M. Mazzarino, M.C. Braganò, S. Turi, F. Botrè

LC-ESI-MS/MS screening method for simultaneous detection in human urine of glucocorticoids, diuretics, some stimulants, some beta2-agonists, some beta-blockers, anti-oestrogens and new designer steroids

Laboratorio Antidoping, Federazione Medico-Sportiva Italiana, Roma, Italy

INTRODUCTION

The excellent suitability of liquid chromatography – mass spectrometry (LC/MS) has been demonstrated for multi-analyte screening of many classes of prohibited substances [1-5]. The appearance of new abused molecules has increased the number of substances in the list of prohibited substances and methods [6] of the World Anti-Doping Agency (WADA), challenging the doping control laboratories to keep their analytical methods updated, also from a logistic point of view: an excessive number of separate analytical procedures renders the laboratory analysis more complex, delays reporting, increases the workload, and raises the cost of a single test. The aim of this paper is to present the development of a general screening method for the detection, in the same analytical run, of chemically and pharmacologically different banned drugs in human urine, requiring a “traditional”, triple quadrupole-based LC-ESI-MS/MS system, using both positive and negative polarity.

EXPERIMENTAL SECTION

LC/MS parameters

All LC/MS-MS experiments were performed using an Agilent 1100 Series HPLC pump with binary gradient system and automatic injector. Reversed-phase liquid chromatography was performed on Supelco Discovery C18 column (2.1 × 150 mm, 5 μm). The solvents were: water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). A gradient program was set up starting at 15 % B and increasing to 60 % B in 7 min and then, after 6 min, to 100 % B in 1 min. The flow rate was set at 250 μL/min. Data were acquired using an Applied Biosystems API4000 triple-quadrupole

analyser with both positive and negative electrospray ionization. For all experiments the collision energy, and the transition used for MRM method are reported in Table 1.

Sample preparation

To 3 mL of urine, 1 mL of phosphate buffer (pH 7.4), 50 µL of β-glucuronidase from E. Coli and 50 µL of the ISTD (17α-methyltestosterone: 200 ng/mL final concentration) were added and incubated for 1 hour at 50 ºC. After hydrolysis 1 mL of carbonate/bicarbonate buffer (pH 9), to alkalinise the sample, was added and the extraction was carried out with 10 mL of *tert*-buthylmethylether. After centrifugation the organic layer was transferred and a second liquid/liquid extraction was carried out with 7 mL of ethyl acetate, after correction of pH to 4-5 by adding 1 mL of formate buffer (pH 3.8) and addition of the second ISTD (furosemide d5: 500 ng/mL final concentration). After centrifugation the organic layer was joined to the first organic layer and evaporated to dryness. The residue was reconstituted in 50 µL of mobile phase and an aliquot of 10 µL were injected on the instrument system.

RESULTS AND DISCUSSION

The experimental data demonstrate that it is possible, using liquid chromatography-mass spectrometry with a triple quadrupole, to carry out a rapid and simultaneous screening in human urine sample of a wide variety of drugs, some of them being also markedly different in terms of their chemical and pharmacological properties. The repeatability of both relative retention times (calculated, for all compounds, using as ISTD 17α-methyltestosterone) and relative abundances are very satisfactory (see Table 1). No significant interferences were found at the expected retention times of the analytes of interest, thus excluding the possibility of false positive results. Carryover signal was not detected in blank urine samples that were injected in sequence after the analysis of the fortified urine samples at the highest concentration (three time the MRPL values). The results presented in this study confirm that LC/MS-MS can be a very powerful analytical tool in toxicological analyses, and especially in anti-doping analyses, also allowing to overcome the difficulties deriving from (1) the need of searching for a great variety of target analytes in a single sample; (2) the necessity of reporting the results in the shortest possible time (24 hours and less in the case of major international sport events); (3) the constraints given by the relatively small volume of urine available.

Table 1: MS parameters, MRM transitions, RRT, LODs and their repeatability (expressed as CV%).

