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New prohibited substances addition to procedures using triple quadrupole LC/MS² technique

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Abstract

New substances are added each year to the different classes of substances of WADA's Prohibited List and have to be included in the test menu of each accredited doping control laboratory. For the analysis of some of these compounds the technique of choice is the LC/MS² technique.
Furthermore, with the decrease of MRPL levels, for stimulants from 500 ng/mL to 100 ng/mL and for narcotics from 200 ng/mL to 50 ng/mL, beginning from the entering in force on 1 January 2013 of the new WADA Technical Document TD2013MRPL, some of the compounds analyzed by the current GC/MS based procedure for stimulants and narcotics needed to be transferred to the LC/MS² based procedure in order to lower their limit of detection.
This paper-work presents the optimal conditions for preparation, separation and identification of new substances introduced to the LC/MS² technique based procedures during the year 2012.

Introduction

Liquid chromatography coupled with mass spectrometry is increasingly used in doping control laboratories [1]. The current work presents the optimization of the LC/MS² detection parameters and the introduction of the prohibited substances in the existent procedures, depending on the characteristics of the studied compounds.
The studied compounds are benzthiazide, cyclopenthiazide, cyclothiazide, epitizide, hydroflumethiazide and polythiazide (thiazide diuretic compounds), tramadol and its O-desmethyl metabolite (narcotic compound from the monitoring program [2]), cyclazodone, pentetrazole, prolintane, propylhexedrine and selegiline (stimulants transferred from the GC/MS- to the LC/MS²-based procedure due to the decreasing of the MRPL levels for stimulants and narcotics [3]).

Experimental

Sample preparation
For the new diuretics the solid phase extraction on XAD-2 resin from the procedure for diuretics was used [4], while for the stimulants and narcotics the liquid-liquid extraction with TBME at pH 9 from the procedure for corticosteroids was used [5,6].

Equipment
The analysis was performed on an Agilent 1200/6410 triple quadrupole LC/MS/MS equipped with ESI source and a Zorbax SB-C18 2.1 x 50 mm 5-Micron column from Agilent. The solvent A of the mobile phase was 5mM ammonium formate, 1‰ formic acid in Millipore ultra-pure water and the solvent B 5mM ammonium formate, 1‰ formic acid in 90% acetonitrile + 10% water. A linear gradient was used increasing from 10% to 40% B in 2 min, then from 40% to 65% B in 3 min; after 4 min at 65% B, the column was reequilibrated for 5 min. The column thermostat was set at 30 °C. The injection volume was 2 µL. The MS was conducted in ESI negative ionization mode for thiazides and ESI posititive ionisation mode for stimulants. The ESI source parameters were Drying Gas 8L N_2/min at 350 °C, Nebulizing 40 psi N_2, Capillary 4000V. Ultrapure N_2 (5.0) was used as collision gas.

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Results and Discussion

The establishment of the MS parameters of each substance was done by the use of solutions with a concentration of 10 µg/mL. The relevant compounds were injected in the MS2SCAN mode and the precursor ion was established for each compound separately. The thiazide diuretic compounds are forming $[M-H]^-$ species as precursor ions, while the stimulant and narcotic compounds are forming $[M+H]^+$ species. 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.
The thiazide diuretic compounds were introduced in the initial testing procedure for diuretics, procedure based on solid phase extraction on XAD-2 resin and LC/MS^2 analysis. The stimulants and narcotics were introduced in the initial testing procedure for corticosteroids, procedure based on liquid-liquid extraction in TBME at pH 9 and LC/MS^2 analysis. In both procedures, two precursor/product transitions were monitored for each compound.
In order to verify the performance of the methods for the newly introduced compounds, two blank urine samples were spiked with 100 ng/mL of each thiazide diuretic compound and, respectively, with 50 ng/mL of each stimulant and narcotic. The spiked concentrations represent 50% of the 200 ng/mL MRPL for diuretics, respectively, of the 100 ng/mL MRPL for stimulants. The spiked samples were analyzed by the relevant procedures. The analysis of the spiked urines resulted for all the studied compounds in abundant signals with signal-to-noise ratio > 3.

