Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography–tandem mass spectrometry with respect to criteria for compound identification

Barbora Maralikova[a,b], Wolfgang Weinmann[b,*]

[a] *Department of Analytical Chemistry, University of Pardubice, FCHT, nam. Cs. legii 565, 53210 Pardubice, Czech Republic*
[b] *Institute of Legal Medicine, Forensic Toxicology, University Hospital, Albertstrasse 9, D-79104 Freiburg, Germany*

Abstract

Recently, high-performance liquid chromatography–tandem mass spectrometry (LC/MS/MS) has become a powerful tool for quantitative confirmatory analysis of drugs of abuse and has begun to spread in the field of forensic toxicology. Guidelines for confirmatory analysis by GC/MS and LC/MS/MS have been published recently by several organizations (WADA, IOC, SOFT, GTFCh, EU). However, these guidelines have not yet been included in procedures for drug analysis with LC/MS/MS. The prerequisites for forensic confirmatory analysis by LC/MS/MS with respect to EU guidelines are chromatographic separation, a minimum number of two MS/MS transitions to obtain the required identification points and predefined thresholds for the variability of the relative intensities of the MS/MS transitions (MRM transitions) in samples and reference standards. LC/MS/MS methods for determination of several classes of drugs of abuse including some basic drugs (opiates, stimulants), cannabinoids and some of their phase-I- and phase-II-metabolites (especially glucuronides) in urine and serum of drug abusers and/or crime offenders or victims have been developed and validated following current recommendations and are presented in this paper. At least two MRM transitions for each substance were monitored to provide sufficient identification of drugs, deuterated analogues of analytes were used as internal standards for quantitation where possible and chromatographic separation has been performed on reversed-phase columns with gradient elution. Validation data obtained and the application to real samples show that the requested criteria for confirmatory analysis of drugs of abuse by EU guidelines can be fulfilled with a total number of four identification points by LC/MS/MS methods using a triple-quadrupole mass spectrometer. Furthermore, the methods are sufficiently sensitive to meet current requirements for confirmatory analysis of drugs of abuse in driving under the influence of drugs (DUID) cases established by the Society of Toxicological and Forensic Chemistry (GTFCh).
© 2004 Elsevier B.V. All rights reserved.

Keyword: Drugs of abuse

1. Introduction

Confirmation of identity of forensically relevant compounds and especially drugs of abuse is a necessary part of the process to decide whether an individual was under the influence of drugs during the particular event, for example medico–legal event controls of people involved in crime, e.g. robberies, rapes, homicides and traffic and workplace accidents, driving under influence of drugs, etc. Because law courts rely on analytical results from toxicological analyses, the confirmation of identity of a particular drug or its metabolites in biological fluid or tissue should be objective and reliable.

According to the SOFT/AAFS Forensic Laboratory Guidelines [1] and to the guidelines of the German Soci-

---

* Corresponding author. Tel.: +49 761 203 6878; fax: +49 761 203 6858.
E-mail address: weinmann@sun11.ukl.uni-freiburg.de
(W. Weinmann).

ety of Toxicological and Forensic Chemistry (GTFCH) [2], the initial detection of drugs and other toxins should be confirmed whenever possible by a second technique based on a different chemical principle. The use of mass spectrometry is recommended as the confirmatory technique, wherever practical. However, most of the recommendations refer to GC/MS and only recently attention has been given to the LC/MS/MS technique. Because this technique is becoming intensively used for drug confirmation, there is a need to establish minimal necessary requirements for LC/MS/MS analyses. Although detailed international guidelines have been established by the EU for confirmation of veterinary drugs in residue control [3,4] as well as for doping-agents by the world anti-doping agency (WADA) and by the IOC, no detailed international guidelines are available for forensic toxicology. Recently, a review article [5] has been published where several new rules and quality assurance criteria are summarized. They concern the identification of organic molecules in biosamples taking into account the latest advances in mass spectrometry. These criteria are expressed as so called identification points (IP) that the particular method has to fulfill to ensure reliable and objective identification of the particular substance. Criteria mentioned in the review article are based on theoretical considerations and no concrete cases tested under these criteria have been presented.

The general criteria for any analytical procedure require that the method has to be able to distinguish between the analyte and all known interfering substances that may possibly occur in the relevant matrix. Since every analytical technique has different identification power a system of IP has been set up to characterize a particular technique. In this study we describe two LC/MS/MS methods for analysis of drugs of abuse that show different fragmentation features and present the results with respect to current recommendations and EU guidelines [4] taking into account the criteria of "minimum number of MRM transitions" and the "relative intensities of the transitions". As examples, we have chosen a method for determination of three forensically relevant cannabinoids Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) and a method for the simultaneous determination of basic drugs and their metabolites: codeine, morphine, morphine-3β-D-glucuronide (M3G), normorphine, 6-O-monoacetylmorphine (MAM), benzoylecgonine (BE), heroin, lysergide (LSD), cocaine and methadone. Even though validated LC/MS methods for determination of drugs of abuse have been published, e.g. for determination of opioids in biological fluids [6–9], methadone in saliva [10], cocaine in urine [11], cocaine in several matrices [12], opiates and cocaine in meconium [13] and opioids, cocaine and metabolites in urine [14], none of the authors has followed the recommendations concerning the necessity to use at least two MRM transition for compound identification and/or they did not take into consideration the

relative intensities of diagnostic ions or transitions. Only one study describes the use of quantifiers and qualifiers for confirmation of cocaine and benzoylecgonine in urine [15] and discusses the quality control requirements established by the Federal Department of Health and Human Services [16]. However, it seems that only quantifiers were used for validation parameters.

Our presented methods have been validated for determination of the mentioned drugs (except of heroin and LSD) in human plasma and have been successfully applied to the identification of these drugs in blood, plasma and urine samples. At least two MRM transitions for each substance were monitored to provide sufficient identification of drugs, deuterated analogues of analytes were used as internal standards for quantitation where possible. The quantifiers were used for all validation parameters except for limit of detection (LOD); for calculation of LOD the qualifiers (with α-error 10%) were used as suggested by the guidelines of the GTFCh for the confirmatory analysis of the drugs of abuse.

