# Fast Quantitative multi method for analysis of prohibited substances using LC–MS/MS

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## Abstract

*A new multi-component method based on LC-ESI-MSMS is developed and presented to screen and confirmation for various categories of prohibited substances with different chromatographic and mass spectrometric characteristics. The method is fast, easy and cheap combined with analytical instruments consisting of liquid chromatographs coupled to sensitive triple-quadrupole or OrbiTrap MS detectors.*

*More than 30 compounds (diuretics, stimulants and narcotics) excreted unchanged could be quantitative determined without pre-concentration step using direct injection of urine samples at concentration levels below the required limits. Additionally for glucuronide conjugated substances, as a morphine group, we used the SPE pre-treatment on the base of new SPE cartridges from SiliCycle. The method has been fully validated according to ISO guidelines[2] and can increases the efficiency of laboratory work.*

## Introduction

Stimulants include psychomotor stimulants, sympathomimetic amines and central nervous system stimulants. For anti-doping purposes, fast analysis can be important during major sports events, where 24-h reporting times are mandatory. From a toxicological point of view, fast analysis facilitates prompt diagnosis which is required in some emergency cases.

Diuretics are drugs that increase the rate of urine floe and sodium excretion to adjust the volume and composition of body fluids. There are several major categories of this drug class and the compounds vary greatly in structure, physicochemical properties, effects on urinary composition and renal hemodynamics, and site and mechanism of action. Because of their abuse by athletes, diuretics have been included on The World Ant-Doping Agency's list of prohibited substances [1]. The use of diuretics is banned both in competition and out of competition.

Liquid chromatography tandem mass spectrometry (LC–MS/MS) has become a powerful tool for the quantitative analysis of drugs that does not require derivatization. DS-LC-MS for the analysis of urine samples has become a trend in the past 10 years in both analytical toxycology and doping control analysis. In particular, the economic benefits (easy sample preparation and omission of time-consuming extraction) are the driving forces behind this trend.

We developed a sensitive and specific confirmation method for more than 25 stimulants and 10 diuretics in urine by LC–MS/MS. The method is fast, easy and cheap combined with modern and powerful analytical instruments consisting of liquid chromatographs.

## Materials and Methods

Reagents and chemicals: Stimulants and narcotics were purchased from Cerilliant (USA) as solutions in methanol or acetonitrile, and diuretics from TRC (Canada). Standard stock solutions of each compound were prepared at 1mg/ml in methanol. HPLC-grade methanol, i-propanol, acetic acid, hexane, ethyl acetate were acquired from Merck (Germany).

*Instrumentation:* The experiments were performed using a Accela LC system (Thermo Fisher Scientific), interfaced with a TSQ Vantage (Thermo Fisher Scientific) with electrospray ionization (ESI). The mass spectrometer was operated in positive and negative ion mode. LC separation was performed on Zorbax RRHD Extend-C18 2.1x100mm, 1.8 µm by gradient elution at a constant flow rate of 300µl/min. The LC eluents were solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The mobile phase gradient was programmed as follows: 0 min 95% A, 0-1 95% A, 01-7,5 min 95-5% A, 7,5-10,5 min 5% A, 10,5-10.51 min 5-95%. Re-equilibration time was 4,5 min. The total chromatographic run time was 15 min.

*Sample preparation :* 3 mL urine sample

2 mL from the urine sample
*Hydrolysis:*
1 β-glucuronidase 20µL
2 1h 55°C
20 µL ISTD mix of d3-morphine, d3-6-monoacetykmorphine, d3-benzoilecgonine
*Solid phase extraction*
1.Conditioned column
2.Loaded the sample
3.Washed the column with 3mL methanol and than 1 mL 0.1M acetic acid
4.First elution-3 mL hexane/ethyl acetate (50:50, v/v)
5.Second elution-3 ml methylene chloride: i-propanol: ammonium 78:20:2

200 µl urine sample diluting with 800 µl mobile phase
10 µL ISTD d5- amphetamine c=10µg/mL
Direct injection at LC/MSMS

Evaporation at 40 °C under gentle nitrogen flow and the residues were re-dissolved with 1 mL of mobile phase. Ten microliters of the final solution was then injected into the LC–MS system

Several solid-phase extraction (SPE) sorbent were evaluated for the extraction of glucuronide conjugated substances and its metabolites from urine. The SPE sorbents used for this study were C18, Servo, Oasis SCX, DrugClean (SiliaPrep) and CleanDrud by SiliCycle. Since several different SPE materials were used in this study, Clean Drug by SiliCycle procedure were used to maximize extraction efficiency and minimize carryover.

