Rosa Bergés[1,2], Montserrat Solé[2], Rosa Ventura[1,2], Jordi Segura[1,2]

Detection of low molecular weight doping agents in urine ultrafiltrates obtained during rEPO determination

[1] Unitat de Recerca en Farmacologia, Institut Municipal d'Investigació Mèdica (IMIM);
[2] Universitat Pompeu Fabra, Doctor Aiguader 80, E-08003, Barcelona, Spain.

Introduction

The procedure used in doping control for the detection of the recombinant erythropoietin isoforms (rEPO α, β and ω) and darbepoetin (NESP) administration is the urinary analysis by isoelectric focusing, double blotting and chemiluminiscent detection [1]. This analysis requires the concentration of a large volume of urine (20 ml) by ultrafiltration through a membrane with a nominal molecular weight cut off of 30,000 Da in order to reach adequate sensitivity for rEPO/NESP detection.

When usual screening procedures must be applied to the sample, including some potential confirmation steps requiring multiple replicates, the volume of urine available can be a limiting factor. The filtrate has no interest for rEPO analysis but can be used for initial screening analyses of low molecular weight drugs such as anabolic agents, diuretics, stimulants or narcotics and this may be useful if small volumes of urine are available.

In this work the usefulness of the urinary filtrate obtained during the rEPO/NESP analysis as the sample for the initial screening of low molecular weight doping agents has been studied.

Experimental

Samples spiked with known concentrations of forbidden substances included in the WADA list [2] were prepared using blank urines obtained from different volunteers. Three replicates of these samples were analyzed directly using the common screening procedures for doping

control. Moreover, three replicates of the spiked samples were analysed after the ultrafiltration step applied during the rEPO/NESP determination.

Different parameter were evaluated to assure that the substances studied can be detected in the urine filtrate: changes in the chromatographic behaviour due to the matrix effect, occurrence of interferences in samples after filtration and differences between area ratios of the compounds vs. the internal standard in samples with and without the filtration step. Percentage of recovery of the different substances studied in urine filtrates were calculated to investigate potential losses by filter retention. All these calculations were done both for spiked compounds and for endogenous substances excreted conjugated in urine.

Results and discussion

The percentages of recovery of the substances studied are presented in Figure 1. In general terms, the substances studied were well recovered after the ultrafiltration performed during rEPO/NESP determination. Losses higher than 20% were observed for some compounds such as strychnine, bendroflumethiazide, buprenorphine and 11-nor-Δ^9-tetrahydrocannabinol carboxylic acid (cannabis metabolite). For some diuretics (i.e. acetazolamide), changes introduced during the filtration step resulted in an increase of the recovery producing a substantial improvement of their detection limits. Contrarily, significant losses (>20%) due to filtration of the urine were observed for a few metabolites of anabolic agents (4-chloroandrost-4-en-3α-ol-17-one, 2α-methyl-5α-androstan-3α-ol-17-one, epimetendiol, 17α-methyl-5α-androstane-3α,17β-diol and 3'-OH-stanozolol). Endogenous conjugated steroids were studied by means of actual excretion urines from non-treated subjects.

Conclusions

The use of the urinary ultrafiltrate obtained during the rEPO/NESP procedure for the initial screening analysis of forbidden drugs of low molecular weight is possible. When rEPO/NESP analysis is requested in addition to a normal screening test and a small urine volume is available, pH, density and peptide hormones (β-hCG and LH) must be measured on the non-filtrated urine but the filtrate is an adequate specimen for the rest of the screening doping

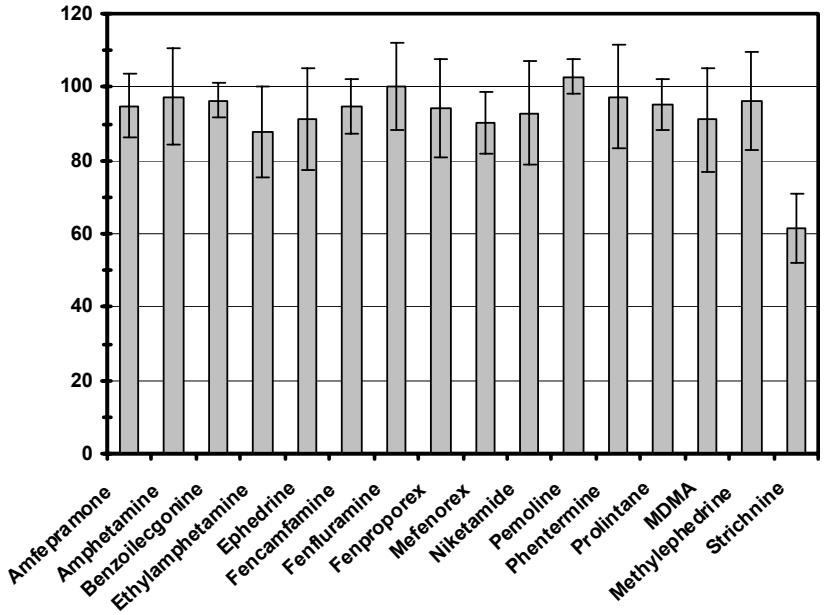
control procedures. The problem of some substances due to filtration of urine can be solved by increasing the sensitivity of the instrumental analysis.