2. Experimental

2.1. Material and chemicals

Standard solutions of codeine (1 mg/ml), morphine (100 μg/ml), M3G (100 μg/ml), normorphine (1 mg/ml), MAM (1 mg/ml), BE (100 μg/ml), heroin (100 μg/ml), LSD (1 mg/ml), cocaine (1 mg/ml), methadone (100 μg/ml), THC (1 mg/ml), 11-OH-THC (100 μg/ml), THC-COOH (1 mg/ml) and deuterated analogues (100 μg/ml) used as internal standards (IS) in methanol were obtained from Promochem/Radian (Wesel, Germany). Acetonitrile (HPLC gradient grade), all solvents for SPE (analytical grade), ammonium carbonate (p.a.), ammonium formate (p.a.) and ammonium acetate (p.a.) were purchased from Merck (Darmstadt, Germany). SPE cartridges Chromabond C18 (3 ml/500 mg) for extraction of cannabinoids and Chromabond C18 (3 ml/200 mg) for extraction of basic drugs were supplied by Macherey-Nagel (Düren, Germany). Deionized water was prepared on a cartridge-deionizer from Memtech (Moorenweis, Germany).

2.2. Instrumentation and methods

SPE was performed with a Zymark RapidTrace SPE Workstation (Zymark, Idstein, Germany). For the evaporation of the extracts the speed vacuum concentrator (Alpha RVC, Martin Christ, Osterode, Germany) was used.

#### 2.2.1. Cannabinoids
In case of cannabinoid analysis LC/MS/MS system consisted of an API 3000[TM] triple-quadrupole mass spectrometer equipped with Turbo IonSpray interface and LINAC[TM] collision cell (Applied Biosystems/Sciex, Langen, Germany)
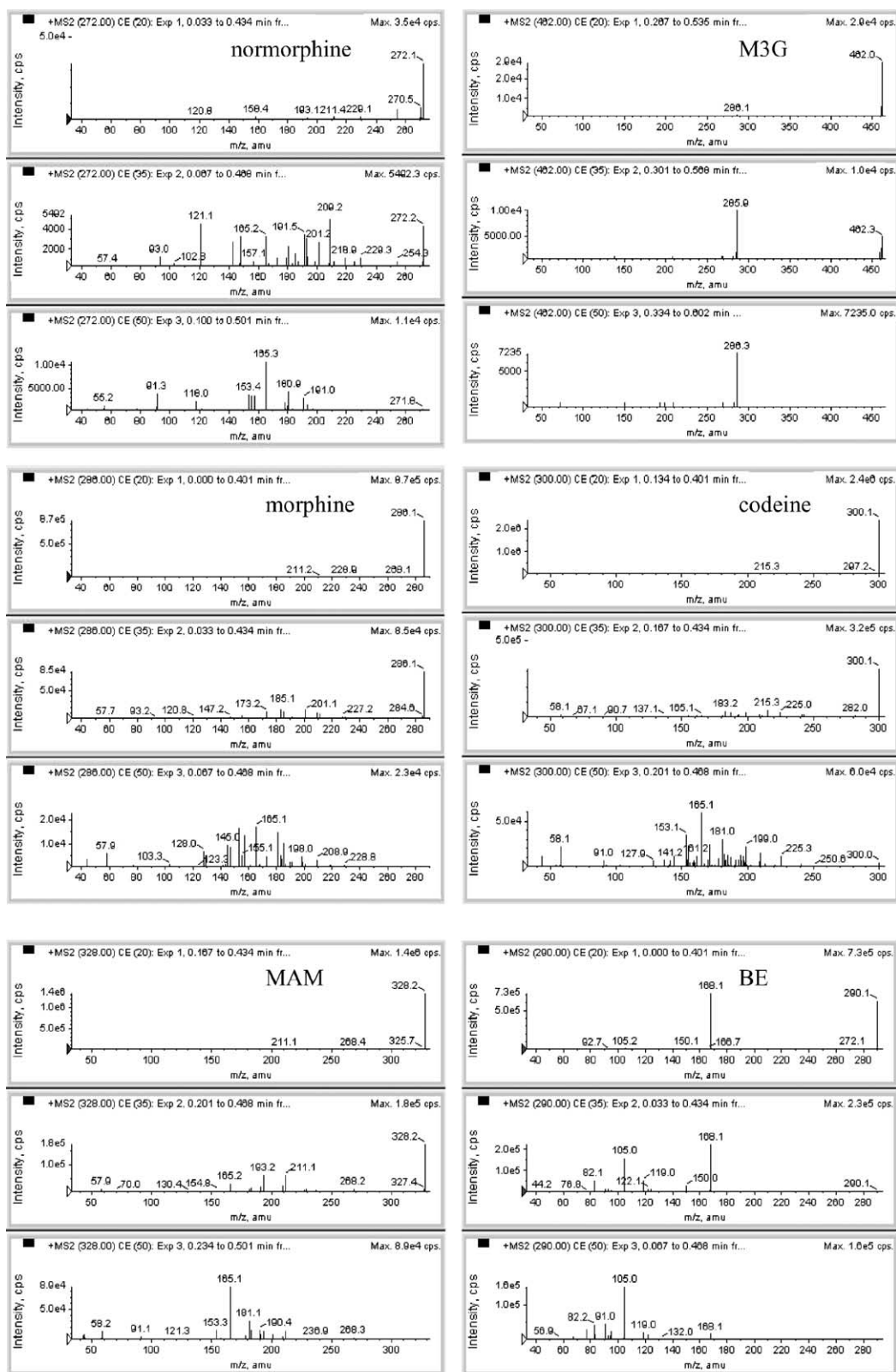
(A)

