Rapid and specific extraction of anabolic steroids (A1, A3, A4) and corticosteroids in urine before detection and identification by UPLC-MS/MS

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I. Introduction

In the European Union, Council Directive 96/23/EC prohibits the use of anabolics as growth promoters for meat production. As the number of molecules abused has increased over the years, "anabolic cocktails" have made their appearance, *i.e.,* mixtures containing several different agents at law but still active concentrations. Because their effects are additive, the resulting mass increases recorded in cattle are similar to those produced by single compounds at higher concentration, but since smaller amounts of each agent are eliminated in biological specimens, they are much more difficult to trace.

Detection requires highly sensitive techniques with law background interference. Immunoaffinity purification techniques offer tremendous and decisive advantages over conventional methods used to pre-treat biological specimens. In this work, we combined multiresidue immunoaffinity chromatography with efficient liquid chromatographic separation. This efficient method, validated for 44 compounds, is widely applicable to urine samples, *i.e.,* the biological specimens that veterinary inspectors usually take in slaughter houses and farms. It could provide a basis for a universal procedure for detecting multiple anabolic agents and their metabolites in such specimens.

II. Methods

II.1. Extraction

2 mL Urine
Hydrolysis
Centrifuge 5 min at 2700 g
Purification on OASIS HLB
Purification on Multi-Immunoaffinity column
Analysis by UHPLC-MS/MS

Injection 1 in ESI – Estrogens

Injection 2 in ESI + All others compounds

II.2. Validation

- According to EU Decision 2002/657/EC
- Specificity and CCβ : analysis of 20 blank and 20 fortified urine samples at the decision limit (ppb-level)

II.3. Chromatography

- Acquity UPLC (Waters)
- Column : Acquity HSS T3 (150 x 2.1 mm, 1.8 µm)
- Mobile phase : ESI+(A) H2O+0.1% FA, (B) MeOH+0.1% FA
  ESI- (A) H2O, (B) ACN + post column delivery of NH_4OH
- Injection volume : 20 µl
- Run time : ESI+ (14 min) ; ESI- (8,5 min)
- Flow rate :0.45 ml/min

II.4. Mass spectrometry

- Sample analysis required two injections per sample ESI-/ESI+ mode
- Xevo TQS (Triple quadrupole mass spetrometer)
- MRM mode : 2 MRM transitions for most compounds
- Acquisition method contained 36 MRM for the injection in ESI- and 78 MRM for the injection in ESI+

III. Results

Table 1 : Decision limits (CCα) and detection capabilities (CCβ)

	CCα (µg L^{-1})	CCβ (µg L^{-1})		CCα (µg L^{-1})	CCβ (µg L^{-1})
Steroids			**Steroids**		
Ethyloestranediol	≤2.26	3	Ethynylestradiol	≤1.50	2
17β-nortestosterone	0.11	0.125	Methenolone	0.69	0.8
17α-nortestosterone	0.20	0.25	Zearalenone	0.91	1
17β-boldenone	0.22	0.25	Zeranol	0.38	0.5
17α-boldenone	0.44	0.5	Taleranol	0.38	0,5
17β-testosterone	0.94	1	Zearalanone	0.92	1
5α-androst-1-en-17α-ol-3-one	0.45	0.5	α-Zearalenol	0.91	1
Methylboldenone	0.45	0.5	β-Zearalenol	0.87	1
Methyltestosterone	0.22	0.25			
Norethandrolone	0.11	0.125	**Corticosteroids**		
Norgestrel	0.12	0.125			
Progesterone	0.93	1	Prednisone	0.88	1
4-chloro-androst-4-en-3,17-dione	0.22	0.25	Prednisolone	0.23	0.25
Stanozolol	0.23	0.25	Methylprednisone	0.43	0.5
16β-hydroxy-stanozolol	0.12	0.125	Methylprednisolone	0.47	0.5
Fluoxymesterone	0.22	0.25	Isoflupredone	0.24	0.25
Medroxyprogesterone acetate	0.11	0.125	Dexamethasone	0.10	0.125
17α-trenbolone	0.46	0.5	Flumethasone	0.12	0.125
17α-methyl-5β-androstane-3α,17-β-diol	2.29	3	Clobetasol	≤0.75	1
Dienestrol	0.76	1	Triamcinolone acetonide	0.04	0.05
Diethylstilbestrol	0.41	0.5	Fluocinolone acetonide	0.34	0.4
Hexestrol	0.39	0.5	Fluorometholone	0.33	0.4
17β-estradiol	≤1.50	2	Beclomethasone	≤3.01	4

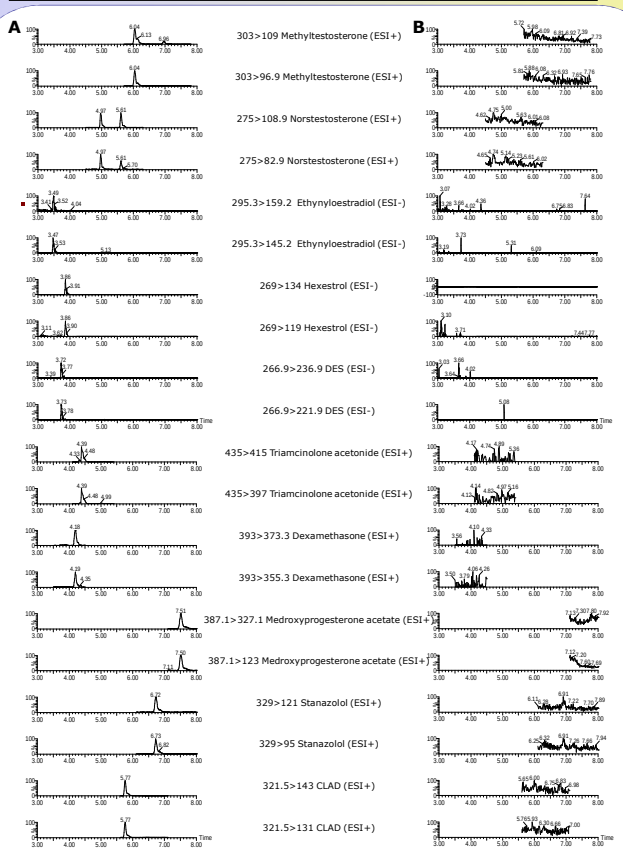

Fig. 1. Selected UHPLC-MS/MS chromatograms in ESI+ and ESI- Mode (A) a treated sample spiked at CCβ and (B) a blank urine

IV. Conclusion

The approach presented here, i.e., combining purification by multi-immunoaffinity chromatography with UPLC-MS/MS detection, has numerous and decisive advantages over conventional purification techniques currently used in most accredited laboratories.

All products initially present could be clearly detected in all processed samples. Application of the new method should considerably reduce time and labour, thereby reducing the cost of the analytical process and increasing sample throughput. This amply justifies using the additional MIAC purification step, which additionally reduces background signals urine and increases detection sensitivity.

V. Aknowledgment

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