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DETECTION OF INTERFERENCES IN URINARY ANABOLIC STEROID ANALYSIS

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ABSTRACT

This paper gives an overview of the research work done in our laboratory on the detection of interferences in urinary anabolic steroid analysis. Anabolic steroids are illegally used in animals for growth promoting purposes. Consumption of meat derived from hormone treated animals, can lead to the presence of anabolic steroids in the urine of the consumer. Results of the research on the doping interference phenomenon are summarized in this paper. More detailed information can be found in the articles, quoted in this survey.

INTRODUCTION

The control of the misuse of anabolic steroids is done by analysis of the athlete's urine. The analytical procedure consists of pretreatment of the urine sample, derivatization and analysis by gas chromatography - mass spectrometry. Anabolic steroids, which are usually administered in the training period and hence are low concentrated in urine at the time of testing at a competition, ask for selected ion monitoring analysis. Selected ion monitoring increases the sensitivity of the assay, but reduces the selectivity. Stringent precautions should be taken in order to avoid misinterpretations due to interfering compounds. The aim of our study was to examine a hypothesis for interference in anabolic steroid analysis not yet reported in the literature, namely the presence of anabolic steroids in urine due to the consumption of hormone contaminated meat. The use of hormonal growth promoters in cattle fattening is forbidden in the European Community since January 1, 1988. These hormonal growth promoters include androgens, estrogens and gestagens. Notwithstanding

the total ban, there is a widespread illegal use. Animals are injected with hormone cocktails, possibly containing some of the anabolic steroids which are on the doping list of the International Olympic Committee (Figure 1).

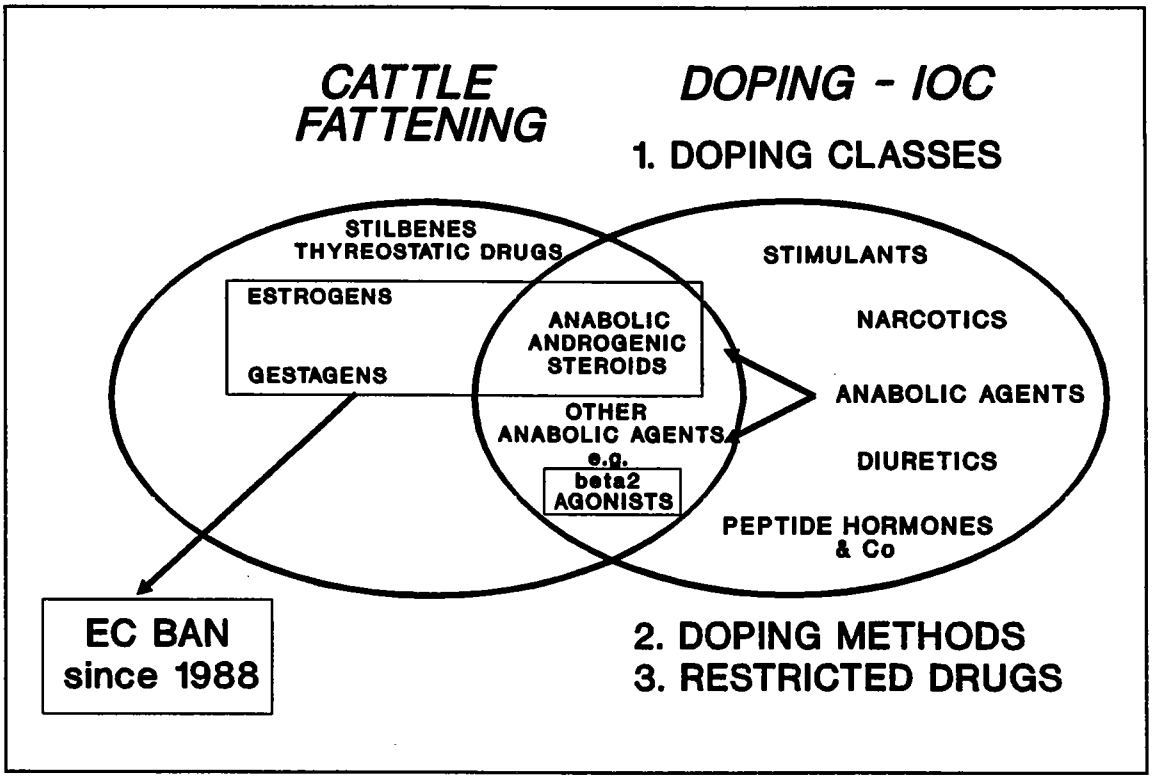

Figure 1: Schematical representation of the overlap of the products on the IOC doping list and the products banned for use in livestock farming

The screening procedures used for urinary doping control of anabolic steroids are very specific and sensitive and could therefore detect residues of anabolic steroids in urine, originating from a single undeliberate consumption of hormone contaminated meat. Results of this doping interference study will be presented.

