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Ultraperformance liquid chromatography tandem mass spectrometric method for direct quantification of salbutamol in urine samples in doping control

R. Ventura[a,b,*], R. Ramírez[a], N. Monfort[a,b], J. Segura[a,b]

[a] Bioanalysis Research Group, IMIM-Hospital del Mar, Barcelona, Spain
[b] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain

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ABSTRACT

A fast and reliable quantitative method for salbutamol using direct analysis of the urine sample by ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) has been developed. Urine samples were spiked with salbutamol-d_6 (internal standard), and, then, they were diluted with ultrapure water (1:1, v/v). Aliquots of 1 μl of the mixture were directly analyzed by UPLC/MS/MS. The chromatographic separation was performed in a UPLC BEH C_{18} (100 mm × 2.1 mm, 1.7 μm) column with a mobile phase contained 0.01% formic acid in ultrapure water (v/v) and 0.01% formic acid in acetonitrile (v/v), using gradient elution at 0.6 ml/min. The temperature of the column was set to 45 °C. The total run time was 3.2 min. Electrospray ionization in positive ion mode was used under multiple reaction monitoring (MRM) at different collision energies. Nitrogen and argon were used as desolvation and collision gas, respectively. The method was shown to be linear from 200 to 5000 ng/ml ($r^2 > 0.99$). The limit of quantitation was estimated in 200 ng/ml. Intra-assay precision and accuracies, evaluated by using quality control samples containing 550 and 1100 ng/ml salbutamol, were always better than 8.4%. The intermediate precision was estimated to be in the range of 5.6–8.9%. The method was shown to be reliable when applying to routine samples, and the short analysis time resulting from a simple sample preparation and a fast instrumental analysis makes it of great interest for antidoping control purposes.

1. Introduction

Salbutamol, a β_2-agonist drug, is one of the most widely used medications in the treatment of bronchial asthma. It has also been demonstrated to be effective for the treatment of the exercise induced asthma [1]. The list of prohibited substances in sports published by the World Antidoping Agency (WADA) specifies that the use of salbutamol is only permitted by inhalation [2]. Administration by the oral or parenteral route or the administration of very large inhaled doses are forbidden due to an strong adrenergic stimulatory effect and an anabolic-like effect [3,4]. In contrast, administration of therapeutic inhaled doses have no ergogenic effect [1,5].

Salbutamol is excreted in urine as a mixture of the unchanged drug and its conjugate metabolite, mainly sulphate. After oral administration, from 24 to 33% of the dose is excreted as unchanged drug in urine, and approximately a 48% of the dose is recovered as the sulphate conjugate [6,7], due to a first-pass metabolism. After inhaled administration, salbutamol is not extensively metabolized in the lungs, and the presence of the metabolite in urine is mainly due to the percentage of salbutamol swallowed during inhalation [8]. Different studies have been performed to compare the concentrations of salbutamol in urine after different oral and inhaled administrations [9,10]. Based on the results of these studies using screening procedures conventionally applied in antidoping control laboratories, that allow the detection of free and glucuronoconjugated metabolites, a threshold concentration of 1000 ng/ml was established for salbutamol to suspect for oral administration [11]. In addition, concentrations between 500 and 1000 ng/ml should be reported as consistent with the use of salbutamol. These threshold concentrations have also been supported by recent studies involving inhaled administration of salbutamol at different doses [12]. The quantitation of the enantiomers of salbutamol allow the discrimination between oral and inhaled administration on the suspicious samples detected after application of the established threshold [9,13].

Recent studies [14] showed similar concentrations of salbutamol in urine after application of procedures without hydrolysis and with hydrolysis using enzymes with β-glucuronidase activity, indicating that the percentage of glucuronide of salbutamol in urine is negligible. For this reason, the threshold concentrations defined by WADA may be applied using quantitation of free salbutamol.

* Corresponding author at: Bioanalysis Research Group, IMIM-Hospital del Mar, Doctor Aiguader, 88, 08003 Barcelona, Spain. Tel.: +34 93 3160471; fax: +34 93 3160499.
E-mail address: rventura@imim.es (R. Ventura).

