°C, at room temperature and 40°C; and analyzed for non-conjugated and total salbutamol contents.

Statistical analysis. The statistical technique that allowed classification of data into groups (inhaled and oral samples) was the discriminant analysis. A function involving both concentration of free salbutamol and the ratio between its enantiomers excreted in urine was obtained using a statistical calculation program (SPSS for Windows version 7.5.2S). A cut-off value for distinguishing between oral or inhaled administration of the drug is established on the basis of the specificity and the selectivity calculated from the distribution of the discriminant values obtained from the function developed.

Results and Discussion

Results obtained for urine samples covering a wide range of salbutamol concentrations showed no significant changes after long storage. Free salbutamol concentrations (individual enantiomers and the addition of both) determined using the enantioselective HPLC procedure, and total salbutamol concentrations (free+conjugated) determined with the ELISA method are

nearly constant after a 45-day storage period at different temperatures: -20° C, 4° C, at room temperature and at 40° C.

The distribution of total salbutamol concentrations determined by ELISA for urine samples obtained in studies 1 and 2 are shown in Figure 1. Concentrations of total salbutamol after oral administration (median 4817 ng/ml, range 1420 to 18993 ng/ml) are extremely higher compared with those detected after inhalation. These differences reflect the dosage differences between oral and inhaled administration and differences in conjugation depending on the route of administration. Taking into account all oral and inhaled data, no urine samples with total salbutamol concentration higher than 1400 ng/ml were obtained after inhaled administration even at the maximum doses considered adequate for asthmatic patients. Thus, urine samples with concentrations higher than this value can be considered highly suspicious of oral (prohibited) salbutamol intake and will require further confirmation analysis. Selecting a cut-off value around 1400 ng/ml of total salbutamol no false positive and no false negative results will be given after the ELISA screening procedure.

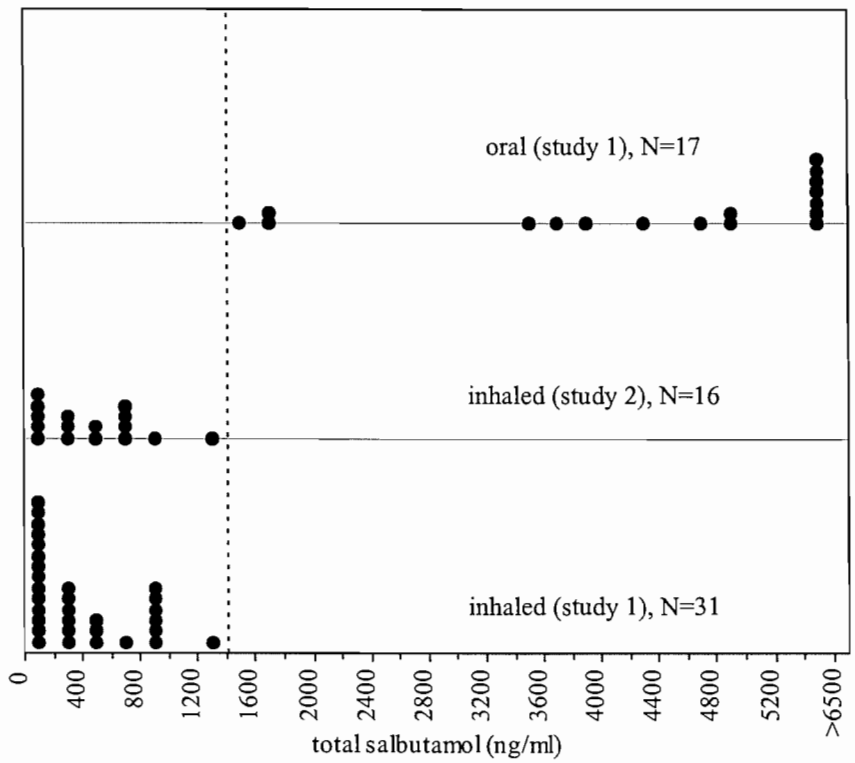

Figure 1. Distribution of total salbutamol concentrations (free+conjugated) determined by ELISA in urine samples obtained in studies 1 and 2.

