TECHNICAL NOTE

Pascal Kintz,[1] *Ph.D.; Véronique Dumestre-Toulet,*[2] *Pharm.D.; Carole Jamey,*[1] *B.S.; Vincent Cirimele,*[1] *Ph.D.; and Bertrand Ludes,*[1] *Ph.D.*

Doping Control for β-Adrenergic Compounds Through Hair Analysis*

REFERENCE: Kintz P, Dumestre-Toulet V, Jamey C, Cirimele V, Ludes B. Doping control for β-adrenergic compounds through hair analysis. J Forensic Sci 2000;45(1):170–174.

ABSTRACT: An original procedure was developed to simultaneously test β_2-agonists (salbutamol and clenbuterol) and β-blockers (atenolol, acebutolol, pindolol, betaxolol, propranolol, timolol, sotalol, metoprolol, tertatolol, bisoprolol, labetalol and oxprenolol) in both human and animal hair. After decontamination with methylene chloride (2 times, 2 min), a 200 mg hair strand is pulverized in a ball mill. Then, a 100 mg portion is incubated overnight in 2 mL 0.1 N HCl, at 56°C, in the presence of carteolol, which was used as an internal standard. After neutralization of the acid phase with 0.1 N NaOH, a 2 mL bicarbonate buffer (pH 8.6) is added to the preparation, which is then purified by solid-phase extraction with Isolute C18 columns. Drugs are derivatized using a mixture of trimethylboroxine-ethyl acetate for 15 min at 80°C to form methaneboronate derivatives. Drugs are detected using GC/MS on an HP 6890-5973 system. A 4 μL portion of the derivatized extract is injected using a pulsed mode in a 30 m HP5 MS capillary column. Linearity was observed for all compounds in the range 25 pg/mg to 10 ng/mg. Limits of detection were in the range 2 to 10 pg/mg. At 1 ng/mg, recoveries were in the range from 37 to 100%, with a within-run precision of 5.9 to 14.1% ($n = 8$).

The application of the method can be documented by the following examples: (1) Hair from asthmatic patients ($n = 11$), including two cases of asthma deaths, tested positive for salbutamol in the range of 27 to 210 pg/mg. (2) A 24-year-old swimmer who tested positive in urine for salbutamol denied the results. Hair analysis confirmed salbutamol exposure, with a concentration of 71 pg/mg. (3) A shooting specialist was assumed to chronically use metoprolol (100 mg/daily, during some periods). Hair concentration of metoprolol was 8.41 ng/mg. (4) An archery specialist was assumed to chronically use sotalol (80 mg/daily, during some periods). Hair concentration of sotalol was 261 pg/mg. (5) Hair from two calves revealed chronic exposure to clenbuterol, which was used to increase the mass of the animals at a concentration of 30 and 48 pg/mg.

KEYWORDS: forensic science, hair, β-adrenergic drug, doping control

[1] Institut de Médecine Légale, 11 rue Humann, 67000 Strasbourg, France.
[2] Laboratoires Ruffié et associés, 30, allées de Tourny, 33000 Bordeaux, France.

* This work was presented at the AAFS meeting of Orlando, 15–20 Feb. 1999.

Received 4 Mar. 1999; and in revised form 10 May 1999; accepted 10 May 1999.

The use of β-adrenergic compounds (agonists and antagonists) is banned in sports by both the International Olympic Committee (IOC) and International Sports Federations (1). 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 writing 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. More recently, clenbuterol was suggested as a countermeasure drug for micro gravity-induced skeletal muscle atrophy in spacemen.

β_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 (2).

In addition to their medical use in the treatment of cardiac arrhythmias and hypertension, β-blockers have found a place in some athletic events, particularly disciplines in which good psychomotor coordination is required. In those sports, athletes can benefit from the peripheral blockade of several symptoms associated with anxiety. β-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 (3), or the more common gas chromatography mass spectrometry (GC/MS) technology (4,5).

However, discontinuing the treatment a few days before the competition, or presenting a medical receipt at the time of the competition, does 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 (6). The major practical advantage of hair testing compared to urine testing for drugs is that it has a larger surveillance window (weeks to months, depending on the length of the hair shaft, against 2 to 4 days for most xenobiotics, with the exception of anabolic steroids in an ester preparation). For practical purposes, the two tests complement each other. Urinalysis provides short-term information of an individual's drug use, whereas long-term histories are accessible through hair analysis. While analysis of urine specimens cannot distinguish between chronic use or single exposure, hair analysis can offer the distinction.