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The list of forbidden substances in sports is increasing continuously and antidoping control laboratories are forced to develop high throughput screening and confirmation methods. The direct analysis of urine samples is one of the approaches used to reduce total analysis time by reducing the sample preparation step. Direct analysis of the urine using liquid chromatography coupled to mass spectrometry or to tandem mass spectrometry (LC/MS or LC/MS/MS) has been described for the analysis of several doping agents, including salbutamol [14–16]. The use of LC systems also avoids the need of derivatization mandatory for GC/MS analysis some compounds, such as salbutamol [17–20]. New LC instrumental strategies have been developed with the aim of reducing analysis time, and increasing separation efficiency, sensitivity and resolution. Ultraperformance liquid chromatography (UPLC) makes it possible by the use of columns packed with small particles (<2 μm) coupled to chromatographic systems specially designed to run at the optimum linear velocities (high pressures and minimal system volumes).

The aim of the present study was to develop a fast and reliable quantitative method for the direct determination of free salbutamol using UPLC/MS/MS allowing for a reduction of sample volume needed, sample manipulation and total analysis time.

2. Experimental

2.1. Chemicals and reagents

Salbutamol and salbutamol-d_6, used as internal standard ISTD, were purchased from Sigma (St. Louis, MO, USA) and RIVM EU Reference Laboratory (Bilthoven, The Netherlands), respectively. Acetonitrile and methanol (LC gradient grade) were purchased from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

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

2.2. Standards solutions

Primary stock solutions of each analyte (1 mg/ml) were prepared by accurately weighing of 10 mg into a 10 ml Grade A volumetric flask, dissolving in methanol and making up to volume. Working solutions of 100 and 10 μg/ml were prepared by 1:10 and 1:100 dilutions of the 1 mg ml^{-1} stock solutions with methanol. All solutions were stored at −20 °C.

2.3. Sample preparation procedure

Aliquots of urine samples (0.5 ml) were added with a methanolic solution of salbutamol-d_6 (final concentration, 500 ng/ml). Samples were, then, diluted with ultrapure water (1:1, v/v) and were shaken for 5 s, centrifuged at 1500 × *g* for 10 min and transferred to vials and aliquots of 1 μl were injected into the UPLC/MS/MS system.

2.4. Ultraperformance liquid chromatography-tandem mass spectrometry conditions

Chromatographic separations were carried out on a Waters Acquity ultraperformance liquid chromatography system, equipped with a quaternary pump system using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters Corporation, Milford, MA). The column temperature was set to 45 °C. Separation was performed with a binary mobile phase at a flow rate of 0.6 ml/min. The mobile phase consisted of: solvent A, ultrapure water with 0.01% formic acid (v/v); and solvent B, acetonitrile water with 0.01% formic acid (v/v). The mobile phase components were daily filtered using filters of 0.22 μm pore size. The gradient elution was as follows: 2% B for 1 min, to 80% B in 1 min; 80% B for 0.2 min, to 2% B in 0.1 min, 2% B for 0.9 min.

The UPLC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation) with an electrospray ionization source working in positive ionization mode. Source conditions were fixed as follows: capillary voltage, 3 kV; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 l/h; desolvation gas flow-rate, 1200 l/h. High-purity nitrogen was used as desolvation gas, and argon was used as collision gas. Acquisition was performed in multiple reaction monitoring (MRM) mode. Electrospray ionization working parameters (cone voltage and collision energies, CE) were optimized for salbutamol and salbutamol-d_6 using infusion of individual standard solutions of the compounds (10 μg/ml) at 10 μl/min with mobile phase (50:50, A:B, v/v) at 200 μl min^{-1}.

Optimum cone voltage was 20 V, and the following transitions were monitored: 240 > 148 (CE 20 eV), 240 > 166 (CE 20 eV), 240 > 222 (CE 10 eV) and 240 > 240 (CE 5 eV) for salbutamol, and 246 > 148 (CE 20 eV), 246 > 228 (CE 10 eV) and 246 > 246 (CE 5 eV) for salbutamol-d_6. Data acquisition was performed in positive mode, with dwell time of 20 ms, and interchannel delays of 5 ms. All data were acquired and processed using MassLynx 4.1 software.