Distribution of concentrations of non-sulfated salbutamol determined by GC/MS in urine samples obtained in the two studies is represented in Figure 2. It can be observed that there is a little overlapping between the distribution of concentrations after oral and inhaled doses. In contrast to the results obtained using ELISA (Figure 1), concentrations of non-sulfated salbutamol in urine after inhalation of maximum appropriate doses collected in Study 2 were higher than those obtained in samples collected in Study 1. Even so, concentrations obtained after oral administration were statistically higher. From all these values it can be deduced that the use of a cut-off concentration around 500 ng/ml of the non-sulfated compound to select suspicious samples of oral intake seems adequate because maximum concentration after inhaled administration, even at the highest dose, was found at 611 ng/ml. However, two false negative and two false positive results (as we will see later, given negative after the confirmatory procedure) are obtained after the GC/MS screening procedure.

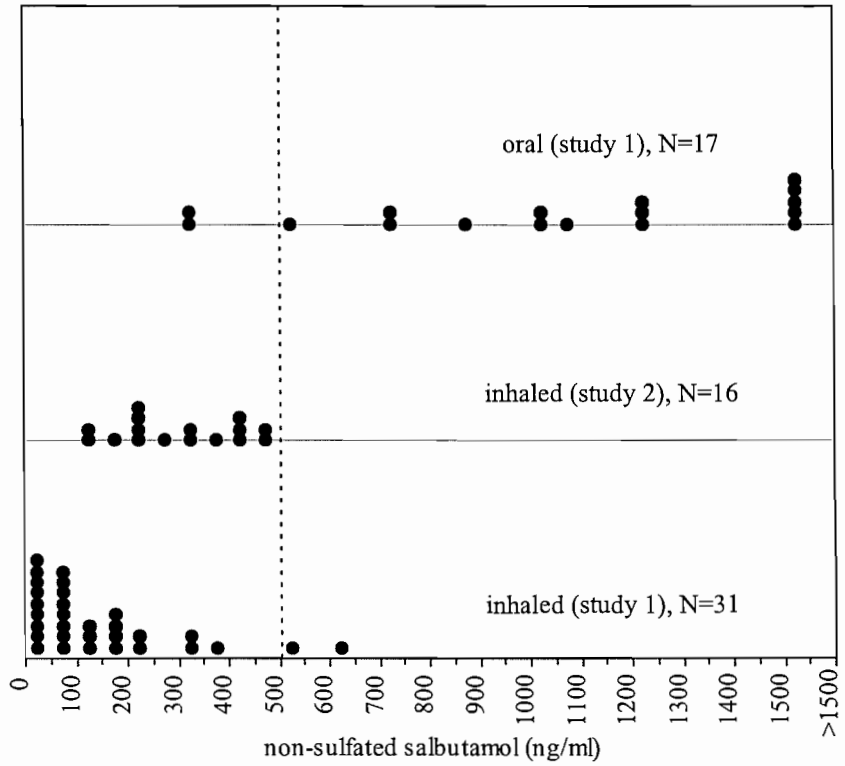

Figure 2. Distribution of non-sulfated salbutamol concentrations determined by GC/MS screening in urine samples obtained in studies 1 and 2.

In summary, considering a cut-off around 1400 ng/ml of total salbutamol (free+conjugated) no false positive and no false negative are obtained. Considering a cut-off around 500 ng/ml of non-sulfated salbutamol 4.3% of false positive (declared finally negative after

confirmation) and 11.8% of false negative results are given. The highest sensitivity and specificity are obtained using ELISA techniques because total salbutamol concentration allows the correct classification of all positive oral samples whereas criteria based on GC/MS data showed some screening false positive results. Therefore, concentration of salbutamol estimated by conventional screening procedures can be used as a preliminary measure for selecting suspicious cases of oral salbutamol administration [10]. These suspicious cases should be further analyzed for free content of S(+) and R(-) salbutamol enantiomers by solid-phase extraction, enantioselective HPLC separation and fluorimetric detection [9].