References

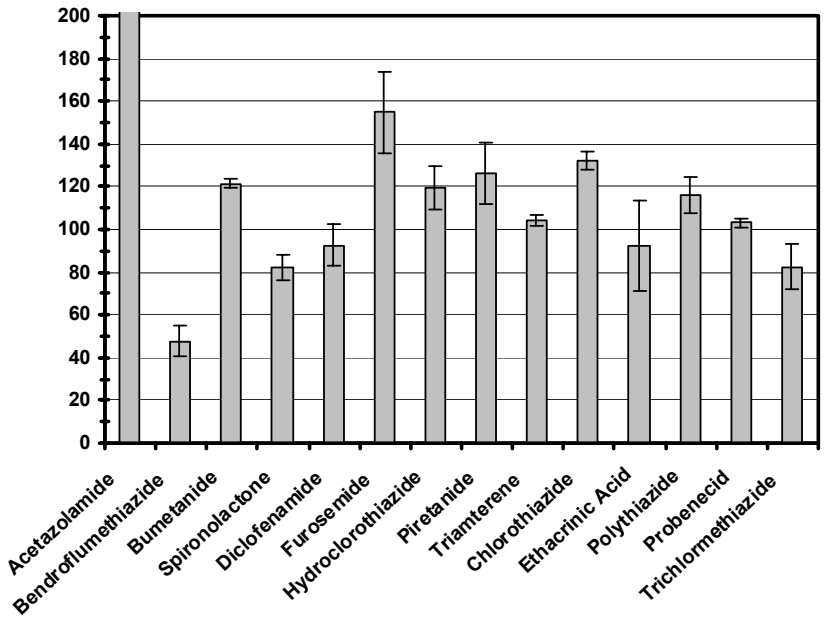
[1] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz. Anal. Biochem., 311 (2002): 119-126.
[2] World Anti-doping Code: The 2005 Prohibited List, International Standard. World Anti-doping Agency.

Figure 1. Percentage of recovery of the different substances studied (A: stimulants; B: diuretics; C: narcotics, β2-agonists and cannabinoids; D: exogenous anabolic agents; E: endogenous anabolic agents).

A:



B:



