ß-ADRENERGIC COMPOUNDS THROUGH HAIR ANALYSIS

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ABSTRACT: ß-adrenergics compounds are sometimes used by sportsmen who wish to improve their performances: β-agonists like salbutamol to increase the respiratory capacity or clenbuterol to increase the muscular mass and β-blockers which supports psychomotor coordination are used. Urine is the mandatory specimen for the International Olympic Comitee for doping control, but urinalysis provides short term information of an individual drug use, cannot distinguish between chronic or single/thérapeutic use and can be adulterated. Hair analysis offers an interesting alternative to solve these problems. An original procedure was developped to simultaneously test 14 β_2-adrenergics in hair. We describe here such a technique with validation procedure and application for doping evidence. A quality control has been realised by several French laboratories with this procedure. Several application of the method are also described.

KEY WORDS: Hair analysis; ß-adrenergic drug; Doping control.

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INTRODUCTION

The use of β-adrenergic compounds (agonists and antagonists) is banned in sports by both the International Olympic Committee (IOC) and International Sports Federations [6] β_2-agonists were banned because of their sympatomimetic properties (stimulant effects) and their activity as anabolic agents at higher dosages, as they decrease lipogenesis and increase lipolysis and glycogenolysis. Simultaneously, they attenuate protein turnover by reducing protein degradation.

These agents are listed in the prohibited classes of substances. Salbutamol and terbutaline are permitted by inhalers only and must be declared in writting prior to the competition to the relevant medical authority.

In humans, salbutamol is extensively used for its therapeutic properties as a bronchodilatator in asthmatic patients. $ß_2$-agonists are also employed successfully in animals. These drugs augment physical performance (horse racing), or increase the mass of animals for consumption, with the potential problem of human poisoning.

In addition to their medical use in the treatment of cardiac arhythmias and hypertension, β-blockers have found a place in some athletic events, particularly disciplines in which good psychomotor coordination is required. ß-blockers are listed in the classes of drugs subject to certain restrictions, and tests will be conducted in some sports, at the discretion of the responsible authorities.

According to the records of the IOC, one positive propranolol urine was identified during the Summer Olympic games in Seoul (1988), two positive clenbuterol cases in Barcelona (1992) and no positive in Atlanta (1996).

Since the first analysis for doping control, urine has been the mandatory specimen. A variety of analytical screening procedures have been described, involving ELISA tests [14] or the more common gas chromatography/mass spectrometry (GC/MS) technology [2, 15].

However, discontinuing the treatment few days before the competition, or presenting a medical receipt at the time of the competition do not prevent the long term illegal abuse of such compounds. Given the retrospective power of the analysis of hair content, the availability of a sensitive and accurate technique for β-adrenergic agents analysis in hair appears highly relevant for detecting previous chronic administration.

For 20 years, hair specimens have been used in toxicology and pharmacology to document repetitive organic drug exposure in various forensic, occupational and clinical situations [7].

Recently, some papers have pointed out the interest of doping control through hair analysis [3, 8]. However, hair is not yet a valid specimen for the IOC, but was accepted by the French courts in the cycling "Tour de France" doping story in 1998.

The international literature is very poor in papers (n = 8) dealing with the identification of β-adrenergic drugs in hair. Most of the papers (n = 6) have been focused on animals. Clenbuterol was identified in rats [1], guinea pigs [11, 13], bovine [10] or calves [4] and salbutamol was identified in guinea pigs [12]. Only Gleixner et al. [5] detected clenbuterol in a 2 bodybuilders by HPLC-EIA. In attempt to better manage hypertensive patients, Kintz and Mangin [9] tested β-blockers in 8 subjects by HPLC/UV.

Neither immunoassay nor HPLC are considered to be specific enough to provide a result that will survive a legal challenge in doping control. Therefore, we have developed a GC/MS method to simultaneously test for 2 β_2-agonists and 12 β-blockers.

MATERIAL AND METHODS

Chemicals

Dichloromethane, ethyl acetate and methanol were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical grade and provided by Merck. Salbutamol hemisulfate, metoprolol tartrate, atenolol, timolol maleate, labetalol

hydrochloride, sotalol hydrochloride, clenbuterol hydrochloride, acebutolol hydrochloride, oxprenolol hydrochloride, pindolol, and propranolol hydrochloride were purchased from Sigma (Saint-Quentin Fallavier, France). Carteolol hydrochloride was a gift of Chauvin laboratories (Montpellier, France). Tertatolol hydrochloride was obtained in pill form (5 mg) by prescription from a local pharmacy. Trimethylboroxine (99%) was purchased from Aldrich (Saint-Quentin Fallavier, France). Isolute C-18 columns were purchased from Touzart et Matignon (Courtaboeuf, France).

