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Liquid chromatography–mass spectrometry as a routine method in forensic sciences: a proof of maturity

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Abstract

The applications of LC–API-MS in routine forensic toxicological casework were presented. This technique has been used for routine determination of several groups of drugs: opiate agonists (like morphine, codeine, dihydrocodeine and their glucuronides, methadone, buprenorphine) cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester), amphetamine and other psychoactive phenethylamines, like MDMA, MDE or MDA, benzodiazepine derivatives (flunitrazepam and metabolites, triazolam, bromazepam), hallucinogens (LSD, psilocybin, psilocin) and olanzapine, A common solid-phase extraction procedure for all drugs (with exception of LSD) has been developed. Among two ionization sources, atmospheric pressure chemical ionization appeared more universal and assured generally higher sensitivity. Only in the case of very polar drugs (e.g. psilocin or psilocybin) electrospray ionization was more sensitive. LC–API-MS became a very powerful and flexible method for dedicated analyses of substances of forensic interest. The use of this technique for general, broad applicable screening depends on the establishing of interlaboratory database of standardized mass spectra.

Keywords: Drugs of abuse

1. Introduction

The term ''forensic'' has particular meaning. All forensic disciplines — among them, forensic toxicology and criminalistics — belong to *forum,* i.e. are subjected — more than any other scientific activities — to public debate and public control. Forensic experts are obliged to explain the smallest details of the methods used, to substantiate the choice of the applied technique and to give their unbiased conclusions — all under critical and often mistrustful look of the servants of the justice, as well as general public including media. This could be perfectly seen during the O.J. Simpson trial, when the forensic expert testimonies were transmitted worldwide. The final result of the work of forensic scientist — an expert evidence — exerts a direct influence on the fate of a given individual, This burden is a most important stimulus, which determines the way of thinking and acting in forensic sciences. Consequently, the methods applied routinely in forensic laboratories should assure very high level of reliability and must be subjected to extensive quality assurance and quality control programs. This concerns par-

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ticularly modem techniques, often noted as ''hyphenated''. The term ''hyphenation'' means that the combination of two techniques did not reach its stage of full maturity, and consequently, such combinations are applicable in forensic sciences when the hyphenation is not noticed anymore [1]. For example, the coupling of gas chromatography with electron-impact ionization quadrupole mass spectrometer became an everyday, robust tool in toxicological analysis and is hardly regarded as a hyphenated technique. Only in the case of coupling of gas chromatography with less frequent used mass analyzers, like magnetic sector, time-of-flight or Fourier transform the term ''hyphenated'' is still in use.

The combination of high pressure liquid chromatography (HPLC) and mass spectrometry (MS) has been used in forensic toxicology for many years. In the 1990s, an important change in development trend occurred. Whilst the earlier interfaces used in LC–MS, like particle beam ionization, thermospray ionization or fast atom bombardment, are gradually vanishing in the scientific literature, the number of scientific contributions devoted to atmospheric pressure ionization (API) LC–MS showed huge increase [2]. According to Willoughby et al. [3] the LC–MS came from the ''innovators'' stage through ''early adaptors'' to the ''early majority'' stage, and is now open to the specialists from various disciplines, also for toxicologists. This was caused by the introduction of robust, user-friendly API-MS instruments at affordable price. Various forensic applications of LC–MS has been recently reviewed [4–6]. All these reviews showed that API is most often used LC–MS interface in forensic toxicology and related disciplines. Both kinds of ionization sources used in API-LC–MS: Atmospheric pressure chemical ionization (APCI) and electrospray (ESI) found many applications in forensic sciences. ESI seems to be more useful for the analysis of highly polar and thermolabile compounds, e.g. polar metabolites, whereas APCI, as an active ionization interface, may give better results for some apolar substances, e.g. lipophilic drugs.

The purpose of this paper was to present a routine approach to the application of LC–API-MS in forensic toxicology, as it is practiced in the forensic toxicological laboratory in Aachen. The concept, proved in three years of daily casework, is based on following points:

- A common solid-phase extraction (SPE) procedure has been used for all groups of basic drags. The possibility of simultaneous isolation of various compounds is of practical value due to the limited amount of available sample and tendency to multiple drug use.
- The mobile phase used for HPLC separation consisted of two identical components, which were mixed in different proportions for appropriate groups of compounds, This greatly facilitates the consecutive analyses of different groups of drugs.
- A single quadrupole instrument with APCI interface has been used in, positive ionization mode. An in-source collision-induced dissociation has been applied in order to measure not only the quasi molecular ion of a given compound, but at least one characteristic fragment ion.

2. Experimental

2.1. Solid phase extraction

SPE cartridges Bond Elut C_{18} (200 mg, Varian Analytichem, Darmstadt, Germany) were rinsed with 1 ml methanol, 1 ml H_2O and 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) before use, Serum, blood or urine samples were centrifuged 5 min at 14 000 *g*. 0.2 ml to 1 ml volumes of supernatant were Vortex mixed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and with appropriate internal standards. After 10 min centrifugation at 5000 *g*, 2 ml of clear supernatant were applied on the SPE cartridge and slowly passed through (ca. 5 min). The SPE cartridge was rinsed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and vacuum dried for 5 min. The retained drugs were eluted with 0.5 ml methanol–0.5 *M* acetic acid (9:1) under gravity force. After addition of 10 μl of 1 mmol HCl the eluates were dried under nitrogen, reconstituted in 100 μl of HPLC mobile phase and centrifuged for 4 min at 14 000 *g*, 5–20 μl of clear supernatant were injected into LC–MS.

2.2. High pressure liquid chromatography

Separations were performed on Superspher RP 18 (E.Merck. Darrnstadt, Germany) or Discovery RP Amide C16 (Supelco, Bellefonte, PA, USA), both columns were 125 mm long and 3 mm wide. Mixtures of acetonitrile (ACN) and 50 m*M* ammonium formate buffer, pH 3.0 (AMF), were used as the mobile phases. The chromatographic conditions used for particular drugs are depicted in Table 1.

2.3. Atmospheric pressure chemical ionization mass spectrometry

A SSQ 7000 single quadrupole mass spectrometer (Finnigan MAT, San Jose, USA) with atmospheric

Table 1
HPLC conditions, typical retention times and detectability of drugs determined with LC–APCI-MS[a]