A optimal purification were carried out for the urine samples, using Clean Drug columns -3 mL; 200 mg (by SiliCycle). Each column was conditioned, sample was loaded, then column washed by the 3ml methanol and 1 ml 0,1M acetic acid and dried under maximum vacuum for 10 min. Elution with 3 mL hexane/ethyl acetate (50:50, v/v). The second elution was with 3 ml methylene chloride: i-propanol: ammonium 78:20:2. After solvent evaporation at 40 °C under gentle nitrogen flow, the residues were re-dissolved with 1 mL of mobile phase. Ten microliters of the final solution was then injected into the LC–MS system.

## Results and Discussion

The performance characteristics for validation according International standard of WADA (1) and Decision 2002/657/ EC are described in table 1. [2]

| Characteristics | WADA requirement | | EU 2002/657/EC | | | |
|---|---|---|---|---|---|---|
| | Non threshold | Threshold substance | Qualitative | | Quantitative | |
| | Screening (S) | Confirmation (C) | S | C | S | C |
| Decision limit ($CC_\alpha$) | | | | | | |
| Detection capability ($CC_\beta$) | + | + | + | + | + | + |
| Limit of quantification (LOQ) | – | + | | | | |
| Precision | – | + | – | – | + | + |
| Accuracy | – | + | – | – | | + |
| Linearity | – | + | – | – | + | + |
| Specificity | + | + | + | + | + | + |
| Robustness | | | | | | |

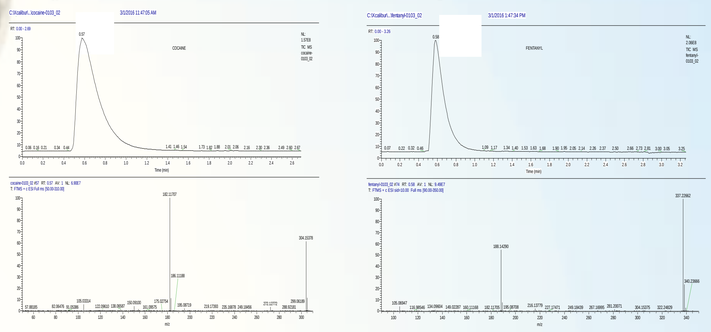
*Screening method:* Minimum criteria for chromatographic-mass spectrometric confirmation of the identity of analytes for doping control purpose are:
1. The retention time(RT) of the analytes chromatographic peak in the sample shall not differ (ΔRT) by more than 1 % or ±0.1 minutes , from that of the same analytes in a spiked sample, reference collection sample, or reference material analyzed in the same analytical batch;
2. The relative abundances of any of the diagnostic ions shall not differ by more than the amount specified in WADA technical document from the corresponding relative abundances of the same ions acquired from a spiked positive control urine, reference collection sample, or reference material [4, 5]. Relative ion intensities were calculated on the basis of ion ratios (quantification transition divided by qualifier transition). These values were compared with the mean relative ion intensity of all analytes.

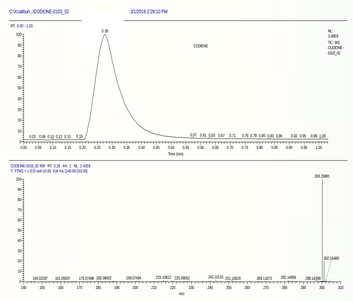
Table 2 contains the optimized parameters for multiple MRM transitions for various prohibited substances [3].