Hair samples

All the specimens were collected in the vertex region and stored at room temperature. Salbutamol positive hair specimens were obtained from asthmatic patients treated by Ventoline™ inhaler (100 µg salbutamol/inhalation). Hair was also collected in 2 asthma deaths. Three specimens were obtained from regional athletes who accepted to provide hair for research purposes. Two calves hair specimens were obtained from Toxlab laboratory (Paris, France).

Sample extraction

Before analysis, samples were decontaminated twice using 5 ml of methylene chloride, for 2 min, at room temperature.

A 4 cm segment from the root was used for the analysis and pulverized in a ball mill.

One hundred milligrams of hair was overnight incubated at 56°C in 1 ml 0.1 M HCl in presence of 50 ng of carteolol (prepared in methanol), used as internal standard. After cooling, the homogenate was neutralized with 1 ml 0.1 M NaOH, and 2 ml of 0.2 M bicarbonate buffer (pH 8.6) were added.

The Isolute C18 columns were conditioned with 3 ml of methanol, followed by 2 ml of bicarbonate buffer-methanol (90:10, v/v). After sample addition, the columns were washed twice with 1 ml of deionized water. After column drying, analyte elution occured with the addition of 3 aliquots of 0.50 ml of methanol. The eluant was evaporated to dryness under nitrogen flow at 40°C. The residue was derivatized by adding 30 µl trimethylboroxine-ethyl acetate (3:1000, v/v), then incubated for 15 min at 80°C.

GC/MS procedure

A 4-µl aliquot of the derivatized extract was injected into the column of a Hewlett Packard (Palo Alto, CA) gas chromatograph (6890 Series) via a Hewlett Packard (7673) autosampler. The flow of carrier gas (helium, purity grade N 55) through the column (HP5-MS capillary column, 5% phenyl-95% methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm film thickness) was 1.2 ml/min.

The injector temperature was 250°C and splitless injection was employed with a split valve off-time of 1.0 min, using the pulsed mode. The column oven temperature was programmed to rise from an initial temperature of 110°C, maintained for 1 min, to

170°C at 20°C/min, then to 225°C at 7°C/min than to 295°C at 24°C/min and maintained at 295°C for the final 10 min.

The detector was a Hewlett Packard 5973 operated in the electron impact mode. The electron multipler voltage was set at 400 V above the EI-tune voltage.

Method validation

Standard calibration curves were obtained by adding 2.5 (25 pg/mg), 10 (100 pg/mg), 25 (250 pg/mg), 50 (500 pg/mg), 200 (2 ng/mg) and 1000 ng (10 ng/mg) of all the adrenergics to 100 mg of pulverized hair, obtained from a drug-free subject of the laboratory. Within-run precisions (n = 8) for the drugs were determined using a strand of hair obtained from a laboratory volunteer, previously pulverized in a ball mill and spiked for a final concentration of 1 ng/mg of each compound. Relative extraction recoveries were determined for the drugs by comparing the representative peak of extracted free-hair (spiked with a final 1 ng/mg concentration) with the peak area of a metanolic standard at the same concentration. The detection limits (LOD) were evaluated with decreasing concentrations of the drugs spiked in drug-free hair, until a response equivalent to 3 times the background noise was observed.

RESULTS AND DISCUSSION

Table I shows the ions monitored for each analyte and the retention times. Carteolol was choosen as internal standard as this drug is rarely prescribed. Deuterated adrenergic compounds are not commercially available.

TABLE I. RETENTION TIMES AND SELECTED IONS FOR EACH ANALYTE

Analyte	Retention time [min]	Ions [m/z]
Oxprenolol	10.35	218, 274, 289
Salbutamol	11.21	188, 230, 272
Metoprolol	11.81	140, 276, 291
Clenbuterol	12.57	243, 285, 300
Propranolol	12.98	128, 268, 283
Pindolol	13.76	124, 257, 272
Bisoprolol	14.11	230, 334, 349
Sotalol	14.24	239, 281, 296
Timolol	14.26	152, 324, 340
Atenolol	14.51	164, 275, 290
Tertatolol	14.61	163, 304, 319
Carteolol (IS)	15.75	218, 301, 316
Acebutolol	17.13	246, 299, 360
Labetalol	17.96 + 18.57	271, 361, 376

IS – internal standard.
Ions underlined are used for quantitation.