Drug	Internal standard	ACN: buffer	Flow (ml/min)	HPLC column	Rt (min)	Recovery (%)	LOD (ng/ml)
Morphine	Mo-d3	10:90	0.3	Super.	4.6	98	0.1
Morphine-3-glucuronide	M3G-d.3	10:90	0.3	Super.	2.4	94	2.5
Morphine-6-glucuronide	M6G-d3	10:90	0.3	Super.	2.7	97	2.5
Codeine	C-d6	10:90	0.6	Disc.	5.8	91	2.5
Codeine-6-glucuronide	C6G-d3	10:90	0.6	Disc.	4.0	90	10
6-Monoacetylmorphine	MAM-d6	10:90	0.6	Disc.	8.5	85	0.5
Dihydrocodeine	C-d6	10:90	0.6	Super.	5.6	73	0.5
Dihydrocodeine-6-lucuronidex	C6G-d3	10:90	0.6	Super.	3.8	75	2.0
Dihydromorphine	M-d3	10:90	0.3	Super.	4.4	78	0.5
Cocaine	Coc-d8	25.75	0.4	Disc.	6.3	85	0.5
Benzoylecgonine	BE-d8	25:75	0.4	Disc.	3.5	88	0.2
Ecgonine methyl ester	EME-d3	5:95	0.2	Disc.	2.3	41	0.2
Buprenorphine	Bu-d4	45:55	0.4	Super.	3.1	90	0.5
Methadone	Me-d3	45:55	0.4	Super.	5.0	87	0.2
Tramadol	BE-d8	15:55	1.0	Super.	5.4	94	0.1
Amphetamine	A-d11	25:75	0.3	Super.	3.4	86	2.0
Methamphetamine	MA-d10	25:75	0.3	Super.	4.1	82	1.0
MDMA	MIDMA-d5	25:75	0.3	Super.	4.2	90	1.0
MDA	MDMA-d5	25:75	0.3	Super.	3.5	96	2.0
MDEA	MDEA-d7	25:75	0.3	Super.	5.2	87	1.0
MBDB	MDEA-d7	25:75	0.3	Super.	5.5	87	1.0
BDMPEA	MTDBA-d7	25:75	0.4	Super.	6.5	86	2.0
Phentermine	MA-d10	25:75	0.3	Super.	4.3	96	1.0
Cathinone	A-d11	25:75	0.3	Super.	2.6	88	5.0
Ephedrine	A-d11	25:75	0.3	Super.	3.1	58	1.0
Phenylpropanolamine	A-d11	25:75	0.3	Super.	2.5	63	1.0
Fenfluramine	BEA	25:75	0.8	Disc.	7.0	96	1.0
Norfenfluramine	BEA	25:75	0.8	Disc.	4.2	90	1.0
Flunitrazepam	Fl-d3	45:55	0.3	Super.	7.6	93	0.2
7-Aminoflunitrazepam	7AF-d3	45:55	0.3	Super.	3.1	92	0.2
N-desmethylflunitrazepam	7AF-d3	45:55	0.3	Super.	5.3	99	1.0
3-OH-Flunitrazepam	7A.F-d3	45:55	0.3	Super.	5.2	96	2.0
Bromazepam	Fl-d3	45:55	0.3	Super.	5.1	90	1.0
Triazolam	Fl-d3	45:55	0.5	Super.	5.1	95	1.0
LSD	LAMPA	25:75	0.5	Super.	5.8	80	0.5
Psilocybin	Mo-d3	15:85	0.4	Super	2.1	90	2.0
Psilocin	Mo-d3	15:85	0.4	Super	5.1	80	2.0

[a] Abbreviations: Mo, morphine; C, codeine; MAM, monoacetylmorphine; Coc, cocaine; Bu, buprenorphine; Me, methadone; A, amphetamine; MA, methamphetamine; Fl, flunitrazepam; Super., Superspher RP 18 column; Disc., Discovery RP C16; Rt, retention time; LOD, limit of detection.

pressure chemical ionization mass spectrometry (APCI) source was used in positive ionization mode. The following inlet conditions were used: sheath gas (nitrogen) pressure 70 p.s.i., auxiliary gas (nitrogen) 20 ml/min, heated capillary temperature 190°C, heated vaporizer temperature 450°C, corona current 5 μA. The selected ion monitoring (SIM) procedures for particular drugs or drug groups were written on the base of in-source fragmentation patterns, observed at given fragmentation energy, measured as octapole offset voltage. These procedures and the diagnostic ions measured were described elsewhere [7–12]. As a rule at least two ions were measured for each substance. The exceptions were morphine, codeine and dihydromorphine, which did not undergo fragmentation before reaching a critical threshold, and then dissipated to very small fragments. For these drugs only protonated molecular ions were measured.

2.4. Biological samples

Forensic blood and urine samples, as well as autopsy samples of body fluids and organs, were subjected to preliminary screening with immunoassays, HPLC (Remedi – Bia-Rad, Hercules, CA) or GC–MS [13]. In the case of positive results the samples were analyzed with LC–APCI-MS, In the last 3 years over 1500 samples, predominantly blood samples, were investigated.

3. Results and discussion

The developed SPE assured very clean extracts and high recoveries for a very broad spectrum of toxicologically relevant substances. The use of acidic mobile phase assured very efficient separation and satisfactory peak shapes of basic drugs, as it was already demonstrated in our previous studies concerning diode array detection system [14]. Two columns used in the study were selected for following reasons: Superspher RP18 packing showed excellent selectivity and was particularly useful for the separation of fast-eluting compounds (e.g. opiate glucuronides), On the other hand, the RP Amide C16 packing allowed fast analysis of compounds of different polarities in one run, e.g. benzoylecgonine and cocaine (Fig. 1) or morphine, codeine and 6-monoacetylmorphine. Since the retention times of late-eluting drugs were distinctly shortened whilst the retention times of fast-eluting drugs were almost unaffected, this column packing may to some extent replace gradient elution, As internal standards, deuterated analogues of drugs with possibly highest extent of deuteration were used. This prevents the cross contribution of ''isotope peaks'' (M+3) to the molecular mass of the triple-deuterated analogue [15]. Unfortunately, for some compounds only triple-deuterated standards are available at present. This situation is steadily improving; e.g. in the last year highly deuterated analogs of morphine and flunitrazepam appeared on the market. For some drugs, however, like ecgonine methyl ester, morphine glucuronides or codeine glucuronide, only d3-analogs are available.

Table 1 presents a summary of the chromatographic conditions applied, observed typical retention times of drugs as well as percent recoveries and limits of detection. These data show, that the total analysis time was never longer than 10 mm and the limit of detection ranged from 0.1 to 5.0 ng/ml for all drugs but codeine-6-glucuronide. Also, the recovery for most drugs was above 80%. When all steps of the analytical procedure are taken into consideration, like simple sample pretreatment, no need for derivatization, analysis speed, adequate selectivity and sensitivity, it may be stated that the LC–API-MS became a real alternative for GC–MS in target analysis. The results obtained for particular groups of drugs will be discussed in turn.

3.1. Opiate agonists

Determination of heroin metabolites, like morphine, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and 6-monoacetylmorphine (MAM) in body fluids belongs to most important and most frequent applications of LC–API-MS in forensic toxicology. In our laboratory, APCI-MS was applied for determination of morphine, its glucuronides and MAM, together with simultaneous determination of codeine and C6G [7–9]. Codeine appears regularly in blood and urine of heroin consumers as a metabolite of 6-acetylcodeine, which is a typical congener of street heroin. The simulta-

