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A unified doping screening procedure based on LLE with ethyl acetate and combined GCMS, GCHRMS and LCQTOFMS analysis

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Abstract

A unified screening procedure for a wide range of prohibited substances (anabolic agents, beta-2 agonists, hormone antagonists and modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticosteroids, beta-blockers) including sulfoconjugated stimulants has been developed. The sample preparation protocol consists of hydrolysis with β-glucuronidase, liquid-liquid extraction (LLE) with ethyl acetate at pH=9.5, moderate salting out, evaporation to dryness, gas chromatography mass spectrometry (GCMS), gas chromatography high-resolution mass spectrometry (GCHRMS) analysis of per-TMS derivatives as well as liquid chromatography-quadrupole time-of-flight mass spectrometry (LCQTOFMS) analysis of reconstituted in mobile phase aliquots. Analytical method validation was performed in accordance with the International Standard for Laboratories for specificity, identification capability, robustness, carryover, matrix interferences, matrix effect, extraction recovery, limit of detection (LOD) and mass accuracy. Regarding endogenous steroids quantitation, additional validation parameters were linearity, within day precision, between days precision and limit of quantitation (LOQ). The developed method was applied successfully for the quantitation of endogenous steroids by GCMS in ten urine samples distributed in two different World Anti Doping Agency (WADA) external quality assessment scheme (EQAS) rounds. The current method permits the identification of more than 210 small molecules from the WADA prohibited list at or below the WADA Minimum Required Performance Level (MRPL). The advantage of using ethylacetate, as extraction solvent with salting out, is the detection of some sulfoconjugated stimulants in liquid chromatography mass spectrometry electrospray ionization (LCMS ESI) positive mode.

Introduction

Several approaches have been published towards the application of universal screening methods for doping control analysis [1-3]. A combined screening method for the analysis of representative prohibited substances using LCTOFMS and GCTOFMS has been developed [2]. A generic LCTOFMS screening method of 241 small molecules from various categories of prohibited substances was also reported [1]. The main objective of the current work was the development of a screening procedure with a generic sample preparation protocol and a simultaneous detection of a wide range of prohibited substances based on a single LLE with ethyl acetate and combined analysis using GCMS, GCHRMS and LCQTOFMS.

Experimental

Chemicals and reagents

A detailed list of chemicals and reagents used for the herewith study is presented elsewhere [1,3,4]. Ethyl acetate was of analytical grade and obtained from Labscan. Stock standard solutions of analytes were prepared in methanol. Urine samples free of prohibited substances were used.

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Sample preparation

To 5.0 mL of urine, 1.0 mL of phosphate buffer (pH 7.0), 100 µL of methyltestosterone (internal standard, ISTD) and 50 µL of β-glucuronidase from *E. coli* were added. After hydrolysis for 1.5 h at 50 °C, pH was adjusted to 9.5 using 1.4 g of $NaHCO_3$/Na_2CO_3 (10/1, w/w). Extraction was carried out with 5 mL of ethyl acetate, using 1 g of Na_2SO_4 as the salting out agent. After centrifugation the organic phase was equally separated. For the LCQTOFMS analysis, the organic phase was acidified with 50 µL of 3M acetic acid, evaporated under nitrogen stream at 50 °C and reconstituted with 100 µL of 80:20 water/acetonitrile (v/v). For the GCMS/GCHRMS analysis, the organic layer was dried under nitrogen stream at 50 °C and derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)/ammonium iodide (NH_4I)/2-propanethiol (2PrSH) 1000:2:3 (v/w/v) for 30 min at 80 °C.

LCQTOFMS instrumentation is described in reference [1]. MS/MS was specifically used on the precursor ion m/z 286, Δm = ± 2 (CID: 25 eV). GCMS and GCHRMS chromatographic and mass spectrometric conditions can be found in reference [4].

Specificity, identification capability, robustness, carryover, matrix interferences, matrix effect, extraction recovery, LOD and mass accuracy were evaluated for method validation. Additionally, the analytical parameters evaluated for the endogenous steroids quantitation were linearity, within day precision, between days precision and LOQ. Moreover, the developed method was applied for the estimation of concentrations for seven endogenous steroids in ten urine samples distributed in two different WADA EQAS rounds. The concentrations obtained were compared with the quantitative results given in the Final Reports of WADA EQAS rounds and the z-scores were calculated.

Validation procedure

Limit of detection

For LCQTOFMS analysis, five different blank urine samples were fortified with standard mixtures at 0.2 times the MRPL, twenty different samples at 0.5 times the MRPL and four different samples at the MRPL. The LOD was determined as the lowest concentration where a substance could be detected in all analyzed samples with S/N>3. For GCMS/GCHRMS analysis, twenty different blank urine samples were fortified with standard mixtures at 0.5 times the MRPL. The LOD was determined as the lowest concentration where a substance could be detected in all analyzed samples with S/N>3.

Extraction recovery/ matrix effect

The mean value of % extraction recovery and the mean value of % matrix effect (ion enhancement/ion suppression) of four different blank urine samples fortified at the MRPL, each two prepared and injected on two different working days were calculated.