2.5. Validation study

The following parameters were evaluated in the method validation: selectivity/specificity, heteroscedasticity, linearity, limits of detection and quantification, recovery, stability and intra-assay and intermediate precision, and accuracy.

The selectivity/specificity of the method was verified by analysing 10 different blank urine samples and checking for the presence of interfering substances at the retention times of the compounds of interest, salbutamol and salbutamol-d_6, in the corresponding ion chromatograms.

For the study of linearity, a calibration curve covering the range defined by WADA rules for threshold substances [21] was studied. Spiked urine samples with concentrations of 250, 500, 1000, 2000 and 5000 ng/ml of salbutamol were prepared. The validation protocol was divided in four assays performed in consecutive days. In the first validation assay, the calibration samples were prepared and analyzed in quadruplicate. The peak area ratios between salbutamol (transition *m*/*z* 240 to *m*/*z* 148) and the ISTD (*m*/*z* 246 to *m*/*z* 148) were plotted against the analyte concentration. The Dixon's test (α = 5%) was applied to detect outliers in the replicates at each concentration level. The behaviour of the variance over the calibration range (homoscedasticity/heteroscedasticity of the procedure) was evaluated by applying the Levene test (α = 5%). In the remaining validation assays, the calibration curves were prepared in duplicate. The evaluation of the goodness of fit to the linear model was demonstrated by an *F* test (α = 5%), in order to compare the variance assigned to the lack of fit with the one related to the random error.

The standard deviation of the estimated concentration values for the lowest calibration point (250 ng/ml) was used as a measure of the noise. The limits of detection and quantification were defined as 3.3 and 10 times the value of the noise, respectively.

Intra-assay stability (influence of time on the response (peak areas) of salbutamol and salbutamol-d_6) was studied with an analysis of the variance (ANOVA) test (α = 5%).

Intra-assay precisions and accuracies were determined by the analysis of three replicates of quality control urine samples with concentrations of 550 and 1100 ng/ml of salbutamol at the same day. Inter-assay precision and accuracy were calculated by the analysis of the control samples in three different days. Precision was

expressed as the relative standard deviation (RSD) of the control samples concentrations, and accuracy was expressed as the relative error (ERR) of these concentrations.

Intermediate precision was estimated by analysis of the quality control urine samples in 7 batches for a period of 8 months, performed by different analysts. Intermediate precision was calculated as the RSD of the mean values of the concentrations obtained in each run for the two quality control samples.

Matrix effect was studied by analysis of 10 different blank urines spiked with 500 ng/ml of salbutamol. Each sample was analyzed in triplicate. An ANOVA test was applied to evaluate the differences in concentrations between the different samples.

The stability of salbutamol was evaluated after going through up to 3 cycles of freeze at −20 °C and thaw. Samples containing 550, 1100 and 3000 ng/ml of salbutamol were studied. For each storage condition, three replicates of each sample were analyzed. An analysis of the variance (ANOVA) test ($\alpha = 5\%$) was applied to compare data.

2.6. Actual urine samples

The method was applied to routine antidoping control, and to samples obtained after oral and inhaled administration of salbutamol to healthy volunteers. Samples from volunteers were collected under controlled conditions and following a clinical protocol approved by the local Ethical Committee (CEIC-IMAS, Barcelona, Spain). In all cases, urine samples were collected and stored at −20 °C until analysis.

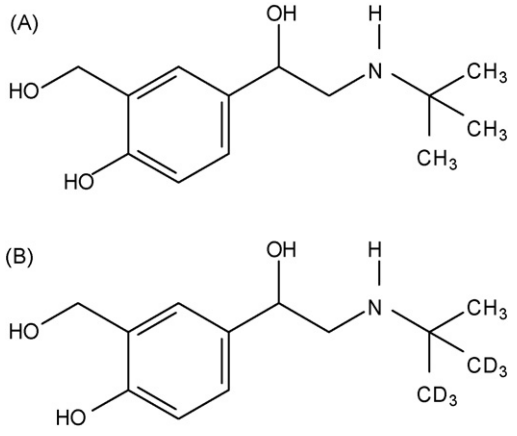

Fig. 1. Chemical structures of salbutamol (A) and salbutamol-d_6 (B).

