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LC-MS/MS method development of an initial testing procedure for prohibited substances in sport

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Abstract

A general screening method based on enzymatic hydrolysis of conjugated substances followed by two stages of (pH 9.6 and 12) liquid-liquid extraction (LLE) and LC-MS/MS identification was developed. In this method 64 different doping agents, including anabolic agents, beta-2-agonists, hormone and metabolic modulators, diuretics, stimulants, narcotics, cannabinoids, glucocorticosteroids and beta-blockers were investigated. Chromatography was based on gradient elution on a rapid resolution C18 column and ionization of the analytes was achieved with electrospray ionization in positive ion mode. The performance of the method was evaluated with regard to specificity, analytical recovery, MRPL-criteria and repeatability. The method was proven specific and sensitive. The minimum required performance limit (MRPL), established by WADA, was attained to all qualitative doping agents. The extraction recoveries were higher than 80% for most of the analytes and above 25% for certain more polar compounds. The repeatability of ion ratios and retention times ranged between 0-17%. The developed method was proven applicable to the analysis of wide selection of prohibited substances, and allows flexible method extensions which may arise from the modifications of WADA prohibited list.

Introduction

The use of performance enhancing substances is prohibited in sports by the World Anti-Doping Agency (WADA). Doping analytics requires fast and reliable monitoring of several hundred substances, and their metabolic products from small sample volume. The purpose of this work was to develop a simple and rapid liquid chromatographic/tandem mass spectrometric (LC-MS/MS) screening method for selected doping agents in human urine and to respond the modifications of WADA prohibited list.

Experimental

Sample pretreatment

To 1 mL urine aliquot, 10 µL internal standard (dibenzepine), 760 µL 0.8 M K/Na-phosphate buffer, pH 7, and 25 µL β-glucuronidase were added. The mixture was incubated at 50 °C for one hour. Then 250 µL 20% (w/v) K_2CO_3/$KHCO_3$ (1:1) and 1 mL saturated NaCl-solution were added and the sample was extracted with 2 mL of ethyl acetate. The organic layer was separated and 600 µL 5 M KOH was added to the aqueous layer and then extracted with 2 mL of ethyl acetate. Organic layers were combined, evaporated to dryness and reconstituted in 100 µL of LC mobile phase (A:B, 90:10).

The performance of the method was evaluated with regard to specificity, analytical recovery, sensitivity at 50% MRPL and repeatability. Samples were analyzed with LC-ESI-MS/MS in Dynamic MRM mode and each analyte was monitored by two MRM-transitions (Table 1 and 2). Glucocorticosteroids were an exception and monitored by only one precursor-product ion-pair.

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Results and Discussion

Method development

Chromatographic conditions were tested concerning mobile phase and column length. Both methanol and acetonitrile were tested with and without buffer. The 50 mm and the 100 mm columns with the same phase material were tested. Best results (peak width, separation and ionization) for most of the compounds were attained with buffer in acetonitrile and 50 mm column. Oxymorphone was the first eluting compound and JWH-122 the last for the total run time being only 12 minutes.
In sample pretreatment diethyl ether and ethyl acetate were compared, and these results were in favor of ethyl acetate. Two-stage liquid-liquid extraction in very alkaline pH extends the method to stimulants.
The initial strategy included polarity-switching of the ionization, and for glucocorticosteroids also negative MRM-transitions were optimized. However, the delay between scans compromized the sensitivity and this feature could not be included in the final method.

Validation results

The method was proven specific and sensitive. The minimum required performance limit (MRPL) criteria, established by WADA, were fulfilled to all investigated doping agents (Table 2). Even clenbuterol, which has the most strict MRPL, was identified reliably in 0.1 ng/mL-level (Figure 1). The repeatability of ion ratios ranged from 0.1 to 12% (intra day) and from 0.1 to 17% (inter day). The repeatability of retention times was between 0 and 5% (intra and inter days). Extraction recoveries are summarized in Table 2.

LC	Agilent 1290, binary pump system
Column	Agilent Zorbax Eclipse Plus C18 2.1 x 50 mm (1.8 µm)
Oven temperature	30 °C
Eluent	A: 2.5 mM Ammonium formiate in 0.1% Formic acid B: 2.5 mM Ammonium formiate, 0.1% Formic acid in 90% ACN
Gradient	5 % B (held for 1 min) → 90 % B (in 8 min; held for 0.5 min)
Run time	9.5 min + 2.5 min post time
Injection volume	5 µl
MS/MS	Agilent 6460 Triple Quad LC/MS, positive mode ESI
Drying gas (N_2)	200 °C, 8 l/min
Sheat gas (N_2)	350 °C, 12 l/min

Table 1. The analysis conditions and parameters for LC-ESI-MS/MS

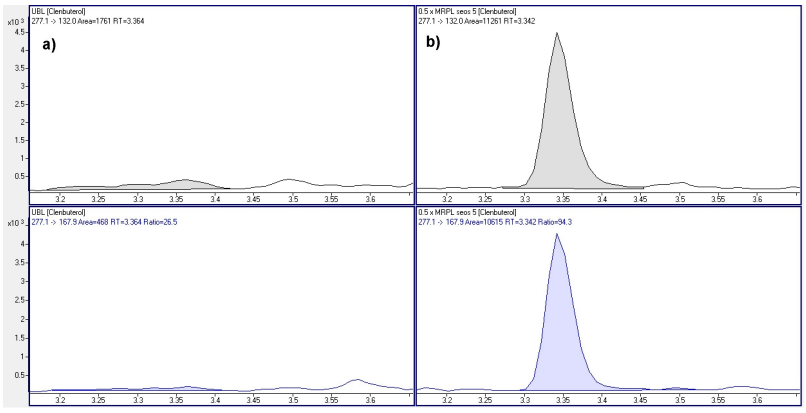

Figure 1: Extracted ion chromatograms of (a) a blank urine specimen and (b) a spiked urine sample at 0.1 ng/ml of clenbuterol.