Prohibited substance	*Section*	*Sample Preparation*	*Molecular Weight*	*Precursor Ion*	*Product Ion (CE, eV)*	*Relative Retention Time*
Benzthiazide	S5	SPE on XAD-2	431	(-)430	(-)308(20) (-)228(40)	0.9895 [1]
Cyclopenthiazide	S5	SPE on XAD-2	379	(-)378	(-)205(30) (-)269(20)	1.0583 [1]
Cyclothiazide	S5	SPE on XAD-2	389	(-)388	(-)322(20) (-)269(30)	1.0175 [1]
Epitizide	S5	SPE on XAD-2	425	(-)424	(-)300(10) (-)310(20)	0.9681 [1]
Hydroflumethiazide	S5	SPE on XAD-2	331	(-)330	(-)239(20) (-)303(20)	0.6146 [1]
Polythiazide	S5	SPE on XAD-2	439	(-)438	(-)398(10) (-)324(20)	1.0400 [1]
Cyclazodone	S6	LLE at pH=9 in TMBE	216	(+)217	(+)146(10) (+)106(25)	0.6718 [2]
Pentetrazole	S6	LLE at pH=9 in TMBE	138	(+)139	(+)96(10) (+)55(25)	0.2943 [2]
Prolintane	S6	LLE at pH=9 in TMBE	217	(+)218	(+)91(25) (+)72(15)	0.7056 [2]
Propylhexedrine	S6	LLE at pH=9 in TMBE	155	(+)156	(+)69(15) (+)41(30)	0.6477 [2]
Selegiline	S6	LLE at pH=9 in TMBE	187	(+)188	(+)91(20) (+)119(5)	0.5487 [2]
Tramadol	S7	LLE at pH=9 in TMBE	263	(+)264	(+)58(15) (+)246(5)	0.6063 [2]
O-Desmethyl-tramadol (Tramadol metabolite)	S7	LLE at pH=9 in TMBE	249	(+)250	(+)58(15) (+)232(5)	0.3211 [2]

Table 1: LC/MS^2 screening analysis analytical parameters of the compounds; [1] SI-Mefruside, RT=6.643 min; [2] SI-Methyltestosterone, RT=8.107 min

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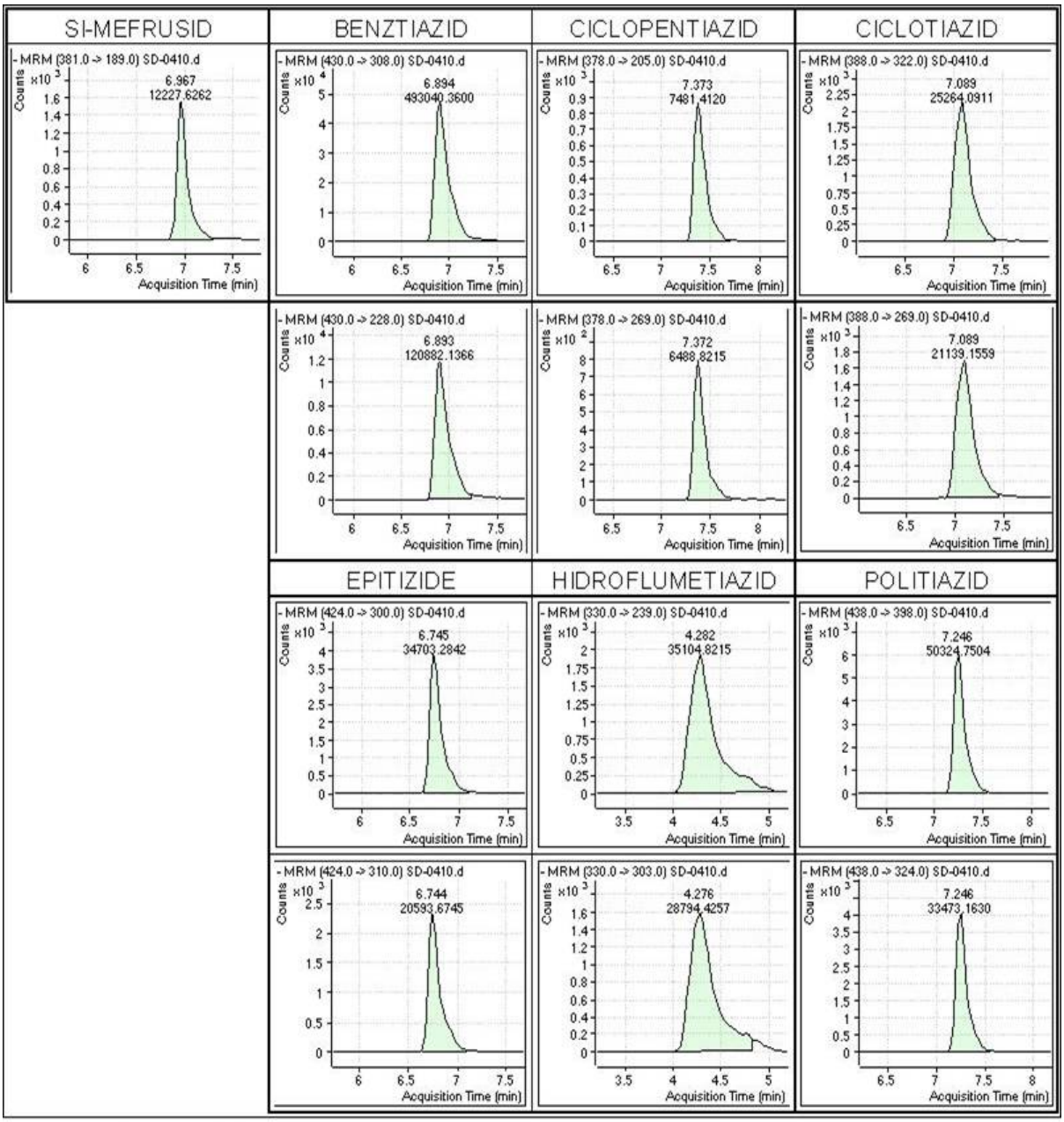