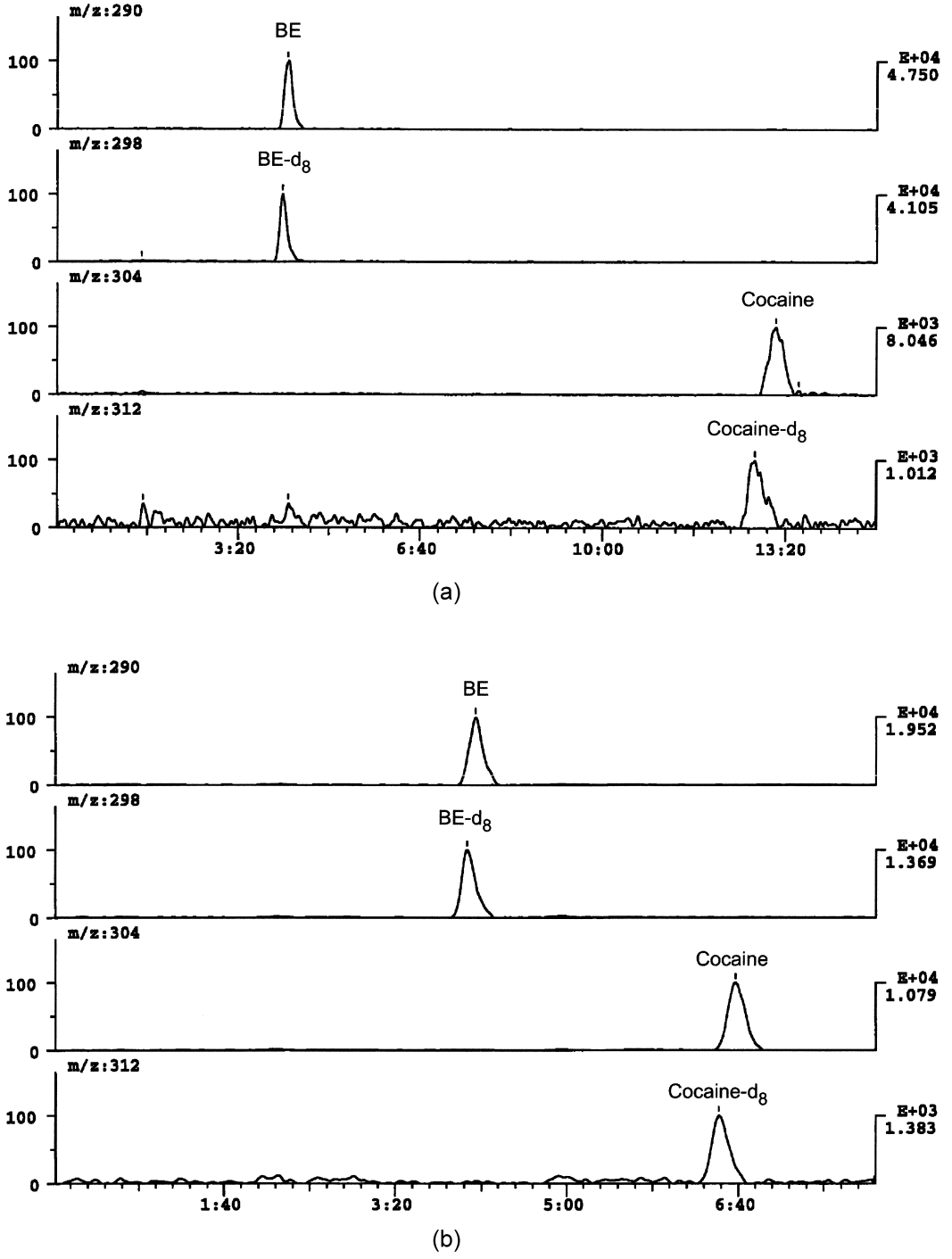

Fig. 1. Comparison of retention times of benzoylecgonine (BE) and cocaine on Superspher RP18 (a) and Discovery Amide C16 (b) columns. The same serum extract was examined, spiked with drugs to the concentration of 20 μg/l each.

neous determination of all metabolites of street heroin is helpful in the assessment of the rapidity of death in fatal cases, in discrimination between heroin, morphine and codeine intake, and in approximation of the time of drug consumption. Table 2 shows the results of opiate determinations in blood samples in 25 selected cases. In the samples 15, 20 and 25 relative high concentrations of C6G and codeine were found, indicating additional codeine intake beside heroin. Figs. 2 and 3 show typical chromatograms of opiates in blood samples taken from a heroin addict. The use of LC–ESI–MS for heroin metabolites was described by other authors [16–20].

LC–APCI-MS was also applied for determination of dihydrocodeine (DHC) and its metabolites in biofluids. DHC is a semisynthetic opioid, which has been extensively used in Germany in the treatment of heroin addicts, with consecutive numerous fatalities due to the accidental overdose [21]. DHC undergoes *N*-demethylation to Nor-DHC and *O*-demethylation to dihydromorphine (DHM). Both demethylated metabolites as well as DHC itself are being then conjugated to appropriate glucuronides (Fig. 4). Since not only DHC but also DHM, DHM-6-glucuronide, and to lesser extent Nor-DHC and DHC-6-glucuronide act as μ-opiate receptor agonists, all these substances play a role in the case of DHC overdose and a should be determined [22,23]. LC–API-MS is a method of choice for simultaneous, selective determination of DHC and metabolites, as shown on the Fig. 5. The sensitivity of assay is comparable with those for morphine and glucuronides.

The determination of methadone in blood samples is important for several reasons. This drug is used as heroin substitute in maintenance therapy and appears also as a street drug. Several reports warned about

Table 2
Concentrations (ng/ml) of morphine (M), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), codeine (C), codeine-6-glucuronide (C6G) and 6-monoacetylmorphine (MAM) in forensic blood samples taken from living subjects (road traffic violators and other offenders)[a]

Sample	M	M3G	M6G	C	C6G	MAM
1	3	39	4	2	5	1
2	4	9	4	4	7	0.9
3	5	167	32	11	45	1.3
4	6	264	24	7	34	5
5	7	124	14	0	0	0.5
6	8	235	34	4	66	0.5
7	9	246	23	5	24	0.5
8	9	540	61	13	79	5
9	9	712	131	6	120	1.1
10	11	197	23	4	11	0.5
11	16	547	174	5	100	4
12	16	386	51	16	80	1
13	18	362	37	0	9	2
14	19	292	73	28	47	2
15	20	1042	182	32	1096	1
16	23	489	55	6	273	2
17	29	401	46	11	50	1.3
18	30	185	55	9	15	0.5
19	33	587	134	8	34	0.8
20	35	710	406	38	1179	1
21	37	242	35	16	110	0.5
22	44	1332	157	8	105	1
23	48	728	80	8	42	2
24	49	654	73	11	97	1
25	82	1648	200	21	1533	13

[a] The samples are arranged according to morphine concentration.

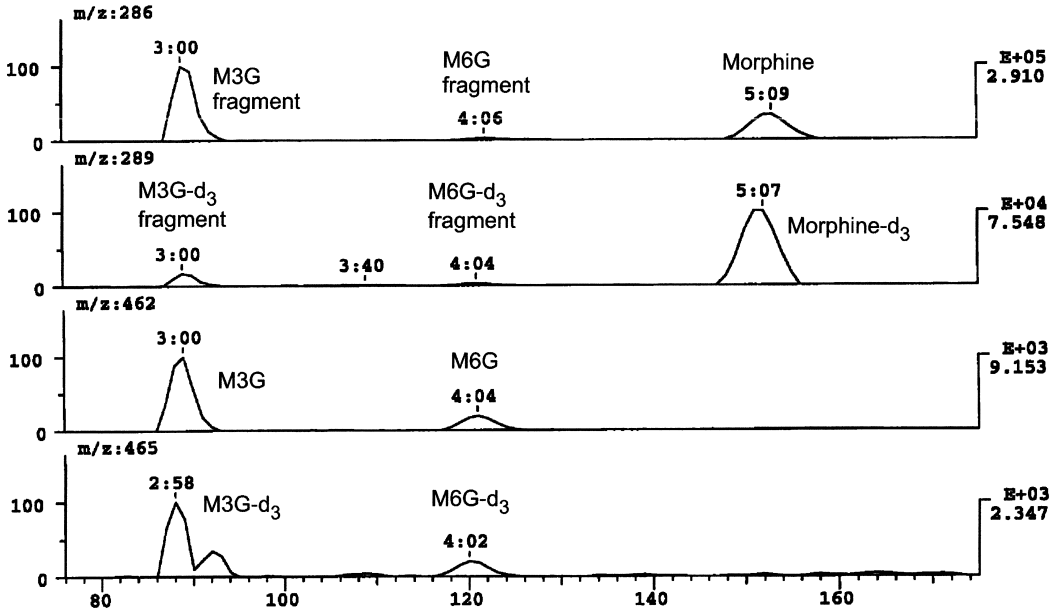

Fig. 2. Chromatogram of blood extract containing M3G 1648 μg/l, M6G 200 μg/1 and morphine 82 μg/l.