Its greatest use, however, may be in identifying users who abstain before the test or try to "beat the test" by diluting urine, as this will not alter the concentration in hair. Urine does not reflect frequency of intake even if they don't abstain prior to the test. Doping during training and abstinence during the competition should therefore be detected, as athletes cannot evade the hair test. However, to avoid conflicting results (positive urine and negative hair), very low limits of detection of drugs in hair are requested to increase the sensitivity of the test.

Recently, some papers have pointed out the interest of doping control through hair analysis (7,8). 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 ($n = 10$) dealing with the identification of β-adrenergic drugs in hair. Most of the papers ($n = 7$) have been focused on animals. Clenbuterol was identified in rats (9), guinea pigs (10,11), bovine (11,12) or calves (14), and salbutamol was identified in guinea pigs (15). Gleixner et al. (16) detected clenbuterol in a two bodybuilders by HPLC/EIA. Clenbuterol was also detected by gas chromatography-high resolution mass spectrometry in hair obtained from four pregnant women who were therapeutically treated with the drug (17). In an attempt to better manage hypertensive patients, Kintz and Mangin (18) tested β-blockers in eight 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 two β_2-agonists and twelve 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 C18 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 two asthma deaths. Three specimens were obtained from regional athletes who agreed to provide hair for research purposes. Hair specimens from two calves 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 mg of hair was incubated overnight at 56°C in 1 mL 0.1 M HCl in the presence of 50 ng of carteolol (prepared in methanol), which was used as an 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 occurred 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 × 0.25 mm i.d. × 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, then 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 and recorded data in scan mode from m/z 100 to m/z 380. 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 from 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 three times the background noise was observed.

Results and Discussion

Table 1 shows the ions monitored for each analyte and the retention times. Carteolol was chosen as the internal standard since this drug is rarely prescribed. Deuterated adrenergic compounds are not commercially available.

This procedure is able to screen for the two major β_2-agonists that are abused, salbutamol and clenbuterol, and for twelve 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 (5). Two peaks, probably corresponding to the two enantiomers, were observed for labetalol. According to Polettini et al. (15), derivatization with trimethylboroxine produces mass spectra which showed more structural information with less chemical noise and better sensitivity than with classic trimethylsilylation.

Analytical validation of the method is presented in Table 2. 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. (16) identified clenbuterol in hair obtained from two bodybuilders at 50 and 92 pg/mg. These concentrations were close to those reported by Machnik et al. (17), as the levels of clenbuterol determined in hair ranged from 2 to 236 pg/mg. In hypertensive patients, Kintz and Mangin (18) identified betaxolol, sotalol, atenolol and propranolol in the range from 0.9 to 5.3 ng/mg. The within-run precisions were in the range from 5.9% (salbutamol) to 14.1% (acebutolol), as determnined by analyzing eight 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 from 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 from 2 (salbutamol and clenbuterol) to 10 pg/mg (acebutolol). Extensive extraction procedures 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.

A critical element in the acceptance of hair analysis for detection of drugs is laboratory performance. Laboratories must be able to demonstrate that they can accurately determine what drugs are present and at what levels in unknown hair samples. A large amount of hair was obtained from an asthmatic patient, treated with salbutamol. The specimen was decontaminated, pulverized in a ball mill and 200 mg were sent to six different French laboratories participating in the Quality Program of the French Society of Analytical Toxicology, along with the here described procedure. Participants were asked to identify the adrenergic drug and to quantify it. According to our tests ($n = 4$), the target concentration was 68 pg/mg. All the laboratories identified salbutamol, with the following concentrations: 31, 55, 62, 96, 130 and 296 pg/mg. The distribution of the results was found similar to that of previous exercises, performed for heroin or cocaine (19, 20). A complete listing of the hair analysis data is given in Table 3.

TABLE 1—*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.

TABLE 2—*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.