Fig. 1. MS/MS spectra of studied drugs obtained at following conditions: flow-injection analysis, 0.25 ml/min; A:B, 1:1 (v/v); (A) 1 mM ammonium formate (pH 2.7); (B) acetonitrile with 1 mM ammonium formate; MS conditions: TurboIonSpray gas, 4 l/min; NEB, 69 kPa; CUR, 76 kPa; CAD, 28 kPa; IS, 5250 V; TEM, 350 °C; DP, 20 V; FP, 230 V; EP, 10 V; CXP, 15 V; collision energies (CE): 20, 35, 50 V.
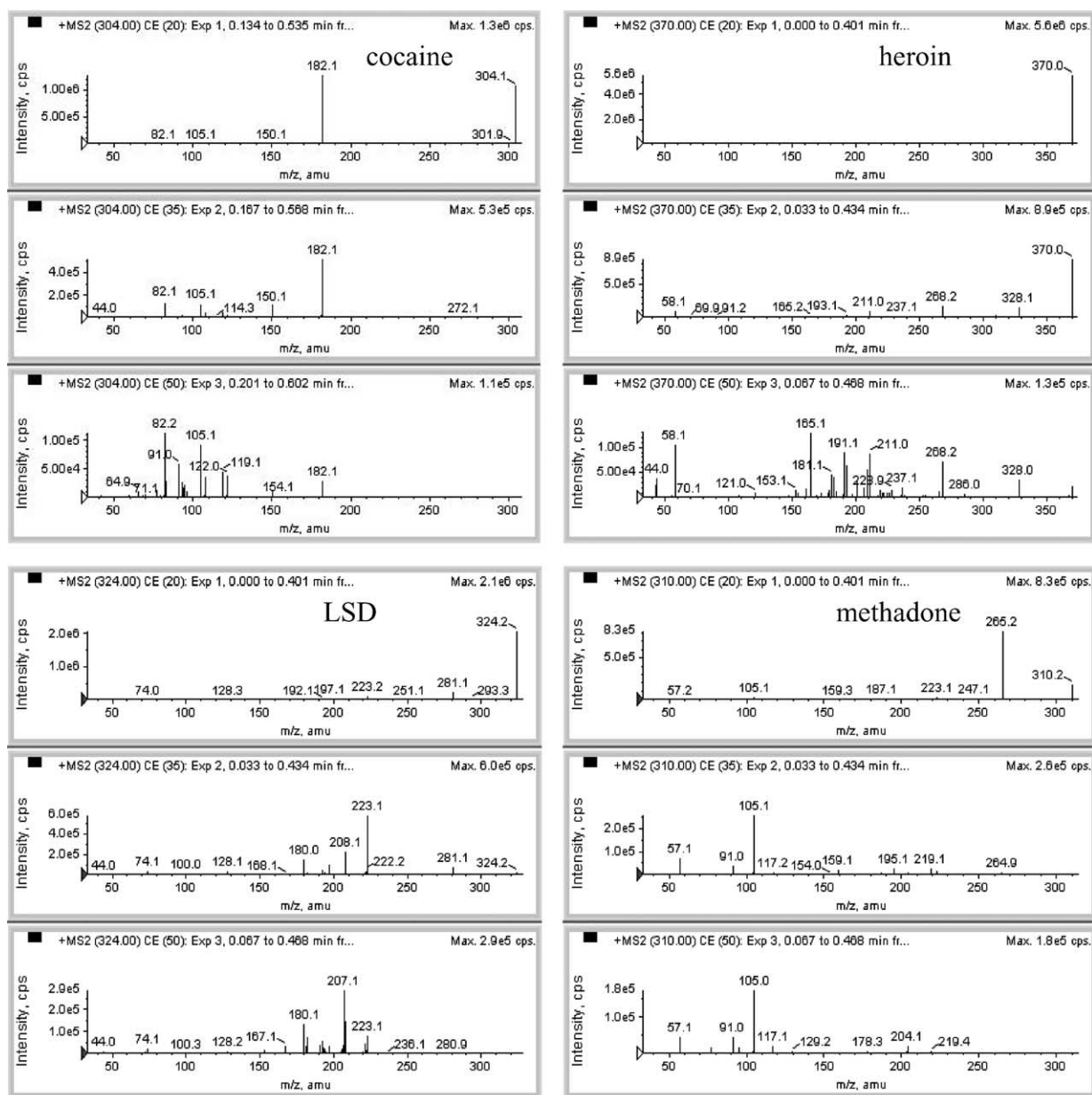
Fig. 1. (*Continued*).

and Series 200 LC Plus system (Perkin-Elmer, Boston, MA, USA). Analytes were separated at 40 °C on a Luna 3µ PhenylHexyl column 50 mm × 2 mm, 3 µm (Phenomenex, Torrance, CA, USA) fitted up with guard cartridge Polar-RP, 4 mm × 2.0 mm (Phenomenex, Torrance, CA, USA) using gradient elution of 5 mM ammonium acetate (pH 6.5) (A) and acetonitrile (B). The flow rate was 0.25 ml/min and the gradient was: 0–0.4 min: 30% B; 0.4–7.8 min: linear from 30% to 90% B; 7.8–8.2 min: 90% B; 8.2–8.5 min: linear from 90% to 30% B; 8.5–11 min: 30% B. Detailed description of MS/MS conditions and MS/MS spectra for selection of MRM transitions are published elsewhere [17].

2.2.2. Opioids and stimulants

In case of basic drugs analysis, LC/MS/MS system consisted of an API 365[TM] triple-quadrupole mass spectrometer equipped with Turbo IonSpray interface (Applied Biosystems/Sciex, Langen, Germany) two HPLC pumps Shimadzu-LC-10AD VP and controller SCL 10Avp (Shimadzu, Duisburg, Germany). Analytes were separated at 40 °C on a Synergi Hydro-RP column 150 mm × 2 mm, 3 µm (Phenomenex, Torrance, CA, USA) fitted up with guard cartridge Polar-RP 4 mm × 2.0 mm (Phenomenex, Torrance, CA, USA) using gradient elution of 4 mM ammonium acetate (pH 4.6) (A) and acetonitrile (B) and post column addition of acetonitrile

Table 1
SPE program of Zymark RapidTrace SPE-Automat for basic drugs

Step	Source	Volume or time	Speed [ml/min]
Condition	Methanol	2 ml	2
Condition	Water	1 ml	2
Condition	AC buffer (pH 9.4)	1 ml	2
Load	Sample	3 ml	1.002
Purge-Cannula	Methanol:water (1:1)	4 ml	30
Rinse	AC buffer (pH 9.4)	1 ml	1.002
Dry	Nitrogen	2 min	–
Collect	2% (v:v) acetic acid in methanol	1.5 ml	1.002
Dry	Nitrogen	2 min	–
Purge-Cannula	Methanol:water (1:1)	4 ml	30
Purge-Cannula	Methanol:water (1:1)	4 ml	30

(0.05 ml/min). The mobile phase flow rate was 0.25 ml/min and the gradient was: 0–1 min: 0% B; 1–13 min: linear from 0% to 60% B; 13–15 min: 60% B; 15–17 min: linear from 60% to 0% B; 17–23 min: 0% B. MS conditions were as follows: TurboIonSpray gas, 4 l/min; nebulizer gas (NEB), 69 kPa; curtain gas (CUR), 76 kPa; collision gas (CAD), 28 kPa; IonSpray voltage (IS), 5250 V; temperature (TEM), 350 °C; focusing potential (FP), 230 V; dwell time, 0.05 s; entrance potential (EP), 10 V; collision cell exit potential (CXP), 15 V; declustering potential (DP), 20 V; collision energies (CE) for particular MRM transitions are mentioned in Table 3.