3. Results and discussion

Electrospray ionization working parameters were optimized for salbutamol and salbutamol-d_6 using infusion of individual standard solutions. Chemical structures of salbutamol and salbutamol-d_6 are described in Fig. 1. Taking into account the mobile phase composition and flow-rate used, in order to improve desolvation efficiency and analyte ionization, a high gas flow rate was used (1200 l/h), and desolvation and source temperatures were set at 450 and 120 °C. Salbutamol and salbutamol-d_6 were predominantly detected as

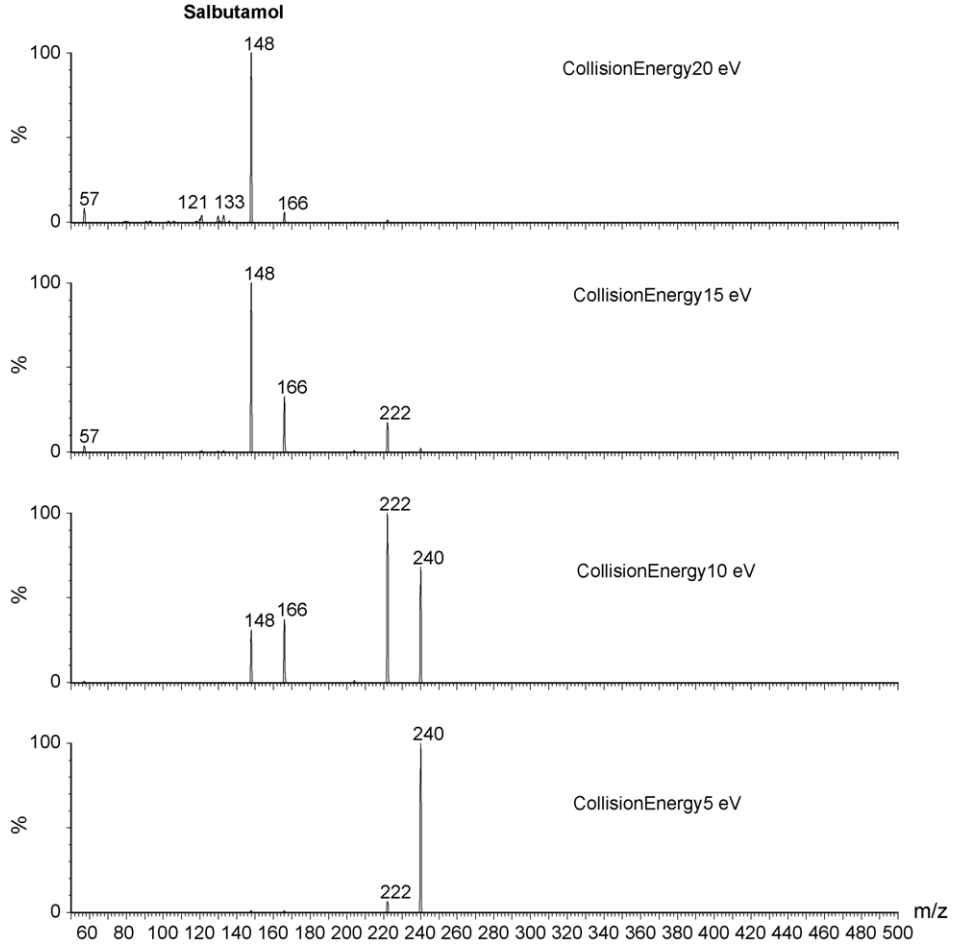

Fig. 2. Product ion mass spectra of the pseudomolecular ion $[M+H]^+$ of salbutamol (m/z 240) at different collision energies.