This procedure is able to screen for the 2 major β_2-agonists that are abused, salbutamol and clenbuterol, and for 12 β-blockers in one single run. Other adrenergics can be added to the current method. Only sotalol and timolol are co-eluting, but can be easily differentiated by their respective mass spectrum. This was also observed in a previous paper dealing with urine [2]. Two peaks, probably corresponding to the 2 enantiomers, were observed for labetalol. According to Polettini et al. [12], derivatization with trimethylboroxine produces mass spectra which showed more structural informations with less chemical noise and better sensitivity than with classic trimethylsilylation.

Analytical validation of the method is presented Table II. Responses for the analytes were linear in the range 25 pg/mg to 10 ng/mg with correlation coefficients (r) in the range 0.992 (acebutolol) to 0.998 (salbutamol and metoprolol). This range corresponds well to the previously reported concentrations of adrenergics in human hair. Gleixner et al. [5] identified clenbuterol in hair obtained from 2 bodybuilders at 50 and 92 pg/mg. In hypertensive patients, Kintz and Mangin [9] identified betaxolol, sotalol, atenolol and propranolol in the range 0.9 to 5.3 ng/mg. The within-run precisions were in the range 5.9 (salbutamol) to 14.1% (acebutolol), as determnined by analyzing 8 replicates of 100 mg of drug-free hair spiked with each drug at a final concentration of 1 ng/mg. At the same concentration, recoveries were in the range 37 (pindolol) to 100% (sotalol). These recoveries were found acceptable for a screening procedure. Using a 100 mg hair sample, the limits of detection were in the range 2 (salbutamol and clenbuterol) to 10 pg/mg (acebutolol). Extensive extraction procedure combined with the injection of 4 µl through the column (pulsed mode) were analytical prerequisites for successful identification of these agents in hair due to the low target concentrations.

TABLE II. ANALYTICAL VALIDATION OF THE PROCEDURE

Analyte	Linearity [r]	Precision [%]	Recovery [%]	LOD [pg/mg]
Oxprenolol	0.996	7.2	62	8
Salbutamol	0.998	5.9	64	2
Metoprolol	0.998	6.8	69	4
Clenbuterol	0.997	6.4	75	2
Propranolol	0.993	7.6	57	8
Pindolol	0.993	9.4	37	8
Bisoprolol	0.993	8.1	57	8
Sotalol	0.994	10.4	100	4
Timolol	0.995	9.9	71	8
Atenolol	0.994	10.6	69	4
Tertatolol	0.993	12.6	45	4
Acebutolol	0.992	14.1	90	10

Labetalol	0.993	13.7	76	8

LOD – limit of detection.

Three regional athletes accepted to provide a hair specimen for research purposes. The first one was a 24-years old swimmer who tested positive for salbutamol in urine. In the proximal 3 cm of his hair, salbutamol was found at 71 pg/mg.

The second specimen was obtained from a specialist of shooting who assumed to chronically use Seloken™ (100 mg/tablet of metoprolol tartrate). The metoprolol concentration in hair was 8.41 ng/mg.

A third specimen was obtained from a specialist of archery who assumed to take Sotalex™ (80 mg/tablet of sotalol chlorhydrate). The sotalol concentration in hair was 261 pg/mg.

These examples illustrate the potential use of hair in controlling the abuse of performance enhancing drugs. According to Ventura et al. [14], sports disciplines where β-blockers are recommended to be tested are archery, bobsleigh, diving, modern pentathlon, shooting and synchronizing swimming.

Apart from their potent abuse in humans, β_2-agonists were detected in animals as they leed to reduced portions of fat and increased portions of lean meat. Owing to the low dosage and the clearance of the drugs, particularly for clenbuterol, discontinuing the administration few days before the animal is slaughtered will produce a negative urine result. Again, considering that hair provides long-term histories, potential veterinary applications have emerged [4]. Two specimens of hair, collected from two 106-days old calves were tested by this procedure. Clenbuterol was identified in both specimens, at 30 and 48 pg/mg. Meat quality control should gain from this new approach.

CONCLUSION

It appears that the value of hair analysis for the identification of drug users is steadily gaining recognition. Hair analysis may be a useful adjunct to conventional drug testing in sports. In anti-doping control, the analysis of hair for β_2-agonists could provide complementary information to urinalysis, allowing the theoretical possibility of discriminating acute administration to treat pulmonary diseases from chronic abuse, necessary to obtain the anabolic effect. Methods for evading urinalysis do not affect hair analysis. Specimens can be more easily obtained with less embarrassment, and hair can provide a more accurate history of drug use. Costs are too expensive for routine use but the generated data are extremely helpful to document positive urine cases. This new technology may find useful applications in doping control, if accepted by the International Olympic Committee.

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