Compounds	LOD (ng/mL)	LOD (ng/mL)	Polarity	RRT	CV%	CE (eV)	MRM Transition (m/z)	CV%
Exemestane	50	30	positive	1.12	0,4	20	297/149 ; 297/279	5
Letrozole	50	30	positive	0.87	0,4	35	286/190 ; 286/177	6
Raloxifene	50	30	positive	0.72	0,3	40	474/112 ; 474/269	8
Aminogluthetimide	50	50	positive	0.20	0,3	40	233/203; 233/131	7
Anastrozole	50	30	positive	0.86	0,2	35	294/225; 294/210	5
Formoterol	100	50	positive	0.53	0,8	20	345/149; 345/121	6
Finasteride metabolite		30	positive	0.76	0,2	38	403/187 ; 403/175	3
Gestrinone	10	5	positive	1.00	0,2	30	309/291; 309/262	3
Tetrahydrogestrinone	10	5	positive	1.12	0,2	30	313/295 ; 313/241	4
3'hydroxystanazolol	2	2	positive	0.88	0.2	65	345/97	4
Amiphenazole	500	300	positive	0.16	0,6	30	192/134; 192/150	8
Famprozanone	500	200	positive	0.74	0,7	30	378/162; 378/91	9
Isometeptene	500	200	positive	0.42	0,6	30	142/41; 142/69	7
Mesocarb metabolite	500	300	positive	0.87	0,6	35	339/193; 339/135	7
Methylphenidate	500	200	positive	0.52	0,4	30	234/174; 234/129	8
Modafinil	500	200	positive	0.75	0,5	60	274/165; 274/128	8
Pentetrazole	500	200	positive	0.29	0,4	40	139/69; 139/96	7
Strychnine	200	100	positive	0.22	0,5	55	335/156; 335/184	7
Tuaminoheptane	500	300	positive	0.28	0,7	30	116/41; 116/57	8
Acebutolol	500	50	positive	0,16	0,7	25	337/116	6
Alprenolol	500	50	positive	0,16	0,7	25	250/91	6
Atenolol	500	50	positive	0,16	0,5	25	267/145	5
Betaxolol	500	50	positive	0,16	0,5	25	308/121	8
Bisoprolol	500	50	positive	0,16	0,5	25	326/116	8
Carteolol	500	50	positive	0,16	0,8	25	293/237	8
Carvedilol	500	50	positive	0,16	0,7	25	407/224	7
Celiprolol	500	50	positive	0,16	0,7	25	380/251	9
Labetalol	500	50	positive	0,16	0.,9	25	329/162	9
Metoprolol	500	50	positive	0,16	0,8	25	268/133	9
Nadolol	500	50	positive	0,16	0,6	25	310/254	4
Oxprenolol	500	50	positive	0,16	0,6	25	266/225	5
Pindolol	500	50	positive	0,16	0,9	25	249/116	4
Sotalol	500	50	positive	0,16	0,8	25	273/133	5
Timolol	500	50	positive	0,16	0,8	25	317/261	7
Beclometasone	30	20	positive	0.85	0,5	20	409/373 ; 409/337	6
Betamethasone	30	5	positive	0.82	0,7	20	393/337 ; 393/355	4
Budenoside	30	10	positive	0.98	0,6	20	431/413 ; 431/341	8
Budenoside metab.	30	20	positive	0.66	0,8	20	377/359 ; 377/341	8
Ciclesonide metab.	30	20	positive	1,20	0,5	20	471/453; 471/323	8
Dexamethasone	30	5	positive	0.82	0,4	20	393/337; 393/355	6
Desonide	30	5	positive	0.86	0,4	20	417/399; 417/341	6
Fluocortolone	30	5	positive	0.89	0,5	20	377/321; 377/303	6
Fludrocortisone	30	15	positive	0.76	0,6	38	381/239; 381/343	5
Flumetasone	30	10	positive	0.86	0,6	20	411/335; 411/253	5
Flunisolide	30	10	positive	0.86	0,6	20	435/397; 435/417	5
Methylprednisolone	30	20	positive	0.81	0,5	20	375/357; 375/339	8
Prednisolone	30	20	positive	0.75	0,7	20	361/343; 361/325	8
Triamcinolone	30	15	positive	0.66	0,9	20	395/357; 395/321	8
Triamcinolone acetonid	30	5	positive	0.86	0,5	20	435/397; 435/415	4
Acetazolamide	250	50	negative	0.77	0.5	-30	221/83; 221/58	3
Ethacrynic acid	250	50	negative	1.13	0.3	-30	301/243	5
Althiazide	250	100	negative	0.78	0.3	-35	382/341; 382/205	4
Bendroflumethiazide	250	100	positive	0.95	0.2	30	422/287; 422/271	5
Bumetanide	250	50	positive	0.99	0.3	30	365/184; 365/240	5
Canrenone	250	100	positive	1.05	0.4	30	341/157; 341/107	6
Clopamide	250	50	positive	0.65	0.4	40	346/169; 346/55	5
Chlortalidone	250	100	positive	0.66	0.2	35	339/1235; 339/193	4

Chlorthiazide	250	100	negative	0.74	0.3	-50	294/214; 294/179	4
Dichlorphenamide	250	100	negative	0.76	0.2	-30	303/224; 303/239	4
Furosemide	250	100	negative	0.78	0.4	-35	329/205; 329/126	4
Hydrochlothiazide	250	100	negative	0.77	0.4	-35	295/205; 295/125	4
Hydroflumethiazide	250	100	negative	0.79	0.4	-40	330/303; 330/160	5
Indapamide	250	50	positive	0.87	0.3	50	366/132; 366/91	5
Metazolone	250	50	positive	0.82	0.4	40	366/259; 366/179	2
Piretanide	250	50	positive	0.96	0.5	40	363/238; 363/196	2
Spironolactone	250	100	positive	1.05	0.4	30	341/157; 341/107	3
Torasemide	250	50	positive	0.69	0.4	40	349/290; 349/183	3
Xipamide	250	50	positive	0.97	0.3	40	355/234;355/274	3

The identification capability of the analytic procedure was proven by the detection of the analytes during the validation process and during the routine activity compared, for almost two months, with the reference GC/MS screening method (diuretics, beta-blockers and stimulants) and the LC/MS-MS method (glucocorticosteroids, anti-estrogenic agents, beta-agonists and anabolic steroids) presently used in our laboratory to detect the compounds here considered [7-8]. The overall performance of the method was also evaluated and discussed taking into account the guidelines of the WADA for the accredited laboratories, with special emphasis on the minimum required performance limit (MRPL). The upgrade of the reference LC/MS-MS procedure was accomplished by taking into account the following issue: due to the acid nature of most diuretics, negative ionization is generally preferred. Hence positive and negative scan events are necessary to cover all compounds in the screening method, the number of data points and sensitivity are lower than in the reference LC/MS-MS reference procedure. However the LODs for most of the target compounds considered in the present study are significantly lower than the minimum performance required limits (MRPL) for laboratories established by WADA (see Table 1) ensuring that the detection of a prohibited substance is often possible also days after administration.

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