Figure 1: MRM transitions extracted from the LC/MS² chromatogram of blank urine spiked with 100 ng/mL of each thiazide diuretic compound

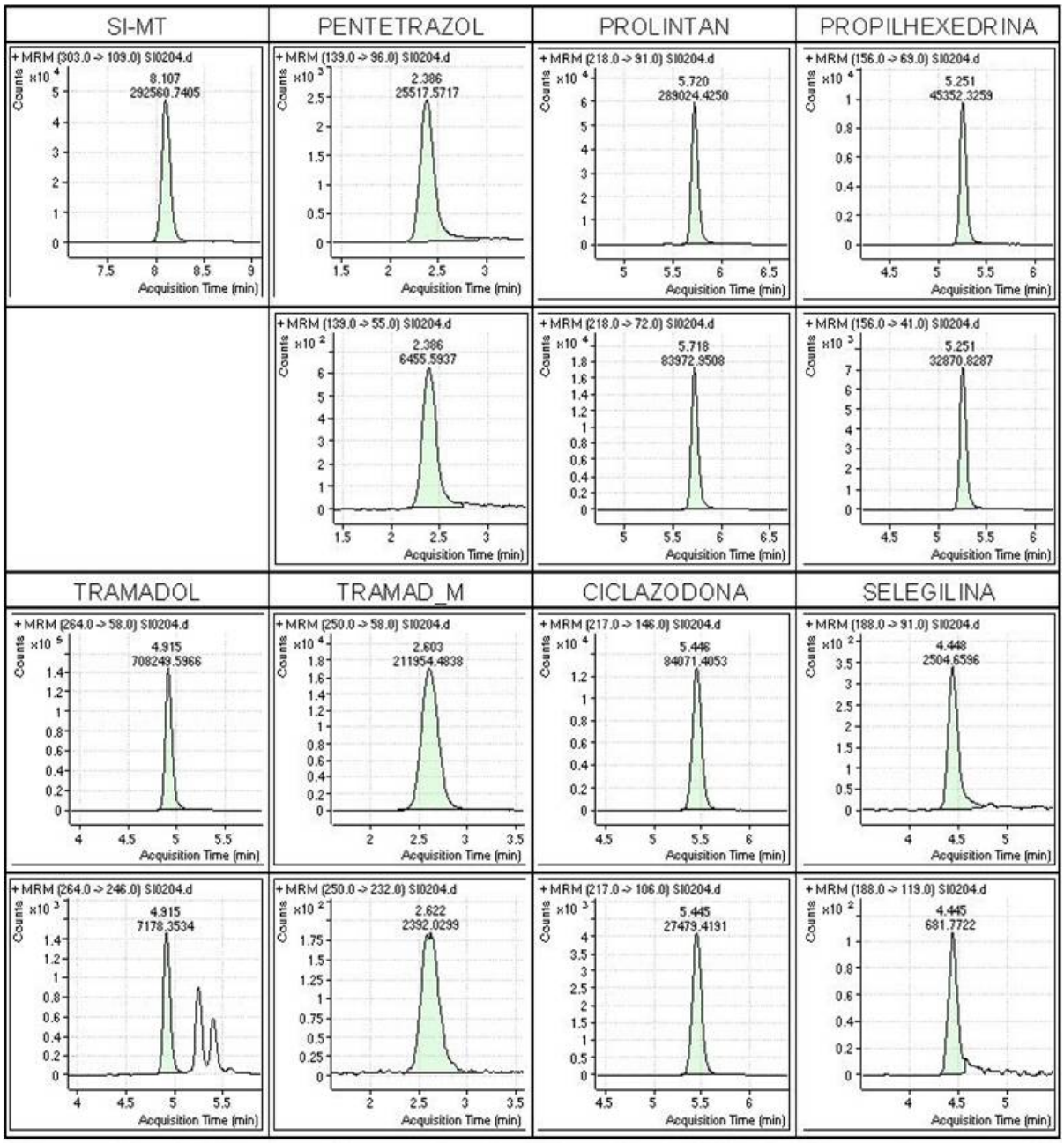

Figure 2: MRM transitions extracted from the LC/MS² chromatogram of blank urine spiked with 100 ng/mL of each stimulant tramadol

Conclusions

The LC/MS² technique described is simple and sensitive, the liquid chromatograph coupled with mass spectrometer in tandem with triple quadruple proving to be an efficient instrument for the doping control analyses. Taking into consideration the structural features, the compounds were analyzed by the use of liquid-liquid and solid phase extraction methods. The optimization of detection parameters of relevant compounds and the establishment of the LC/MS² methods were done. Six thiazide diuretics were introduced in the initial testing procedure for diuretics and five stimulants and one narcotic were introduced in the initial testing procedure for corticosteroids. All compounds can be easily detected at 50% MRPL.

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References

1. DEVENTER, K. (2006). In: Nautilus Academic Books (eds.) *Liquid Chromatography – Mass Spectrometry, an evolution in doping analysis*.