The simultaneous evaluation of concentration of unchanged salbutamol and ratio between its S(+) and R(-) enantiomers measured in urine is useful to establish the correct or incorrect use of the drug. Plot of total free salbutamol concentration excreted in urine *versus* S(+)/R(-) ratio for samples considered is shown in Figure 3. 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 after inhalation. Our object is to classify these data into two groups and identify rules for deciding in which class a sample of unknown position should be placed according to a combination of the two variables, total concentration of free salbutamol excreted in urine and the ratio between its enantiomers.

The discriminant function developed [11] using the experimental data (N=45) for samples obtained in Studies 1 and 2 after oral ingestion and after inhalation of salbutamol is:

$$D = -3.776 + 1.46 \cdot 10^{-3} \{[S(+)] + [R(-)]\} + 1.012 [S(+)]/[R(-)] \qquad (1)$$

The line that separates the two distributions of points is plotted together with the experimental data set in Figure 3. The combination of variables defined by the concentration of free salbutamol ([S(+)]+[R(-)]) and the ratio between its enantiomers ([S(+)]/[R(-)]) excreted in urine could distinguish between authorized inhaled and prohibited oral salbutamol much better than individuals markers could. The larger the discriminant score (D), calculated from equation 1, the more likely it is that the sample was in the oral group rather than in the inhaled distribution.

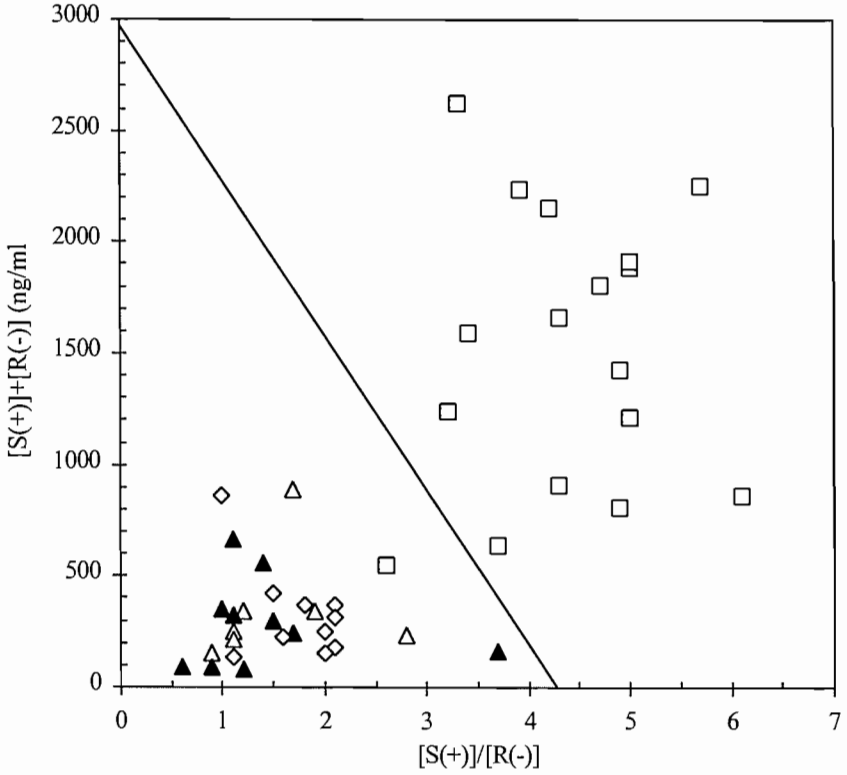

Figure 3. Plot of total free salbutamol *versus* the ratio between S(+) and R(-) enantiomers. Symbols: □, oral non-asthmatic (NA); Δ, inhaled NA (study 1); ▲, inhaled asthmatics (A); ◊, inhaled NA (study 2); ——— , discriminant function.

In Figure 4 the distribution of values of function D calculated for all urine samples obtained after inhalation and oral ingestion of salbutamol in studies 1 and 2 is represented. The bottom points show the distribution for inhaled samples with the vertical lines indicating the mean inhaled D value and distances of one, two, three and four standard deviations from that mean value. Taking a decision limit of D=1.06 (four standard deviations from the mean D value of the inhaled distribution) 15 of the 17 subjects that were administered oral salbutamol would be declared positive, we assume a 11.8% of false negative but nearly no false positives are obtained. In fact, from the theoretical normal distribution of D values for negative samples 1 false positive every 33000 assays are obtained. Therefore, this cut-off value implies a very good specificity and a sensitivity of about 99.997%.

