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EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Simultaneous Estimation of Prohibited Substances in Human Urine by GC-MS/MS: Application to Doping Analysis

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Received: June 04, 2018; **Published:** June 29, 2018

Abstract

A simple and rapid GC-MS/MS method for the simultaneous determination of threshold/endogenous drugs (epitestosterone, testosterone, Morphine, salbutamol and 19 nor androsterone) was developed and validated. Out of these five drugs, two drugs are threshold as per WADA TD2018DL. Further, one of the drug/metabolite named 19 nor androsterone was threshold substance in WADA TD2017DL, which is presently removed from the Threshold drug list of WADA Technical documents. A solid phase extraction (SPE) procedure using XAD2 columns followed by derivatization using N-methyl N-(trimethylsilyl) trifluoroacetamide. The precision values of all analytes were expressed in %RSD (< 10), which ranged between 2 - 10%. The accuracy values for all analytes were ranged from 90.3 - 109.5%. Furthermore, this method could be used for therapeutic drug monitoring purposes.

Keywords: *GC-MS/MS; Threshold Substances; Doping Control; WADA*

Introduction

The use of performance enhancing drugs in sports is prohibited by the World Anti-Doping Agency and a wide range of pharmacological classes of drugs figures on the Prohibited List 2018 [1,2]. In the framework of the fight against doping, mostly urine samples were collected and analyzed for the presence of prohibited drugs or metabolites. In general, except for peptide hormones, screening for the misuse of doping substances is carried out using chromatography coupled with mass spectrometry [1].

The prohibited substances are analyzed mostly from urine. In urine many drugs exist as their metabolites, and for some drugs the metabolites are the only detectable compounds. For a long time, conventional gas chromatographic-mass spectrometric methods (GC/MS) have been used extensively in doping analysis [3,4].

Although, several gas chromatographic mass spectrometry [2-4] and high performance liquid chromatographic tandem mass spectrometry (LC-MS/MS) [5] methods have been reported for these drugs. A simultaneous determination of the prohibited substances [6] viz., epitestosterone, testosterone, Morphine, salbutamol and 19 nor androsterone (Figure 1) using GC-MS/MS is lacking. The aim of this study was to develop and validate an GC-MS/MS method, followed by ISO/IEC 17025:2005 guidelines for the simultaneous determination five prohibited drugs viz epitestosterone, testosterone, Morphine, salbutamol and 19 nor androsterone, within a single run of less than 8 min was investigated.

Experimental

Chemicals and reagents

All reagents and chemicals used were of analytical grade or HPLC grade and purchased from different manufacturers. Methanol, Potassium dihydrogen phosphate and Potassium carbonate were obtained from Merck (Mumbai, India), Tertiary Butyl Methyl Ether (TBME) from J.T.Baker (Phillipsburg, USA), β-Glucuronidase (*E. coli*) enzyme from Roche Diagnostics Corporation (Indiapolis, USA). XAD2 from Supelco, Acetone, Anhydrous Sodium Sulphate, MSTFA, Iodo-trimethylsilane and Dithioerythritol were obtained from Sigma Aldrich (Mumbai, India). The certified reference standards of steroids and/or their metabolites were obtained from established sources like Sigma-Aldrich, USA, National Measurement Institute, Australia, Cerilliant, USA.