Mass accuracy

For LCQTOFMS analysis, the average absolute mass error of two different blank urine samples fortified at the MRPL was calculated. Each sample was prepared and injected on a different working day.

Results and Discussion

Classes of prohibited substances detected with the current method are listed in Table 1.

LCQTOFMS Analysis

Beta-2-agonists, b-blockers, stimulants, narcotics and diuretics were detected below half of the MRPL, whereas all glucocorticosteroids were detected at least at half of the MRPL. A QTOF MS/MS method was applied within the same run specifically for boldenone, methyldienolone and methyltrienolone due to matrix interfering peaks. Mass errors for 148 analytes were within 0-5 ppm. 138 analytes showed extraction recoveries over 60%. Fluticazone propionate carboxy metabolite and dichlorphenamide, previously reported as non-detectable with the use of diethyl ether as extraction solvent [1] showed increased extraction and were detected with the current method. Ethyl acetate permitted the detection of the conjugated stimulants ethamivan sulphate and p-OH-mesocarb sulphate from excretion urine in positive ESI mode. The chromatograms and the diagnostic ions of the respective molecules are illustrated in Figure 1.

GCMS / GCHRMS Analysis

Analytes from S1, S4, S5, S6 and S8 classes were detected at or below the MRPL. Extraction recoveries were estimated over 80% for 81% of the detected substances. No matrix interferences were recorded with the exemption of oxandrolone and aminoglutethimide. Eventually oxandrolone was successfully identified by its metabolite epioxandrolone by GCHRMS and

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aminoglutethimide was detected by LCQTOFMS.
Validation data for endogenous steroids by GCMS analysis are presented in Table 2. The developed method was linear at the tested range for each analyte and the quality control samples demonstrated intra and inter day precision and accuracy. The results obtained for the endogenous steroids were comparable with the values given in the Final Reports of WADA EQAS rounds and the calculated z-scores were in all cases less than two. Overall the developed method complied with the WADA's ISL method validation criteria in terms of specificity, identification capability, robustness, absence of carryover contamination and matrix interferences.

Prohibited substances	Number of compounds detected	Instrumentation
(S1) Anabolic agents	40	LCQTOFMS, GCHRMS, GCMS
(S1) Endogenous steroids	11	GCMS
(S3) Beta-2 Agonists	6	LCQTOFMS
(S4) Hormone and metabolic modulators	11	LCQTOFMS, GCHRMS, GCMS
(S5) Diuretics and other Masking Agents	28	LCQTOFMS, GCHRMS
(S6) Stimulants	70	LCQTOFMS, GCHRMS
(S7) Narcotics	12	LCQTOFMS
(S8) Cannabinoids	1	LCQTOFMS, GCMS
(S9) Glucocorticosteroids	15	LCQTOFMS
(P2) Beta-Blockers	19	LCQTOFMS
(M1) Enhancement of Oxygen Transfer	1	LCQTOFMS

Table 1: Classes of prohibited substances detected with the current method

S1 Endogenous steroids	Derivative	Ion (m/z)	RT (min)	Linearity (ng mL^{-1})	r^2	%[Er]*	%RSD$_{intra\ day}$	%RSD$_{inter\ day}$	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
11-OH-Androsterone	tris-OTMS	432	12.71	16 – 1,000	> 0.9991	≤ 3.8	≤ 3.8	≤ 7.2	2.6	7.8
5α-androstane-3α,17β-diol	bis-OTMS	241	10.15	8 – 1,000	> 0.9987	≤ 6.8	≤ 2.7	≤ 8.8	5.1	15.6
5β-androstane-3α,17β-diol	bis- OTMS	241	10.28	8 – 1,000	> 0.9996	≤ 5.1	≤ 2.9	≤ 8.6	2.9	8.8
5α-androstane-3,17-dione	bis-OTMS	432	11.44	3 – 200	> 0.9984	≤ 6.6	≤ 6.6	≤ 7.1	1.0	3.3
5β-androstane-3,17-dione	bis-OTMS	432	8.58	6 – 200	> 0.9988	≤ 4.3	≤ 4.3	≤ 5.3	1.2	3.9
Androsterone	bis-OTMS	434	9.82	78 – 10,000	> 0.9996	≤ 7.5	≤ 1.8	≤ 6.2	24.2	73.5
Dehydroepiandrosterone	bis-OTMS	432	11.10	3 – 200	> 0.9994	≤ 8.8	≤ 3.0	≤ 8.7	0.9	2.8
Dehydrotestosterone	bis-OTMS	434	11.81	6 – 200	> 0.9989	≤ 9.8	≤ 5.8	≤ 6.1	1.3	4.1
Epitestosterone	bis- OTMS	432	11.59	2 – 200	> 0.9992	≤ 5.1	≤ 3.8	≤ 7.9	0.6	2.0
Etiocholanolone	bis-OTMS	434	10.00	78 – 10,000	> 0.9991	≤ 6.3	≤ 2.2	≤ 6.9	28.7	87.1
Testosterone	bis- OTMS	432	12.40	2 – 200	> 0.9992	≤ 6.2	≤ 1.9	≤ 8.3	1.0	3.1

For the evaluation of linearity, within day precision (%RSD$_{intra\ day}$) and between days precision (%RSD$_{inter\ day}$), four seven-point calibration curves were obtained on four different working days. LOD and LOQ were calculated using the following equations: LOD = [3.3 x SD]/b and LOQ = [10 x SD]/b.