Having selected the cut-off (four standard deviations from the mean D value of the inhaled distribution) it is necessary to demonstrate that it is valid. This procedure will be used to confirm suspicious samples with concentrations higher than 1400 ng/ml of total salbutamol

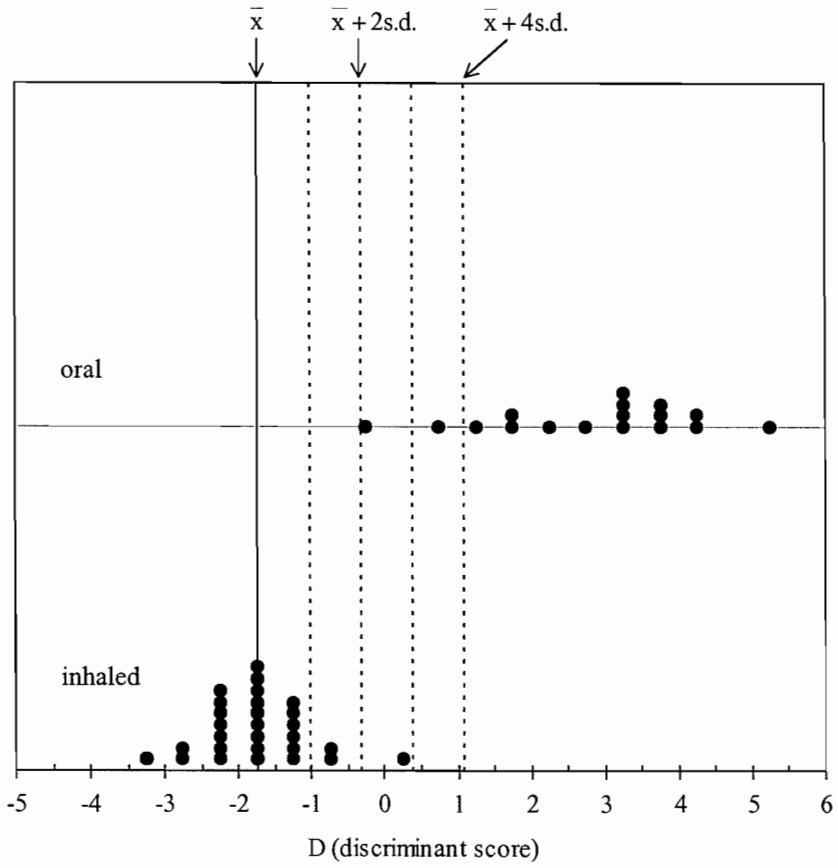

Figure 4. Distribution of discriminant score values (D) calculated for urine samples obtained after inhalation and oral ingestion of salbutamol in studies 1 and 2.

and higher than 500 ng/ml of non-sulfated salbutamol detected after application of preliminary screening procedures. In Figure 5 the distribution of the discriminant score calculated from S(+)+R(-) and S(+)/R(-), equation 1, for samples involved in the three studies presenting suspicious concentrations by GC/MS screening (top) and suspicious concentrations after ELISA screening procedure (bottom) are represented. Moreover, all screening suspicious samples collected after competition at the Nagano Winter Olympic Games (inhaled salbutamol being declared) were analyzed with the enantioselective HPLC procedure developed. As can be observed, the D cut-off values established allow classification of all the inhaled samples into their correct group (experimental or declared) and no false positive are obtained. Conversely, a doping urine sample collected in a routinely doping test from an athlete who declared the ingestion of oral salbutamol is also classified according his declaration. Therefore, a definitive distinction between prohibited oral and authorized inhaled salbutamol in doses that are adequate for all asthmatic athletes to compete can be achieved by urinalysis of screening suspicious samples.