the danger of uncontrolled methadone therapy, particularly due to the tendency to mixed ingestion of methadone and other drugs of abuse [24–27]. Therefore, it is of forensic relevance to determine not only the concentration of methadone but also the whole spectrum of other relevant drugs. Table 3 presents blood methadone concentrations in selected cases, as well as other drugs of abuse found. Methadone exists

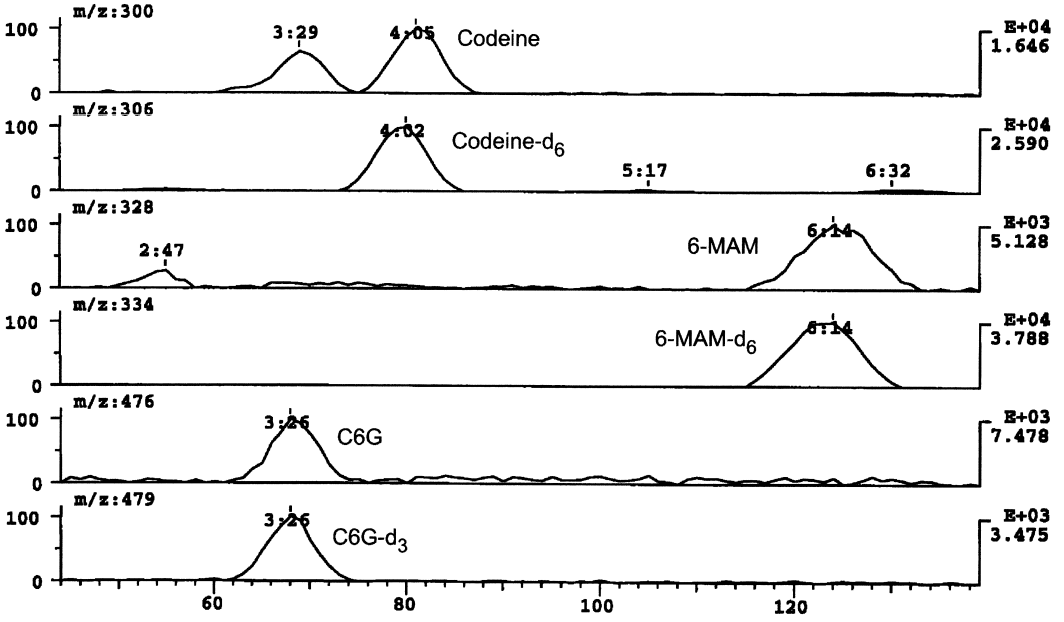

Fig. 3. Chromatogram of blood extract containing C6G 1533 μg/1, codeine 21 μg/l and 6-MAM 13 μg/l.

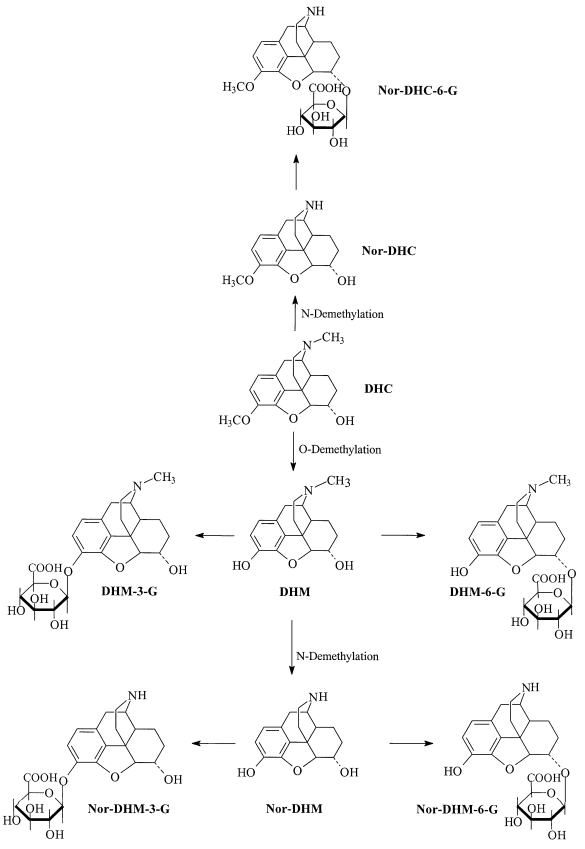

Fig. 4. Metabolic pathways of dihydrocodeine. DHC, dihydrocodeine; DHM, dihydromorphine; DHM3G, DHM-3-glucuronide; DHM6G, DHM-6-g1ucuronide, DHC6G, DHC-6-glucuronide.

in two enantiomeric forms: more active (*R*)-(−)-methadone, known as levomethadone and almost inactive (*S*)-(+)-methadone. In some countries the ''legal'' methadone preparations (e.g. Polamidon) contain only levomethadone, Also, the bioavailability of methadone stereoisomers may show large inter-individual variability. Therefore, it is advisable to perform stereoselective determination of the drug. An LC–ESI–MS enantioselective procedure was described by Kintz et al. and applied for detection of methadone and its main metabolite EDDP in hair [28].

The determination of buprenorphine with LC–APCI-MS deserves special comment. The validated assay of this drug has been published by us [9], but applied in only one forensic case. This drug is not frequently used in Germany. In other countries, however, e.g. in France, buprenorphine has been broadly applied in substitution therapy of opiate addicts, and several LC–API-MS methods for buprenorphine has been published [29–31]. These methods demonstrated the superiority of LC–MS determination of this drug.

3.2. Cocaine and metabolites

Cocaine and its two most important metabolites: benzoylecgonine (BE) and ecgonine methyl ester (EME) show widely differing polarities. Therefore, simultaneous determination of these compounds is feasible in gradient HPLG. Alternatively, the drugs may be determined in two isocratic runs; first for EME and second for cocaine and BE. This option was selected for practical purposes. Table 4 presents the results in 50 selected cocaine-positive blood samples. There was a lot of cases with very low cocaine concentration, but with high level of metabolites, particularly of beuzoylecgonine. It is difficult to differentiate between in vivo and in vitro metabolism; it has been demonstrated, that cocaine present in blood is very unstable in vitro and undergoes enzymatic hydrolysis to EME and chemical hydrolysis to BE [32]. Therefore, for the assessment of the severity of cocaine consumption, its possible acute influence at a given time or signs of tolerance, the whole analytical spectrum, i.e. active cocaine and its both inactive metabolites should be taken into consideration, The sum of molar concentrations of all analytes, shown in the Table 4, may provide additional information in a given case.