Positive electrospray ionisation was used in all cases and all analyses were performed in multiple reaction monitoring mode (MRM) [18–20].

2.3. Sample preparation and validation

For validation of both methods two series of nine calibration standards were prepared by adding aliquots of stock solution into 1 ml of drug-free plasma. The validation of linearity, accuracy and precision were done by Quantitation Wizard of the Analyst® software. The program for statistical data evaluation B.E.N. Version 2.0 was used for calculation of limits

Table 2
Validation data for human plasma samples evaluated from "quantifiers data"; linearity and correlation coefficients of calibration standard samples and accuracy and precision (R.S.D.) of quality control samples

Analyte	Retention time	Linearity (dynamic range, ng/ml)	r^2	Concentration of QC [ng/ml]	Mean[a] [ng/ml]	Accuracy[a] [%]	R.S.D.[a] [%]
THC	7.0	$y = 0.237x + 0.460$ (1–100)	0.998	8	7.63 ± 0.14	95.47 ± 1.73	2.0
				20	20.73 ± 0.82	103.67 ± 4.09	4.0
11-OH-THC	5.5	$y = 0.129x + 0.054$ (1–100)	0.998	8	8.62 ± 0.13	107.7 ± 1.62	1.6
				20	19.52 ± 0.71	97.62 ± 3.53	4.2
THC-COOH	3.9	$y = 0.038x + 0.163$ (5–250)	0.998	15	16.85 ± 0.23	112.34 ± 1.53	1.4
				40	39.80 ± 2.71	99.50 ± 6.78	7.6
Codeine	6.8	$y = 0.076x + 0.144$ (1–250)	0.998	10	10.93 ± 0.64	109.34 ± 9.37	6.4
				50	50.25 ± 3.23	100.50 ± 6.46	7.2
Morphine	5.5	$y = 0.056x + 0.140$ (2–250)	0.998	5	5.83 ± 0.25	116.73 ± 4.96	4.8
				10	9.14 ± 0.65	91.37 ± 6.53	8.1
M3G	4.7	$y = 0.069x + 0.181$ (2–250)	0.998	10	11.51 ± 0.45	115.09 ± 4.46	4.2
				50	51.32 ± 3.48	102.64 ± 6.96	6.9
Normorphine	5.2	$y = 0.004x + 0.009$ (2–250)	0.998	5	4.51 ± 0.11	90.26 ± 2.12	2.6
				10	10.75 ± 0.56	107.56 ± 5.63	5.9
MAM	7.3	$y = 0.002x + 0.005$ (1–250)	0.994	10	10.81 ± 1.01	108.11 ± 10.18	10.2
				50	54.95 ± 4.47	110.12 ± 9.17	11.5
BE	7.9	$y = 0.069x + 0.002$ (1–500)	0.998	5	4.79 ± 0.13	95.98 ± 2.67	2.9
				50	50.97 ± 1.88	101.94 ± 3.76	4.1
Cocaine	10.2	$y = 0.068x + 0.229$ (2–500)	0.996	10	11.19 ± 0.42	111.92 ± 4.17	4.3
				50	45.63 ± 0.87	91.26 ± 1.74	2.2
Methadon	13.4	$y = 0.055x + 0.033$ (1–500)	0.998	10	10.08 ± 0.29	100.81 ± 2.88	3.2
				50	50.22 ± 1.36	100.43 ± 2.71	2.9

[a] $n = 5$.

Table 3
Summarized MRM transitions, their relative intensities (qualifiers 100%), LODs and LOQs for selected drugs from plasma samples

Analyte	MRM transitions (collision energy (CE) [V])					LOD[a] [ng/ml]	LOQ[b] [ng/ml]
	Quantifier	Qualifier 1	Relative intensity [%]	Qualifier 2[c]	Relative intensity [%][d]		
M3G	$462 \rightarrow 462$ (20)	$462 \rightarrow 286$ (50)	34.6 ± 10.1[d]	$462 \rightarrow 268$ (50)	1.4 ± 21.4	2.4	6.6
Normorphine	$272 \rightarrow 272$ (20)	$272 \rightarrow 121$ (35)	3.6 ± 13.9[d]	$272 \rightarrow 165$ (50)	3.4 ± 11.7	3.1	8.0
Morphine	$286 \rightarrow 286$ (20)	$286 \rightarrow 201$ (35)	3.5 ± 18.5[d]	$286 \rightarrow 165$ (50)	2.7 ± 22.2	4.0	6.3
Codeine	$300 \rightarrow 300$ (20)	$300 \rightarrow 165$ (50)	2.7 ± 18.5[d]	$300 \rightarrow 215$ (35)	3.5 ± 14.3	2.3	3.1
MAM	$328 \rightarrow 165$ (50)	$328 \rightarrow 193$ (35)	53.1 ± 13.9[d]			1.2	5.3
LSD	$324 \rightarrow 223$ (35)	$324 \rightarrow 207$ (50)	41.9 ± 7.4[d]			_[f]	_[f]
BE	$290 \rightarrow 168$ (20)	$290 \rightarrow 105$ (35)	37.6 ± 7.7[d]			0.3	0.9
Cocaine	$304 \rightarrow 182$ (20)	$304 \rightarrow 150$ (35)	10.9 ± 8.3[d]			1.5	4.7
Methadone	$310 \rightarrow 265$ (20)	$310 \rightarrow 105$ (35)	28.9 ± 6.9[d]			0.7	1.8
THC-COOH	$345 \rightarrow 327$ (21)	$345 \rightarrow 299$ (29)	32.8 ± 7.0[e]			1.6	4.3
11-OH-THC	$331 \rightarrow 313$ (21)	$331 \rightarrow 193$ (37)	11.4 ± 7.9[e]			0.2	0.8
THC	$315 \rightarrow 193$ (33)	$315 \rightarrow 259$ (29)	22.9 ± 9.6[e]			0.2	0.8

[a] α-Error, 10% for qualifier 1.
[b] α-Error, 1% for quantifier.
[c] Second qualifier is used when the unfragmented precursor ion is used for quantification.
[d] $n = 12$ (at 5 ng/ml).
[e] $n = 10$ (at concentration levels used for establishing LODs and LOQs); for LOD and LOQ, relative uncertainty 33% ($k = 3$).
[f] No validation performed.

of detection (LOD) and limits of quantitation (LOQ). Six different sources of blank plasma were tested for matrix interferences. For determination of relative intensities of ion ratios (qualifier-to-quantifier) and determination of percent deviations of relative intensities of MRM transitions for basic drugs duplicate analysis of six different plasma samples spiked with 5 and 50 ng/ml were performed on two separate days. For calculation of relative intensities of ion ratios (qualifier-to-quantifier) and determination of percent deviations of relative intensities of MRM transitions for cannabinoids the same calibration samples were used as for the LOD and LOQ determination.