EXPERIMENTS

Preliminary findings[1]

In 1989, research work was done to study nandrolone metabolism in nandrolone treated patients. They were intramuscularly injected with Deca Durabolin® (nandrolone decanoate, 25 or 50 mg). Nandrolone metabolites were detected in those urine samples. Pretreatment of the urine samples was done by solid phase extraction. Before gas chromatographic-mass spectrometric analysis, MO-TMS derivatization was carried out.

Urine samples obtained from male control samples ("blank urine samples") were screened for nandrolone interferences. The aim of the screening was to become familiar with the endogenous steroid profile and to become vigilant for eventual interferences due to endogenous substances. Two of the 20 blank urine samples showed interferences with nandrolone metabolites. The results obtained by MO-TMS derivatization were confirmed by HFB derivatization and TMS-enol-TMS-ether derivatization.

Since no dietary control was performed of the persons yielding the nandrolone positive urine samples, the presence of nandrolone metabolites in the urine of untreated males due to consumption of contaminated meat could only be presumed. This presumption was however strengthened by the fact that nandrolone was very popular in cattle fattening at the time that the nandrolone positives were found (1989). Further attempts to investigate the possible presence of anabolic steroids in the urine of untreated persons due to consumption of hormone contaminated meat, will be discussed in the following.

Random experiments[2,3,4]

The possible influence of the consumption of hormone contaminated meat on routine doping tests was essentially investigated through the so-called random experiments. The set-up of these experiments will be summarized. Volunteers consumed minced beef meat, which was bought in butchers' shops in Ghent. An aliquot of the meat was stored at - 20 °C prior to consumption. Before consumption, 1 urine sample was obtained from the volunteer (pre-consumption urine sample). Several post-consumption urine samples were obtained up to 18 to 24 hours after consumption. For screening purposes the first two post-consumption urine samples were analyzed for anabolic steroids which were known to be used in cattle fattening. Three of 60 volunteers were positive in a first screening. 19-Norandrosterone (19-NA), considered to be evidence of the administration of the anabolic steroid nandrolone, was demonstrated in the urine of untreated persons after meat consumption. 4-Chloro-androst-4-en-3α-ol-17-one (CLOS-MET), being considered to be evidence of the administration of the anabolic steroid clostebol, was demonstrated in the urine of 2 untreated persons after meat consumption. After the screening of the first two post-consumption urine samples, all available urine samples from the "positive volunteers" were analyzed. No anabolic steroid metabolite was detected in the pre-consumption urine samples of the respective volunteers. Anabolic steroid metabolite could however be detected in the urine samples taken up to several hours after meat consumption. These

findings, pre-consumption urine samples being negative and post-consumption urine samples being positive, triggered us to examine the aliquots of the meat samples which were connected to the "positive volunteers". Nandrolone was detected in an aliquot of the meat sample connected to the nandrolone positive volunteer. Clostebol acetate was detected in an aliquot of the meat samples connected to the 2 clostebol positive volunteers. The random experiments were in a way blinded, since aliquots of the meat samples were only analyzed after results of the urine analysis were interpreted. Pharmacokinetics of the anabolic steroids nandrolone and clostebol, obtained from control experiments (controlled administration of known amounts), enabled us to further interpret the quantitative results. Quantification showed that concentrations of the anabolic steroid metabolite gradually declined after the meat consumption. This too points to the fact that the anabolic steroid metabolites were present due to consumption of hormone contaminated meat. Highest clostebol metabolite concentration after consumption of contaminated meat were 32.2 ng/ml and 22.7 ng/ml respectively. 19-Norandrosterone concentrations found in untreated volunteers ranged from 3.8 ng/ml (highest concentration in the volunteer involved in the random experiment) to 37 ng/ml (highest concentration found in a "blank urine sample").

Control experiments[3,4,2]

Experiments in which known amounts of clostebol acetate and nandrolone were administered to volunteers were carried out. Clostebol acetate and nandrolone were selected since they were found in the urine samples of some volunteers involved in the random experiments, as described earlier. The amounts were comparable to the amounts which could be expected in meat samples containing an injection site. Results of the control experiments involving oral administration are represented in Figure 2 for nandrolone and Figure 3 for clostebol acetate. The urinary excretion of 19-NA in terms of the administered dose of nandrolone amounts to 29.4 $\pm$ 1.6 % (n = 3, C.V. 5.3 %). The urinary excretion of CLOS-MET in terms of the administered dose of clostebol acetate amounts to 18.8 $\pm$ 2.2 % (n = 3, C.V. 11.7 %).