3.3. Amphetamine and ''ecstasy''

The determination of amphetamine and related compounds, particularly methylenedioxyamphetamines known as ''Ecstasy'', belong to most important tasks of forensic toxicologist nowadays. These drugs present very serious risk, due to high exposition in discos and at rave parties [33–37]. In a previous report [38], we described a procedure for determination of thirteen psychoactive phenethylamines with LC–APCI-MS or HPLC with diode array detector (DAD). The drugs were extracted with ether and derivatized with phenylisothiocyanate in

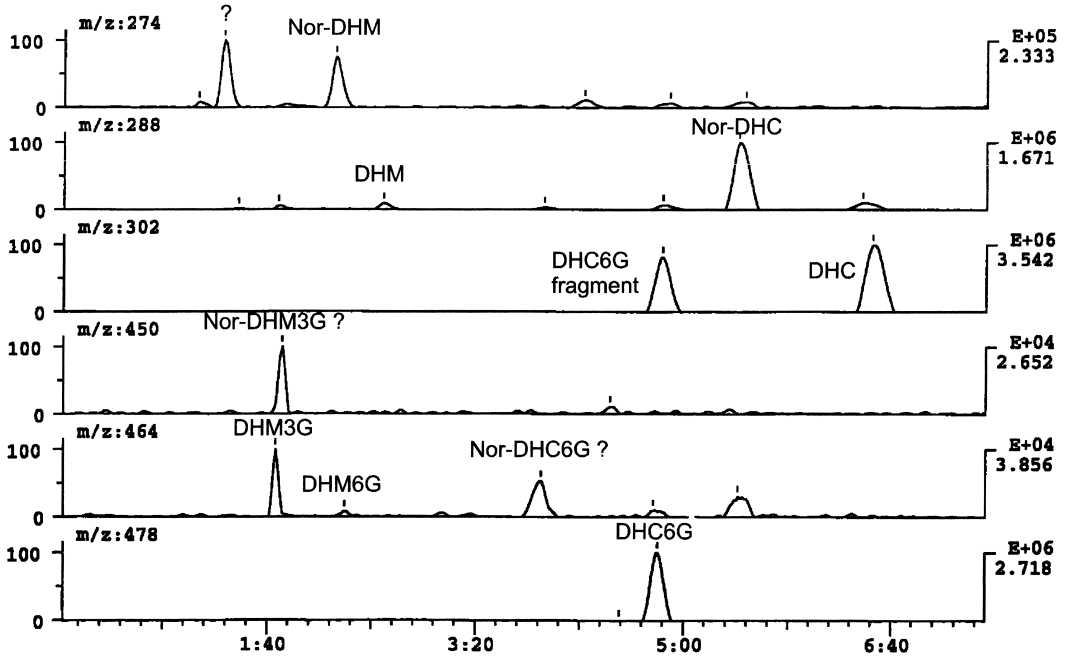

Fig. 5. Chromatogram of urine extract from a sample taken 1 h after administration of 10 mg dihydrocodeine. DHC, dihydrocodeine; DHM, dihydromorphine; DHM3G, DHM-3-glucuronide; DHM6G, DHM-6-glucuronide; DHC6G, DHC-6-glucuronide.

order to enhance the sensitivity of DAD. However, the detection limits for DAD were about 20 to 50 times higher than these for LC–MS. Therefore, the derivatization procedure was later abandoned in favor to the present assay [10], consisting of common solid-phase extraction and LC–APCI-MS. The latter procedure has been applied for detection and quantitation of sixteen phenethylamines, among them illicit drugs, like MDMA, MDA, MDE, MBDB, BDMPEA, and allowed an unequivocal differentiation of all drugs involved, Particularly, the problem of misidentification of some over-the-counter sympathomimetic amines with amphetamine or methamphetamine, which may exist in some GC–MS procedures [39–41], was avoided. During routine casework it was stated that Ecstasy-positive blood samples very often contain also amphetamine. Table 5 presents amphetamine, MDMA, MDE and MDA concentrations in 20 selected cases from 1998/1999 casework. Simultaneous determination of MDMA and MDE together with MDA, which is a metabolite of both compounds, may be helpful in assessment of the time span elapsing between the drug intake and blood sampling. Heimlin et al. [42] administered MDMA to volunteers (oral dose of 1.5 mg/kg and demonstrated that MDA was detectable after 90–135 min and its peak concentration was measured after 150–380 min. Therefore, it is advisable to determine the molar ratio MDMA:MDA. Among the samples presented in the Table 5, the samples 2, 5 and 7 show low MDMA:MDA ratio what may indicate drug consumption several hours before blood sampling. On the other hand, the ratio MDMA/MDA is particularly high in samples 13, 15 and 20, evidencing acute exposition to drug. The data presented are concordant with the observations of Moeller and Hartung [43], who measured MDMA, MDE and MDA in serum of impaired drivers and found median values of 76 μg/l for MDMA, 87 μg/l for MDE and 13 μg/l for MDA, respectively, Fig. 6 shows typical chromatogram of blood extract from the case 16, where amphetamine, MDMA, MDE and MDA were found.

3.4. Benzodiazepines

Among benzodiazepine derivatives, the detection of flunitrazepam issues particular analytical chal-

Table 3
Concentrations (ng/ml) of methadone in forensic blood samples taken from living subjects (road traffic violators and other offenders)[a]

Sample	Methadone	Other illicit drugs
1	25	THC, THC–COOH, A
2	52	M, M3G, M6G, C, C6G
3	71	EtOH
4	84	M, M3G, M6G, MAM, C, C6G
5	108	M, M3G, M6G, MAM, C6G, Co, BE
6	110	EtOH,D
7	111	EtOH, Fl, D, ND, Co, BE, EME
8	118	EtOR
9	161	M, M3G, M6G
10	181	Co, BE, EME, D, ND
11	198	M, M3G, M6G, MAM, C. C6G
12	296	M, M3G, M6G
13	297	–
14	308	CO, BE, EME, D, NDJF1
15	310	M, M3G, M6G, C, C6G
16	368	D, ND, Fl, THC, THC–COOH
17	376	M, M3G, M6G, MAM, C, C6G
18	434	D, ND, FL, THC, THC–COOH
19	441	–
20	671	–

[a] Abbreviations: A, amphetamine; BE, benzoylecgonine; C, codeine; C6G, codeine-6-glucuronide; Co, cocaine; D, diazepam; EME, ecgonine methyl ester; EtOH, ethyl alcohol; F1, flunitrazepam; M, morphine; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; MAM, 6-monoacetylmorphine; ND, nordiazepam; THC, tetrahydrocannabinol; THC–COOH, THC–carboxylic acid.

lenge, due to low therapeutic concentration range in serum (in low μg/l range), and extensive metabolism to active, polar metabolites, particularly to 7-aminoflunitrazepam. Flunitrazepam is frequently involved in drug-facilitated sexual assaults as so-called ''knock-out'' drug [44]. The determination of flunitrazepam and metabolites with GC–MS requires tedious derivatization [45,46], whilst HPLC with ultraviolet detection is of questionable specificity [47]. For these reasons, LC–API-MS is a method of choice for determination of flunitrazepam and nietabolites (7-aminoflunitrazepam, *N*-desmethylflunitrazepam, 3-OH-flunitrazepam) in biological material, Such a method was developed in our laboratory [11] and applied in the routine casework. The results of routine determinations of flunitrazepam and metabolites are shown in Table 6. In Fig. 7 a typical chromatogram of blood extract in flunitrazepam-positive case is depicted. LC–API-MS may be used for other low-dosed benzodiazepines, e.g. triazolam (Fig. 8). Other authors applied electrospray LC–MS for determination of benzodiazepine derivatives, like flunitrazepam in urine [48], midazolam and 1-OH-midazolam in serum [49]. Kanazawa et al. [50] and McClean et al. [51] presented methods for assay of several benzodiazepines and their metabolites with LC–ESI–MS.

