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Evaluation of ambroxol as a masking agent in doping control

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Introduction: The history of sport shows that athletes tend to try to overcome their limits, not measuring efforts to achieve their goals. Sometimes, athletes use methods or substances that might compromise not only their health, but also their teammates and their opponents besides being against the ethics in sport **[1].** A substance is considered doping when it improves athlete's performance beyond what could supposedly be achieved through training. Since doping control is a routine procedure, sometimes athletes use doping substances along with masking agents, which might turn analysis of some substances into a more difficult or impossible task. Thus masking agents are also a part of the prohibited list of the World Anti-doping Agency (WADA). Ambroxol **(Figure 1)** is an expectorant substance that influences parameters considered to be the basis for the physiological production and transport of the bronchial mucus, with a secretolytic effect helping to eliminate the pulmonary secretion **[2].** It is an over the counter drug in Brazil which can be found in syrups and tablets. Approximately 90% of its excretion is urinary, being found in both metabolized and unchanged forms. Previous analyses performed by our research group showed that ambroxol has a great impact on the chromatographic behavior of samples submitted to a screening procedure for anabolic steroids by gas chromatography coupled to mass spectrometry (GC-MS). This impact occurs because of the high concentrations in which ambroxol is generally found in the urine of its users **(Figure 1).** It can also interfere on the mass spectra of some steroids by changing the ion ratios and thus preventing it from fulfilling the identification criteria required by WADA. These results raised some questions about the masking potential of this expectorant agent. There is no published data about the extent of usage of ambroxol by athletes. Declarations of usage received in our laboratory show that this is a commonly used drug either in this form (Mucosolvan®) or as a pro-drug (Bromexine - Bisolvon®). The goals

of this study were to evaluate actual ambroxol interference on the screening procedure for anabolic agents and to elaborate an alternative sample extraction procedure which could be applied to urine samples that contain ambroxol.

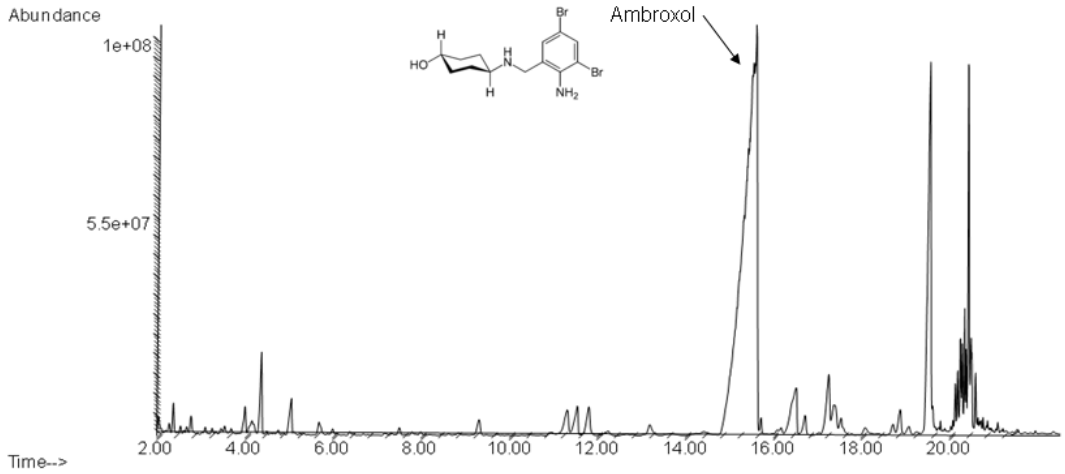

Figure 1: Structural formula of ambroxol and total ion chromatogram obtained by GC-MS for anabolic steroid analysis of an urine sample containing ambroxol.

