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Review

Liquid chromatography–mass spectrometry in forensic and clinical toxicology[1]

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Abstract

This paper reviews liquid chromatographic–mass spectrometric (LC–MS) procedures for the identification and/or quantification of drugs of abuse, therapeutic drugs, poisons and/or their metabolites in biosamples (whole blood, plasma, serum, urine, cerebrospinal fluid, vitreous humor, liver or hair) of humans or animals (cattle, dog, horse, mouse, pig or rat). Papers published from 1995 to early 1997, which are relevant to clinical toxicology, forensic toxicology, doping control or drug metabolism and pharmacokinetics, were taken into consideration. They cover the following analytes: amphetamines, cocaine, lysergide (LSD), opiates, anabolics, antihypertensives, benzodiazepines, cardiac glycosides, corticosteroids, immunosuppressants, neuroleptics, non-steroidal anti-inflammatory drugs (NSAID), opioids, quaternary amines, xanthins, biogenic poisons such as aconitines, aflatoxins, amanitins and nicotine, and pesticides. LC–MS interface types, mass spectral detection modes, sample preparation procedures and chromatographic systems applied in the reviewed papers are discussed. Basic information about the biosample assayed, work-up, LC column, mobile phase, interface type, mass spectral detection mode, and validation data of each procedure is summarized in tables. Examples of typical LC–MS applications are presented.

Keywords: Reviews; Therapeutic drugs; Drugs of abuse; Poisons

Contents

[1]Dedicated to Prof. Dr. Gottfried Blaschke, Münster, Germany, on the occasion of his 60th birthday.

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1. Introduction

In forensic and clinical toxicology analytical methods must provide high reliability and accuracy. The combinations of mass spectrometry with suitable chromatographic procedures are the methods of choice, because they are very sensitive, precise, specific, universal and fast. Today, GC–MS is the *golden standard* for detection and quantification of drugs and poisons volatile under GC conditions ([1] and other papers of this Special Volume), whereas non-volatile compounds require LC–MS [2–18]. While GC can easily be coupled with MS, LC can only be coupled with MS via sophisticated interfaces.

1.1. LC–MS interfaces

Since the 1970s, different LC–MS interface types, such as moving belt, direct liquid introduction, continuous-flow or frit-terminated fast atom bombardment (FAB), particle beam (PB), thermospray (TS), electrospray (ES) or atmospheric-pressure chemical ionization (APCI) were developed to remove the mobile phase and to ionize the analyte. The technical principles, and the advantages and disadvantages of these interfaces were recently described in the reviews of Gelpi [15], Careri et al. [16] or Hoja et al. [17], so that this topic needs not be repeated here. In Fig. 1 the relation of the molecular mass range and the polarity of analytes that can be analyzed by GC–MS and different LC–MS interface techniques are sketched. As shown, LC–MS with ES is the most universal of these analytical techniques.

In the last 2 years only few papers using PB [10,19–21], FAB [3,5,12,22] or TS [7,23–31] have appeared. These interfaces have several limitations, such as less sensitivity or less universality. Today, two relatively robust LC–MS interface types seem to have become the *golden standards* of LC–MS, the atmospheric-pressure ionization techniques, ES and APCI. The majority of the papers reviewed here deal already with ES [2,8,9,11,32–52] or APCI [4,6,53–69]. Modern (benchtop) apparatus supply both techniques. They allow very sensitive, gentle and universal procedures.

ES enables the determination of analytes of high molecular mass up to several hundred thousand units such as peptides or proteins [14,15,18,70,71], and/or very high polarity such as quaternary amines, sulfate conjugates, nucleotides or phospholipids [9,28,70,72–74]. A prerequisite is that the analyte must be ionizable in solution, so that the mobile phase often contains a small amount of a volatile acid or base. If such additives impair the chromatographic separation, they can be added after the separation before the eluent enters the ES interface [25,29,43].

APCI allows very sensitive determination of analytes with moderate polarity and molecular mass. Since ES and APCI are soft ionization techniques, they usually produce quasi-molecular ions. If more information on the chemical structure is needed, fragmentation can be obtained either by collisionally induced dissociation (CID) in the ion source or by using tandem mass analysis (MS–MS) [16,17]. However, interpretation of the resulting mass spectra may be difficult, since on the one hand the frag-

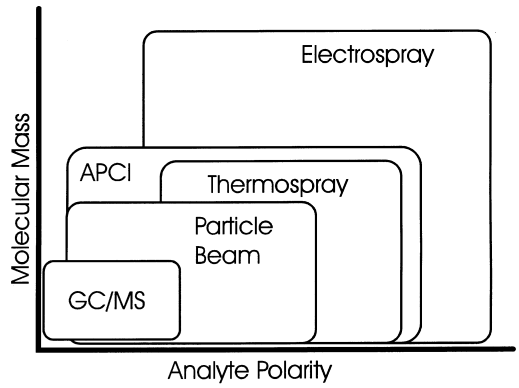

Fig. 1. Relation of the molecular mass range and the polarity of analytes analyzable by GC–MS and different LC–MS interface techniques.

mentation depends on several parameters (e.g. CID voltage) and on the other hand adduct ions can be formed, e.g. by ammonia or sodium present in the eluent.

1.2. LC–MS detection modes

Mass analysis of the substances ionized by the different interfaces is performed using one or two mass analyzers (MS–MS), which consist of ion traps, sector field instruments or predominantly of quadrupoles. They can operate in the full scan mode or in the more sensitive selected-ion monitoring mode (SIM) detecting positive or negative ions. The MS–MS combination provides additional possibilities, because both analyzers can be operated in a scan or in a selected-ion mode resulting in four combinations: parent-ion scanning (scan mode in the first analyzer, SIM in the second), daughter-ion scanning (DIS; SIM in the first analyzer, scan mode in the second), constant neutral-loss scanning (CNLS; scan mode in both analyzers) or selected-reaction monitoring (SRM; SIM in both analyzers) [16,17]. DIS is preferable for the identification of drugs and/or their metabolites in complex matrices, since separation is performed on the LC and in the first mass analyzer, while structural information is obtained by fragmentation in the second analyzer. SRM (sometimes named multiple-reaction monitoring, MRM) is the most powerful technique for quantification of small amounts of analyte in complex matrices. Especially pharmaceutical companies are using this technique for series of quantifications during pharmacokinetic studies of new (polar and low dosed) drugs [75–86].