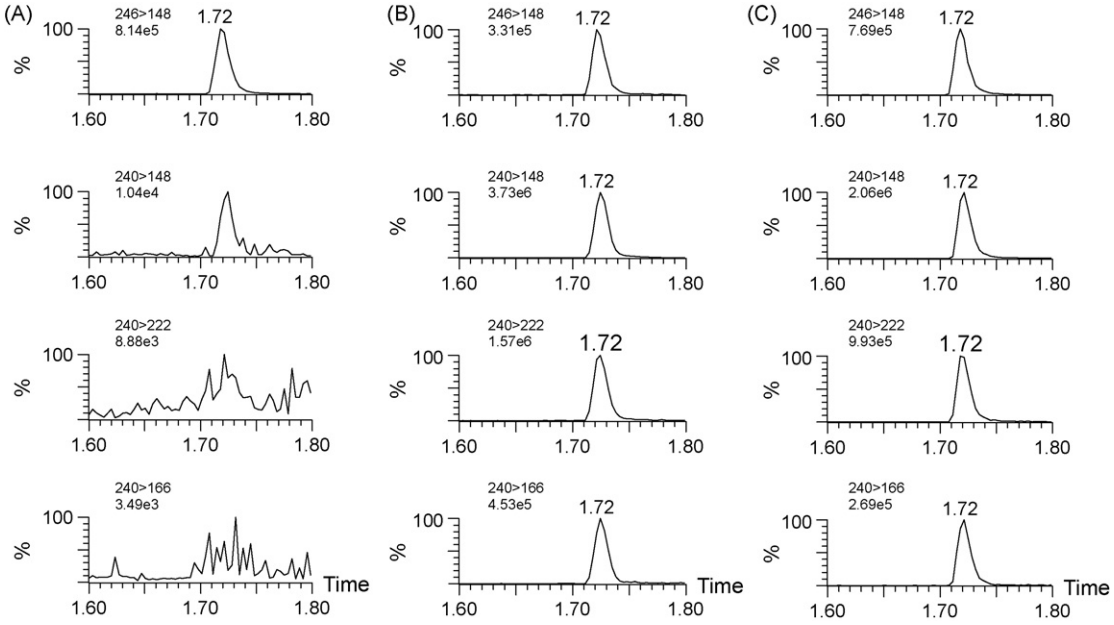

Fig. 3. UPLC/MS/MS chromatograms obtained after analysis of a blank urine (A), an actual urine of routine antidoping control with an estimated concentration of 2509 ng/ml (B), and a quality control urine spiked with 550 ng/ml of salbutamol (C). Transitions monitored: 246 > 148 for salbutamol-d_6; and 240 > 148, 240 > 222 and 240 > 166 for salbutamol.

$[M+H]^+$ in positive ion mode due the high proton affinity, as described by using other mobile phase compositions [14,15]. Apart from formic acid, no other mobile phase additive was needed either to promote ionization or to improve chromatographic behaviour. Cone voltage was optimized to obtain maximum signal for the protonated molecular ions $[M+H]^+$. Data acquisition was performed in MRM mode, and different collision energies were studied in order to select at least three transitions for salbutamol, according to identification criteria defined by WADA [22]. Different collision energies were evaluated to obtain the maximum response for each transition. Product ion mass spectra of $[M+H]^+$ of salbutamol at different collision energies are shown in Fig. 2. At low collision energy, only product ions resulting from the neutral loss of water were obtained at m/z 222. At higher collision energies, additional elimination of isobutene and subsequent water loss yielded the most important product ions at m/z 166 and 148, respectively. For salbutamol-d_6, the fragmentation profile was similar.

The gradient was optimized to obtain a reasonable short chromatographic time. The total run time was 3.2 min, and the retention time of salbutamol and its deuterated analog was 1.7 min. Although not needed for chromatographic separation, the final content of organic solvent in the mobile phase was increased to 80% in order to have a good clean-up of the chromatographic column to avoid problems of blocking and to extent its life. Also, the sample dilution and the small volume of sample injected contributed to extent the life of the column. In our conditions, a reduction between two- and five-folds of the analysis time was achieved compared with previous methods using columns packed with particles of bigger size [14,15]. As for other methods using direct analysis of the sample, a significant simplification in the sample preparation procedure was accomplished compared with previous methods using GC/MS [17,19,20], because only dilution of the sample is needed before instrumental analysis. Taking into account the composition of the batch (calibration samples, quality control samples, replicates of the sample), the quantitation of salbutamol can be performed in 1 h analysis time.