3.5. Hallucinogens

Among hallucinogens, two drugs are of particular importance due to the wide availability: LSD and psilocybin. LSD, together with metabolites nor-LSD and 2-oxo-3-hydroxy LSD may be determined in urine with LC–ESI–MS [52–56] or GC–MS [57–59] Chromatographic-mass spectrometric methods for determination of LSD and its metabolites have been recently reviewed [60]. In our laboratory, LC–APCI-MS was applied for determination of LSD in urine (Fig. 9), In the case of this drug, the standard SPE procedure was replaced by immunoaffinity extraction with ImmunElute columns (Microgenics Corp., USA). The latter procedure was clearly superior, due to much lower matrix peak interference and resulting higher signal-to-noise ratio.

A second important hallucinogenic drug — psilocybin — belongs to very polar and thermal labile substances and is after ingestion (or after injection into GC) immediately dephosphorylated to psychoactive psilocin. Both psilocybin and psilocin may be determined in hallucinogenic *Psilocybe* mushrooms with EPLC. We have previously applied LC–APCI-MS for determination of psilocybin and psilocin in honey mixed with ''magic mushrooms'' [61].

In the present study, ESI was applied for routine determination of psilocin in serum and urine of subjects who consumed ''magic mushrooms''. ESI was used instead of APCI since this ionization mode assured much better sensitivity (Fig. 10). A LOD of 1 μg/l serum was achieved, which is sufficient for forensic practice, since the reported peak levels of psilocin after administration of 0.2 mg/kg psilocybin ranged from 6 to 21 μg/l [62], The levels of psilocin

Table 4
Concentrations (ng/ml) of cocaine (C), benzoylecgonine (BE) and ecgonine methyl ester (EME) (ng/ml) as well as sum of molar concentrations (C+BE+EME) in forensic blood samples taken from living subjects (road traffic violators and other offenders)[a]

Sample	C (ng/ml)	BE (ng/ml)	EME (ng/ml)	C+BE+EME (nmol/l)
1	0	123	5	450
2	0	404	9	1400
3	0	441	67	1860
4	0	447	279	2950
5	1	216	23	840
6	1	289	11	1020
7	1	694	143	3040
8	1	743	51	2740
9	2	254	73	1220
10	3	490	85	2070
11	4	291	25	1100
12	4	319	10	1130
13	4	553	32	2020
14	4	2579	79	9030
15	6	717	102	2930
16	6	1811	82	6490
17	8	467	29	1830
18	8	789	48	2910
19	9	2845	146	10280
20	10	364	64	1570
21	12	488	162	2490
22	13	89	18	430
23	13	916	106	3540
24	14	706	34	2580
25	14	887	82	3420
26	15	182	50	910
27	15	569	108	2500
28	16	698	108	2930
29	16	1522	72	5500
30	17	1694	187	6660
31	21	129	21	610
32	25	1222	86	4600
33	27	1050	133	5100
34	36	3074	218	11500
35	40	386	49	1670
36	41	133	34	900
37	43	442	122	2230
38	49	894	61	3620
39	57	1378	73	5320
40	58	1217	47	4500
41	63	1147	155	4820
42	64	1697	138	6580
43	69	2570	360	10630
44	87	2058	130	7820
45	110	1705	202	7080
46	115	1685	19	6110
47	130	1774	161	7170
48	156	2131	382	9560
49	237	3988	741	17840
50	391	2772	1851	19860

[a] Samples are arranged according to cocaine concentration.

Table 5
Concentrations (ng/ml) of amphetamine (A), MDMA, MDE and MDA as well as molar ratios MDMA:MDA and MDE:MDA in forensic blood samples taken from living subjects in cases of poly-drug abuse. The cases are arranged according to the MDMA concentration

Sample	A	MDMA	MDE	MDA	MDMA/MDA	MDE/MDA
1	128	0	266	58		3.97
2	87	10	0	3	3.09	
3	98	20	0	6	3.09	
4	80	25	0	2	11.59	
5	721	28	0	8	3.25	
6	260	30	0	4	7.34	
7	196	45	0	13	3.21	
8	55	59	0	7	8.72	
9	483	97	0	6	14.99	
10	13	117	0	9	12.05	
11	120	122	0	13	8.70	
12	125	138	64	16		11. 46
13	34	143	0	5	26.52	
14	300	159	0	18	8.19	
15	60	159	0	5	29.49	
16	134	223	135	44	12.04	
17	17	228	0	17	12.44	
18	197	330	10	30		10.49
19	280	340	0	30	10.51	
20	130	344	0	15	21.27	

in serum and urine after β-glucuronidase hydrolysis, measured with GC–MS (MSTFA derivatization) were much higher that those extracted directly [63]. This indicated the presence of psilocin glucuronide, which may be directly determined with LC–API-MS.

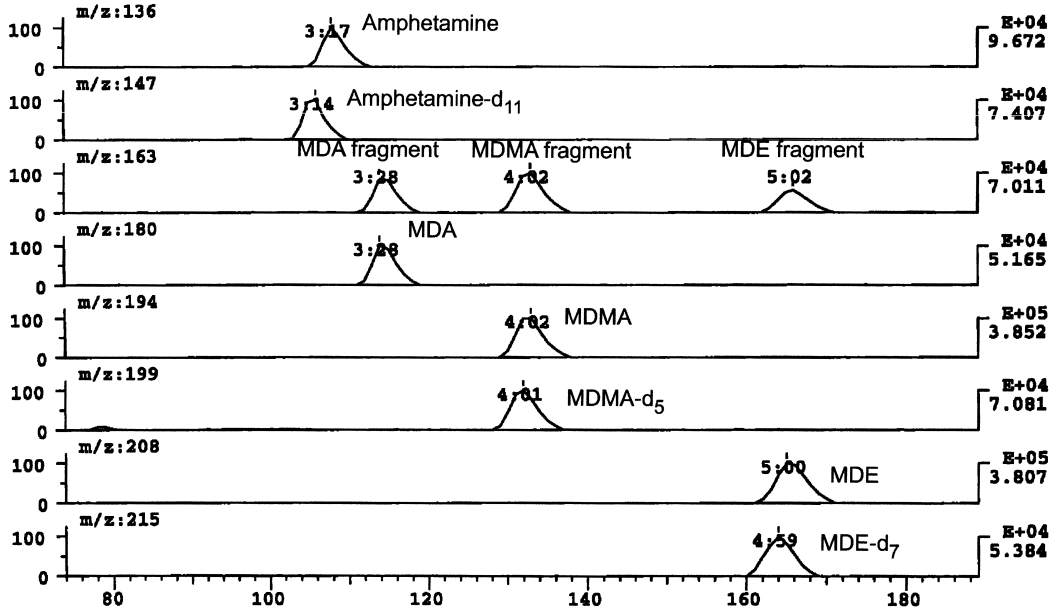

Fig. 6. Chromatogram of blood extract containing amphetamine (60 μg/l), MDA (44 μg/l), MDMA (223 μg/l) and MDE (135 μg/1).