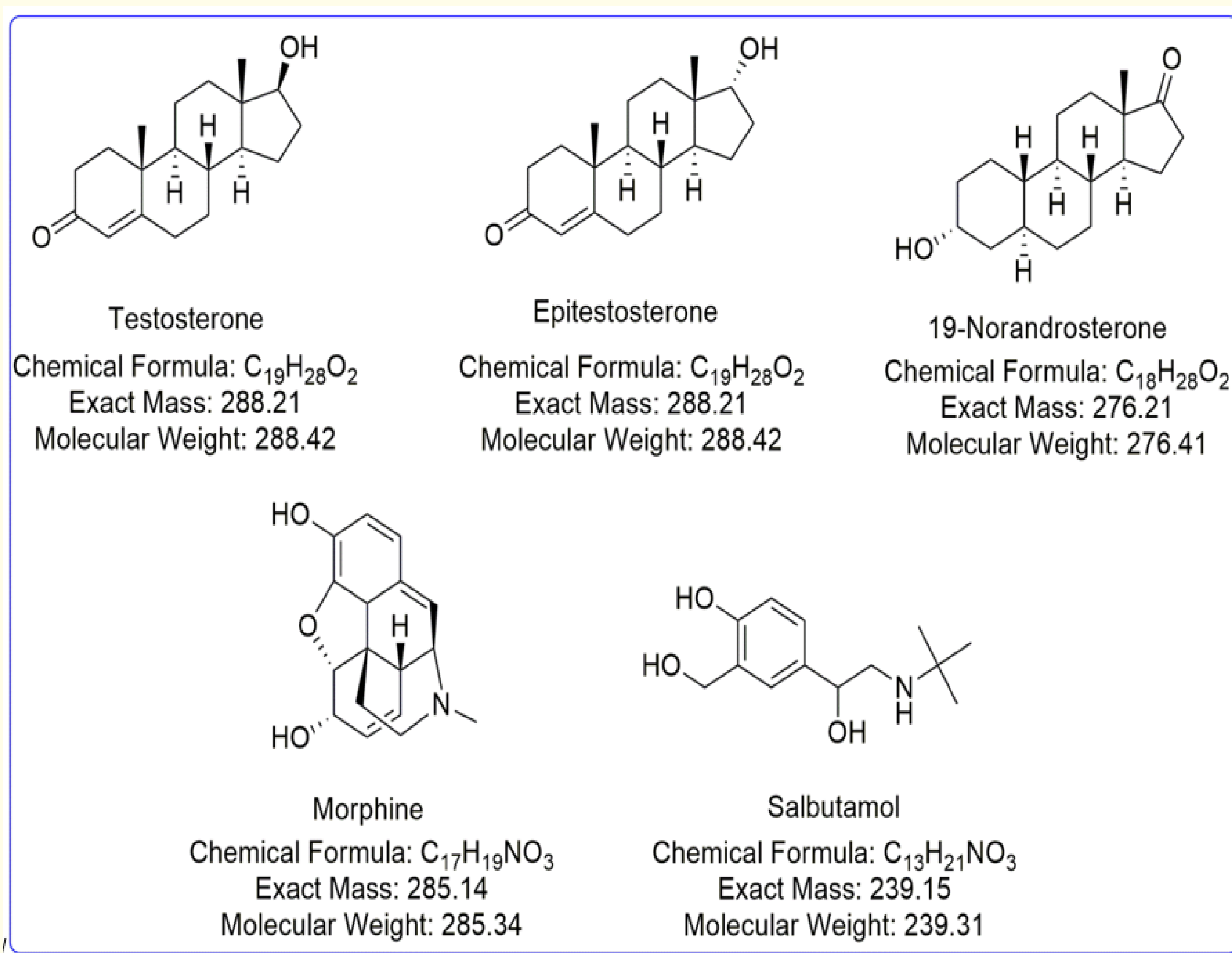

Figure 1: *Structure of prohibited substances.*

Instrumentation and chromatographic conditions

GC-MS/MS Analysis was performed using Thermo QqQ Mass spectrometer TSQ 8000, Thermo 1300 GC equipped with AI 1310 Automatic liquid sampler (ALS). HP Ultra-1(17m X 220 µm X 0.11 µm) was used to separate the analyte. Spectral detection was carried out on TSQ 8000. The GC-MS/MS data were acquired and processed using Thermo Trace Finder software.

The temperature program was as follows: the initial temperature was 100°C (0.2 minutes), increased at 90°C/min to 190°C (0.2), then at 10°C/min to 225°C and at 90°C/min to a final temperature of 310°C (held for 0.95 minutes). The transfer line was set at 280°C. Helium (Air Liquid) was used as a carrier gas at a split flow rate of 16.5 mL/min followed by split mode at a ratio 11:1. The total run time of the method was found to be less than 8 minutes.