1.3. Applicability of LC–MS in forensic and clinical toxicology

The choice of the method in analytical toxicology depends on the problems which have to be solved. Usually, the compounds which have to be analyzed are unknown. Therefore, before quantification, the drug or poison must first be identified. The analytical strategy – especially in drugs of abuse testing – includes a screening test and a confirmatory test. If only a single drug or a single category of drugs must be monitored, immunoassays, if available, can be used for screening in order to differentiate between negative and presumptively positive samples. Positive results must be confirmed by a second independent method that is at least as sensitive as the screening test and that provides the highest level of confidence in the result. Some recent LC–MS papers on drugs of abuse testing deal with confirmation of positive immunoassay results [23,24,33–36,41,53,55] or with the detection of drugs of abuse not detectable by immunoassay [25,26,37–40,53–57]. Some authors describe procedures for identification and/or quantification of therapeutic drugs relevant to analytical toxicology in human or animal samples [2–11,19–22,25–28,42–52,58–66,87], while others describe the detection of non-volatile poisons relevant to diagnosis of an acute or chronic intoxication [12–14,67,69]. Further papers deal with the quantification of (future) therapeutic drugs as part of pharmacokinetic or metabolic studies [9,23,29–31,37,38,55,72,73,75–86,88–98]. Such papers are not reviewed in detail, but they are mentioned in Section 2.3.4. They were not omitted for two reasons: on the one hand such therapeutic drugs may also occur in future toxicological cases, and on the other hand they may give hints for developing new procedures for similar compounds of toxicological interest.

1.4. Choice of the references

The reviewed references were selected by on-line searching the *Medline* database, the *Chemical Abstract Services (CAS)* and *Current Contents*. Since Hoja et al. [17] most recently published a review considering papers until the beginning of 1995, the period from January 1995 to April 1997 was taken into consideration. Only papers written in English were considered.

2. LC–MS determinations of drugs, poisons and/or their metabolites in biosamples

LC–MS procedures for the identification (ID) and/or quantification (QU) of drugs or poisons relevant to forensic or clinical toxicology published in the last 2 years are critically reviewed in this chapter. Most of the papers concerning this topic

have been published in early 1997 [2,12,14,32,33,39,40,44,50,53,55,61,99]. This fact indicates that LC–MS is a new but upcoming technique also in this field of scientific research and application.

The principal information on each procedure is summarized in Tables 1–3 to simplify the rapid selection of a method suitable for an actual analytical problem. The information, whether a paper deals with a quantitative assay, can be taken from the *Validation* column. Retention time and mass spectral data are not listed in the tables to save space.

2.1. Sample preparation

Suitable sample preparation is an important prerequisite for chromatography of biosamples. It involves isolation after cleavage of conjugates by enzymatic (EHY) or acid hydrolysis (if required), and/or derivatization of the drugs and their metabolites. Isolation was performed by liquid–liquid extraction at a pH at which the analyte is uncharged (see LLE in *Work-up* column in Tables 1–3) or by solid-phase extraction (see SPE) preceded or followed by clean-up steps. Afterwards, the extract is concentrated and finally dissolved in an appropriate volume of mobile phase. The pros and cons of both types of extraction are discussed in detail in the review of Franke and De Zeeuw in this Special Volume [100].

Most analytes do not require derivatization for LC separation or MS detection. However, some analytes such as amines show only poor chromatographic behaviour without derivatization. Therefore, Bogusz et al. [53] have derivatized their analytes (amphetamine derivatives) to improve the LC separation and the MS or the diode array detection (DAD). However, if derivatization was necessary, the advantage of such an LC–MS procedure seems to be questionable in comparison to GC–MS procedures as reviewed by Kraemer and Maurer in this Special Volume [101].

An excellent way to isolate analytes from biosamples is immunoaffinity extraction (IAE), especially if coupled on-line with the LC–MS [34,35,61,102]. Cai and Henion [61], for example, have recently described an automated on-line clean-up and enrichment procedure using a commercially available workstation to perform the complete process. Three different columns were used in the column switching system: an IAE column packed with the bound antibodies, a trapping column and an analytical column. The work-up process consisted of the following steps: preconditioning of the IAE column, loading of the urine sample, elution of the unbound matrix to waste, equilibration of the trapping and analytical column with mobile phase, elution of the bound analytes and trapping in the trapping column, back-flushing of the trapped analytes onto the analytical column, separation and introduction into the MS(–MS). The advantage of such a procedure is not only the full automation but also the highly selective and sensitive detection. For example, the LOD for the IAE-LC–LC–MS–MS detection of LSD in urine was 20-fold below that using off-line SPE and LC–MS–MS [35]. However, a disadvantage of IAE is that overloading the antibodies with the analyte (e.g. in overdose cases) leads to poor linearity. This is in accordance with our studies on IAE of amanitins [103,104].

A simpler alternative way (e.g. for determination of plasma levels) is the direct injection of the biosample onto a pre-column, washing the matrix from this column to waste and back-flushing the analyte onto the analytical column [64]. On-line extraction was also described using two analytical columns (LC–LC–MS–MS) [27] or using an SPE column connected to the analytical column (SPE–LC–MS–MS) [19]. By applying such techniques, the analysis cycle time can drastically be reduced (e.g. <5 min), especially in combination with MS–MS [64], in which the analyte can be separated further by its mass in the first mass analyzer and specifically detected in the last.

2.2. Liquid chromatographic systems

As shown in Tables 1–3, the most commonly used stationary phases were – as usual – reversed-phase C_{18} columns. For enantioselective determination, normal-phase columns with alkane–alcohol mixtures as mobile phase were used [43,47]. Ammonium acetate must be mixed into the non-aqueous eluent [47] or it must be introduced post-column to the eluent [43] to improve the ionization in the MS and to reduce the risk of explosion in the heated nebul-

Table 1
LC–MS procedures for the identification and/or quantification of illicit drugs of abuse and/or their metabolites in biosamples