2. World Anti-Doping Agency. The 2013 Prohibited List. International Standard, Montreal (2013) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf (access date 24.07.2013).
3. WADA Technical Document-TD2013MRPL. Minimum required performance levels for detection and identification of Non-Threshold substances (2013) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Summary-modifications-EN.pdf (access date 24.07.2013).
4. BICAN, G.; POP, V.; SUBAŞU, R.; LAMOR, M; VÂJIALĂ, I. (2008) Improved detection of banned doping substances by triple quadrupole LC/MS^2 technique. In Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analyses* (16), Köln, pp 281-284.
5. MARECK, U.; THEVIS, M.; GUDDAT, S.; GOTZMANN, A.; BREDEHOFT, M.; GEYER, H.; SCHÄNZER, W. (2004) Comprehensive Sample Preparation for Anabolic Steroids, Glucocorticosteroids, Beta-Receptor Blocking Agents, Selected Anabolic Androgenic Steroids and Buprenorphine in Human Urine. In Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analyses* (12), Köln, pp 65-68.
6. POP, V.; VÂJIALĂ, G.; LAMOR, M.; BICAN, G.; ŢONE, M. (2005) Comparative analytical techniques for the identification of some androgenic anabolic steroids (GC/ MS – LC/MS/MS). In Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analyses* (13), Köln, pp 369-374.

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