G. Bican, V. Pop, R. Subaşu, M. Lamor, I. Vâjială

Improved detection of banned doping substances by triple quadrupole LC/MS^2 technique

Doping Control Laboratory, Bucharest, Romania

Introduction

In the last years, some new substances belonging to different classes of substances have been included on WADA's Prohibited List [1]. Due to this fact and the necessity to increase the number of analyzed samples, we have optimized the procedures used within the laboratory.

For many compounds in our GC/MS screening, which were difficult to detect [2], we have developed new methods of analysis by using 1200 Agilent liquid chromatograph coupled with 6410 Agilent mass spectrometer with triple quadrupole.

The substances requiring hydrolysis were separated by combined extraction for steroids and corticosteroids, and for the compounds included in Section S6 of the Prohibited List it was used the extraction for the identification of diuretic substances, so that these substances have been included in the procedures already existent in the laboratory.

This work presents the optimal conditions for preparation, separation and identification by LC/MS^2 technique of some compounds analyzed traditionally by GC/MS together with the substances studied by LC/MS^2.

Materials and Methods

The substances have been studied by using standard solutions of 2-aminoheptane, etilefrine, isometheptene, methylecgonine (metabolite of cocaine), sibutramine, strychnine, hydromorphone, pemoline, ritalinic acid (metabolite of methylphenidate), benzoylecgonine (metabolite of cocaine), oxymorphone, oxycodone, monoacetylmorphine (metabolite of heroin) in concentrations of 10ppm in methanol. The reference materials were purchased from Sigma-Aldrich, AK Scientific, Promochem, National Measurement Institute – Australian Government, the Amberlite XAD-2 resin from Supelco, the beta-glucuronidase from E. Coli K12 from Roche Diagnostics, Germany, and the ultra-pure water was obtained by using the filtration system Simplicity 185, Millipore, Great Britain.

1. Sample preparation

For some of the analyzed compounds, we have used the combined extraction method [3,4] developed for the compounds from the androgenic anabolic steroids and corticosteroids classes. For the other compounds, we have used the solid phase extraction on XAD-2 resin, an extraction method used for diuretics: 2ml urine passed through a column filled with 2cm of XAD-2, washed with 2ml water, eluted with 2x1ml methanol, evaporated and reconstituted in 100µL methanol.

2. Analytical parameters

The instrumental analysis has been carried-out on LC/MS^2 with triple quadrupole Agilent 1200/6410 equipped with ESI source. The analytic conditions are presented in table 1.

Table 1. LC/MS^2 analytic parameters

LC Parameters				
Column	Zorbax SB-C18 (50x2.1mm, part.size 5µm)			
Mobile phase	*Solvent A* 5mM ammonium formate in water *Solvent B* 5mM ammonium formate in 95% acetonitrile + 5% water			
LC Program	Time (min:sec)	%A	%B	Flow (ml/min)
	0:00	90	10	0.3
	2:00	60	40	0.3
	5:00	35	65	0.3
	9:00	35	65	0.3
	9:10	90	10	0.3
	14:00	90	10	0.3
Injection Volume	1µL			

MS Parameters	
Ionization Mode	ESI positive
Scan Type	MRM
Dwell time	30ms
Source Parameters	
ESI Drying Gas	N_2
Gas Temp	350^0C
Gas Flow	12l/min
ESI Nebulizing Gas	50 psi N_2
Collision Gas	ultrapure (5.0) N_2
Capillary	4000V

Results and discussion

The establishment of the MS parameters for each substance was established by the use of solutions with concentration 10ppm. The relevant compounds were injected in the MS2SCAN mode and the precursor ion was established for each compound separately. The analyzed compound are forming $[M+H]^+$ species as precursor ions. The optimization of the fragmentor energy (capillary voltage, declustering potential) for the precursor ion was done in MS2SIM mode for the highest abundance. Then, compounds were injected in the Product Ion Scan

mode. After choosing the specific MRM transitions for each compound, the collision energies were optimized and the final MRM method was established.

Two precursor/product transitions were monitored in the screening analysis for each compound.

The next step in establishing the analysis method was the sample preparation of the urine samples spiked with the relevant standards and the establishment of the optimal extraction method.

In Table 2 the analyzed substances as well as the extraction methods used, the relative retention times and the specific transitions, each with its optimized collision energy are shown.

In Figure 1, the LC/MS^2 chromatogram of a mixture of standards prepared by solid phase extraction is shown, with Mefruside as internal standard (retention time 6,641), while in Figure 2, the LC/MS^2 chromatogram of a mixture of standards prepared following the liquid-liquid extraction is shown, with Methyltestosterone internal standard (retention time 7,72).

Two metabolites of cocaine were studied. For benzoylecgonine, very good results were obtained by the use of solid phase extraction on Amberlite XAD-2 resin, while for methylecgonine the liquid-liquid extraction in TMBE gave better results.

Also, the metabolite of methylphenidate, ritalinic acid, had very good results when using the extraction in solid phase on Amberlite XAD-2 resin.