Compound	Sample	Work-up	Stationary phase	Mobile phase (mixtures in v/v)	Interface	Detection mode	Validation data	Ref.
Amphetamines: Amphetamine (AM), methamphetamine (MA), methylenedioxy-AM (MDA), methylenedioxy-ma (MDMA), methylenedioxyethyl-AM (MDE); ephedrine, fenfluramine, norfenfluramine, phentermine, phenylethylamine, phenylpropanolamine, propylhexedrine	S, U	LLE, phenylisocyanate derivatization	Superspher Select Ba ECOcart (125×3 mm I.D.)	50 m*M* ammonium formate (pH 3)–ACN (55:45)	APCI	ID: scan QU: SIM	REC: 70–75% LOD: 5 ng/ml (AM, MDA); 1 ng/ml (others) LIN: 5–1000 ng/ml	[53]
Amphetamines: Amphetamine (AM), methamphetamine (MA)	U	SPE	Ultron ES-PhCD (150×6 mm I.D., 5 μm); chiral separation	100 m*M* ammonium acetate (pH 6)–MeOH–ACN (60:30:10)	TS	ID: scan, SIM; QU: UV, 220 nm	REC: 96.5–98.5% LOD_{scan}: 10 ng/ml (AM); 20 ng/ml (MA) LOD_{SIM}: 0.5 ng/ml (AM); 0.8 ng/ml (MA) *For HPLC–UV*: LIN: 0.5–10 ng/ml	[24]
Amphetamines and other drugs of abuse: Amphetamine (AM), methamphetamine (MA), (methyl)ephedrine; morphine+M (M3G, M6G), cocaine+M	U	LLE	L-column ODS (150×4.6 mm I.D.)	gradient elution: 100 m*M* ammonium acetate--ACN (100:0–60:40)	TS	ID: scan QU: SIM	REC: 88–99% LOD_{scan}: 50–400 ng/ml LOD_{SIM}: 2–40 ng/ml LIN:?–2000 ng/ml (M3G, M6G) LIN: 40–500 ng/ml (others)	[23]
Cocaine: Cocain-M (BZE)	Blood spots	Elution of BZE from the spot by aqueous ammonium acetate, deproteination by MeOH, evaporation	2 Perkin-Elmer C_{18} in series (30×4.6 mm I.D., 3 μm)	25 m*M* ammonium acetate in MeOH–water (50:50)	APCI	MS-MS, SRM	REC:? LOD: 2 ng/ml LIN: 4–166 ng/ml	[54]
Cocaine and other drugs of abuse: Cocaine+M (benzoylecgonine, BZE); amphetamine (AM), methamphetamine (MA), (methyl)ephedrine, morphine+M (M3G, M6G)	U	LLE	L-column ODS (150×4.6 mm I.D.)	gradient elution: 100 m*M* ammonium acetate--ACN (100:0–60:40)	TS	ID: scan QU: SIM	REC: 88–99% LOD_{scan}: 50–400 ng/ml LOD_{SIM}: 2–40 ng/ml LIN:?–2000 ng/ml (M3G, M6G) LIN: 40–500 ng/ml (others)	[23]
Lysergide: LSD+M (nor-)	U	SPE	Nucleosil C_{18} (150×1 mm I.D.)	2 m*M* ammonium formate (pH 3)–ACN (70:30)	ES	SIM	REC: 93%, 80% (M) LIN: 0.05–20 ng/ml LOD: 0.025 ng/ml, 0.035 ng/ml (M) LOQ: 0.1 ng/ml (metabolite)	[32]
Lysergide: LSD+M (nor-)	U	SPE	Hypersil C_{18} (125×3 mm I.D., 3 μm)	100 m*M* ammonium acetate (pH 8)–ACN (75:25)	ES	SIM	REC: ? LIN: 0.5–10 ng/ml	[33]

(Cont.)

Table 1. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase (mixtures in v/v)	Interface	Detection mode	Validation data	Ref.
Lysergide: LSD	U	SPE, IAE	Hypersil C_{18} (125×3 mm I.D., 3 μm)	100 m*M* ammonium acetate (pH 8)-ACN (75:25)	ES	SIM	REC: 83–100% (SPE); 64–166%! (IAE) LIN: 0.5–10 ng/ml	[34]
Lysergide: LSD+M	U	IAE (on-line extraction)	*IAE column*: antibodies bound to protein G (2.1×3.3 mm I.D.) *Trapping column*: LC Packings C_{18} (15×0.5 mm I.D., 5 μm) *Analytical column*: LC Packings C_{18} (150×0.3 mm I.D., 3 μm)	ACN–MeOH–HOAc–5 m*M* ammonium acetate (30:30:0.1:39.9)	ES	MS-MS, SRM	REC: ? LOD: 0.0025 ng/ml	[35]
Lysergide: LSD+M	U, human liver microsomes	SPE	Spherisorb ODS-2 (100×1 mm I.D., 5 μm)	Gradient elution from 44 to 100%B: (A) ACN–MeOH–HOAc–5 m*M* ammonium acetate (10:10:0.1:79.9) (B) ACN–MeOH–HOAc–5 m*M* ammonium acetate (49.5:49.5:0.1:0.9)	ES	MS-MS, CNLS (microsomes), SRM (U)	REC: 45–74% (U) LOD: 0.05 ng/ml	[36]
Opiates: Morphine, morphine-3-glucuronide (M3G) morphine-6-glucuronide (M6G), 6-monoacetylmorphine (MAM)	B, U, cerebro-spinal fluid, vitreous humor	SPE	Superspher Select B (125×3 mm I.D.)	50 m*M* ammonium formate (pH 3)–ACN (95:5) or (90:10) for MAM	APCI	QU: SIM	REC: 82–89% LOD: 0.1–1 ng/ml LIN: 5–500 ng/ml	[55]
Opiates: Morphine+M (M3G, M6G)	S	SPE	Supelcosil ABZ (250×4.6 mm I.D., 5 μm)	Gradient elution: water-MeOH (85:15–40:60)	ES	QU: SIM	REC: 70% (M3G) REC: 95% (others) LIN: 10/100/50–1000 ng/ml (morphine/M3G/M6G)	[37]
Opiates: Morphine+M (M3G, M6G)	S	SPE	YMC ODS-AL (100×4.6 mm I.D.)	gradient elution: 3 m*M* formic acid in water–3 m*M* formic acid in ACN (4:96–70:30)	ES	QU: SIM	REC:? LIN: 0.84–17/5–500/2–100 ng/ml (morphine/M3G/M6G)	[38]
Opiates and other drugs of abuse: Morphine+M (M3G, M6G, MAM); amphetamine (AM), methamphetamine (MA), (methyl)ephedrine, cocaine+M (BZE)	U	LLE	L-column ODS (150×4.6 mm I.D.)	Gradient elution: 100 m*M* ammonium acetate–ACN (100:0–60:40)	TS	ID: scan QU: SIM	REC: 88–99% LOD_{scan}: 50–400 ng/ml LOD_{SIM}: 2–40 ng/ml LIN:? –2000 ng/ml (M3G, M6G) LIN: 40–500 ng/ml (others)	[23]

izer. Some authors recommended a short guard column with the same type of stationary phase [2,14,27,42,45,47,49,52,59,63,64,68]. Others used two analytical columns in series [29,54]. If the column was heated, the column temperature is given in the tables. It is incomprehensible, why referees and editors accept papers in which neither the length nor the diameter of the column were reported [4].