2.3.1. Cannabinoids

The stock solutions of cannabinoids (1 µg/ml and 100 ng/ml for THC and 11-OH-THC, 5 µg/ml and 500 ng/ml for THC-COOH; and mixture of deuterated analogues 1 µg/ml THC-d$_3$, 1 µg/ml 11-OH-THC-d$_3$, 5 µg/ml THC-COOH-d$_3$) were prepared by diluting the standard solution of drugs in methanol. Two series of calibration standards were prepared by adding aliquots of stock solution into 1 ml of drug-free plasma. The concentration in plasma calibration standards were 1, 2, 3, 4, 5, 6, 8, 10, 20, 50, 100 ng/ml for THC and 11-OH-THC and 5, 10, 15, 20, 25, 30, 40, 50, 100, 250, 500 ng/ml for THC-COOH. The validation included also blank plasma samples. Sets of five quality control samples at two concentration levels (8 and 20 ng/ml of THC and 11-OH-THC, 15 and 40 ng/ml of THC-COOH) were prepared by adding aliquots of stock solution into 1 ml of drug-free plasma and were used to determine the accuracy and the precision of the method. Linear regressions with equidistant calibration levels (0,1, 2, 3, 4, 5 ng/ml for THC and 11-OH-THC and 0, 5, 10, 15, 20, 25 ng/ml for THC-COOH) were used for the calculation of LOD and LOQ with a relative uncertainty of 33% ($k = 3$) [21].

Aliquots (10 µl) of deuterated standard-mix stock solution were added into every sample (calibration, quality control, real sample) in the beginning of the sample preparation process. Two millilitres of 0.1 M acetic acid was added into 1 ml of sample and this mixture was vortex-mixed for 20 s. This solution was applied onto preconditioned SPE cartridge for extraction. Detailed SPE program of Zymark RapidTrace SPE-Automat is published elsewhere [17]. The extracts were evaporated to dryness in a speed vacuum concentrator at 40 °C, 500 Pa. The residue was reconstituted in 100 µl of the HPLC mobile phase (70% 5 mM ammonium acetate and 30% acetonitrile) and 20 µl aliquots were injected for LC/MS/MS analysis.

2.3.2. Opioids and stimulants

The stock solutions of drugs (1 µg/ml and 100 ng/ml each; 1 µg/ml of deuterated analogues) were prepared by diluting the standard solution of drugs in methanol. Two series of nine calibration standards were prepared by adding aliquots of stock solution into 1 ml of drug-free plasma to cover the concentration range 1–250 ng/ml for normorphine, morphine and M3G; 1–250 ng/ml for codeine and MAM; 1–500 ng/ml for BE and methadone; 2–500 ng/ml for cocaine. Sets of five quality control samples at two concentration levels (summarized in Table 2) were prepared by adding aliquots of stock solution into 1 ml of drug-free plasma as well and were used to determine the accuracy and the precision of the method. Linear regressions with following calibration levels were used for the calculation of LOD and LOQ with a relative uncertainty of 33% ($k = 3$) [21]: 0, 2, 5, 10, 20, 30 ng/ml for normorphine, morphine and M3G; 0,1, 2, 3, 5, 10 ng/ml for codeine and MAM; 0, 2, 4, 6, 8, 10 ng/ml for cocaine, BE and methadone.

Aliquots (20 µl) of deuterated standards stock solution were added into every sample (calibration, quality control, real sample) in the beginning of the sample preparation

Table 4
Selected examples of the identification points (IP) for some hyphenated MS techniques (adapted from [4,5])

Technique	Number of ions	IP
LC/MS	n	n
GC/MS/MS or LC/MS/MS	1 precursor and 2 products	4
GC/MS/MS or LC/MS/MS	2 precursors, each with 1 product	5
LC/MS^3	1 precursor, 1 product and 2 of its fragments	5.5
HRMS[a]	n	$2n$
GC/MS and LC/MS	2 + 2	4
GC/MS and HRMS	1 + 1	4

[a] HRMS, here, means LC–time-of-flight mass spectrometry; n, an integer.

process. Two millilitres of 4 mM ammonium carbonate buffer (AC buffer) (pH 9.4) were added into 1 ml of sample and this mixture was vortex-mixed for 20 s. This solution was applied onto preconditioned SPE cartridge for extraction. The operation program of automated SPE was as described in Table 1. The extracts were evaporated to dryness in a speed vacuum concentrator at 40 °C, 500 Pa. The residue was reconstituted in 100 μl of the HPLC mobile phase (4 mM ammonium acetate) and 20 μl aliquots were injected for LC/MS/MS analysis.

3. Results and discussions

The MRM transitions of basic drugs have been chosen based on measurement of product ion spectra (MS/MS) (Fig. 1) at three different collision energies (CE) to ensure different fragmentation of compounds. Three collisions energies 20, 35, 50 eV have been chosen for responding to already existing MS/MS libraries [19,20]. Selection of MRM transitions of cannabinoids has already been published [17], MRM transition for THC-COOHglu m/z 521 → 345, 345 → 327 have been chosen according to the literature [21] and the transitions for codeine–glucuronide m/z 476 → 300, 476 → 215 were based on theoretical calculations that opiate-glucuronides fragment yielding an ion $[M - 176]^+$. The chosen MRM transitions are summarized in Fig. 2 and Table 3. The most prominent MRM transitions were used for quantification and the other ones were used as so called qualifiers, except of opioids. Here, the unfragmented precursor ions were used for quantification. The transitions for deuterated analogues were m/z 348 → 330 for THC-COOH-d$_3$, m/z 334 → 316 for 11-OH-THC-d$_3$, m/z 318 → 196 for THC-d$_3$, m/z 303 → 303 for codeine-d$_3$, m/z 289 → 289 for morphine-d$_3$, m/z 331 → 168 for MAM-d$_3$, m/z 465 → 465 for M3G-d$_3$, m/z 293 → 171 for BE-d$_3$, m/z 307 → 185 for cocaine-d$_3$, m/z 313 → 268 for methadone-d$_3$. The validation parameters for particular compounds are summarized in Tables 2 and 3. The validation for LSD has not been performed because the developed SPE method has proved to be unsuitable for LSD isolation. The linear regression model was used to calculate regression lines. The relative intensities of selected MRM transitions are summarized together with particular collision energies in Table 3 and corresponding LODs and LOQs are shown here as well. The relative intensities are presented in

