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Recovery of polar and non polar substances from the ultrafiltrate fraction of the EPO aliquot

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INTRODUCTION

The procedure used in doping control for the detection of recombinant human erythropoietin (rhuEPO) is based on the urinary analysis of the EPO isoforms by isoelectric focusing, double blotting and chemiluminiscent detection. This method requires a large volume of urine (20 ml), to be concentrated by ultrafiltration through a membrane with a nominal weight cut off of 30,000 Da. In the case of samples requiring a confirmation analysis, with multiple replicates, the volume of urine could be a limiting factor; for this reason the goal of this study was to verify whether the ultrafiltrate fraction produced at the first stages of the pretreatment of urine samples for EPO analysis can be used for the analysis of low molecular weight drugs (anabolic agents, diuretics, stimulants, narcotics and beta blockers). The recovery of the different substances in the ultrafiltrate fraction was evaluated on spiked and real urines, analysed according to the ISO 17025 screening procedures of the laboratory of Rome.

EXPERIMENTAL SECTION

Isolation of ultrafiltrate fraction

To 20 ml of spiked urine 400 µl of protease inhibitor ("Complete") and 2 ml of tris-(hydroxymthyl)-aminomethane hydrochloride (Tris-HCl) 3.75 M were added; the sample was then centrifugated for 10 min, filtrated by Steriflip and the filtrate fraction transferred to a Centricon plus 20 and centrifuged for 20 minutes.

Anabolic agents (steroids and beta-agonists) and beta-blockers

Two 3 ml aliquots of the filtrate fraction and two aliquots of 3 ml of the same spiked sample but without filtration were analysed using the following procedure: to 3 ml of urine 50 µl of internal standard (17α-methyltestosterone), 1 ml of 0.2 M phosphate buffer pH 7.4 and 30 µl of beta-glucuronidase from E. coli were added and hydrolysis was performed for 1 h at 50 °C. The buffered solution was alkalinised with 1 ml of 0.1 M potassium carbonate solution to pH 8-9 and the anabolic agents were extracted with 10ml of tert-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation, the etheral layer was transferred and evaporated to dryness under vacuum; the residue was derivatized by 50 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA):NH_4I:Dithioerythreitol (1000:2:4 v/w/w).

Diuretics

Two aliquots of 3 ml of the filtrate fraction and two aliquots of 3 ml of the same spiked sample but without filtration were analysed using the following procedure: to 3 ml of urine 50 µl of internal standard (indomethacine/mefruside), 800 µl of 4 M carbonate/bicarbonate buffer solution to pH 10 and the basic drugs were extracted with 6ml of a mixture of chloroform:isopropanol:tert-butylmethyl ether (80:10:10) on a mechanical shaker for 5 minutes. After centrifugation, the etheral layer was transferred and evaporated to dryness under vacuum; the residue was dissolved in 100 µl of formate/formic acid buffer 5 M and the acid agents were extracted with 6ml of a mixture of chloroform:isopropanol: tert-butylmethyl ether (80:10:10) on a mechanical shaker for 5 minutes. After centrifugation, the etheral layer was transferred and evaporated to dryness under vacuum; the residue was derivatized by 200 µl of a mixture of acetone/iodomethane (9/1) and 50 mg of anhydrous potassium carbonate for ten minutes at 100 °C.

Stimulants and narcotics

Two aliquots of 2 ml of the filtrate fraction and two aliquots of 2ml of the same spiked sample but without filtration were analysed using the following procedure: to 2 ml of urine 50 µl of internal standard (diphenylamine), 0.2 ml of soda and 1 g of sodium chloride were added and stimulants were extracted with 2 ml of tert-butyl methyl ether on a mechanical shaker for 5 minutes. After centrifugation, the etheral layer was transferred and evaporated to dryness under vacuum and diluted in 50 µL of tert-butylmethyl ether.