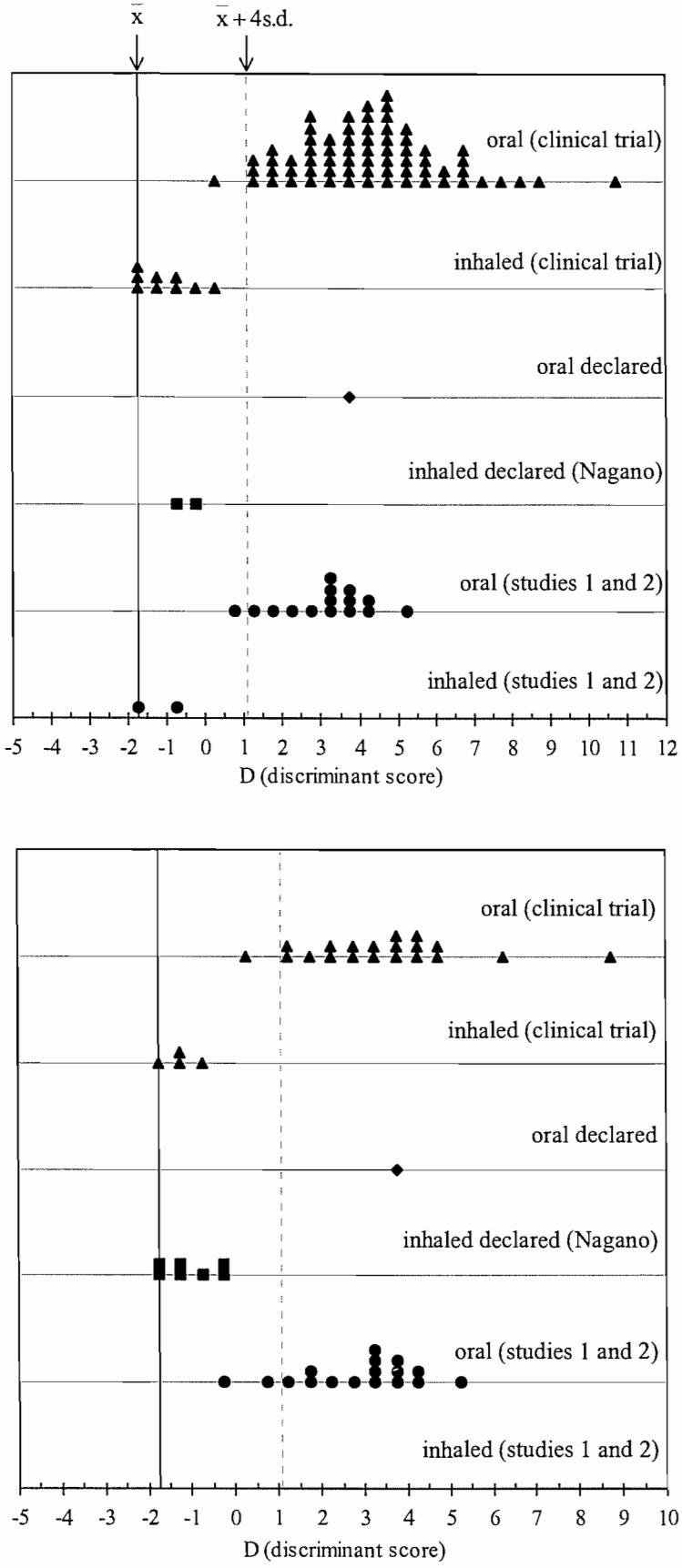

Figure 5. Distribution of D values for urine samples collected in studies 1 and 2 (•), in the clinical trial (study 3, ▲) and in the Nagano Winter Olympic Games (■) having shown concentrations of non-sulfated salbutamol higher than 500 ng/ml (top) or concentrations of total salbutamol higher than 1,400 ng/ml (bottom). A doping real sample from an athlete who declared oral salbutamol is included (◆).

Definitive identification of salbutamol enantiomers by mass spectrometry, usually required in doping control, is accomplished by collection of the fractions containing salbutamol enantiomers after enantioselective HPLC separation. The elution fractions containing the enantiomers are collected, the solvent is evaporated and the salbutamol enantiomers are identified by GC/MS analysis methylboronate derivatives [12]. The potential use of coupled chiral HPLC-MS should be also investigated.

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