Sample preparation

A 2 mL aliquot of urine was applied into the XAD2 column and spiked with 50 µL of the internal standard mixture. For hydrolysis samples were incubated in an oven at 60°C for 1h after adding 1 mL of 0.1M phosphate buffer solution (pH = 7) and 50 µL of b-glucuronidase. After adding 250 µL of 7% potassium carbonate solution (pH = 9 - 10), the mixture was extracted for 10 minutes with 5 mL Tert Butyl methyl ether (TBME) by using horizontal shaker. The organic phase was centrifuged for 5 minutes at approximately 2000 rpm, dried with Na2SO4 and evaporated under nitrogen at room temperature.

A derivatization procedure of the dried residue using 50 µl of MSTFA/IODO-TMS/DTE mixture (1000/2/2: v/v/w) for 30 minutes at 60°C was used to achieve optimum derivatization efficiency. A 2 µL aliquot of this mixture was injected into the GC for analysis.

Method validation

The method was validated in accordance with ISO:IEC 17025/2005 guidelines. Method validation was performed under the following headings: selectivity, sensitivity, recovery, accuracy, precision, linearity, specificity, inter-day and intra-day precision. The five-level calibration curve was made by using defined volumes of ethanolic solution of the reference standards of analytes as depicted in table 1. A linear regression was used with a weighting factor of 1/x. To assess precision, three injections of three different concentrations were made on the same day and intra-day precision was determined as relative standard deviation. The specificity of the method was evaluated by analyzing human urine samples collected from six different humans to investigate the potential interferences at the LC peak region for analytes and IS using the proposed extraction procedure and chromatographic-MS conditions. Accuracy was evaluated by spiking a known concentration of analyte in comparison with post-extracted concentration. To assess precision, three injections of five different concentrations were made on the same day and intra-day precision was determined as relative standard deviation. These studies were also repeated on different days to determine inter-day precision. Repeatability and reproducibility was determined in multiple measurements of the samples under the same analytical conditions. The limits of detection (LOD) and quantification (LOQ) were determined experimentally, by analysis of samples spiked with decreasing concentrations of the analytes. LOD was defined as the concentration yielding a signal-to-noise ratio of 3. LOQ was calculated as the smallest concentration of analyte that could be measured with a signal-to-noise ratio of 10.

Name	Precursor(m/z)	Product ion	MRM transitions (quantifier)	CE
19 Norandrosterone	405	315.3, 239.3, 225.1, 183.1, 155.1	405 > 225.0	18
Morphine	429	220, 287	429 > 286.9	32
Salbutamol	369	191,207	369 > 206.9	20
Epitestosterone	432	209,327	432 > 208.9	24
Testosterone	432	209,327	432 > 208.9	24
Methyl testosterone (ISTD)	446	301, 198	466 > 301	24

Table 1: *Diagnostic MRM transitions used for quantitation of analytes.*

Results and Discussion

Method Development and optimisation

This method could analyse 5 compounds of different chemistries prohibited in sports (anabolic steroids, narcotic, and β2 agonists). The method was validated for all the analytes. The column allowed separation of all of the analytes within a run time of 8 minutes. Two analogues viz testosterone and epitestosterone, show an identical fragmentation pattern resulting in similar base mass spectra. However, they have different retention time in our developed method. All compounds were identified within 8 minutes of GC elution with solvent delay of 1.0 minute. The selected ion transitions of all five substances are shown in table 1. The column was injected with 2 µl of sample volume through split liner at the split ratio of 11:1 at 280ºC avoid saturation of liner (leading to overloading of column) and ensuring vaporization of all the analytes of interest. Good chromatographic resolution was achieved for all of the compounds as depicted in figure 2 and 3.