RESULTS AND DISCUSSION

The recovery data for some of the compounds studied are reported in Table 1. All data were normalized to the recovery values obtained by the reference procedure (no ultrafiltration). As it can be seen, the recovery was very low for the most polar substances. Some examples of the quantitative values obtained for threshold substances are given in Figure 1.

TABLE 1 Recovery data for representative anabolic agents, diuretics, beta-blockers, stimulants and narcotics.

ANABOLIC AGENTS (steroids, beta2-agonists and antioestrogens)

Substances	Traditional (%)	Filtrate (%)
Steroids		
Bolasterone	100	73
Boldenone	100	72
Chlormetandienone	100	59
4-chloro-4-androsten-3α-ol-17-one	100	55
Danazol m.	100	5
2α-methyl-5α-androstan-3α-ol-17-one	100	71
Epioxandrolone	100	88
Epitestosterone	100	77
Epitrenbolone	100	62
9α-fluoro-17,17-dimethyl-18-norandrosta-4,13-diene-11β-ol-3-one	100	53
16β-OH-furazabol	100	41
Epimetendiol	100	80
6β-OH-metandienone	100	85
17α-methyl-5α-androstene-3α,17β-diolo	100	67
17α-methyl-5β-androstene-3α,17β-diolo	100	78
Mibolerone	100	75
Norandrosterone	100	79
17α-ethyl-5β-estrane-3α,17β-diolo	100	70
Testosterone	100	95
3'-OHstanozolol	100	85
Beta2-agonists		
Salbutamol	100	90
Bambuterol	100	82
Terbutaline	100	60
Salmeterol	100	70
Fenoterol	100	88
Procaterol	100	55
Zeranol	100	68
Clenbuterol	100	95
Antioestrogens		
Anastrozol	100	90
Formestane	100	70
Exemestane	100	65

DIURETICS AND BETA-BLOCKERS

Substances	Traditional (%)	Filtrate (%)
Diuretics		
Acetazolamide	100	95
Amiloride	100	76
Althiazide	100	53
Bendroflumethiazide	100	47
Benthiazide	100	41
Brinzolamide	100	95
Bumetanide	100	97
Butizide	100	38
Canrenone	100	91
Clamide	100	55
Chlorthalidone	100	71
Chlorothiazide	100	94
Dichlorphenamide	100	92
Dorzolamide	100	96
Fenquinizone	100	94
Furosemide	100	94
Hydrochlorthiazide	100	88
Indapamide	100	89
Methylchlorthiazide	100	56
Piretanide	100	96
Probenecid	100	94
Torasemide	100	87
Triamterene	100	88
Xipamide	100	81
Beta-blockers		
Acebutolol	100	69
Alprenolol	100	67
Atenolol	100	68
Betaxolol	100	69
Bisoprolol	100	77
Carteolol	100	51
Carvedilol	100	32
Celiprolol	100	77

STIMULANTS AND NARCOTICS

Substances	Traditional (%)	Filtrate (%)
Stimulants		
Bromantan	100	49
Ethamivane	100	77
Ethylefrine	100	80
Pholedrine	100	74
Pemoline	100	87
Amphetamine	100	55
Fentermine	100	62
Cathine	100	20
Niketamide	100	79
Ephedrine	100	65
Fencamfamine	100	60
MDMA	100	85
Fenetilline	100	80
Pentazocine	100	68
Pipradol	100	94
Caffeine	100	88
Pentetrazol	100	65
Methafetamine	100	38
Narcotics		
Morphine	100	69
d,l-11-nor-9-carboxy-D9-THC	100	33
Methylphenidate	100	98
Oxycodone	100	46
Oxymorfone	100	30

Conclusions

- Our results show that the concentration of some substances, and specifically those of the more polar compounds, decreased significantly following the ultrafiltration process.
- Our findings also suggest that the ultrafiltration fraction can be used, without the risk of false negative, only for the screening analysis of diuretics, some stimulants and beta-blockers.
- Finally, the data here presented suggest that the ultrafiltration fraction should not be used for confirmation of threshold substances (primarily norandrosterone, d,l-11-nor-9-carbossi-D9-THC and ephedrine).