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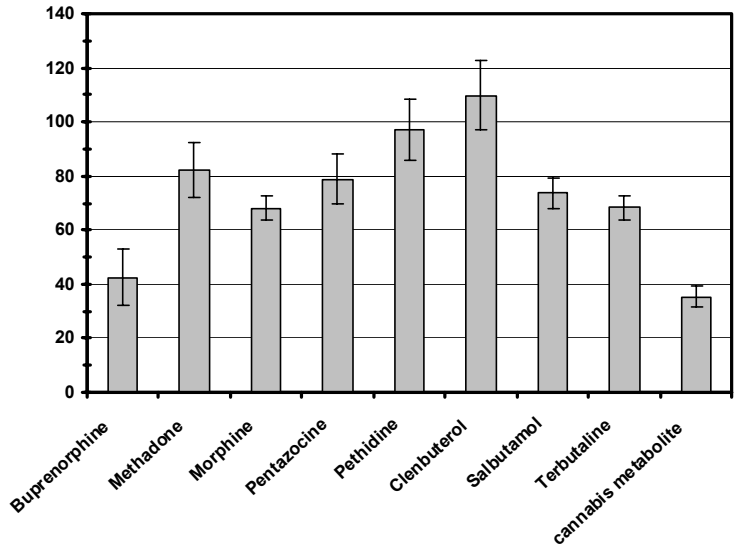
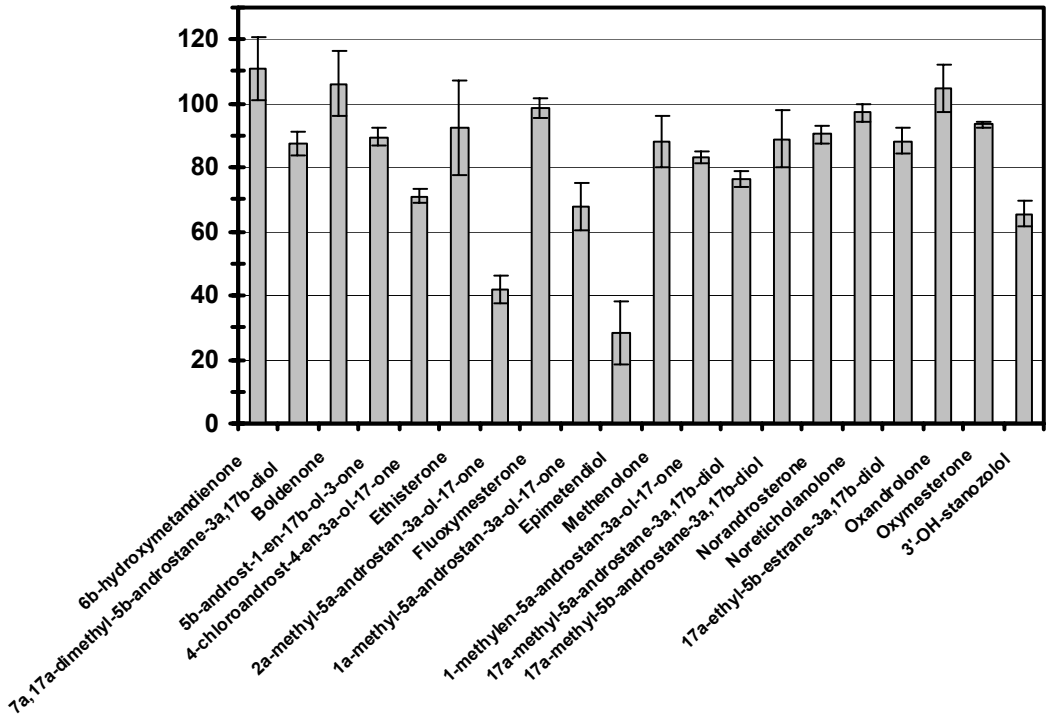
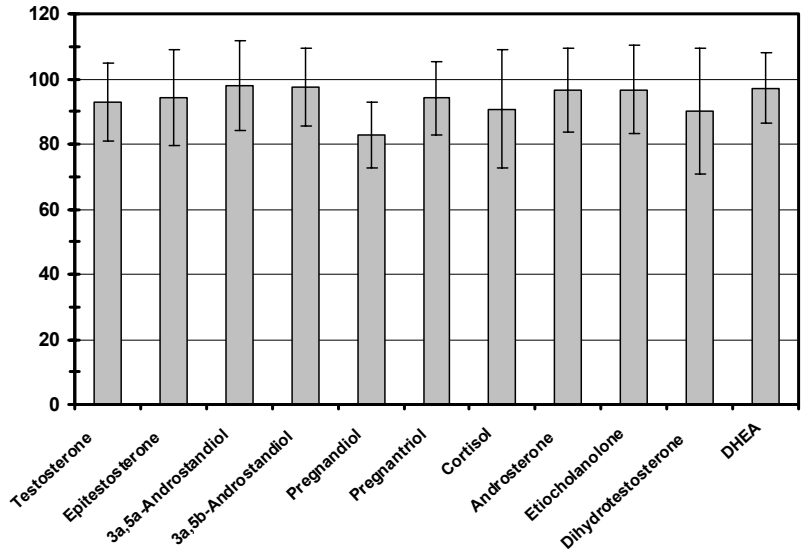

140
120
100
80
60
40
20
0
Buprenorphine
Methadone
Morphine
Pentazocine
Pethidine
Clenbuterol
Salbutamol
Terbutaline
cannabis metabolite

D:


120
100
80
60
40
20
0
6b-hydroxymetandienone
7a,17a-dimethyl-5b-androstane-3a,17b-diol
Boldenone
5b-androst-1-en-17b-ol-3-one
4-chloroandrost-4-en-3a-ol-17-one
Ethisterone
2a-methyl-5a-androstan-3a-ol-17-one
Fluoxymesterone
1a-methyl-5a-androstan-3a-ol-17-one
Epimetendiol
Methenolone
1-methylen-5a-androstan-3a-ol-17-one
17a-methyl-5a-androstane-3a,17b-diol
17a-methyl-5b-androstane-3a,17b-diol
Norandrosterone
Noreticholanolone
17a-ethyl-5b-estrane-3a,17b-diol
Oxandrolone
Oxymesterone
3'-OH-stanozolol

E: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100
80
60
40
20
0
Testosterone
Epitestosterone
3a,5a-Androstandiol
3a,5b-Androstandiol
Pregnandiol
Pregnantriol
Cortisol
Androsterone
Etiocholanolone
Dihydrotestosterone
DHEA