percent of the intensity of corresponding quantifier (the most abundant MRM transition) and the percent deviation of relative intensites. The percent deviations of relative intensities of MRM transitions for basic drugs were determined from 12 measurements at concentrations 5 and 50 ng/ml and the results for 5 ng/ml are shown in Table 3. However, the results for MRM intensities at 50 ng/ml were in the same ranges. For identification of codeine, morphine, M3G and normorphine two qualifiers have been used because the quantitation of these have been done with the unfragmented precursor ions. It is also apparent from results in Table 3 that the relative intensities of qualifiers vary significantly for selected drugs. However, the results (LOD) are satisfactory for all substances, also for those such as morphine and codeine that have very low intensities of MRM transition used as qualifiers.

As was already mentioned criteria that the particular method has to fulfill to ensure reliable and objective identification of the particular substance are expressed as so called identification points (IP). Examples of this IP attributed to some methods are shown in Table 4. Generally two multiple reaction monitoring (MRM transitions) transitions (correspond to three diagnostic ions) are required using LC/MS/MS method, first one (usually the most intensive) as so called "quantifier" and the second one as so called "qualifier" for confirmation of identity of substance. This gives for our developed methods four IP. Table 5 shows then the required relative intensities of the detected ions using various LC/MS/(MS) techniques expressed as a percentage of the intensity of the most intense ion or transition. Comparing required relative intensities stated in Table 5 with our results mentioned in Table 3, it is apparent that our LC/MS/MS methods easy fulfill criteria for permitted relative intensities of MRM transitions.

LC/MS/MS chromatograms of studied drugs are shown in Fig. 2. The most intense peak corresponds to the quantifier (most intense MRM transition) and the other ones to qualifiers.

Table 5
Maximum permitted tolerances for relative ion intensities using LC/MS techniques (adapted from [4,5])

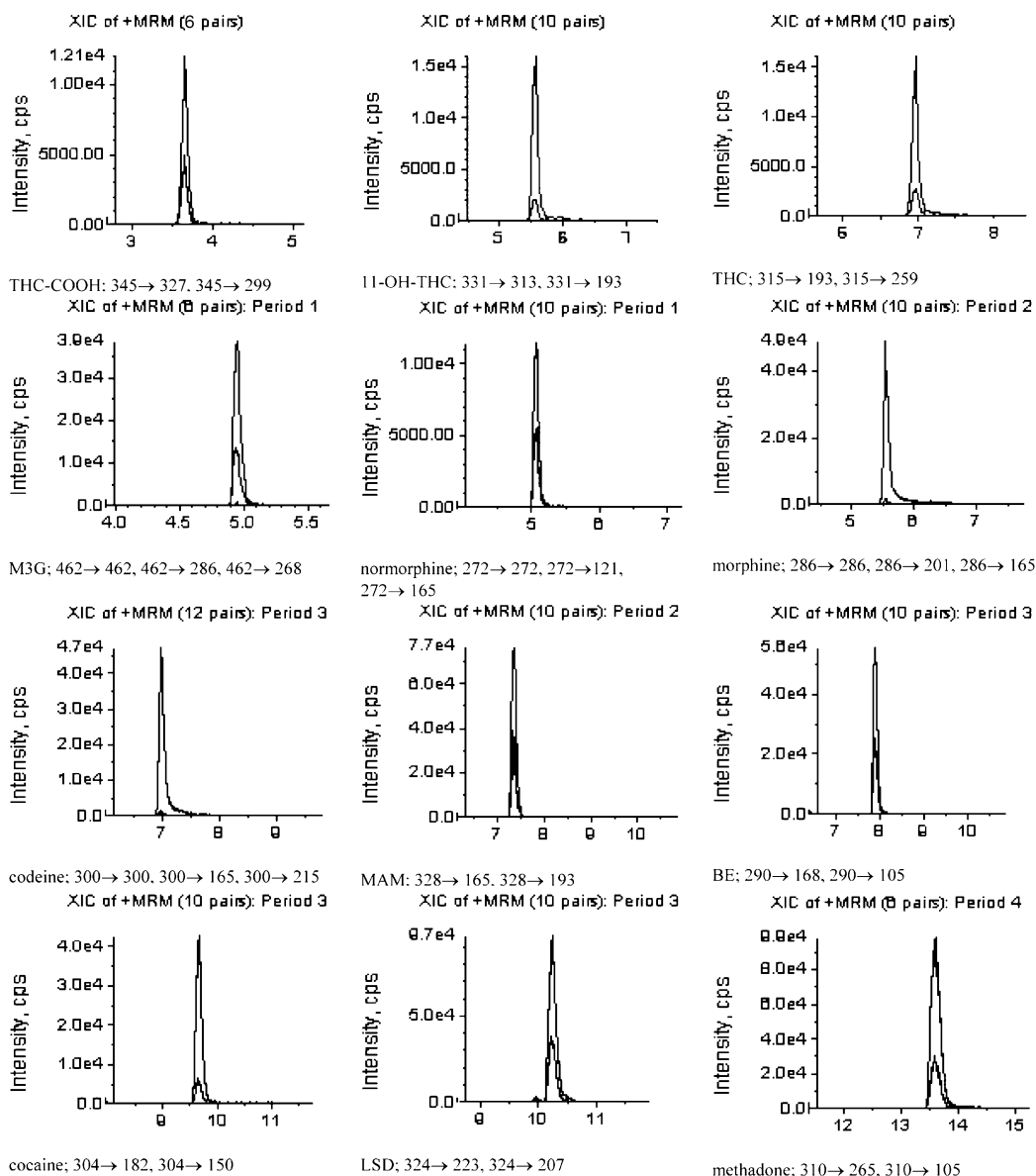| Relative intensity (% of base peak)	LC/MS, LC/MS^n (relative, %)
>50	±20
20–50	±25
10–20	±30
≤10	±50

Fig. 2. Typical LC/MS/MS chromatograms of calibration standard samples (plasma spiked at level of 100 ng/ml) for demonstration of intensities of selected MRM transitions.