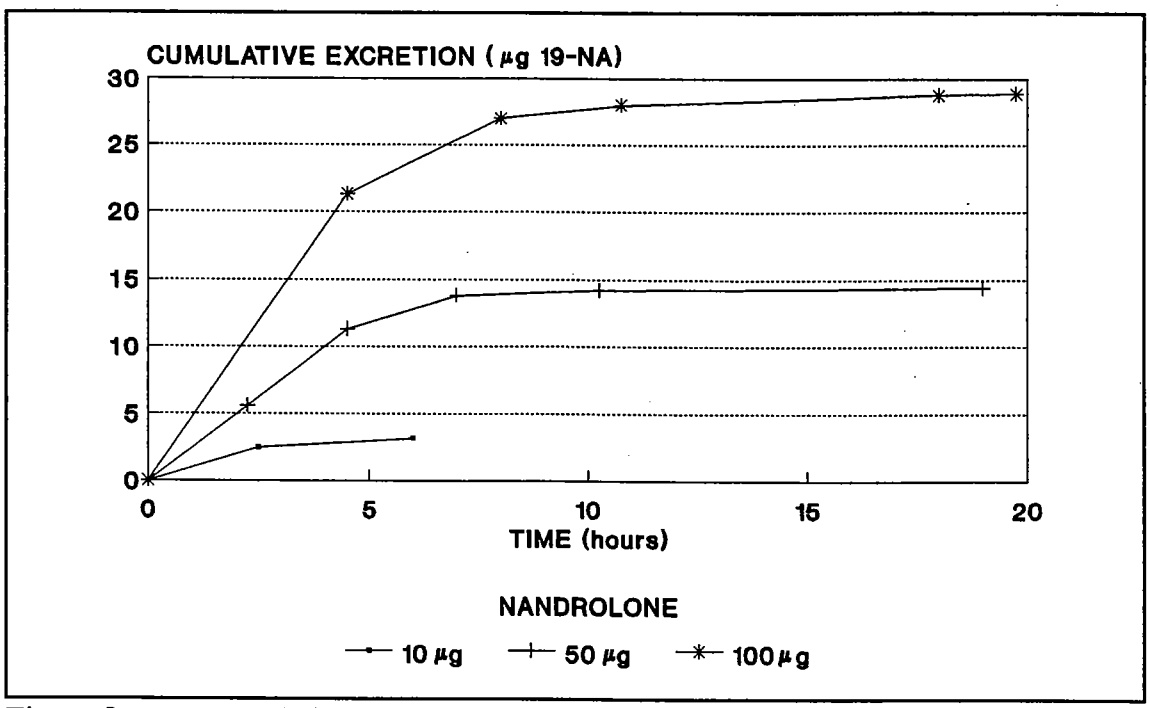

Figure 2: Cumulative excretion curves for 19-NA excreted by the volunteers involved in the control experiments (oral administration of nandrolone)