TABLE 3—*Listing of the hair analysis data.*

Type of Subject	Demographic	Hair Color	β-adrenergic
Asthmatic patient	37 years	blond	salbutamol, 27 pg/mg
Asthmatic patient	52 years	grey	salbutamol, 42 pg/mg
Asthmatic patient	unknown	brown	salbutamol, 48 pg/mg
Asthmatic patient	18 years	brown	salbutamol, 59 pg/mg
Asthmatic patient	44 years	blond	salbutamol, 63 pg/mg
Asthmatic patient	31 years	black	salbutamol, 92 pg/mg
Asthmatic patient	unknown	dark brown	salbutamol, 106 pg/mg
Asthmatic patient	51 years	black	salbutamol, 124 pg/mg
Asthmatic patient	27 years	brown	salbutamol, 192 pg/mg
Asthma death	52 years	dark brown	salbutamol, 210 pg/mg
Asthma death	58 years	grey	salbutamol, 87 pg/mg
Swimmer	24 years	brown	salbutamol, 71 pg/mg
Shooter	unknown	black	metoprolol, 8.41 ng/mg
Archer	31 years	brown-blond	sotalol, 261 pg/mg

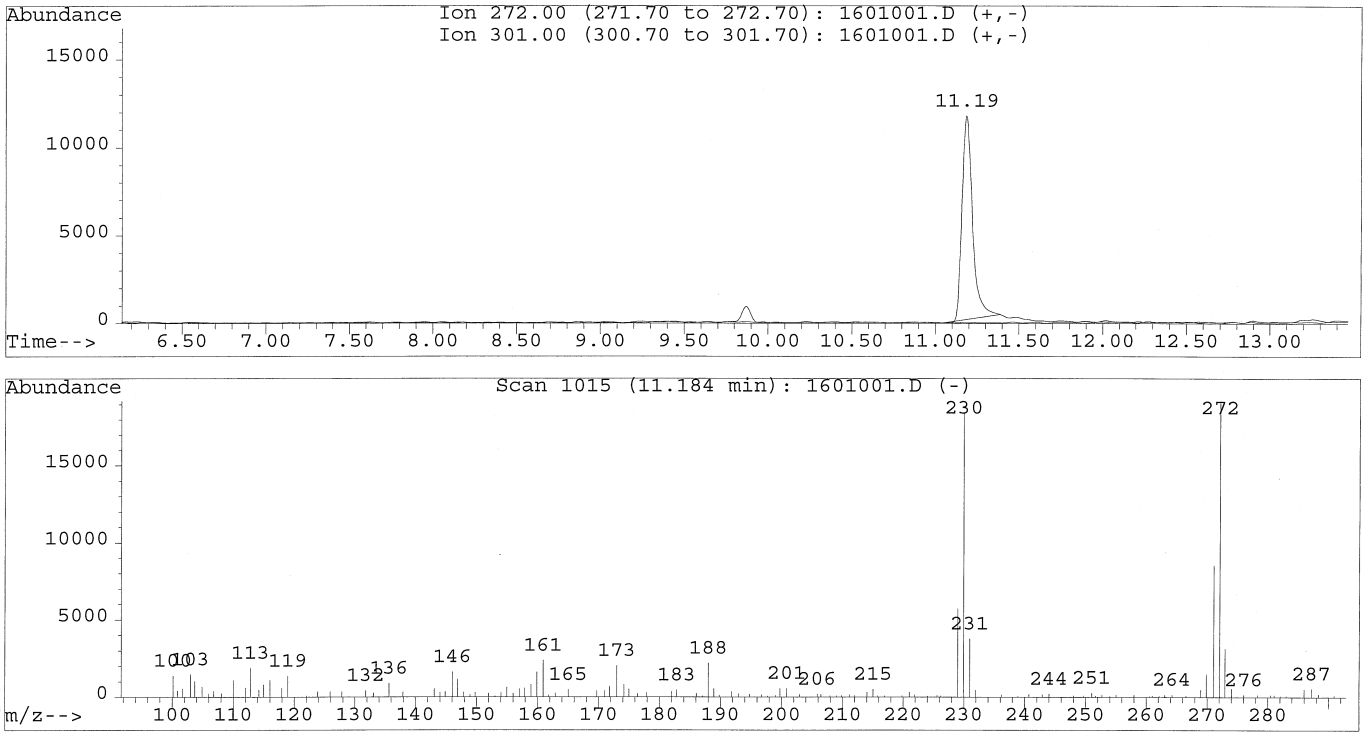

FIG. 1—*Selected ion chromatogram, along with electron impact, after extraction of a hair specimen positive for salbutamol at 71 pg/mg.*

