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Comprehensive screening of urinary free fraction using HPLC-MS/MS

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Abstract

Two high-throughput high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods were developed for the screening of five important classes of drugs in sports, namely 23 corticosteroids, 12 anabolic agents, 23 diuretics, 49 stimulants and narcotics. Sample preparation included liquid-liquid extraction, evaporation of organic layer in nitrogen stream and reconstitution of dry residue. Thereafter two HPLC-MS/MS injections from the same vial (aliquot) were done with different mode of registration: negative ions for corticosteroids and most diuretics, and positive ions for some diuretics, anabolic steroids, narcotics and stimulants.

Introduction

Corticosteroids, diuretics, narcotics, stimulants and anabolic agents are listed in the Prohibited List issued by WADA (World Anti-Doping Agency) list [1]. Several publications referring to simultaneous determination of these classes using HPLC-MS/MS or GC-MS have been appeared recently [2-6]. This approach is very promising, and we have developed uniform screening procedure including two high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) methods for the comprehensive screening of corticosteroids, diuretics, stimulants, narcotics, and anabolic steroids. "Two methods" means that after single sample preparation step two injections from the same vial have been done in two different acquisition modes for the registration of negative ions for corticosteroids and diuretics, and positive ions for all the others. Detection was performed in selective reaction monitoring (SRM) mode with two segments for each method.

Materials and Methods

Sample pretreatment: 50 µL of internal standards solution (fluoxymesterone 10 µg/mL, mefruside 10 µg/mL, methyltestosterone 10 µg/mL) was added to 5 mL urine followed by addition of 500 mg K₂CO₃/KHCO₃ (2:1) for to adjust pH to 9.5. Liquid-liquid extraction was performed for 10 min by rolling with 5 mL diethyl ether in presence of 1 g

ammonium sulphate. After centrifugation (3000 rpm) the organic layer was evaporated to dryness. The remaining residue was reconstituted in 100 µL of the initial mobile phase.

HPLC-heated electrospray ionization-tandem mass spectrometry: HPLC-HESI-MS/MS was performed using Thermo Finnigan TSQ Quantum triple stage quadrupole mass spectrometer equipped with a heated ESI ion source connected to HPLC Surveyor Plus system with autosampler and a quaternary pump (Thermo Electron). Analytical column Eclipse XDB-C18 (150 mm × 2.1 mm I.D., 5 µm, Zorbax, Agilent) was connected to C18 guard-cartridge. The mobile phase was methanol (solvent A) and 0.05% formic acid in water with 20 mM ammonium acetate pH 3 (solvent B) at flow rate of 200 µL/min. The solvent gradient program was as follows: initial methanol at 40%, then linearly increased to 90% for 8 min, isocratic for 1 min, linearly decreased to 40% for 12 min, and finally maintained at 40% for 18 min. Total run time was 18 min, the injection volume was 15 µL.

Results and Discussion

All target compounds were detected at low ng/mL levels (Table 1). Extraction recovery was ranged 40-90 % for most target analytes. Ion suppression for all compounds was within 10-25 % range. Validation data showed that both positive and negative ion methods have acceptable sensitivity, selectivity, and LOD sufficient for routine screening analysis, and no significant interference from matrix had been observed. These methods were also validated through their application to the analyses of real samples and post administration urine specimens. For samples that are suspicious after screening analysis, the confirmation of the analytes was done from another aliquot by matching the retention time and full-scan product-ion spectra with those of the corresponding reference standards. Since detection is based on mass-spectrometry, both methods can be easily modified to accommodate a number of new compounds.

References

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Table 1. Summary of corticosteroids, diuretics, stimulants, narcotics and anabolic agents screening by HPLC-MS/MS (IS* – ion suppression, %; R** – recovery, %; LD*** – limit of detection, ng/mL)

Substance	IS	R	LOD	Substance	IS	R	LOD
Amiloride	27	11	50	Adrafinil	16	43	10
Acetazolamide	39	23	50	Amitriptyline	8	55	50
Bendroflumethiazide	14	84	10	Amiphenazole	39	28	50
Benzthiazide	10	78	10	Amfepramone	33	16	75
Bumethanide	18	86	20	Amphetamine	16	31	50
Hydrochlorothiazide	30	89	10	Bupropion	30	54	50
Dichlorphenamide	15	92	20	Butorphanol	33	61	10
Indapamide	17	77	10	Heptaminol	4	19	50
Clopamide	11	87	10	Hydrocodone	38	72	10
Xipamide	33	85	10	Dimethylamphetamine	33	44	50
Metolazone	25	75	10	Carphedon	6	39	10
Mefruside	12	84	10	Codeine	23	83	10
Polythiazide	14	82	10	Cocaine	57	63	10
Probenecide	14	96	20	Caffeine	5	74	10
Spirolactone	8	62	20	Cropropamide	6	51	10
Torasemide	19	79	20	Crotethamide	20	53	10
Triamterene	27	88	10	MDMA	25	53	50
Trichlormethiazide	8	67	10	MDEA	25	53	50
Furosemide	17	64	10	Methadone	11	49	10
Chlorthalidone	24	88	10	Methylamphetamine	18	43	20
Chlorthiazide	17	20	50	Methylphenidate	15	49	50
Cyclopenthiiazide	16	83	10	Methylephedrine	35	45	10
Ethacrynic acid	6	74	20	Mephentermine	39	42	50
Beclomethasone	13	81	1	Modafinil	12	49	50

Betamethasone	16	91	1	Morphine	35	40	20
Budesonide	14	72	1	Nikethamide	27	65	10
Desoxymethasone	12	90	1	Norpseudoephedrine	23	36	10
Desonide	19	89	1	Norfenfluramine	19	46	10
Dexamethasone	16	91	1	Norephedrine	23	16	50
Diflorasone diacetate	24	73	5	Octopamine	27	10	150
Clobetasol	31	97	5	Pemoline	26	39	50
Cortisol	12	88	1	Pethidine	31	45	50
Cortisone	13	88	1	Prolintane	40	61	50
Methylprednisolone	23	80	5	Pseudoephedrine	18	32	50
Prednisolone	12	92	1	Selegiline	30	14	50
Prednisone	13	96	1	Sydnocarb	20	52	50
Triamcinolone	18	82	5	Sydnophenum	15	64	50
Triamcinolone acetonide	11	95	3	Synephrine	25	43	50
Fludrocortisone	14	89	1	Strychnine	32	67	50
Fludrocortisone	23	89	1	Famprophasone	17	53	10
Flumethasone	10	93	2	Phendimetrazine	24	60	50
Flunisolide	16	92	0.5	Phenmetrazine	19	55	50
Fluoxymesterone	25	97	10	Phentermine	19	41	50
Fluocinolone	7	87	5	Phenfluramine	23	45	50
Fluocortolone	19	85	5	Pholedrine	31	20	10
Fluorometholone	15	85	5	Ethamivan	8	56	10
Ephedrine	18	32	10	Ethylamphetamine	33	44	10
Gestrinone	14	54	1	4 β -OH-STR	5	45	0.5
Clenbuterol	47	85	0.5	Tetrahydrogestrinone	13	51	1
Oxandrolone	9	47	1	Testosterone	10	57	1
Oralturinabol	12	49	1	Trenbolone	12	54	1
3'-OH-STR	9	47	0.5	Epitestosterone	10	57	1
16 β -OH-STR	1	43	0.5	Methyltestosterone	12	88	1
4 α -OH-STR	5	49	0.5				