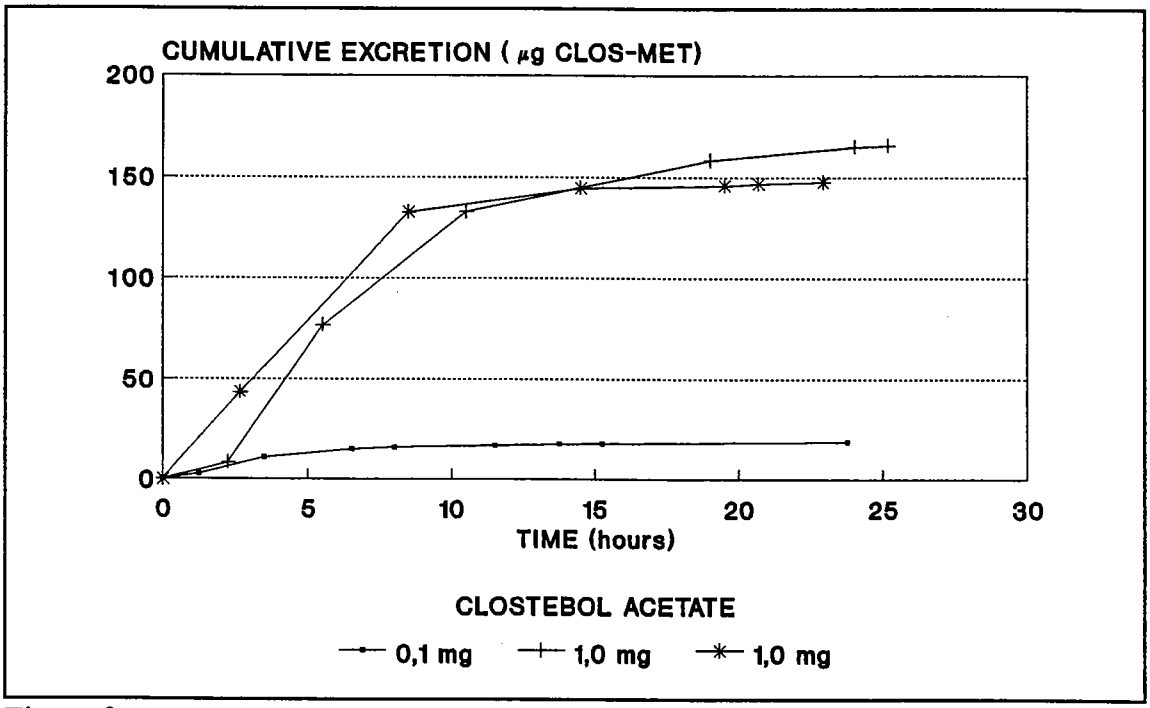

Figure 3: Cumulative excretion curves for CLOS-MET excreted by the volunteers involved in the control experiments (oral administration of clostebol acetate)

CONFIRMATION

The elaboration of confirmation techniques was primarily done on clostebol positive urine samples. Both low resolution mass spectrometry (LRMS) and high resolution mass spectrometry (HRMS) were used after gas chromatographic separation. LRMS was used to determine the relative abundances of the ions. With HRMS, selectivity was increased by recording exact masses instead of nominal masses.

Confirmation by comparison of the relative abundances of the ions[5]

For detection of residues of hormonal substances, EC legislation prescribes confirmation techniques. The intensities of preferably at least four diagnostic ions should be measured and the relative abundances of all diagnostic ions monitored from the analyte should match those of a reference standard, preferably within a range of ± 10 % (EI mode)[6]. In our study, seven ions were chosen to calculate ion ratios. Ion ratios of the "presumed positive" were compared to the ion ratios of reference standard and reference urine samples. Mean ion ratios for reference urine samples obtained after intramuscular injection of Steranabol® and for a presumed positive urine sample are presented in Figure 4.