Experimental: Preliminary analyses showed that three steroids could potentially be masked by ambroxol and then excretion samples containing ambroxol were spiked with these steroids. The steroids investigated were 17α-ethyl-5β-estrane-3α,17β-diol (norethandrolone metabolite), 7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metabolite) and 4-chloroandrost-4-en-3α-ol-17-one (clostebol metabolite). The excretion study conducted was authorized by the research ethics committee of Universitary Hospital Clementino Fraga Filho protocol 011/00. Urine samples were collected during 3 days by a female volunteer, who had taken the therapeutical dose (30 mg) of ambroxol syrup each 3 hours during daylight. The screening procedure for anabolic steroids used consists of the method described by Schänzer and Donike **[3]** with few modifications which is: spiking of a 2 mL aliquot of each urine sample with internal standard (17α-methyltestosterone) at a concentration of 500 ng/mL, enzymatic hydrolysis at pH 7,0 (β-glucuronidase from *E.coli*), followed by a liquid-liquid extraction with *tert*-butylmethylether (TBME) at pH 10 and derivatization with MSTFA:NH_4I:2-mercaptoethanol (1000:2:6, v:w:v). A 3 µL aliquot of this extract is analyzed by GC-MS. An alternative extraction method was developed changing two steps: the extraction pH which was changed from 10 to 3 with formate buffer and the extraction solvent which changed from TBME to n-pentane.

The analyses were performed in an Agilent system (GC 6890/MS 5973). Carrier gas was helium 1 mL/min, in constant flow mode. Ultra-1® capillary column (17 m x 0.2 mm x

0.11 µm; J&W Scientific, Agilent Technologies Inc.); injector temperature was 280°C; injection mode: pulsed split; split ratio 1:10, pulse pressure 50 psig/0.80 min; injection volume: 3 µL the GC oven temperature was programmed to raise from 140°C to 180°C (rate of 40°C/min), then, set to raise from 180°C to 230°C (rate of 3°C/min) and finally set to raise from 230°C to 300°C (rate of 40°C/min) (hold 3 min). MS parameters: electron ionization, ionization voltage, 70 eV; ion source temperature, 220°C; interface temperature, 280°C; quadrupole temperature, 150°C; transfer line temperature, 280°C. Mass spectra were obtained in both full scan and selected ion monitoring modes.

Results and discussion: Figures 3 A to 5 A show blank urine samples spiked in the MRPL with norethandrolone metabolite, bolasterone metabolite and clostebol metabolite respectively, while figures 3 B to 5 B show excretion samples from ambroxol spiked with the same compounds in the MRPL. Figures 3 to 5 C show excretion samples from ambroxol spiked in the MRPL with the three steroids and analyzed by the alternative method developed in this work.

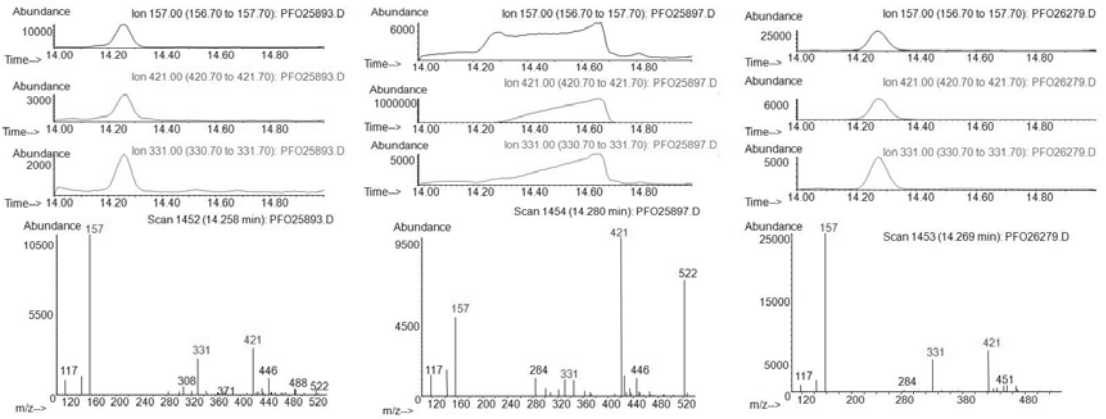

Figure 3: Extracted ion chromatograms and selected ion mass spectra from17α-etil-5β-estrane-3α,17β-diol (norethandrolone metabolite). Left (A); Center (B); Right (C)