Table 6
Concentrations (ng/ml) of flunitrazepam (F), 7-aminoflunitrazepam (7-AF), *N*-desmethylflunitrazepam (N-DF) and 3-OH-flunitrazepam (3-OH-F) in forensic blood samples taken from living subjects (road traffic violators and other offenders)

Sample	F	7-AF	N-DF	3-OH-F
1	0	9	0	0
2	0	8	2	0
3	0	15	2	0
4	4	4	0	0
5	5	26	5	0
6	8	13	0	0
7	9	21	2	2
8	11	12	3	0
9	11	24	10	2
10	12	29	5	0
11	13	16	2	0
12	14	9	0	0
13	20	11	5	0
14	27	32	31	12
15	48	36	0	10

3.6. Other drugs

Among other applications of our standard SPE followed by LC–APCI-MS the determination of olanzapine may be mentioned [64]. This assay has been developed basically for therapeutic monitoring of drug during therapy; however, it may be also of forensic relevance, since several reports concerning olanzapine-associated fatal cases appeared in the last years [65–67].

4. Concluding remarks

Experience gathered during 3 years of routine application of the LC–API-MS in forensic toxicology showed that this technique became a standard, robust and very reliable tool in everyday casework. The sensitivity of LC–API-MS for drugs which has been traditionally determined with GC–MS, like for cocaine and metabolites or amphetamines, is comparable. From the practical point of view it is important that no derivatization is necessary for LC–MS analysis, and the extracts may be frozen and reexamined weeks or even months later. The spectrum of substances may be easily broadened, especially concerning polar active metabolites of numerous drugs, which are not accessible for GC–MS. In other words, LC–API-MS proved to be an excellent technique to confirm the identity of target com-

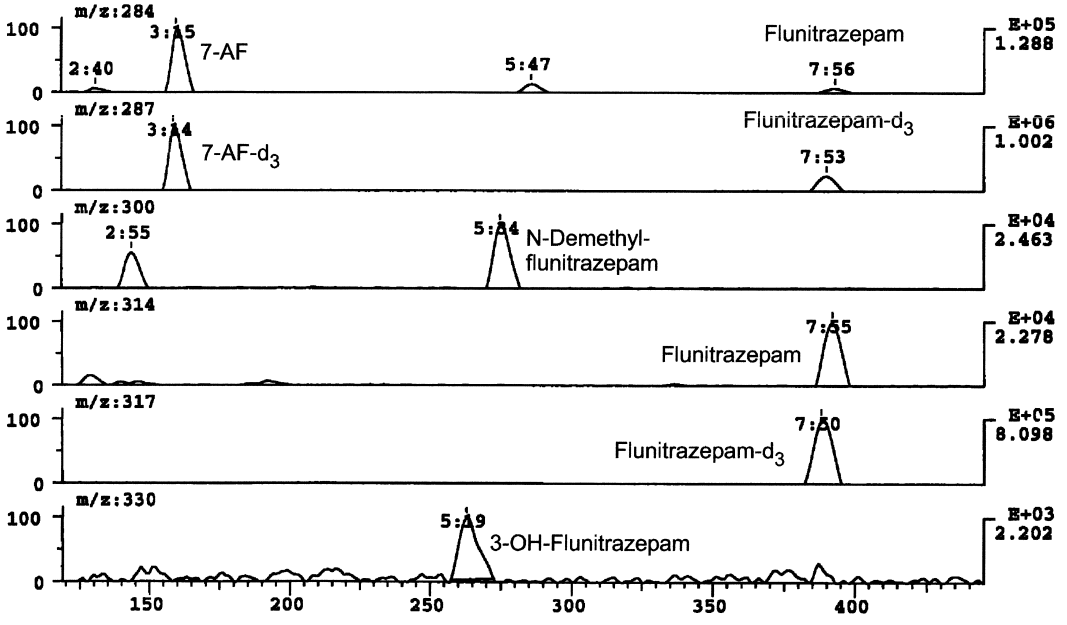

Fig. 7. Chromatogram of serum extract containing flunitrazepam (11 μg/l), 7-aminoflunitrazepam (7-AF, 24 μg/l), *N*-desmethylflunitrazepam (10 μg/l) and 3-OH-flunitrazepam (2 μg/1).

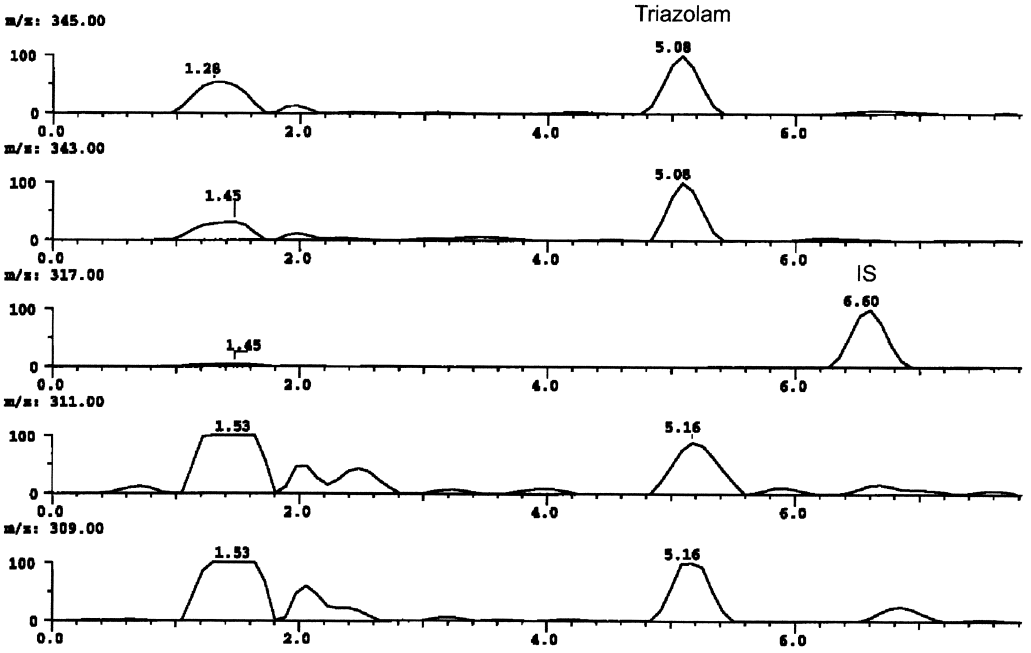

Fig. 8. Chromatogram of serum extract containing 8 μg/l of triazolam. Flunitrazepam-d3 was used as internal standard.