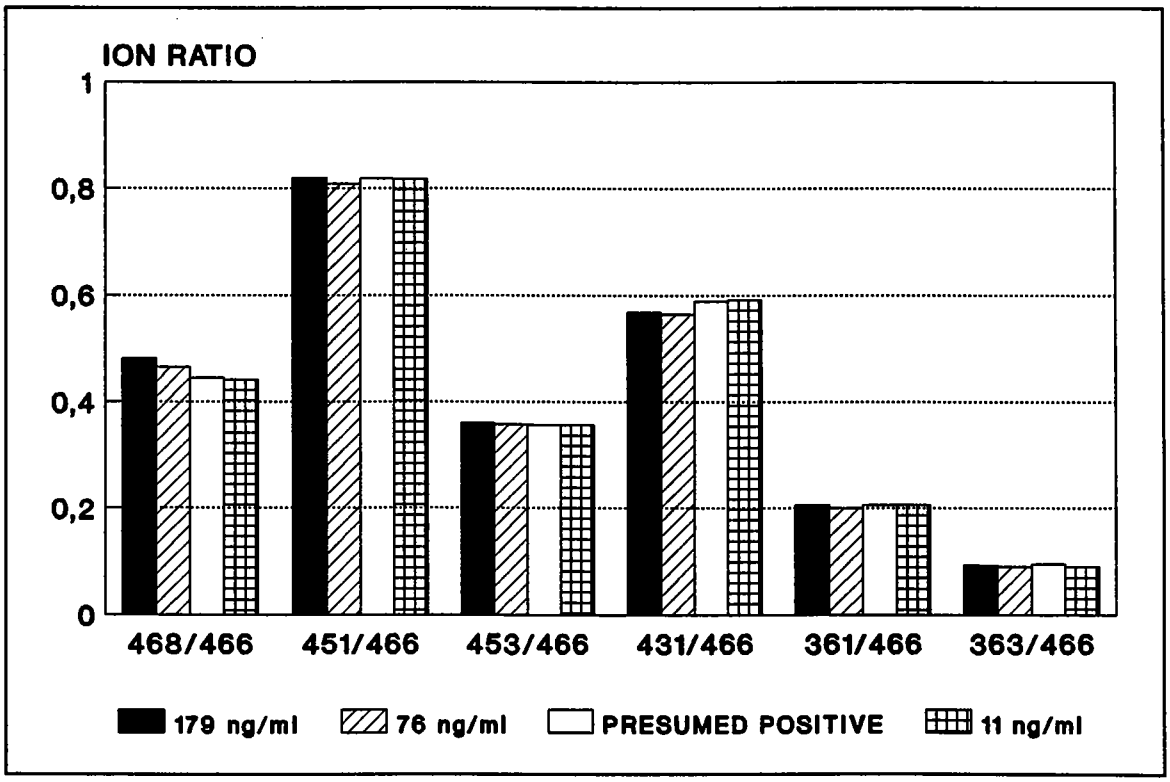

Figure 4: Mean ion ratios for the reference urine samples, obtained 25 (179 ng/ml), 30 (76 ng/ml) and 35 (11 ng/ml) days after injection of Steranabol® and for the presumed positive urine sample (23 ng/ml)

Confirmation by GC - HRMS[7]

For pretreatment of the urine sample, solid phase extraction was followed by an additional HPLC purification step. The ion chromatograms for the reference urine sample are shown in Figure 5A. The 5 selected ions appear simultaneously at retention time 15.55. The ion chromatograms of the urine sample of one of the volunteers involved in the random experiments ("presumed positive") are shown in Figure 5B.