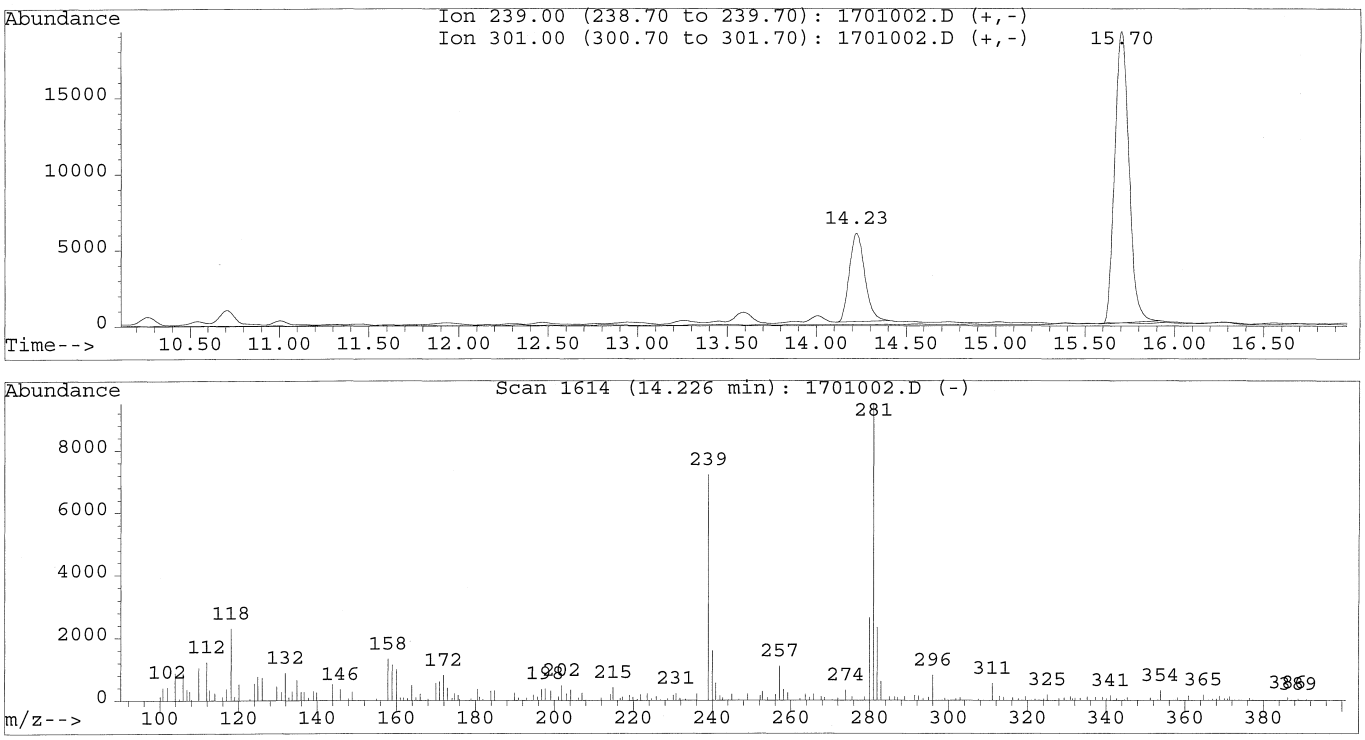

FIG. 2—*Selected ion chromatogram, along with electron impact, after extraction of a hair specimen positive for sotalol at 261 pg/mg. Carteolol (RT 15.70 min) is used as internal standard.*

As specimens from athletes are difficult to obtain, particularly because hair is not recognized by the IOC, the practical validation of the procedure was achieved by testing nine asthmatic patients. In all cases, subjects used a Ventoline™ inhaler (100 μg salbutamol/inhalation). The salbutamol concentrations in hair were in the range from 27 to 192 pg/mg. As no medical reports were available (retrospective study), no attempt to evaluate the correlation between salbutamol dosage and hair concentration was done.

In two asthma deaths (as recorded by the medical examiner), salbutamol concentrations were 56 and 77 ng/mL and 210 and 87 pg/mg for whole blood and hair, respectively.

Three regional athletes agreed to provide a hair specimen for research purposes. The first one was a 24-year-old swimmer who tested positive for salbutamol in urine. In the proximal 3 cm of his hair, salbutamol was found at 71 pg/mg. Figure 1 is the chromatogram of his hair extract, along with the mass spectrum of the drug. Under the chromatographic conditions used, there was no interference by any extractable endogenous material in hair.

The second specimen was obtained from a shooting specialist who was 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 an archery specialist who was assumed to take Sotalex™ (80 mg/tablet of sotalol chlorhydrate). The sotalol concentration in hair was 261 pg/mg. Figure 2 is the chromatogram of his hair extract, along with the mass spectrum of sotalol.

These examples illustrate the potential use of hair in controlling the abuse of performance-enhancing drugs. According to Ventura et al. (3), sports disciplines where β-blockers are recommended to be tested are archery, bobsleigh, diving, modern pentathlon, shooting and synchronized swimming.