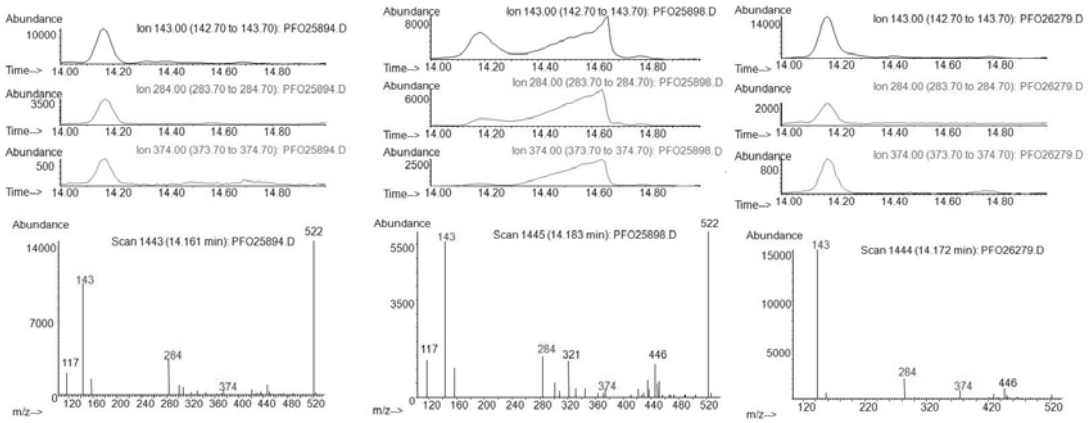

Figure 4: Extracted ion chromatograms and selected ion mass spectra from 7α,17α-dimetil-5β-androstane-3α,17β-diol (bolasterone metabolite) Left (A); Center (B); Right (C)

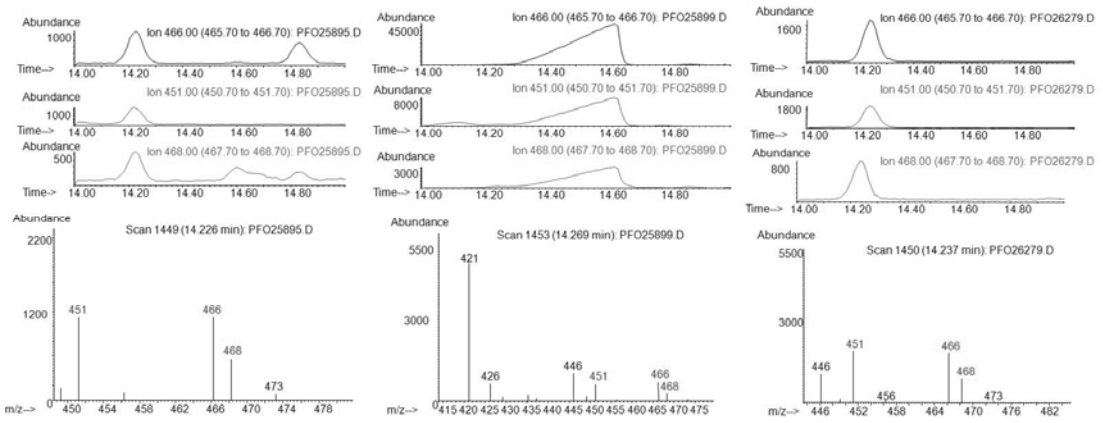

Figure 5: Extracted ion chromatograms and selected ion mass spectra from 4-chloroandrost-4-en-3α-ol-17-one (clostebol metabolite) Left (A); Center (B); Right (C)

These chromatograms show that ambroxol has great impact on the chromatographic behavior of these steroids, completely disabling their analysis by overloading the chromatographic column. Since ambroxol has two amino groups in its structure, extraction was performed at a low pH value, in which the steroids extraction would not be prejudiced while ambroxol would be in a charged state, remaining preferably in the aqueous phase. By the application of the extraction method developed herein, excretion samples of ambroxol could be easily analyzed and three diagnostic ions with signal-to-noise ratio greater than three could be obtained for the three steroids which meets WADA´s criteria for identification.

Conclusion: Samples containing ambroxol revealed to be an actual problem when analyzed by the screening procedure employed for anabolic steroids, since this substance overloads the chromatographic column, generating a wide signal in the retention time of three of the steroid metabolites monitored, namely clostebol metabolite, norethandrolone metabolite and bolasterone metabolite. The developed extraction method was able to eliminate this interference and the steroids could be analyzed with no further problems meeting the identification criteria and MRPL defined by WADA.

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