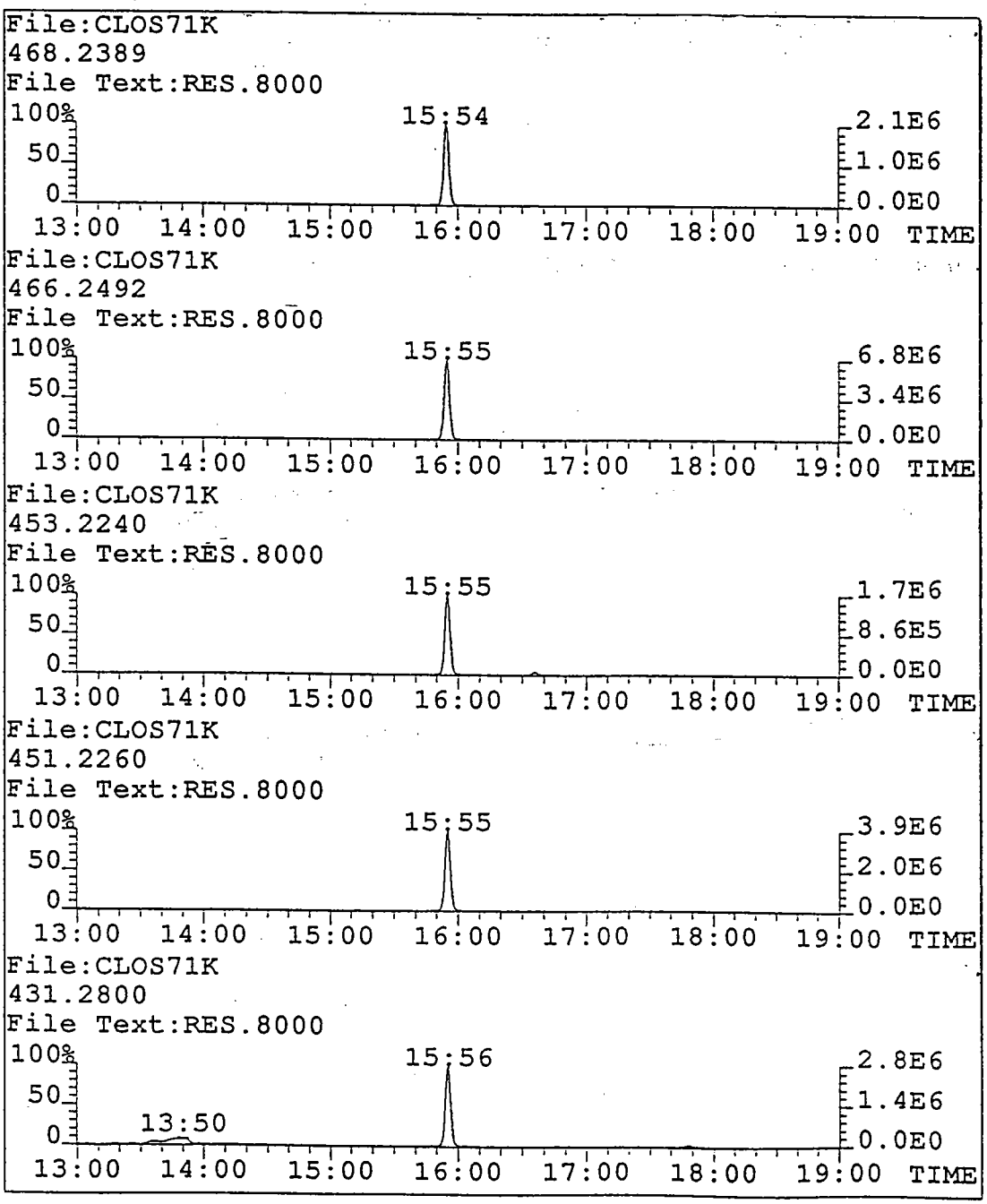

Figure 5A: Ion chromatograms for the clostebol positive reference urine sample (30 days after injection of Steranabol®)

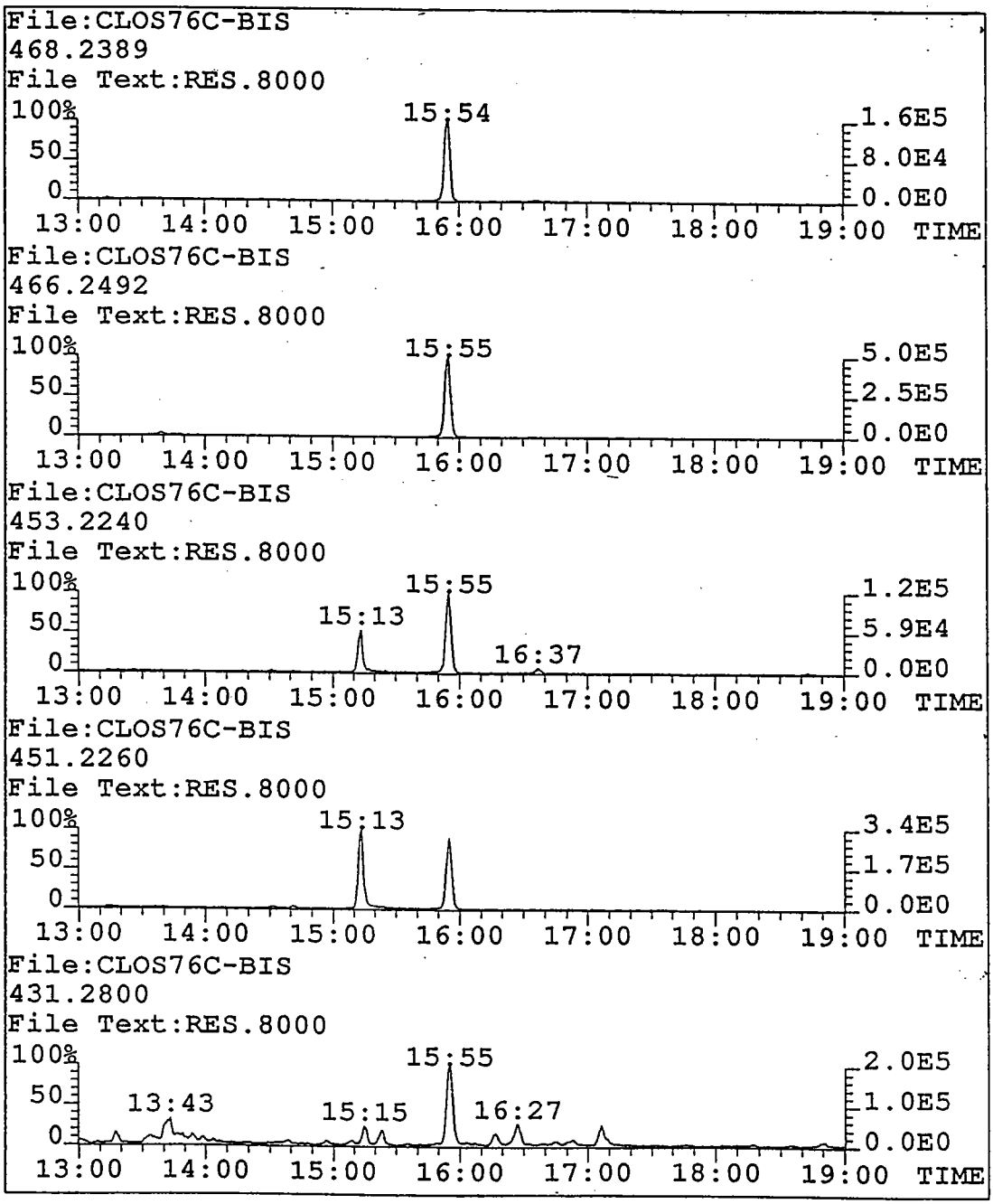

Figure 5B: Ion chromatograms for the urine sample obtained 7.40 hours after consumption of meat contaminated with clostebol acetate