The mobile phases consisted of mixtures of volatile buffers (e.g. ammonium acetate or formate) with

Table 2. LC–MS procedures for the identification and/or quantification of toxicologically relevant drugs and/or their metabolites in biosamples

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Anabolic steroids: Excretion studies on testosterone, boldenolone, methenolone, methyltestosterone, trenbolone; for further 10 steroids and 13 of their metabolites only t_R and MS data are given	U	EHY, SPE-LC (on-line extraction)	Ultrasphere ODS (250×2.1 mm I.D., 5 μm)	10 m*M* ammonium acetate (pH 7.35)–ACN (55:45)	PB	scan	REC: >80% LOD: th. conc.	[19]
Anabolic steroids: Sulfate and glucuronide conjugates of (epi)-testosterone, (epi)-androsterone, dehydroepiandrosterone, estrone, etiocholanolone	U	SPE? (not described!)	Hypersil BDS C_{18} (150×1 mm I.D.) or Hypersil BDS C_{18} (250×0.3 mm I.D., 3 μm)	Gradient systems: (A) 0.11% acetic acid–7.5 m*M* ammonium acetate in water; (B) 0.11% acetic acid–7.5 m*M* ammonium acetate in MeOH or ACN	ES	MS-MS, SRM	No data recorded in urine samples were given!	[9]
Antihypertensive drugs: Amlodipine	S	LLE	Puresil C_{18} (150×2.1 mm I.D., 5 μm)	10 m*M* ammonium acetate (pH 4)–MeOH (33:67)	APCI	MS-MS, SRM	REC: ? LIN: 0.014–7.2 ng/ml	[58]
Antihypertensive drugs: Doxazosin	P	LLE	Chiralpak AD CSP (100×2.1 mm I.D., 10 μm), 30°C; chiral separation	*n*-Hexane–2-propanol–diethylamine (70:30:0.1)	ES	MS-MS, SRM	The authors state that validation is in progress	[43]
Antihypertensive drugs: Nimodipine (the method could be transfered to other dihydropyridines like felodipine, nisoldipine, nitrendipine)	P	LLE	Chira OJ MOD (250×2 mm I.D., 8 μm) with a guard column (10×2 mm I.D.), 35°C; chiral separation	2 m*M* ammonium acetate in ethanol–*n*-heptane (20:80)	ES	MS-MS, SRM	REC: 90% LOD: 0.25 ng/ml LIN: 0.25–75 ng/ml LOQ: 0.5 ng/ml	[47]
Antihypertensive drugs: Reserpine	P (horse)	LLE, SPE	Betasil C_{18} (100×1 mm I.D., 5 μm)	5 m*M* ammonium acetate–ACN (20:80) *For LLE extracts:* pH 7.12 *For SPE extracts:* pH 5.47	ES	MS-MS, SRM	*For LLE extracts*: REC: 68–76% LOD: 0.01 ng/ml LIN: 0.01–5 ng/ml) LOQ: 0.05 ng/ml *For SPE extracts*: REC: 44–58% LOD: 0.01 ng/ml LIN: 0.1–5 ng/ml LOQ: 0.2 ng/ml	[50]
Antihypertensive drugs: Sotalol, verapamil+M (nor-)	P	LLE	Chiralpak AD CSP (100×2.1 mm I.D., 10 μm), 20°C; chiral separation	Ethanol–*n*-hexane–2-propanol–diethylamine (30:63:7:0.17), addition of 800 μl/min of 25 m*M* ammonium acetate–2-propanol (25:75) post-column to improve the ionization and to reduce the risk of explosion	ES	MS-MS, SRM	The authors state that validation is in progress	[43]

(Cont.)

Table 2. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Benzodiazepines: Diazepam, nordazepam, nitrazepam, flunitrazepam, medazepam	S, U	SPE	LiChrospher 60-RP (100×2 mm I.D., 5 μm)	MeOH–water–ACN (1:1:1), pH 6	ES	MS-MS, SRM	REC: 91–110% (S), 90–104% (U) LIN: 1–1000 ng/ml)	[41]
Benzodiazepines: Flunitrazepam+M (nor-), lormetazepam (for brotizolam, clonazepam, diazepam, ketazolam, loprazolam, nitrazepam and triazolam only t_R and on-column LOD data are given)	B	SPE	Nova-Pak C18 (150×3.9 mm I.D., 4 μm)	50 m*M* ammonium acetate–MeOH (40:60), addition of 800 μl/min of 50 m*M* ammonium acetate post-column to improve the ionization	TS	MS-MS, SRM	REC: about 50% LOD: not reported for biosamples	[25]
Benzodiazepines: Midazolam, (atipamezole, medetomidine)	P (pig)	SPE	Hitachi C_{18} (150×4.6 mm I.D., 5 μm)	100 m*M* ammonium acetate–MeOH (35:65)	APCI	SIM	REC: 93–98% LIN: 0.5–100 ng/ml) LOQ: 1–2 ng/ml	[56]
Benzodiazepines: Triazolam+M	P, U	LLE	Nomura ODS-HG-5 (150×4.6 mm I.D., 5 μm)	Gradient: 50 m*M* ammonium acetate (pH 4)–MeOH (50:50–0:100)	APCI	SIM	REC: 77–84% (P), 79–85% (U) LIN: 0.02–300 ng/ml	[57]
β_2-Agonists: Clenbuterol, fenoterol, metaproterenol, salbutamol, terbutaline	P	SPE	Upchurch α-Chrom C_{18} (250×3 mm I.D., 5 μm) with a guard column (C_{18} silica (5×2 mm I.D., 10 μm))	Gradient: 10 m*M* ammonium acetate (pH 7)–water–ACN (12:11:77) to 25 m*M* ammonium acetate (pH 3.5)–ACN (35:65)	APCI	MS, SIM; MS-MS, SRM for salbutamol	REC: 62–104% LOD: 2.5 ng/ml LIN: 2.5–50 ng/ml	[59]
β_2-Agonists: Cimaterol, clenbuterol, mabuterol, salbutamol, terbutaline	U, liver (cattle)	EHY, SPE	Upchurch α-Chrom C_{18} (250×3 mm I.D., 5 μm)	Gradient: (A) 10 m*M* ammonium acetate (pH 7)–water–ACN (12:11:77) to 25 m*M* ammonium acetate (pH 3.5)–ACN (35:65)	APCI	MS-MS, SRM	REC: 60–80% (U); 45–55% (liver) LOD: 0.05 ng/ml (U); 0.05–0.1 ng/g (liver) LIN: 0.5–25 ng/g (liver)	[60]
β_2-Agonists: Clenbuterol, salbutamol	U (cattle)	LC-LC (on-line sample enrichment)	2 columns Microspher C_{18} (50×5.6 mm I.D., 3 μm) with a guard column each (Chrompack (10×3 mm I.D.))	MeOH–water containing 100 m*M* ammonium acetate, 10 m*M* triethylamine and 170 m*M* formic acid (30:70) for clenbuterol, (18:82) for salbutamol	TS	MS-MS, SRM	REC: 102–107% LOD: 0.05 ng/ml LIN: ? LOQ: 0.1 ng/ml	[27]
β_2-Agonists: Clenbuterol, mabuterol, mapenterol, methylclenbuterol, tolubuterol	U (cattle)	IAE (on-line extraction)	*IAE column*: antibodies bound to protein G (10×2 mm I.D.) *Trapping column*: pellicular C_{18} (20×1 mm I.D., 30–40 μm)	ACN–MeOH–0.1% aqueous acetic acid (A) 2.5:2.5:95 (B) 47.5:47.5:5 ID: gradient 90%A–100B; QU: 28%A, 72%B	APCI	MS-MS, SRM	REC: 94–108% LIN: 0.05–250 ng/ml	[61]