* $\%\,[Er] = 100 \times \frac{Cexp - Ctheor}{Ctheor}$

Table 2: Validation data for endogenous steroids quantitation obtained by GCMS analysis

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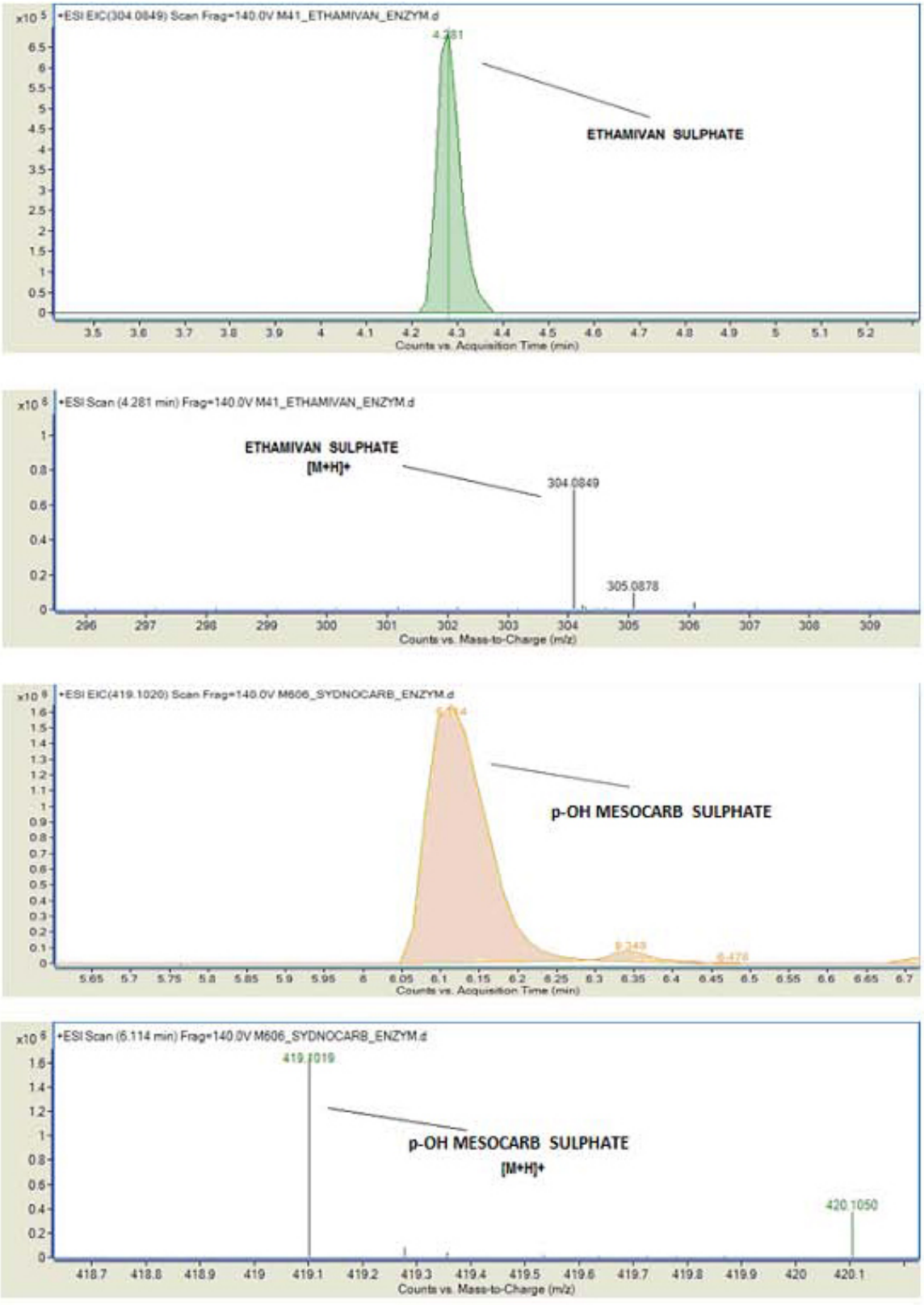

Figure 1: Ion chromatograms of ethamivan sulphate and p-OH mesocarb sulphate and their respective diagnostic ions obtained from excretion urine samples by LCQTOFMS analysis

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Conclusions

A rapid and cost-efficient screening method, based on a single LLE with ethyl acetate, moderate salting out and combined analysis using LCQTOFMS, GCMS and GCHRMS was developed and validated. The main advantages of the current method are the detection of more than 210 small molecules from various categories of the WADA's prohibited list and the quantification of steroid parameters required in WADA EQAS rounds. In addition, generic extraction with ethyl acetate allowed the detection of two sulfoconjugated molecules within the same injection run (+ESI). Further investigation is being carried out for the detection of the sulfoconjugated steroids with the application of the current method.

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