References

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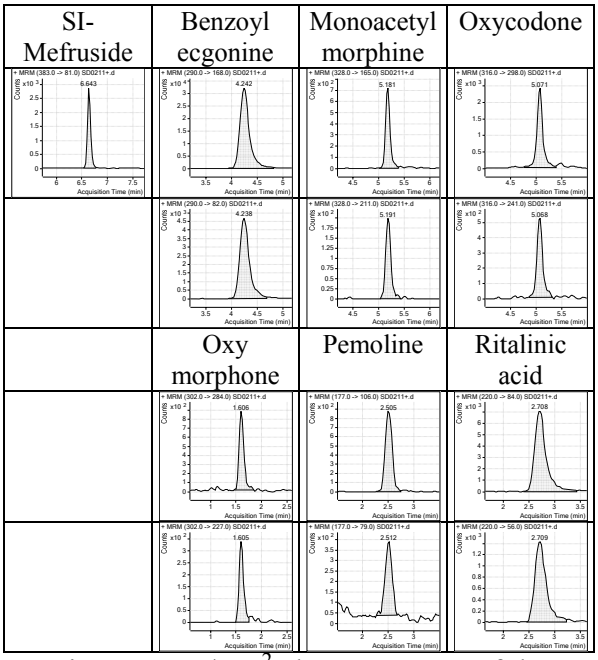

Figure1. LC/MS^2 chromatogram of the mixture of standards (Sample preparation: SPE on XAD-2)

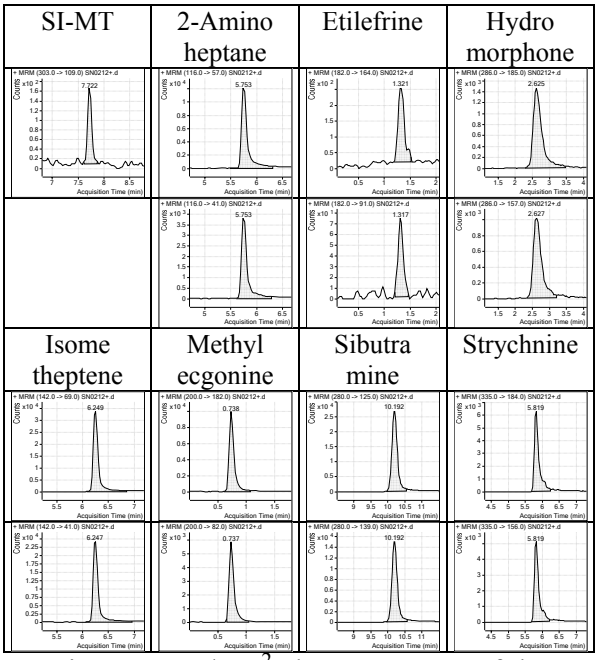

Figure2. LC/MS^2 chromatogram of the mixture of standards (Sample preparation: LLE at pH=9 in TMBE).

Table 2. LC/MS^2 Screening analysis – analytic parameters of the compounds.

Prohibited substance	*Section*	*Sample Preparation*	*Molecular Weight*	*Precurson Ion*	*Product Ion (CE, eV)*	*Relative Retention Time*
2-Aminoheptane	S6	LLE at pH=9 in TMBE	115	116	(+)57(10) (+)41(20)	0,7450[1]
Etilefrine	S6	LLE at pH=9 in TMBE	181	182	(+)164(10) (+)91(30)	0,1710[1]
Isometheptene	S6	LLE at pH=9 in TMBE	141	142	(+)69(15) (+)41(30)	0,8092[1]
Methylecgonine (Cocaine metabolite)	S6	LLE at pH=9 in TMBE	199	200	(+)182(15) (+)82(30)	0,0955[1]
Sibutramine	S6	LLE at pH=9 in TMBE	279	280	(+)125(30) (+)139(10)	1,3198[1]
Strychnine	S6	LLE at pH=9 in TMBE	334	335	(+)184(40) (+)156(50)	0,7535[1]
Hydromorphone	S7	LLE at pH=9 in TMBE	285	286	(+)185(30) (+)157(50)	0,3399[1]
Pemoline	S6	SPE on XAD-2	176	177	(+)106(15) (+)79(30)	0.3781[2]
Ritalinic acid (Methylphenidate metabolite)	S6	SPE on XAD-2	219	220	(+)84(20) (+)56(50)	0.4077[2]
Benzoylecgonine (Cocaine metabolite)	S6	SPE on XAD-2	289	290	(+)168(15) (+)82(30)	0.6385[2]
Oxymorphone	S7	SPE on XAD-2	301	302	(+)284(15) (+)227(30)	0.2417[2]
Oxycodone	S7	SPE on XAD-2	315	316	(+)298(15) (+)241(30)	0.7633[2]
Monoacetylmorphine (Heroin metabolite)	S7	SPE on XAD-2	327	328	(+)211(30) (+)165(40)	0.7814[2]

(1: SI-Methyltestosterone, RT=7.722 min.; 2: SI-Mefruside, RT=6.643 min.)