Table 2. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
			Analytical column: LC Packings C_{18} (150×0.3 mm I.D., 3 μm)					
Cardiac glycosides: Digoxin, digitoxin, α-, β-acetyldigitoxin, lanatoside C, oleandrin	P	LLE	Nova-Pak C_{18} (150×2 mm I.D., 4 μm) with a guard column (Optiguard C_{18} (15×1 mm I.D., 5 μm))	Gradient: 2 m*M* ammonium acetate (pH 3)–ACN (20:80–70:30)	ES	Scan, SIM	REC: 67–90% LOD: 0.15–0.6 ng/ml	[2]
Cephem antibiotics: 24 Antibiotics	S	SPE	Pre-column: Develosil PhA (30×0.5 mm I.D., 10 μm), analytical column: Develosil PhA (150×0.5 mm I.D., 5 μm)	*For pre-column*: 10 m*M* acetic acid–glycerol (or diethanolamine) (99.5:0.5); water–MeOH–acetic acid–glycerol (59:40:0.5:0.5) or water–MeOH–acetic acid–diethanolamine (57:40:2.5:0.5)	FAB	Scan, SIM	REC: >60% LOD: th. conc.	[3]
Corticosteroids: Betamethasone, cortisone, dexamethasone, flumethasone, flunisolide, hydrocortisone, methylprednisolone, prednisolone, prednisone	U (cattle)	EHY, SPE	Spherisorb ODS2 (250×4.6 mm I.D., 5 μm)	100 m*M* ammonium acetate (pH 6.8)–ACN (60:40)	APCI	MS-MS, SRM	REC: 80–86% LOD: 0.05 ng/ml	[62]
Corticosteroids: Triamcinolone	S, U (horse)	LLE	Lichrospher 100 C_{18} (125×4 mm I.D., 5 μm) with a guard column (C_{18} (4×4 mm I.D., 5 μm))	Gradient: 10 m*M* ammonium acetate–MeOH (50:50–0:100)	ES	MS-MS, SIM	*For serum*: REC: 83% LOD: 0.1 ng/ml LIN: 0.3–12 ng/ml *For urine*: REC: 81% LOD: 0.1 ng/ml LIN: 0.5–50 ng/ml	[52]
Immunosuppressants: Sirolimus+M	B	SPE	Resolve C_{18} (150×3.9 mm I.D., 5 μm), 40°C	MeOH–1% formic acid (90:10)	ES	SIM (MS-MS for identification of M)	REC: 85–91% LIN: 0.25–250 ng/ml	[8]
Immunosuppressants: Tacrolimus+M	B, U	SPE	Spherical C_{18} (150×3.9 mm I.D., 5 μm)	MeOH–water (90:10)	PB	SIM	REC: 90% LIN: 0.2–100 ng/ml	[10]
Immunosuppressants: Tacrolimus	B	SPE	Brownlee C-4 Column (30×2.1 mm I.D.)	40 m*M* ammonium acetate (pH 5.1)–MeOH (20:80)	ES	MS-MS, SRM	REC: 54.3% LIN: 0.2–100 ng/ml	[11]
Neuroleptics (Butyrophenones): Haloperidol+M (dihydro-)	P	LLE	Nucleosil C_{18} (150×1 mm I.D.)	2 m*M* ammonium formate (pH 3)–ACN (55:45)	ES	SIM	REC: 58%; 70% (M) LOD: 0.075 ng/ml; 0.1 ng/ml (M) LIN: 0.1–50 ng/ml; 0.25–50 ng/ml (M)	[44]

(Cont.)

Table 2. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Neuroleptics (Butyrophenones): Haloperidol (for penfluridol, pimozide, benperidol, droperidol and pipamperone only t_R and on-column LOD data are given)	B	SPE	Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm)	Gradient: 50 m*M* ammonium acetate–MeOH (35:65–25:75)	TS	MS-MS, SRM	REC: 80–90% LOD: 0.05 ng/ml LIN: 0.5–12.5 ng/ml	[26]
Neuroleptics (Butyrophenones): Haloperidol (for penfluridol, pimozide, benperidol, droperidol and pipamperone only t_R and on-column LOD data are given)	B	SPE	Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm)	50 m*M* ammonium acetate–MeOH (25:75), addition of 800 μl/min of 50 m*M* ammonium acetate post-column to improve the ionization	TS	MS-MS, SRM	REC: 80–90% LOD: not reported for biosamples	[25]
Neuroleptics (Phenothiazines): Chlorprothixene (for flupentixol, thiothixene and clopenthixol only t_R and on-column LOD data are given)	B	SPE	Asahipak OPD-50 (125×4 mm I.D., 5 μm)	50 m*M* ammonium acetate–ACN (15:85), addition of 800 μl/min of 50 m*M* ammonium acetate post-column to improve the ionization	TS	MS-MS, SRM	REC: 80–90% LOD: not reported for biosamples	[25]
Non-steroidal anti-inflammatory drugs (NSAIDs): Flunixin+M (hydroxy-); for a further 40 NSAIDs only t_R and MS data are given	U (horse)	SPE	Hypersil ODS (100×2.1 mm I.D., 5 μm), 45°C	*For PB and UV:* gradient: hexane–95% 2-propanol–water (98:2–0:100), 400 μl/min *For ES:* gradient: 1% aqueous acetic acid–ACN (70:30–0:100)	PB, ES	scan	REC: 88.5% LOD: 10 ng/ml (PB)	[21]
Opioids: Dextromoramide (for dextropropoxyphene and methadone only t_R and on-column LOD data are given)	B	SPE	Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm)	50 m*M* ammonium acetate–MeOH (25:75), addition of 800 μl/min of 50 m*M* ammonium acetate post-column to improve the ionization	TS	MS-MS, SRM	REC: 80–90% LOD: not reported for biosamples	[25]
Opioids: Dextromoramide (for dextropropoxyphene and methadone only t_R and on-column LOD data are given)	B	SPE	Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm)	50 m*M* ammonium acetate–MeOH (25:75)	TS	MS-MS, SRM	REC: 80–90% LOD: 0.05 ng/ml LIN: 0.5–12.5 ng/ml	[26]
Opioids: Buprenorphine+M (nor-)	B	SPE	Nucleosil C_{18} (150×1 mm I.D.)	2 m*M* ammonium formate (pH 3)–ACN (55:45) or for MAM (90:10)	ES	SIM	REC: 56–60% LOD: 0.05 ng/ml LIN: 0.1–100 ng/ml	[39]
Opioids:	B, U, hair	LLE	Nova-Pak C_{18} (150×2 mm I.D., 4 μm)	2 m*M* ammonium acetate (pH 3)–ACN (20:80)	ES	SIM	REC: 87–94%;	[40]