DISCUSSION

Anabolic steroid detection

Documentation of the positive findings was done by the use of reference urine samples (nandrolone and clostebol), by combined use of different derivatization reagents (nandrolone), by determination of the relative abundances of the ions (clostebol), matching of SIM spectra (clostebol) and use of high resolution mass spectrometry (nandrolone and clostebol).

Anabolic steroids in urine due to consumption of hormone contaminated meat

Illegal use of hormonal growth promoters in cattle frequently occurs via injection of hormone cocktails. These hormone cocktails can contain anabolic androgenic steroids which are on the doping list. Large concentrations can be found at or around the injection site. Injection sites are often found in edible tissues of the animal carcass and hence can be processed into meat or meat products. The possible influence of the consumption of hormone contaminated meat on routine doping tests was investigated through the earlier described random experiments. Anabolic steroid metabolites were detected in urine of untreated persons after consumption of hormone contaminated meat.

Perspectives to the future

Notwithstanding the IOC statement that the presence of the drug in urine constitutes an offence, irrespective of the way of administration, it is a scientific challenge to look for a way to distinguish between oral undeliberate intake of anabolic steroids via the meat and deliberate doping. Some considerations are described in the following. They could serve as an initial guideline for further research on the doping interference problem.

Determination of a cut-off level[2]

Some authors proposed a cut-off level for the control of the illegal use of nandrolone. This cut-off level was installed since endogenous presence of nandrolone metabolites could not be excluded. Hence, levels under this cut-off value could be due to the endogenous presence of 19-norandrosterone and higher levels would be considered as doping positive. Björkhem and Ek[8] proposed a cut-off level of 100 ng/ml for 19-norandrosterone and Kicman and Brooks[9] proposed the same value, however for combined 19-norandrosterone

and 19-norepiandrosterone. For control of clostebol, no cut-off level was proposed in the literature. If we would adopt the 100 ng/ml cut-off level for 19-norandrosterone and CLOS-MET, none of the untreated persons in which anabolic steroid metabolites were found after undeliberate consumption of contaminated meat would be considered as doping positive. However, if also considering the concentrations found after controlled intake of nandrolone and clostebol acetate (control experiments), highest concentrations of 19-norandrosterone and CLOS-MET in urine approach or even exceed the proposed cut-off level. Control experiments were carried out with amounts which might be present in injection sites. Hence, the cut-off level approach for distinction between oral undeliberate ingestion and deliberate intramuscular injection is not defendable, since it would be unrealistic to further increase the cut-off level.

Determination of the retrospectivity[2]

Instead of establishing a cut-off level value, retrospectivity of the presence of the metabolites in urine could be considered. The main difference between a non-deliberate oral intake and a deliberate injection, is that a positive urine sample, originating from the ingestion of contaminated meat, is only a random indication. In our experiments, metabolites of nandrolone and clostebol were detectable for 1 or 2 days. A new urine sample taken afterwards will give negative results. With deliberate administration of pharmaceutical preparations as Deca Durabolin® or Steranabol® with the intention to improve the athletic performance, metabolites can be detected for weeks or even months. Another urine sample taken 24 h or 48 h after the first will under those circumstances most certainly give a positive result. The problem becomes more complicated when intramuscular injection has occurred weeks or months before or when shorter acting formulations are used. In both cases, it might be possible that a sample taken a few days after a first positive sample, is negative, despite the deliberate administration.

Detection in blood[2,5]

There is a tendency towards the use of blood samples in doping analysis, especially for the control of peptide hormones. If blood samples are available next to urine samples, there is a possibility that detection in blood of orally inactive anabolics could differentiate between deliberate and undeliberate intake. Orally inactive drugs are injected intramuscularly under the form of their esters in an oily solution or suspension. The esters migrate to the

systemic circulation, where hydrolysis occurs under the influence of esterases. As such the parent compound can be detected in the blood stream. Literature on the pharmacokinetics of anabolic steroids in healthy men is scarce. For nandrolone, plasma levels are reported in the detectable lower ng/ml level during the first days after injection of nandrolone esters[10,11]. When an orally inactive anabolic is orally ingested, the compound is transported via the portal vein to the liver. Since for orally inactive compounds as testosterone and analogues, a considerable proportion of the hormone is altered by passage through the liver before reaching the systemic circulation[12], levels of the parent compound after oral consumption will be much lower than those after deliberate injection. Only a minor part of the already small intake will be present in the plasma as parent compound and therefore in a much lower concentration, presumably far below the nowadays limit of detection. Therefore positive findings in urine (metabolite) and negative findings in blood (parent compound) could suggest that the drug is present in urine as a result of the consumption of contaminated meat. However, even when the scientific data were sound, the legal aspects of the doping interference problem remain problematic.