The selectivity/specificity was evaluated after analysis of 10 different blank urines and no matrix interferences were detected at the retention times of salbutamol and salbutamol-d_6 for the ions monitored. In Fig. 3, the chromatograms obtained after analysis of a blank urine sample are compared with those obtained after analysis of a quality control urine spiked with 550 ng/ml of salbutamol, and a sample of routine antidoping control (2509 ng/ml).

Regarding the validation study, the procedure was found to be heteroscedastic, so peak area ratios between salbutamol and the ISTD (salbutamol-d_6) were subjected to a proportional weighted least-square regression analysis. Determination coefficients (r^2) greater than 0.99, confirmed the linearity of the method over the range between 250 and 5000 ng/ml. The test of comparison of variances was not significant ($\alpha = 5\%$), indicating adequate adjustments of the data to the proposed model. Four replicates of the low concentration value of the calibration curve of salbutamol (250 ng/ml) were processed for the calculation of the detection and quantification limits. The estimated values for these limits were 66 and 200 ng/ml, respectively. Taking into account the estimated limit of quantitation, the linearity of the method in the range between 200 and 5000 ng/ml was verified as described above. A calibration curve from 200 to 5000 ng/ml is used for the quantitation of free salbutamol in routine analysis.

No correlation was found between the response (values of area) of salbutamol and salbutamol-d_6 and the time elapsed up to the moment of the analysis of each sample. The results of the analysis of variance test were not significant for this parameter. Thus, the method showed intra-assay stability.

Results obtained for intra-assay precision and accuracy, and intermediate precision of the replicates of quality control urine samples were satisfactory and are presented in Table 1. Intra-assay precision and accuracy ranged from 0.4 to 5.6% and 1.8 to 8.4%, respectively. Intermediate precision was estimated in 8.9 and 5.6%, depending on the concentration of the quality control sample.

Regarding the matrix effect, no statistical differences were detected in concentrations of salbutamol obtained after analysis of 10 blank samples spiked with 500 ng/ml of the compound, indicating that the matrix effect was negligible.

The stability of free salbutamol was evaluated with samples spiked with 550, 1100 and 3000 ng/ml. No significant modification in concentrations was observed compared to initial concentration confirming the stability of free salbutamol after three freeze at

Table 1
Intra-assay precision and accuracies, and intermediate precision.

Concentration (ng/ml)	Intra-assay study				Intermediate precision		
	n	Estimated concentration (mean ± SD) (ng/ml)	Precision (RSD%)	Accuracy (ERR%)	*n*	Estimated concentration (mean ± SD) (ng/ml)	Precision (RSD%)
550	3	571.8 ± 12.2	2.1	4.0	21	553.7 ± 49.3	8.9
	3	503.7 ± 1.9	0.4	8.4			
	3	510.0 ± 12.4	2.4	7.3			
1100	3	1080.0 ± 12.2	5.6	1.8	21	1046.3 ± 58.8	5.6
	3	1045.6 ± 31.9	3.1	5.0			
	3	1149.1 ± 7.6	0.7	4.5			

−20 °C and thaw cycles. Salbutamol is excreted in urine in free form and conjugated with sulphate [6,7]. For this reason, it is also important to know the stability of the conjugate in real positive samples. Previous results obtained in our group, demonstrated the stability of free and sulphated salbutamol for a period of at least 45 days after storage at −20 °C and 4 °C in actual samples obtained after administration of salbutamol to healthy volunteers [9]. The long-term stability of free salbutamol was also evaluated by other groups using spiked samples. After 60 days of storage at −18 °C the concentrations of free salbutamol decreased slightly (2–4%) [20].

The method developed was applied to samples obtained after administration of salbutamol to healthy volunteers and to routine samples of antidoping control and it has demonstrated to be reliable.

In summary, a method to quantify free salbutamol in urine has been optimized and validated. The small volume of sample used and the short analysis time resulting from a simple sample preparation and a fast instrumental analysis makes it of great interest for antidoping control purposes.

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