Table 2. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Buprenorphine+M (nor-)							61–66% (M) LOD_{blood}: 0.1 ng/ml; 0.05 ng/ml (M) LOD_{hair}: 4 pg/mg; 2 pg/mg (M)	
Quarternary ammonium drugs: Oxyphenonium bromide (antrenyl), (for methylbenactyzine, mepenzolate, pipenzolate, clidinium, neostigminium, valethamate, isopropamide and propantheline only LC and MS data are given)	P	LLE	Hamilton PRP-1 (resin) (150×4.1 mm I.D., 5 μm)	50 m*M* ammonium acetate–ACN (70:30)	TS	MS-MS, SRM	REC: 80% LIN: 1–100 ng/ml	[28]
Xanthins: Theophylline, theobromine, caffeine	P, U	SPE	*Pre-column:* Develosil ODS-HG-15/30 (35×0.3 mm I.D., 15–30 μm) *Analytical column:* Develosil ODS-HG-5 (150×0.3 mm I.D., 5 μm)	*For pre-column*: 17 *M* acetic acid–glycerol–water (0.1:0.5:99.4); 17 *M* acetic acid–glycerol–water (0.5:0.5:99–99:89–0)	FAB	Scan	REC: 82–98% LOD: 2–10 ng/ml LIN: 10–1000 ng/ml	[22]
Miscellaneous drugs:								
Bromisovalum	P, B	SPE	Hypersil ODS (100×2.1 mm I.D., 5 μm), 40°C	MeOH-water (50:50)	PB	SIM	REC:? LOD: 100 ng/ml LIN: 500–5000 ng/ml)	[20]
Colchicine	B, P, U	LLE	Alltech C_{18} (250×1 mm I.D., 5 μm) with a guard column (MGU-80 C_{18} (1×0.8 mm I.D., 5 μm))	2 m*M* ammonium acetate (pH 3.0)–ACN (25:75)	ES	SIM	REC: 86–91% LOD: 0.6 ng/ml LIN: 5–200 ng/ml	[42]
Cyclobenzaprine	P, U	EHY (U), LLE	Keystone BDS C_{18} (50×4.6 mm I.D., 5 μm) with a guard column (BDS C_{18} (20×4.6 mm I.D.))	10 m*M* ammonium acetate/0.1 formic acid–ACN (10:90)	APCI	MS-MS, SRM	REC: 92–104% (P, U) LIN: 0.1–50 ng/ml (P); 10–1000 ng/ml (U)	[63]
Glibenclamide	S	LLE	Nucleosil C_{18} (length and I.D. not reported, 7 μm)	0.05% aqueous acetic acid–MeOH (20:80)	APCI	SIM	REC: 98–100% LOD: 5 ng/ml LIN: 50–1500 ng/ml	[4]
Granisetron+M (hydroxy-)	P (dog)	LC-LC (on-line sample enrichment)	Inertsil C_8 (50×4.6 mm I.D., 5 μm) with two guard columns: Regis Chemicals ISRP GFF II (10×3 mm I.D.)	50 m*M* ammonium acetate (pH 5)–ACN (73:27); initial loading solvent: water–ACN (95:5)	APCI	MS-MS, SRM	REC: 93–105% LIN: 0.05–50 ng/ml (from 80 μl P!)	[64]
Hyoscyamine (L-atropine)	P	LLE	Keystone BDS C_{18} (50×3 mm I.D., 3 μm) with a guard column (BDS C_{18} (10×2 mm I.D., 5 μm))	10 m*M* ammonium acetate–MeOH–ACN (13:32.7:54.3)	ES	MS-MS, SRM	REC: 110% LIN: 0.02–0.5 ng/ml LOQ: 0.05 ng/ml	[45]

(Cont.)

Table 2. Continued

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Ifosfamide+M	S	LLE	Spherisorb C_{18} (20×1.6 mm I.D.)	*For FAB:* MeOH–water–glycerol (50:40:10), 12 μl/min; *For ES:* ACN–water (90:10)	FAB, ES	scan, SIM	REC: 93–99% LOD_{scan}: 35 ng/ml LOD_{SIM}: 1.5 ng/ml LIN: 50–120.000 ng/ml	[5]
Iloperidone+M (dihydro-)	P	SPE	Hypersil C_{18} (100×4.6 mm I.D., 5 μm)	2.5 m*M* ammonium formate (pH 3.5)–ACN (30:70)	ES	SIM for QU; MS-MS, DIS for ID	REC: 82–101%, 73–97 (M) LIN: 0.25–20 ng/ml	[46]
Indinavir	P	LLE	Hypersil BDS C_8 (50×2 mm I.D., 3 μm)	7 m*M* ammonium acetate (pH 4.9)–ACN (60:40)	APCI	MS-MS, SRM	REC: 94% LIN: 1–200 ng/ml	[6]
Mesocarb+M	U (rat)	LLE	Capcell PAK C_{18} (60×4.6 mm I.D., 3 μm)	Gradient: 150 m*M* ammonium acetate–MeOH (80:20–40:60)	TS	ID: scan QU: SIM	REC: ? LOD: ?	[7]
Oxybutynin+M (desethyl-)	P	LLE	Chiralpak AD CSP (100×2.1 mm I.D., 10 μm), 20°C; chiral separation	*n*-Hexane–2-propanol--diethylamine (90:10:0.1), addition of 800 μl/min of 25 m*M* ammonium acetate–2-propanol (25:75) post-column to improve the ionization and to reduce the risk of explosion	ES	MS-MS, SRM	The authors state that validation is in progress	[43]
Oxymetazoline	B (rat)	LLE	Zorbax C_{18} (150×2.1 mm I.D., 5 μm)	5 m*M* ammonium acetate (pH 6.5)–ACN (40:60)	ES	SIM	REC: 68–89% LIN: 0.67–167 ng/g LOQ: 1ng/g	[48]
Pirenzepine	P	LLE	BDS-Hypersil C_{18} (50×4.6 mm I.D., 5 μm) With a guard column (BDS-Hypersil C_{18} (20×4.6 mm I.D., 5 μm))	100 m*M* ammonium acetate–ACN (60:40)	ES	MS-MS, SRM	REC: 78% LIN: 1–100 ng/ml	[49]
Pramipexole	P	LLE	Zorbax SB-CN (150×4.6 mm I.D., 5 μm)	Water–0.1 m*M* ammonium acetate–MeOH (15:5:80)	APCI	MS-MS, SRM	REC: 82% LIN: 0.05–5 ng/ml	[65]
Sumatriptan	P	SPE	Beckman CN (250×4.6 mm I.D., 5 μm)	0.1% aqueous trifluoroacetic acid–MeOH–ACN (58:6:36)	APCI	MS-MS, SRM	REC: 87–113% LIN: 0.5–50 ng/ml	[66]
Terfenadine	P	LLE	TSK gel ODS-80TS (150×2 mm I.D., 5 μm)	10 m*M* ammonium acetate (pH 4)–formic acid (1%)–ACN (2:13:85)	ES	MS-MS, SRM	REC: 125% LIN: 0.2–50 ng/ml	[51]

variable pH and organic modifiers such as methanol (MeOH) or acetonitrile (ACN) used for isocratic or gradient elution. Since basic drugs often give poor peak shapes on reversed-phase columns, minor amounts of an amine such as triethylamine were added [27,43]. Volatile acids, such as formic [8,27,38,51], acetic [4,9,21,61,67] or trifluoroacetic acid [66], were used to improve the ionization. In our experience, there is no general rule for the choice of the acid. It should be tested for every analyte and