In case of cannabinoids the influence of mobile phase was studied. Fig. 3 shows chromatograms of standard mixtures obtained by using mobile phases with different pH. No bigger differences have been observed for 11-OH-THC when using mobile phase I. (A) 1 mM ammonium formate (pH 2.7) and as (B) acetonitrile with 1 mM ammonium formate (pH 5.5) or mobile phase II: (A) 5 mM ammonium acetate (pH 6.5) and (B) acetonitrile, but on the other hand, the analysis and ionization of THC-COOH and especially THC have been influenced by the pH of mobile phase more significantly. The peak areas of analytes were approximately 1.3× smaller for 11-OH-THC, 2× for THC-COOH and 6.5× for THC when using more acidic mobile phase I. As can be seen (Fig. 3), there was practically no separation of 11-OH-THC and THC-COOH using these conditions.

The method developed for basic drugs shows very good selectivity. As an example one of the six different blank plasma samples is shown in Fig. 4 and a calibration plasma sample spiked at level 10 ng/ml is shown here as well. No matrix interferences have been observed during any other analysis. Examples of analysis of real case samples are illustrated in Fig. 5. These show chromatograms of urine sample after heroin and cocaine intake and urine sample after codeine intake. The second example demonstrates the possibility of application of this method for the identification of codeine–glucuronide, the major phase-II-metabolite of codeine. Ion suppression effects, which have been reported to be a problem in some cases with ESI of drugs and have been investigated in several studies, were not further investigated here. It was because the deuterated internal analogues of the
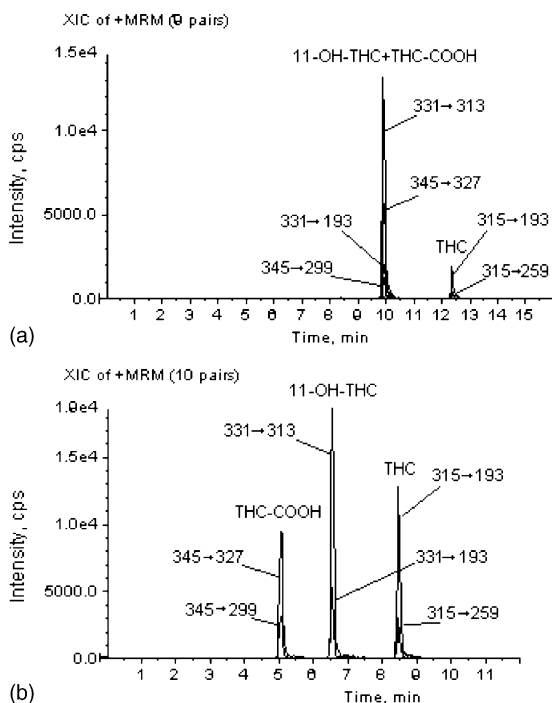
Fig. 3. MRM chromatograms of analysis of cannabinoid standard mixture (concentration, 10 μg/ml) using different mobile phases: (a) A: 1 mM ammonium formate (pH 2.7), B: acetonitrile with 1 mM ammonium formate (pH 5.5); (b) A: 5 mM ammonium acetate (pH 6.5), B: acetonitrile; flow: 0.25 ml/min, gradient: 0–1.0 min, 30% B, 1.0–10 min: linear from 30% to 90% B, 10–11 min: 90% B, 11–12 min: linear from 90% to 30% B, 12–16 min: 30% B; Luna 3μ PhenylHexyl column.

Fig. 4. Reconstructed (from MRM transitions of quantifiers, see Table 3 or Fig. 2) MRM chromatograms: (a) of calibration standard mixture of selected basic drugs (plasma spiked at concentration level of 10 ng/ml); t_r [min]: 4.83, M3G; 5.08, normorphine; 5.36, morphine; 6.71, codeine; 7.11, MAM; 7.93, BE; 8.62, heroin; 9.15, cocaine; 10.3, LSD; 12.07, methadone; individual MRM chromatograms shown in Fig. 2 MRM (b) same MRM traces of blank plasma.
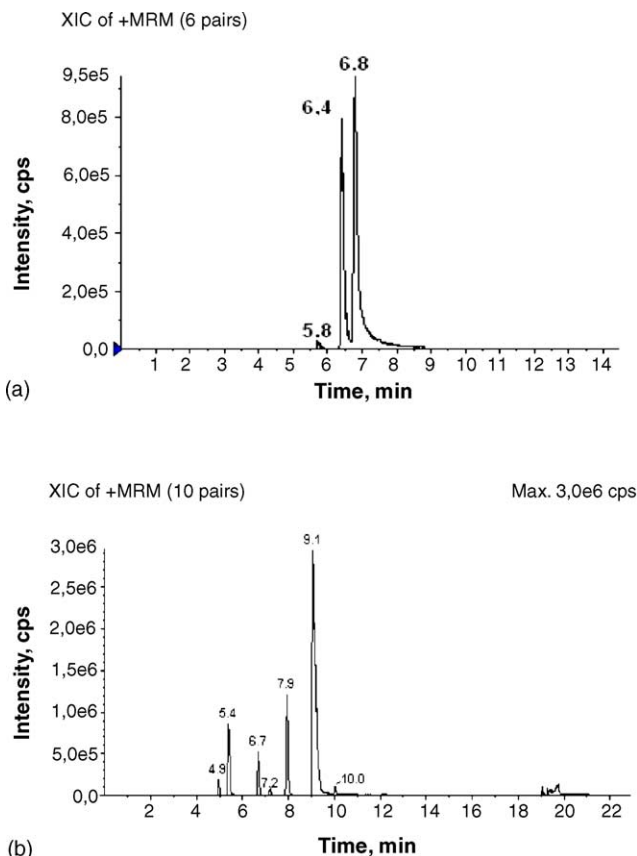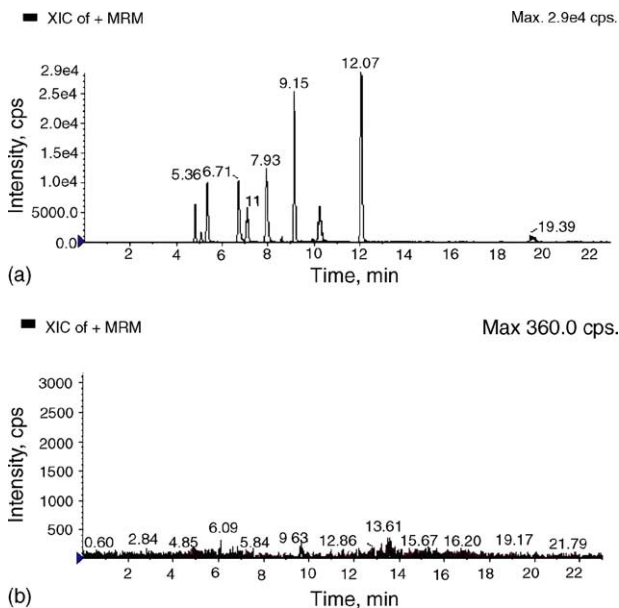