CONCLUSION

Anabolic steroid metabolites have been detected in the urine of untreated persons. The anabolic steroid metabolites were present due to consumption of hormone contaminated meat. These findings can have implications for routine doping control.

For us, the before-mentioned proposed strategies for distinguishing deliberate use (doping) from undeliberate consumption of hormone contaminated, are not yet scientifically feasible. The scientific challenge remains. Hence, taking into consideration the IOC statement "the presence of the drug in urine constitutes an offence, irrespective of the way of administration", we propose to warn the athletes not to eat any risky beef meat during the 24 hours preceeding competition. Consumption of hormone contaminated meat occurs at own risk until new scientific data would arise distinguishing undeliberate use from doping.

REFERENTIES

1. Debruyckere, G., de Sagher, R., De Leenheer, A. and Van Peteghem, C., The impact of nandrolone metabolites, occurring normally in male urines, on the cut-off level stipulated for nandrolone doping. In : "Advances in Steroid Analysis '90". Proceedings of the 4th Symposium on the Analysis of Steroids, Pécs (Hungary), April 24-26, 1990. S. Görög (Ed.), Akadémiai Kiado, Budapest (1991) pp. 363-370.

2. Debruyckere, G., Gas chromatographic - mass spectrometric detection of interferences in urinary anabolic steroid analysis. Thesis presented in fulfilment of the degree of Doctor in Pharmaceutical Sciences, University of Ghent, Belgium, February 1, 1994.

3. Debruyckere, G., de Sagher, R. and Van Peteghem, C., Clostebol positive urine after consumption of contaminated meat. Clin. Chem., 38 (1992) 1869-1873.

4. Debruyckere, G., de Sagher, R. and Van Peteghem, C. Influence of the consumption of meat contaminated with anabolic steroids on doping tests. Anal. Chim. Acta, 275 (1993) 49-56.

5. Debruyckere, G., de Sagher, R., Van Peteghem, C., Confirmation of clostebol positive urine samples. In: "Blood Samples in Doping Control". Proceedings of the Second International Symposium on Drugs in Sports, Towards the Use of Blood Samples in Doping Control? Lillehammer, Noorwegen, 29-31/8/93, P. Hemmersbach and K.I. Birkeland (Ed.), Hormone Laboratory, Aker Hospital, Oslo (1994) pp. 117-129.

6. Commission Decision of 14 April 1993 laying down the methods to be used for detecting residues of substances having a hormonal or a thyrostatic action (93/256-/EEC). Official Journal of the European Communities, No 118/64, 14.5.93.

7. Debruyckere, G., de Sagher, R., Van Peteghem, C., Van Vyncht, G., Maghuin-Rogister, G. and De Pauw, E., Gas chromatographic - mass spectrometric confirmation of a clostebol metabolite in urine. Anal. Chim. Acta, 291 (1994) 155-160.

8. Björkhem, I. and Ek, H., Detection and quantitation of 19-norandrosterone in urine by isotope dilution - mass spectrometry. J. Steroid Biochem., 17 (1982) 447-451.

9. Kicman, A.T. and Brooks, R.V., A radioimmunoassay for the metabolite of the anabolic steroid nandrolone. J. Pharm. Biomed. Anal., 6 (1988) 473-483.

10. Belkien, L., Schürmeyer, T., Hano, R., Gunnarsson, P.O. and Nieschlag, E., Pharmacokinetics of 19-nortestosterone esters in normal men. J. Steroid Biochem., 22 (1985) 623-629.

11. Courtot, D., Forichon, F. and Paris, J., Pharmacokinetics of 19-nortestosterone in man, Chromatography in Biochemistry, Medicine and Environmental Research, 1. A. Frigerio (Ed.), Elsevier Scientific Company, Amsterdam (1983) pp. 95-110.

12. Murad, F. and Gilman, A.G., Androgens and anabolic steroids. The Pharmacological Basis of Therapeutics. Goodman, L.S. and Gilman, A. (Ed.), Macmillan, New York (1975) p. 1451.