Table 3
LC–MS procedures for the identification and/or quantification of poisons and/or their metabolites in biosamples

Compound	Sample	Work-up	Stationary phase	Mobile phase	Interface	Detection mode	Validation data	Ref.
Aconitum alkaloids: Aconitine, mesaconitine, hypaconitine, jesaconitine	B, U	SPE	Inertsil ODS-2 (150×4.6 mm I.D.), 40°C	Tetrahydrofuran–0.3% trifluoroacetic acid–glycerol (19:81:0.3)	FAB	SIM	REC: 94–100% LOD: 50 ng/ml (UV) LIN: 100–10 000 ng/ml (UV)	[12]
Aflatoxins: B_1, B_2, G_1 and G_2	U (dust)	LLE	Supelco C_{18} (250×4.6 mm I.D., 5 μm)	ACN–MeOH–water (20:20:60)	ES	MS-MS, SRM	REC: ? LOD: 0.05 ng/ml LIN: 0.05–0.2 ng/ml	[13]
Amanita toxins: α-, β-Amanitin	U	SPE	Kromasil C_{18} (125×2 mm I.D., 5 μm) with a guard column (Kromasil C_{18} (10×2 mm I.D., 5 μm))	20 m*M* ammonium acetate (pH 5)-MeOH (78:22)	ES	SIM	REC: 63–65%; LOD: 10 ng/ml	[14]
Nicotiana alkaloids: Nicotine+M (cotinine)	P	LLE	BDS-Hypersil C_{18} (100×3 mm I.D., 3 μm) with a guard column (BDS-Hypersil C_{18} (10×2 mm I.D.))	10 m*M* ammonium acetate–MeOH–ACN (15:32:53)	APCI	MS-MS, SRM	*For nicotine*: REC: 106% LIN: 1–50 ng/ml *For cotinine*: REC: 86% LIN: 10–500 ng/ml	[68]
Pesticides: Alachlor+M (mercapturate)	U	LLE (ID), alkaline HY to form diethyl aniline SPE (QU)	Whatman ODS-3 (250×4.6 mm I.D.)	0.1% aqueous acetic acid–MeOH (30:70)	APCI	MS-MS, DIS (ID), SRM (QU)	REC: 75% LOD: ?	[67]
Pesticides: Propoxur, and other organophosphorus or carbamate pesticides	S, U	LLE	Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm)	Gradient: 100 m*M* ammonium acetate–MeOH (35:65–55:45)	APCI	scan, SIM	REC: ? LOD: ?	[69]

chromatographic system. In some papers, post-column addition of ammonium acetate solutions was recommended to improve the ionization in the MS without influencing the chromatographic separation [25,29,43].

2.3. LC–MS procedures

The drugs and poisons are listed in the tables according to drug classes and to the international non-proprietary names (INN) or the common names. If only metabolites were determined '−M' is added to the name. If metabolites were determined additionally, '+M' is given in the *Compound* column. The information concerning quantification, if available, can be found in the *Validation* column. The type of biosample used is given in the *Sample* column (B, blood; P, plasma; S, serum; U, urine, etc.). If samples from animals were studied, the species is given in brackets. The sample preparation is concisely summarized in the *Work-up* column. The principal information on the stationary and mobile phases is given. The flow-rate data were omitted, since in many cases the eluent was split before entering the interface, but the split ratio was not reported. The interface type and the mass spectral detection mode are listed. Since in most papers both mass spectra and chromatograms/retention times are given, these data were not specified in the tables. Validation data, such as recovery (REC), limit of detection (LOD) or linearity (LIN), are summarized for easy evaluation, whether or not a procedure can be useful to solve an actual toxicological case. However, only data recorded in biosamples are given, since data recorded in methanolic solutions are of minor value for toxicological analysis. The limit of quantification (LOQ) is given only if not identical to the lowest linearity value. Since precision of all the reviewed procedures was better than 20% as recommended for analysis in biosamples, these data were omitted in order to save space.

2.3.1. Illicit drugs of abuse

2.3.1.1. *Amphetamines*. Amphetamine derivatives are often misused as central stimulants. Immunoassays are available for amphetamine, methamphetamine and some ring or side chain-modified derivatives, such as the methylenedioxy designer drugs. For confirmation of the immunoassay result or

for differentiation, series of GC–MS procedures were published [101], since amphetamine derivatives are volatile in GC. Nevertheless, three LC–MS procedures for serum or urine analysis have been recently published [23,24,53]. Bogusz et al. [53] described the APCI-LC–MS identification and quantification of amphetamines, including designer drugs, in serum and urine after derivatization. The mass spectra of the 12 drugs tested are quite different allowing their differentiation even if the chromatographic peaks are not completely separated. Due to the use of deuterated internal standards, the validation data promise reliable quantification in the SIM mode. However, Bogusz himself has warned, that in APCI-LC–MS large amounts of analyte may considerably influence the peak areas of their coinjected deuterated analogues used as internal standard [105].

Katagi et al. [24] have described enantioselective TS-LC–MS detection and LC–UV quantification of amphetamines in urine for differentiation between ingestion of illicit and therapeutic drugs. Again, such differentiation can also be performed by GC–MS [101].

Tatsuno et al. [23] have developed a reliable TS-LC–MS procedure for simultaneous determination of different illicit drugs, such as amphetamines, cocaine, morphine and their metabolites, in urine. The mass spectra of the 10 drugs tested are quite different allowing their differentiation with the exception of that of morphine-3- and -6-glucuronide. Both glucuronides can only be differentiated by their retention time.

2.3.1.2. *Cocaine*. As just discussed, cocaine and its main metabolite benzoylecgonine (BZE) can be determined in urine by TS-LC–MS [23]. Sosnoff et al. [54] have described APCI-LC–MS–MS detection of traces of BZE eluted from blood spots of newborns collected on filter paper for epidemiological studies of the prevalence of cocaine abuse in late pregnancy. The LOD was about 2 ng/ml based on a 12-μl sample volume.