| Compound | Polarity | Parent ion | Product ion | CE | RT min | S-lens |
|---|---|---|---|---|---|---|
| Amphetamine | ESI+ | 136 | 119 | 5 | 3.16 | 39 |
| | | | 91 | 16 | | |
| Methylamphetamine | ESI+ | 150 | 91 | 19 | 3.25 | 48 |
| | | | 119 | 8 | | |
| Ethylamphetamine | ESI+ | 164 | 65 | 42 | 3.44 | 52 |
| | | | 119 | 11 | | |
| MDA | ESI+ | 180 | 133 | 17 | 3.20 | 43 |
| | | | 163 | 7 | | |
| MDMA | ESI+ | 194 | 163 | 12 | 3.26 | 55 |
| | | | 135 | 20 | | |
| Codeine | ESI+ | 300 | 165 | 41 | 2.90 | 93 |
| | | | 215 | 25 | | |
| Oxycodone | ESI+ | 316 | 298 | 19 | 2.98 | 75 |
| | | | 241 | 28 | | |
| Morphine | ESI+ | 286 | 165 | 38 | 1.53 | 93 |
| | | | 152 | 58 | | |
| Hydromorphone | ESI+ | 286 | 185 | 29 | 2.26 | 70 |
| | | | 157 | 40 | | |
| 6-Acetylmorphine | ESI+ | 328 | 165 | 37 | 3.89 | 98 |
| | | | 211 | 25 | | |
| Benzoilecgonine | ESI+ | 290 | 168 | 18 | 4.80 | 82 |
| | | | 105 | 30 | | |
| Oxymorphone | ESI+ | 302 | 284 | 18 | 1.82 | 93 |
| | | | 227 | 28 | | |
| Hydromorphone | ESI+ | 272 | 152 | 55 | 1.33 | 93 |
| | | | 165 | 38 | | |
| Heroine | ESI+ | 370 | 165 | 48 | 4.76 | 109 |
| | | | 268 | 27 | | |
| Fentanyl | ESI+ | 337 | 188 | 24 | 5.60 | 89 |
| | | | 105 | 42 | | |
| Cocaine | ESI+ | 304 | 182 | 18 | 3.77 | 81 |
| | | | 82 | 31 | | |
| Mepethydine | ESI+ | 248 | 220 | 20 | 3.95 | 94 |
| | | | 174 | 19 | | |
| Methadone | ESI+ | 310 | 265 | 14 | 4.88 | 74 |
| | | | 105 | 29 | | |
| Ephedrine | ESI+ | 166 | 148 | 11 | 6.10 | 50 |
| | | | 115 | 27 | | |
| Pseudoephedrine | ESI+ | 166 | 148 | 11 | 6.45 | 50 |
| | | | 115 | 27 | | |
| Cathine | ESI+ | 134 | 115 | 21 | 4.86 | 64 |
| | | | 117 | 27 | | |
| Norephedrine | ESI+ | 134 | 115 | 21 | 5.36 | 64 |
| | | | 117 | 27 | | |
| Methylephedrine | ESI+ | 180 | 91 | 34 | 6.50 | 66 |
| | | | 162 | 13 | | |
| Methcathinone | ESI+ | 164 | 131 | 28 | 3.40 | 76 |
| | | | 130 | 40 | | |
| THC | ESI+ | 315 | 193 | 22 | 7.2 | 79 |
| | | | 123 | 32 | | |
| THC-COOH | ESI+ | 345 | 327 | 15 | 7.21 | 104 |
| | | | 299 | 18 | | |
| JWH-018 | ESI+ | 342 | 155 | 24 | 7.85 | 110 |
| | | | 127 | 44 | | |
| JWH-250 | ESI+ | 336 | 121 | 24 | 7.40 | 97 |
| | | | 91 | 39 | | |
| Acetazolamide | ESI+ | 223 | 181 | 14 | 2.80 | 60 |
| | | | 164 | 22 | | |
| Piretanide | ESI+ | 363 | 236 | 30 | 5.48 | 83 |
| | | | 282 | 20 | | |
| Tolvaptane | ESI+ | 451 | 252 | 17 | 6.03 | 97 |
| | | | 119 | 36 | | |
| Metazolamide | ESI+ | 237 | 195 | 14 | 3.59 | 62 |
| Benzylhydrochlorothiazide | ESI– | 386 | 294 | 24 | 4.74 | 87 |
| | | | 296 | 24 | | |
| Diclofenamide | ESI– | 303 | 239 | 19 | 3.73 | 73 |
| | | | 267 | 17 | | |
| Polythiazide | ESI– | 438 | 324 | 22 | 4.98 | 95 |
| | | | 398 | 17 | | |
| Furosemide | ESI– | 329 | 205 | 23 | 4.90 | 77 |
| | | | 285 | 16 | | |
| Indapamide | ESI– | 364 | 189 | 27 | 5.01 | 117 |
| | | | 191 | 27 | | |
| Xipamide | ESI– | 353 | 274 | 27 | 5.43 | 113 |
| | | | 127 | 33 | | |

Triple-quadrupole mass spectrometry with multiple reaction monitoring (MRM) is the most commonly adapted technique for confirmatory and quantitative drug analysis. We are developing a confirmatory method for the analysis of drugs of abuse in human urine by using a high resolution and high mass accuracy hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap-MS). This method allows for the detection of different drugs of abuse, including amphetamines, cocaine, opiate alkaloids, synthetic cannabinoids, hallucinogens and their metabolites [6-8].