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Compound	MRPL (ng/ml)	Precursor	Products	CE (V)	RT	Recovery%
S1. ANABOLIC AGENTS						
16β-OH-Stanozolol	2	345	81, 95	40, 40	4.84	86
3'-OH-Stanozolol	2	345	97, 107	40, 40	4.98	77
6β-OH-Methandienone	2	317	299, 281	4, 8	4.37	95
Clenbuterol	0.2	277	132, 168	28, 32	3.25	86
Fluoxymesterone-M	5	337	95, 67	24, 40	3.02	88
Formebolone-M	5	347	281, 147	12, 32	4.29	72
Gestrinone	5	309	291, 165	16, 30	6.19	93
Methyltrienolone	5	285	227, 198	24, 32	5.61	85
Oxandrolone	5	307	271, 229	12, 15	5.82	59
Tetrahydrogestrinone	5	313	295, 241	10, 20	6.82	90
Trenbolone-M	5	271	199, 179	24, 30	5.52	97
Zeranol	5	323	189, 149	24, 32	5.80	79
Zilpaterol	5	262	244, 185	8, 24	1.27	83
S3. BETA-2-AGONISTS						
Formoterol	40*	345	327, 91	12, 40	3.32	77
S4. HORMONE AND METABOLIC MODULATORS						
Anastrozole	20	294	210, 142	40, 40	5.18	92
Androst-1,4,6-triene-3,17-dione	20	283	147, 265	24, 10	5.72	92
Clomiphene	20	406	100, 58	24, 37	6.33	88
Exemestane	20	297	121, 149	20, 10	6.37	87
Exemestane-M	20	299	121, 147	28, 16	6.04	82
Fulvestrant	20	607	467, 159	28, 40	8.19	99
Fulvestrant-M	20	605	587, 377	20, 36	8.63	40
GW 1516	20	454	257, 188	32, 56	8.43	88
Raloxifene	20	474	84, 147	40, 50	4.44	87
Toremifene	20	406	45, 205	40, 40	6.32	95
S5. DIURETICS						
Amiloride	200	230	171, 116	16, 36	1.69	30
Canrenone	200	341	187, 169	24, 28	6.51	91
Eplerenone	200	415	121, 397	40, 12	5.10	95
Triamtrene	200	254	237, 104	28, 40	2.86	96
S6. STIMULANTS						
p-OH-Mesocarb	100	339	91, 193	40, 8	4.93	81
P2. BETA-BLOCKERS						
Carvedilol	100	407	100, 224	32, 24	4.76	100
Celiprolol	100	380	74, 251	36, 24	3.63	114

Compound	MRPL (ng/ml)	Precursor	Products	CE (V)	RT	Recovery%
S7. NARCOTICS						
3-Methylfentanyl	2	351	202, 105	24, 40	4.55	89
6-Monoacetylmorphine	50	328	193, 191	28, 50	2.54	87
Fentanyl	2	337	188, 105	24, 40	4.22	112
Norfentanyl	2	233	84, 55	16, 40	3.00	62
Hydrocodone	50	300	241, 183	28, 32	2.61	96
Hydromorphone	50	286	157, 227	40, 28	1.23	83
Oxycodone	50	316	241, 256	32, 28	2.42	82
Oxymorphone	50	302	198, 161	40, 40	0.95	66
S8. CANNABINOIDS						
JWH-018-COOH	1	372	155, 127	24, 40	6.81	85
JWH-018-OH	1	358	155, 127	20, 40	6.94	87
JWH-073-M	1	344	155, 127	24, 40	6.64	89
JWH-122	1	356	169, 141	28, 40	9.50	27
JWH-200-M	1	401	114, 155	32, 24	5.59	129
JWH-250-M	1	352	121, 91	24, 40	7.42	75
THC-COOH	15**	345	327, 299	16, 20	8.12	85
S9. GLUCOCORTICOSTEROIDS						
Beclomethasone	30	409	391	8	5.26	87
Betamethasone	30	393	279	16	5.09	92
Budesonide	30	431	171	40	6.14	97
Budesonide-M	30	377	226	24	4.01	46
Deflazacort-M	30	400	142, 173	30, 32	4.65	78
Deoxycorticosterone	30	331	123	32	6.10	87
Desonide	30	417	323	12	5.38	94
Dexamethasone	30	393	355	8	5.12	77
Fludrocortisone	30	381	239	28	4.65	78
Flumethasone	30	411	371	5	5.14	84
Flunisolide	30	435	339	12	5.42	81
Fluocortolone	30	377	303	12	5.55	85
Fluticasone propionate-M	30	453	275	28	6.08	35
Methylprednisolone	30	375	357	8	5.01	93
Prednisolone	30	361	221	20	4.57	90
Prednisone	30	359	341	8	4.58	89
Triamcinolone	30	395	357	12	4.02	69
Triamcinolone acetonide	30	435	339	12	5.36	98

* threshold substance, not MRPL **previous threshold

Table 2: Summarized results (MRPLs, transitions, MS-conditions, retention times and extraction recoveries).

Conclusions

The developed method was proven applicable to the analysis of wide selection of prohibited substances, and allows flexible method extensions which may arise from the modifications of WADA prohibited list. The method is simple, rapid and suitable for initial testing procedure.

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