Ultrafiltrate fraction **Whole urine**

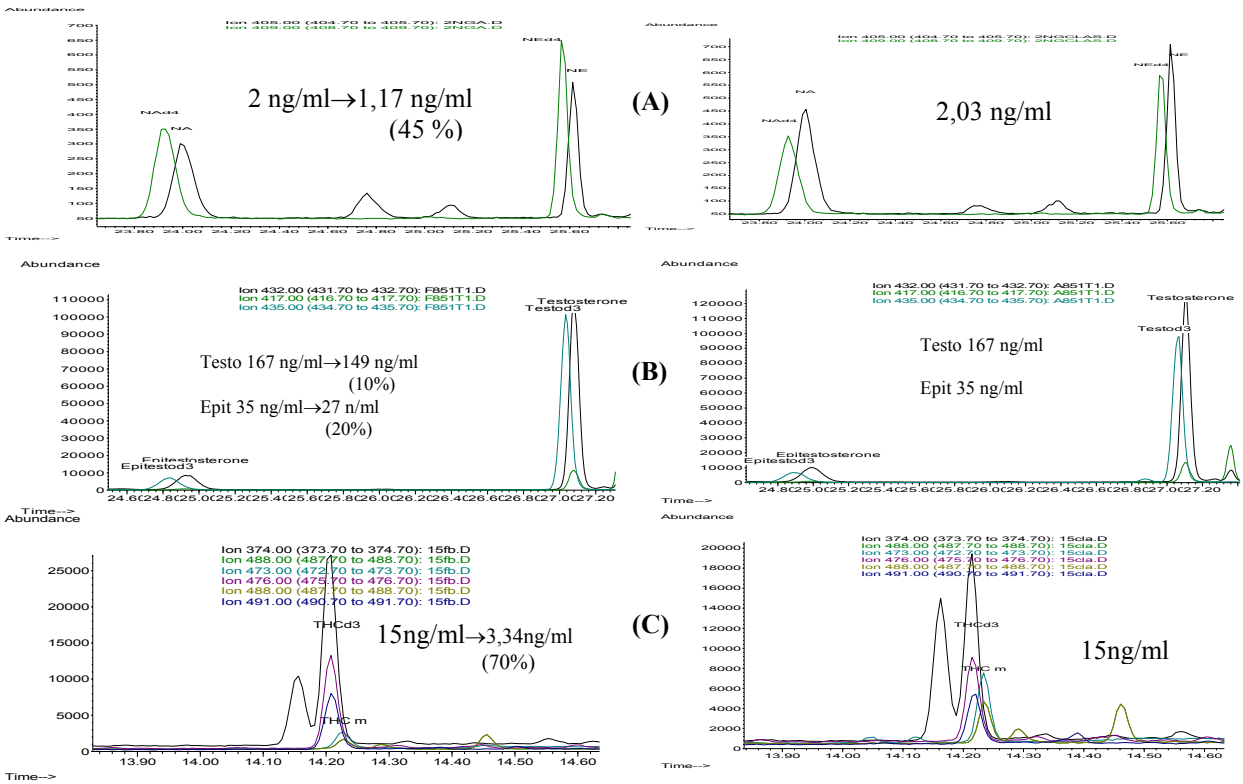

Figure 1. Quantitation of threshold substances: comparison between the data obtained on the whole urine and on the EPO ultrafiltrate fraction. Urine spiked with 2 ng of norandrosterone/ml and 2 ng of noretiocholanolone/ml **(A)**; routine sample, with an elevated T/E ratio **(B)**; urine spiked with 15 ng of d,l-11-nor-9-carboxy-D9-THC /ml **(C).**