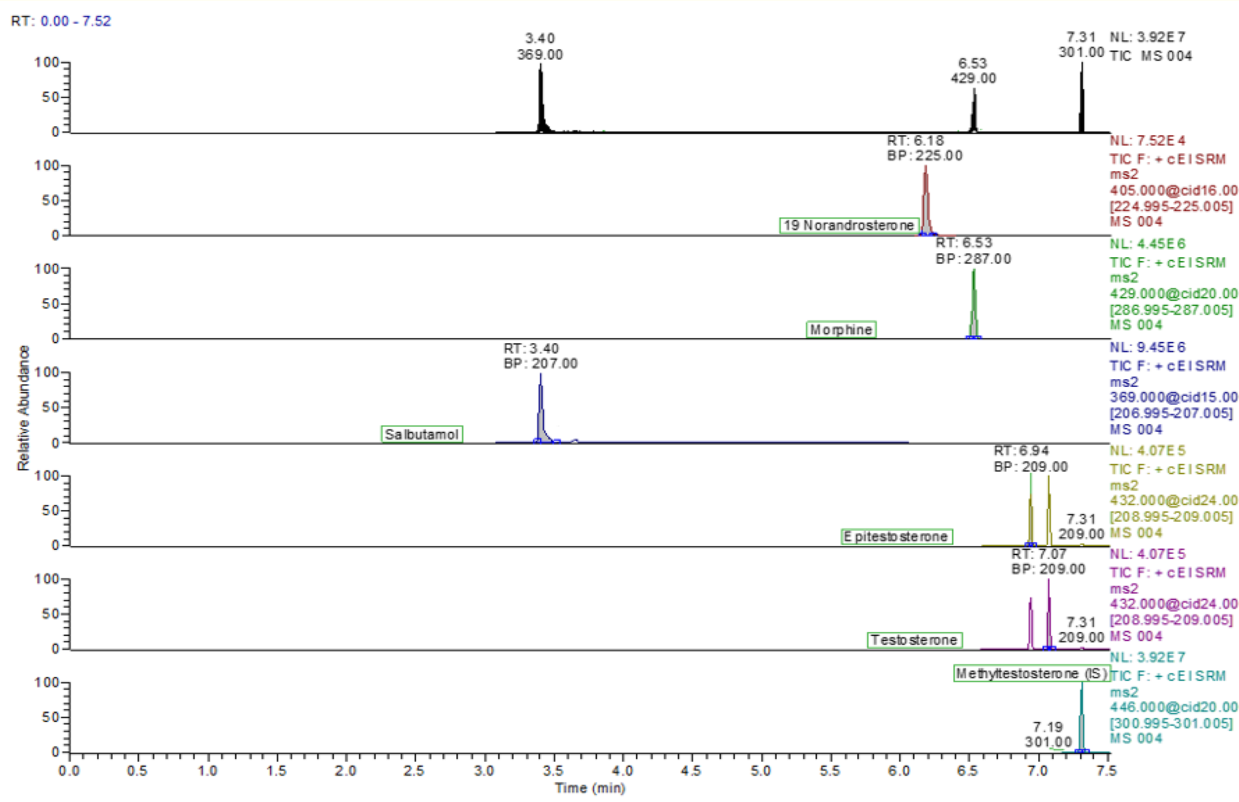

Figure 2: *Representative SRM ion chromatograms of prohibited substances in human urine.*