Apart from their potent abuse in humans, β_2-agonists were detected in animals as they lead 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 a 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 (14). Two specimens of hair collected from two 106-day-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.

References

1. International Olympic Committee. Prohibited classes of substances and prohibited methods. In IOC Medical Code and Explanatory Document. IOC Lausanne, Switzerland, 1995.
2. Martinez Navarro JF. Food poisoning related to consumption of illicit β-agonist in liver. Lancet 1991;336:1311.
3. Ventura R, Gonzalez G, Smeyers MT, de la Torre R, Segura J. Screening procedure for β-adrenergic drugs in sports drug testing by immunological methods. J Anal Toxicol 1998;22:127–34.
4. Zamecnik J. Use of cyclicboronates for GC/MS screening and quantitation of beta-adrenergic blockers and some bronchodilatators. J Anal Toxicol 1990;14:132–6.
5. Branum GD, Sweeney S, Palmeri A, Haines L, Huber C. The feasibility of the detection and quantitation of β-adrenergic blockers by solid-phase extraction and subsequent derivatization with methanboronic acid. J Anal Toxicol 1998;22:135–41.
6. Kintz P. Drug testing in hair. Boca Raton: CRC Press, 1996.
7. De Boer D, Höld KM, Crouch DJ, Wilkins DG, Rollins DE, Maes RAA. The potential use of hair in doping control in general and in the detection of stanozolol in particular. Proceedings of the 15th Cologne Workshop on Dope Analysis; 1997 Feb 23–28, Koln. Koln:Sport und Buch, 1998.
8. Kintz P. Hair testing and doping control in sport. Toxicol Letters 1998;102:109–13.
9. Adam A, Gervais N, Panoyan A, Ong H, Beliveau L, Ayotte C. Detection of clenbuterol residues in hair. Analyst 1994;119:2663–6.
10. Polettini A, Segura J, Gonzalez G, de la Torre X, Montagna M. Clenbuterol and β-adrenergic drugs detected in hair of treated animals by ELISA. Clin Chem 1995;41:945–6.
11. Segura J. Possibilities of ELISA methodologies for hair analysis. Proceedings of the 1995 International Conference and Workshop on Hair Analysis in Forensic Toxicology; 1995 Nov 19–23, Abu Dhabi. Abu Dhabi: The General Directorate of Abu Dhabi Police, 1995.
12. McGath GJ, O'Kane E, Smyth WF, Tagliaro F. Investigation of the electrochemical oxidation of clenbuterol at a porous carbon electrode, and its application to the determination of this β-agonist in bovine hair by liquid chromatography with coulometric detection. Anal Chimica Acta 1996;332:159–66.
13. Fente CA, Vazquez BI, Franco C, Cepeda A, Gigosos PG. Determination of clenbuterol residues in bovine hair by using diphasic dialysis and gas chromatography-mass spectrometry. J Chromatogr B 1999;726:133–9.
14. Gaillard Y, Balland A, Doucet F, Pépin G. Detection of illegal clenbuterol use in calves using hair analysis. Application in meat quality control. J Chromatogr B 1997;703:85–95.
15. Polettini A, Montagna M, Segura J, de la Torre X. Determination of β_2-agonists in hair by gas chromatography/mass spectrometry. J Mass Spectrom 1996;31:47–54.
16. Gleixner A, Sauerwein H, Meyer HHD. Detection of the anabolic β_2-adrenoceptor agonist clenbuterol in human scalp hair by HPLC/EIA. Clin Chem 1996;42:1869–71.
17. Machnick M, Geyer H, Horning S, Breidbach A, Delahaut P, Schänzer W. Long-term detection of clenbuterol in human scalp hair by gas chromatography-high-resolution mass spectrometry. J Chromatogr B 1999;723:147–55.
18. Kintz P, Mangin P. Hair analysis for detection of beta-blockers in hypertensive patients. Eur J Clin Pharmacol 1992;42:351–2.
19. Welch MJ, Sniegoski LT, Allgood CC. Interlaboratory comparison studies on the analysis of hair for drugs of abuse. Forensic Sci Int 1993;63:295–303.
20. Sachs H. Quality control by the society of hair testing. Forensic Sci Int 1997;84:145–50.

Additional information and reprint requests:
Dr. P. Kintz, Ph.D.
Institut de Médecine Légale
11, rue Humann
F-67085 Strasbourg Cedex
France