Fig. 5. Reconstructed (from MRM transitions of quantifiers, see Table 3 or Fig. 2) MRM chromatograms of analysis of urine samples (a) after codeine intake: 5.8, morphine; 6.4, codeine–glucuronide; 6.8, codeine and (b) after heroin and cocaine intake: 4.9, M3G; 5.4, morphine; 6.7, codeine; 7.2, MAM; 7.9, BE; 9.1, cocaine.

analytes were used for quantification and good linearity was found throughout the calibration range for every analyte.

4. Conclusion

LC/MS/MS is a very powerful analytical tool for confirmatory analysis of drugs of abuse especially of polar, thermally labile and not volatile compounds and their metabolites. However, only the most recent criteria concerning confirmation of identity of substances in forensic toxicology also take into consideration LC/MS and LC/MS/MS methods. We have presented two validated methods taking into account recently established criteria for confirmation of identity using LC/MS/MS. Presented methods can fulfill the requirement for a minimum of four identification points with two MRM transitions for confirmation of compound identity. Furthermore, the requirements of the EU guidelines concerning the variability of relative ion intensities of the MRM transitions could be met by these methods even at low analyte concentrations. Both methods are sufficiently sensitive to fulfill current recommendations of the German Society of Toxicological and Forensic

Chemistry [22] for confirmatory analysis of drugs of abuse in driving under the influence of drugs (DUID) cases. Furthermore, they can also be used for the fast and simultaneous detection of illegal drugs in drug related deaths.

References

[1] Forensic Toxicology Laboratory Guidelines, Society of Forensic Toxicologists Inc., American Academy of Forensic Sciences, 2000, p. 10 (http://www.soft-tox.org/docs/Guidelines.2002.final.pdf).

[2] R. Aderjan, T. Briellmann, T. Daldrup, U. Demme, K. Harzer, M. Herbold, H. Käferstein, G. Kauert, L. von Meyer, M. Möller, F. Musshoff, G. Schmitt, W. Weinmann, Toxichem + Krimtech 65 (1998) 18.

[3] A.A.M. Stolker, R.W. Stephany, L.A. van Ginkel, Analusis 28 (2000) 947.

[4] EU-Amtsblatt der Europaeischen Gemeinschaften, 17 August 2002, 2002/657/EG.

[5] L. Rivier, Anal. Chim. Acta 492 (2003) 69.

[6] N. Tyrefors, B. Hyllbrant, L. Ekman, M. Johansson, B. Långström, J. Chromatogr. A 729 (1996) 279.

[7] A.D. Nagy, L. Rivier, C. Giroud, M. Augsburger, P. Mangin, J. Chromatogr. A 854 (1999) 109.

[8] S. Pichini, I. Altieri, M. Pellegrini, P. Zuccaio, R. Pacifici, Mass Spectrom. Rev. 18 (1999) 119.

[9] W. Naidong, J.W. Lee, X. Jiang, M. Wehling, J.D. Hulse, P.P. Lin, J. Chromatogr. B 735 (1999) 255.

[10] M.E.R. Rosas, K.L. Preston, D.H. Epstein, E.T. Moolchan, I.W. Wainer, J. Chromatogr. B 796 (2003) 355.

[11] S.R. Needham, P.M. Jeanville, P.R. Brown, E.S. Estape, J. Chromatogr. B 748 (2000) 77.

[12] K. Srinivasan, P.P. Wang, A.T. Eley, C.A. White, M.G. Bartlett, J. Chromatogr. B 745 (2000) 287.

[13] S. Pichini, R. Pacifici, M. Pellegrini, E. Marchei, E. Pérez-Alarcón, C. Puig, O. Vall, O. García-Algar, J. Chromatogr. B 794 (2003) 281.

[14] R. Dams, C.M. Murphy, W.E. Lambert, M.A. Huestis, Rapid Commun. Mass Spectrom. 17 (2003) 1665.

[15] P.M. Jeanville, E.S. Estapé, S.R. Needham, M.J. Cole, J. Am. Soc. Mass Spectrom. 11 (2000) 257.

[16] Mandatory Guidelines for Federal Workplace Drug Testing Programs, Federal Reister, Department of Health and Human Services Administration, Part V, Notice, 1994, p. 29908.

[17] B. Maralikova, W. Weinmann, J. Mass Spectrom. 39 (2004) 526.

[18] W. Weinmann, B. Maralíková, C.A. Mueller, LC-ESI-MS/MS Library of Drugs 2004/1. http://www.chemicalsoft.de/.

[19] W. Weinmann, B. Maralíková, C.A. Mueller, ESI-Product Ion Mass Spectra Library of Drugs 1.0, MS/MS Library of Drugs and Pharmaceuticals for Sciex^{TM} API and Analyst^{TM} software (1.3 or higher), Product Code: WWMSMS01, 2002–2003.

[20] W. Weinmann, S. Vogt, R. Goerke, C. Müller, A. Bromberger, Forensic Sci. Int. 113 (2000) 381.

[21] R. Aderjan, B. Babel, T. Briellmann, T. Daldrup, U. Demme, J. Hallbach, M. Hartung, K. Harzer, M. Herbold, L. von Meyer, M. Moeller, F. Musshoff, G. Schmitt, W. Weinmann, Toxichem. Krimtech. 67 (2000) 13.

[22] F. Mußhoff, T. Daldrup, R. Aderjan, L. Meyer, Toxichem. Krimtech. 69 (2000) 32.