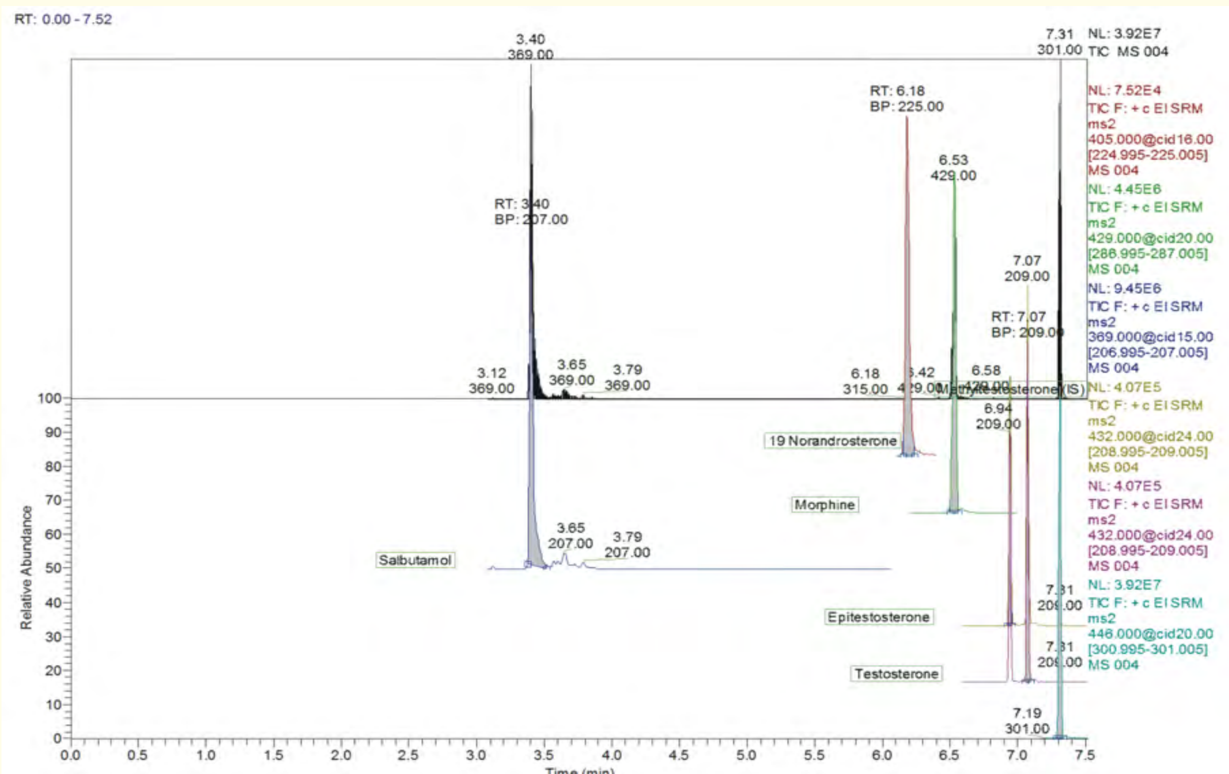

Figure 3: *Overlay chromatograms of prohibited substances representing specificity and selectivity in human urine.*

Validation

Under the conditions described above, the response to the all substances were a linear function of concentration within the range 1 - 2000 ng/ml. Results from the linearity study are given in table 2. The correlation coefficients (R2) ranging from 0.828 to 0.999 showed the method linearity for all analytes over the specified concentrations. The RSD for intra-day and inter-day precision were < 15%, indicating the method was sufficiently precise. The accuracy values on both the occasions (intra- and inter-day) were ranged from 90.3% to 109.7%. The peaks of the substances were resolved from one another, indicating the method was selective. the specificity has been studied by using independent plasma samples from six different humans. The LOQ and LOD were depicted in table 2.

Analytes/linearity equation	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	R^2	RRT	LOQ (ng/ml)	LOD (ng/ml)
19- NA 2.11e-3X+6.175e-5	0.5 ng/mL	1 ng/mL	2 ng/mL	4 ng/mL	10 ng/mL	0.99	0.88	0.5	0.2
Epitestosterone 1.072e-3X-9.393e-4	2 ng/mL	5 ng/mL	10 ng/mL	20 ng/mL	40 ng/mL	0.98	0.99	2	0.5
Morphine 6.612e-5X+2.172e-4	100ng/mL	500 ng/mL	1000 ng/mL	1500 ng/mL	2000 ng/mL	0.99	0.93	20	5
Salbutamol 4.511e-4X-1.114e-2	100ng/mL	500 ng/mL	1000 ng/mL	1500 ng/mL	2000 ng/mL	0.99	0.48	20	5
Testosterone 1.47e-3X-1.940e-3	2 ng/mL	5 ng/mL	10 ng/mL	20 ng/mL	40 ng/mL	0.98	1.01	2	0.5

Table 2: *Linearity Concentration levels (ng/mL) used for the construction of calibration curves.*

R^2: Correlation Co-Efficient; RRT: Relative Retention Time; LOD: Limit of Detection; LOQ: Limit of Quantitation

Conclusion

A simple and fast GC-MS/MS method for the quantitative estimation of five thresholds drug viz 19 norandrosterone, salbutamol, morphine, testosterone and epitestosterone was developed, validated in accordance with the requirements of ISO:IEC 17025. In sports, it is covered in WADA prohibited list and its quantitation is required as per WADA TD2018DL. The total run time of the method is 7.5 minutes followed by the constant pressure. Further, studies are in progress to assess the effectiveness of this method in other threshold drugs/ endogenous steroids.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Acknowledgments

The author wishes to acknowledge the Ministry of Youth Affairs and Sports, Govt. of India for their continuous support.

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Volume 6 Issue 7 July 2018
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