In fig. 1 we demonstrated confirmation of some opioid substances:





*Quantitative analysis:*

We developed an LC–MS/MS method for the simultaneous identification and quantification of stimulants, narcotics and diuretics, and we validated the method for linearity, sensitivity, carryover, extraction efficiency, matrix effects, precision, accuracy, process efficiency, and selectivity. Linear regression with 1/x weighting was used to construct the calibration curves, coefficients of determination (R2), extraction efficiency, limit of detection, limit of quantification and uncertainty of measure were determined and summarized in Table 3.

| Compound | Calibration Concentration [ng/ml] | $R^2$ | Extracting Efficiency/ Output [%] | LOD [ng/ml] | LOQ [ng/ml] | Uncertanty [%] |
|---|---|---|---|---|---|---|
| Amphetamine | 10 – 200 | 0.9985 | 94 | 0.150 | 10.5 | 4.48 |
| Methylapmphetamine | 10 – 200 | 0.9966 | 104 | 0.149 | 10 | 4.89 |
| Ethylamphetamine | 10 – 200 | 0.9982 | 104 | 1.480 | 10.5 | 4.55 |
| MDA | 15 – 200 | 0.9985 | 102 | 1.550 | 15.5 | 4.37 |
| MDMA | 15 – 200 | 0.9958 | 102 | 0.149 | 14.9 | 5.73 |
| Codeine | 10 – 200 | 0.9980 | 99 | 1.520 | 10 | 5.88 |
| Oxycodone | 10 – 200 | 0.9996 | 94 | 1.490 | 10 | 7.72 |
| Morphine | 5 – 100 | 0.9989 | 81 | 0.350 | 5 | 5.50 |
| Hydromorphone | 5 – 105 | 0.9989 | 39 | 0.362 | 5.8 | 9.41 |
| 6-Acetylmorphine | 5 – 100 | 0.9982 | 68 | 0.350 | 5 | 7.97 |
| Benzoilecgonine | 5 - 100 | 0.9958 | 87 | 0.350 | 5 | 6.74 |
| Oxymorphone | 5 - 100 | 0.9981 | 58 | 0.350 | 5 | 7.10 |
| Heroine | 5 – 100 | 0.9858 | 57 | 0.344 | 5.5 | 5.83 |
| Fentanyl | 0.22 – 4 | 0.9965 | 64 | 0.034 | 0.22 | 5.26 |
| Cocaine | 10 – 200 | 0.9981 | 99 | 1.490 | 10 | 5.22 |
| Mepethydine | 10 – 200 | 09985 | 100 | 1.480 | 10 | 5.16 |
| Methadone | 15 – 200 | 0.9991 | 97 | 0.151 | 15 | 5.42 |
| Ephedrine | 20 – 403 | 0.9962 | 103 | 10.3 | 20.2 | 6.40 |
| Pseudoephedrine | 20 – 400 | 0.9981 | 108 | 9.6 | 20 | 7.95 |
| Cathine | 20 – 400 | 0.9969 | 104 | 7.56 | 19.7 | 8.34 |
| Norephedrine | 20 – 400 | 0.9966 | 110 | 8.56 | 19.8 | 6.31 |
| Methylephedrine | 20 - 300 | 0.9910 | 104 | 14.62 | 20 | 7.10 |
| Methcathinone | 100 - 400 | 0.9976 | 76 | 35.5 | 100 | 6.82 |
| Acetazolamide | 50 - 200 | 0.9984 | 92 | 9.97 | 50 | 7.58 |
| Piretanide | 50 – 202 | 0.9971 | 94 | 1.01 | 50.4 | 10.3 |
| Tolvaptane | 50 – 200 | 0.9951 | 109 | 0.987 | 49.3 | 12.63 |
| Metazolamide | 50 - 200 | 0.9948 | 106 | 9.91 | 50 | 9.92 |
| Benzylhydrochlorothiazide | 50 – 200 | 0.9984 | 89 | 2.97 | 49.6 | 10.18 |
| Diclofenamide | 51 – 204 | 0.9965 | 78 | 10.2 | 51 | 17.26 |
| Polythiazide | 50 – 200 | 0.9960 | 79 | 2.96 | 49.5 | 12.9 |
| Furosemide | 51 - 204 | 0.9996 | 73 | 10.2 | 51 | 8.58 |
| Indapamide | 50 - 202 | 0.9960 | 105 | 3.03 | 50.5 | 11.5 |
| Xipamide | 50 – 200 | 0.9943 | 75 | 2.98 | 49.8 | 14.02 |

Selectivity was determined by comparing responses of 10 different spiked urine samples with analytes at the WADA MRPL level with a spiked standards at the same concentration prepared in water. No interference being detected et the expected retention times of the analytes. For all compounds the matrix effect was less than 15% apart from oxycodone which gave a value of 23%; hydromorphone – 24%; nor morphine – 27%.

## Conclusions

The present investigation confirms that direct injection of urine in combination with electrospray LC-MS/MS and a.m. spectrometry can be used for confirmation of various classes of drug residues and prohibited substances in urine. LC/MSMS multi-compound analysis combined with a simple sample preparation procedure have been developed and validated for the measurement of target substances in urine samples. Based on the results of the proficiency tests, the methods seem to be useful for the rapid and accurate quantitative determination of substances in urine samples.

**References**

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