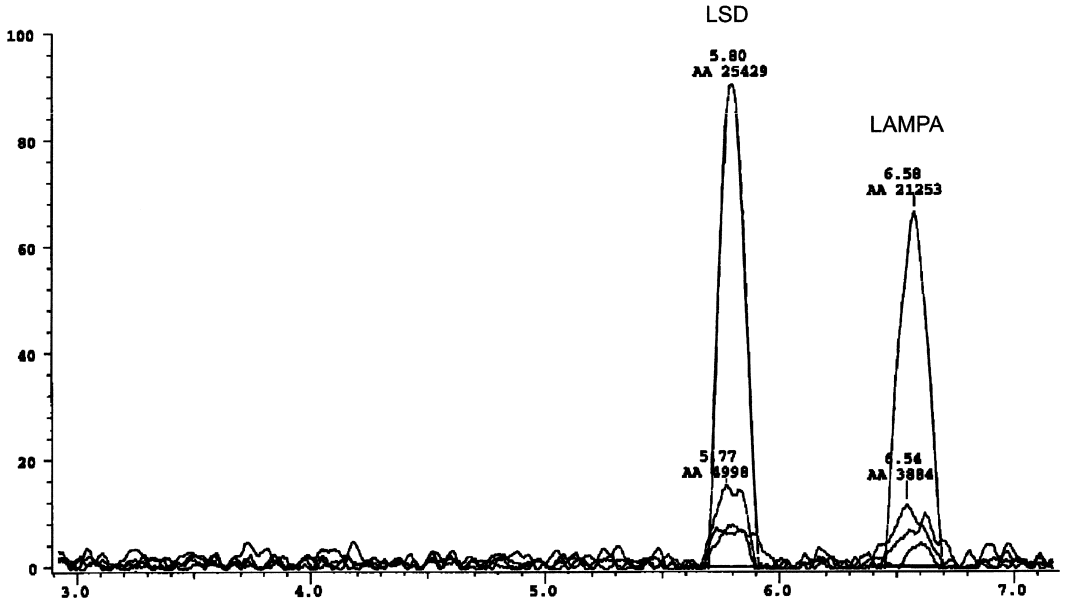

Fig. 9. Chromatogram of urine extract containing 0.8 μg/l LSD. Immunoaffinity extraction.

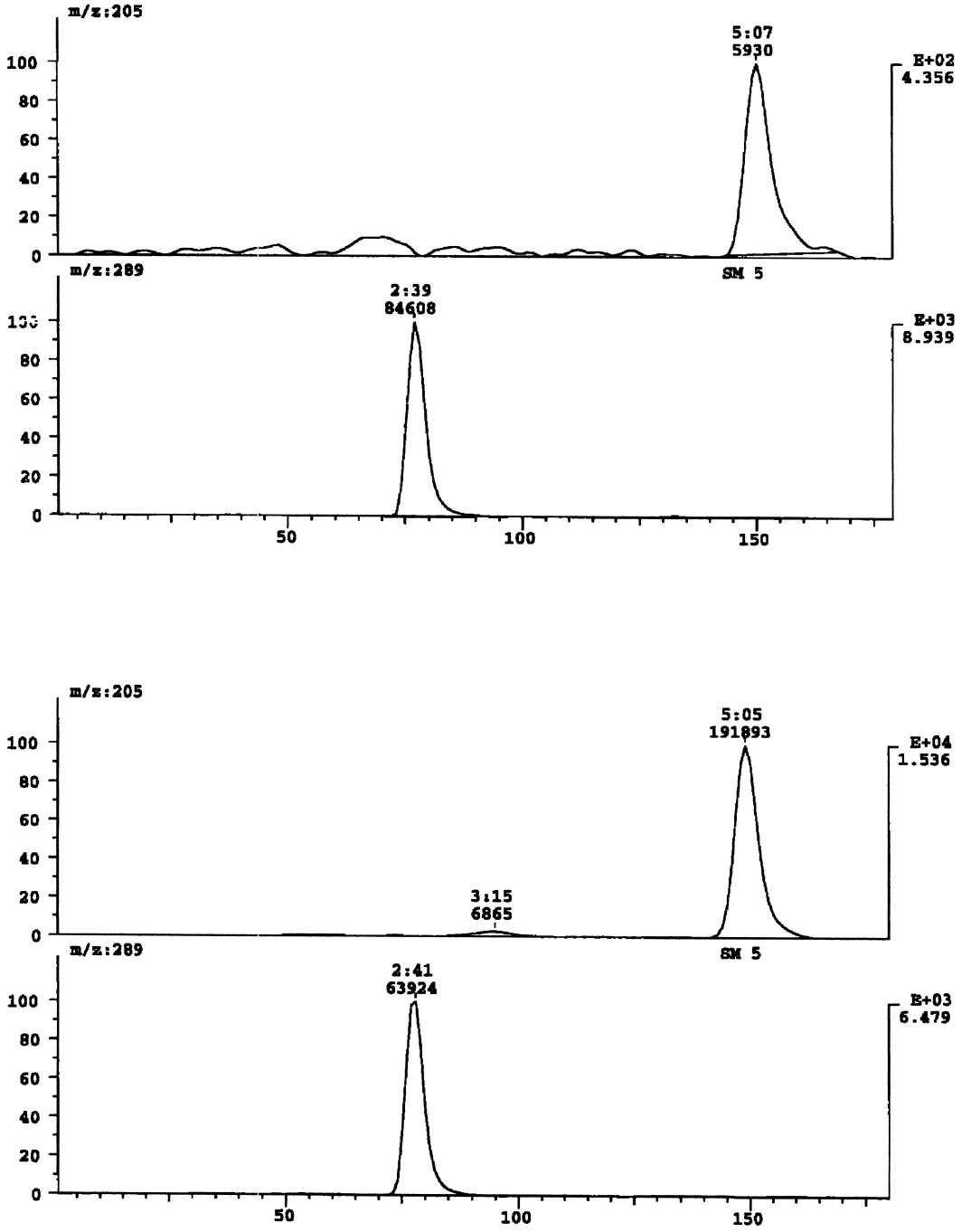

Fig. 10. Comparison of sensitivity of APCI (upper chromatogram) and ESI (lower chromatogram) for determination of psilocin in serum extract. Mass traces off protonated molecular ion of psilocin (m/z 205) and of internal standard (morphine-d3, m/z 289) were monitored. The concentration of drug was 5 μg/l serum.

pounds, not only in forensic sciences [68]. On this field LC–API-MS may replace GC–MS.

The most important and actual question at the moment is: to what extent may be LC–API-MS applied for systematic toxicological analysis, i.e. in the search for unknown substance? The role of GC–EIMS as ''golden standard'' of toxicological screening [5] is unquestionable, due to the availability of large databases of mass spectral containing usually more than 10 peaks per spectrum [69]. Could the further development of LC–API-MS change this situation? In the last years some studies have been performed which demonstrated very high potential of LC–ESI–MS for systematic toxicological screening. Shushan et al. [70] established a database of ESI-generated mass spectra of over 1200 substances, using positive and negative ionization modes and different fragmentation energies. Also, Weinmann et al. [71] described a similar database for over 600 drugs, and Fitzgerald et al. [72] applied HPLC (Remedi system) coupled with MS–MS (ion trap) for identification of drugs in urine. It must be stated, however, that all above-mentioned studies were performed in a single laboratory. The interlaboratory comparison of ESI- and APCI-generated mass spectra of selected drugs showed large variabilities in fragmentation intensities and sometimes in fragmentation patterns [73]. Therefore, the study on standardization of MS procedures is of critical importance for further, broad application of LC–API-MS-based toxicological screening system. A novel approach to enhance the identification potential of LC–API-MS is the introduction of a quadrupole-orthogonal acceleration time-of-flight instruments, which may enable the measurement of ions with an accuracy better than 1 ppm [74]. When the problem of interlaboratory reproducibility of mass spectra will be solved, LC–API-MS may become a ''golden standard'' both for identification and quantitation in forensic toxicology.

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