2.3.1.3. *Lysergide* (*LSD*). LSD is less often abused than other illicit drugs, but with increasing frequency, at least in Europe. Immunoassays are available for screening (cut-off value, 0.5 ng/ml) and some GC–MS procedures are described for confirmation as reviewed by Kuffer et al. in this *Special volume* [106]. However, the detection of LSD is rather complicated because the concentrations are very low and the molecule is not very volatile. Therefore, since 1996 five papers appeared describing ES-LC–MS(–MS) detection in urine [32–36]. After solid-phase extraction, the detection limits ranged between 0.5 and 0.025 ng/ml, which is sufficient to confirm positive immunoassay results. However, after immunoaffinity extraction (IAE) and LC–MS–MS detection the LOD was 10 times lower (0.0025 ng/ml) [35]. As discussed above, IAE and/or MS–MS detection are powerful procedures for the detection of very low concentrations of analytes in biomatrices.

2.3.1.4. *Opiates*. Heroin is widely abused by drug addicts for euphoriant and anxiolytic effects, while morphine is therapeutically used as a potent analgesic especially in the final stage of cancer diseases. If heroin is not available, addicts often take morphine or other opioid medicaments. For legal reasons, the application of heroin must be differentiated analytically from an intake of other opioids. Therefore, 6-monoacetylmorphine (MAM), the only heroin-specific metabolite, must be detected in body samples. The TS-LC–MS procedure of Tatsuno et al. [23], as well as the recent APCI-LC–MS procedure of Bogusz et al. [55], allow the detection of MAM as well as morphine and its glucuronides. Determination of morphine and its two glucuronides in serum was further described using ES-LC–MS [37,38]. Although only the 6-glucuronide is pharmacologically active and therefore toxicologically relevant, Bogusz et al. [55] have discussed the possible relevance in correlating the blood levels of morphine and its two glucuronides, e.g. in fatal cases for the estimation of the survival time after drug intake.

2.3.2. Therapeutic drugs relevant to forensic or clinical toxicology

2.3.2.1. *Anabolic steroids*. Anabolic steroids are misused in sports and in cattle breeding. Since GC–MS procedures are time-consuming, Barron et al. [19] have developed a fully automated LC–MS procedure for anabolics in urine. Twenty-eight steroids and metabolites could be detected after enzymatic hydrolysis, on-line SPE, reversed-phase chromatography and PB interfacing by electron

impact mass spectrometry. The relatively low sensitivity of PB was sufficient to detect therapeutic concentrations of the tested anabolics even if only small amounts of urine were available, because the whole sample could be injected onto the on-line SPE column. Bowers and Sanaullah [9] described the direct detection of several steroid glucuronide and sulfate conjugates in urine by ES-LC–MS and ES-LC–MS–MS to avoid the enzymatic hydrolysis of the conjugates, which is less reproducible and may lead to artifacts. Considering the measured on-column LODs of the reference solutions the authors conclude that they were readily compatible with testing in urine. In the MS–MS mode spectral differences appeared to be sufficient for differentiation.

2.3.2.2. *Antihypertensive drugs*. Antihypertensive drugs, such as α_1-blockers, β-blockers or calcium channel blockers, may lead to cardiovascular disorders when they are incorrectly taken. For monitoring such patients, procedures published for pharmacokinetic studies can be used. The calcium channel blocker, amlodipine, can very sensitively be quantified in serum by APCI-LC–MS–MS [58], so that only small amounts of blood are needed. Chiral determination in plasma by normal-phase ES-LC–MS–MS was described for the α_1-blocker, doxazosin [43], the β-blocker, sotalol, and the calcium channel blockers, verapamil and nimodipine [47]. Problems arising from normal-phase LC were already discussed in Section 2.2. Reserpine used in humans as antihypertensive drug is also used as a tranquillizer in horses. To monitor plasma levels for both indications, the ES-LC–MS–MS procedure validated for equine plasma can be applied [50].

2.3.2.3. *Benzodiazepines*. Benzodiazepines are widely used and they may lead to addiction or severe intoxication, especially in combination with alcohol. Therefore, screening for benzodiazepines is necessary in clinical and forensic toxicology. While benzodiazepines can easily be screened in urine, e.g. after acid hydrolysis by GC–MS [1,107], GC–MS quantification in blood suffers, e.g. from thermal instability and low volatility of some of the parent compounds. LC–MS provides good precision, specificity and sensitivity. The LOQs ranged between 1 and 2 ng/ml using ES-LC–MS–MS [41] or APCI-LC–MS [56]. Senda et al. [57] have published a very sensitive APCI-LC–MS–MS procedure for the low-dosed triazolam with a LOD of 0.02 ng/ml. Verweij et al. [25] described TS-LS–MS–MS quantification of benzodiazepines, neuroleptics and opioids in whole blood. Unfortunately, this paper is of less value for toxicological analysis since no validation data recorded in biosamples were reported. The conversion of on-column LODs of methanolic solutions to LODs in blood is very daring. Every bioanalyst knows that this is unrealistic.

2.3.2.4. *β_2-Agonists*. β_2-Adrenoceptor agonists (β_2-agonists) are widely used as bronchodilators or tocolytics. Because of their acclaimed anabolic effect at higher doses, they are misused in sports or in livestock production. Doerge et al. [59] have described APCI-LC–MS and LC–MS–MS procedures for sensitive and specific determination of five β_2-agonists in human plasma. Salbutamol could not be determined by LC–MS–SIM because of matrix interferences. Therefore, MS–MS was tested for determination of clenbuterol, fenoterol and salbutamol. The authors stated that lower LODs could be reached by LC–MS–MS, but no detailed data were given.

Determination of β_2-agonists in bovine urine was described using APCI-LC–MS–MS [60,61] or TS-LC–MS–MS [27] with LODs of 0.05 ng/ml. Hagendoorn et al. [27] and Cai and Henion [61] have developed on-line extraction as discussed in detail in Section 2.1. Further methods for β_2-agonists were recently reviewed by Polettini [108].

2.3.2.5. *Cardiac glycosides*. Cardiac glycosides are used in the treatment of congestive heart failure. Because of their narrow margin of therapeutic safety they are routinely monitored in plasma by immunoassay. In some circumstances (e.g. forensic cases), the immunoassay results must be confirmed and/or the glycoside that was actually applied must be identified. Tracqui et al. [2] developed an ES-LC–MS procedure for the detection of the most important cardiac glycosides in plasma. The precision of the method is acceptable. Although the LC–MS LODs were higher than the immunoassay LODs, they should be sufficient for overdose cases. As shown in Fig. 2, the authors were able to identify oleandrin in a plasma extract of a patient who ingested a tea made

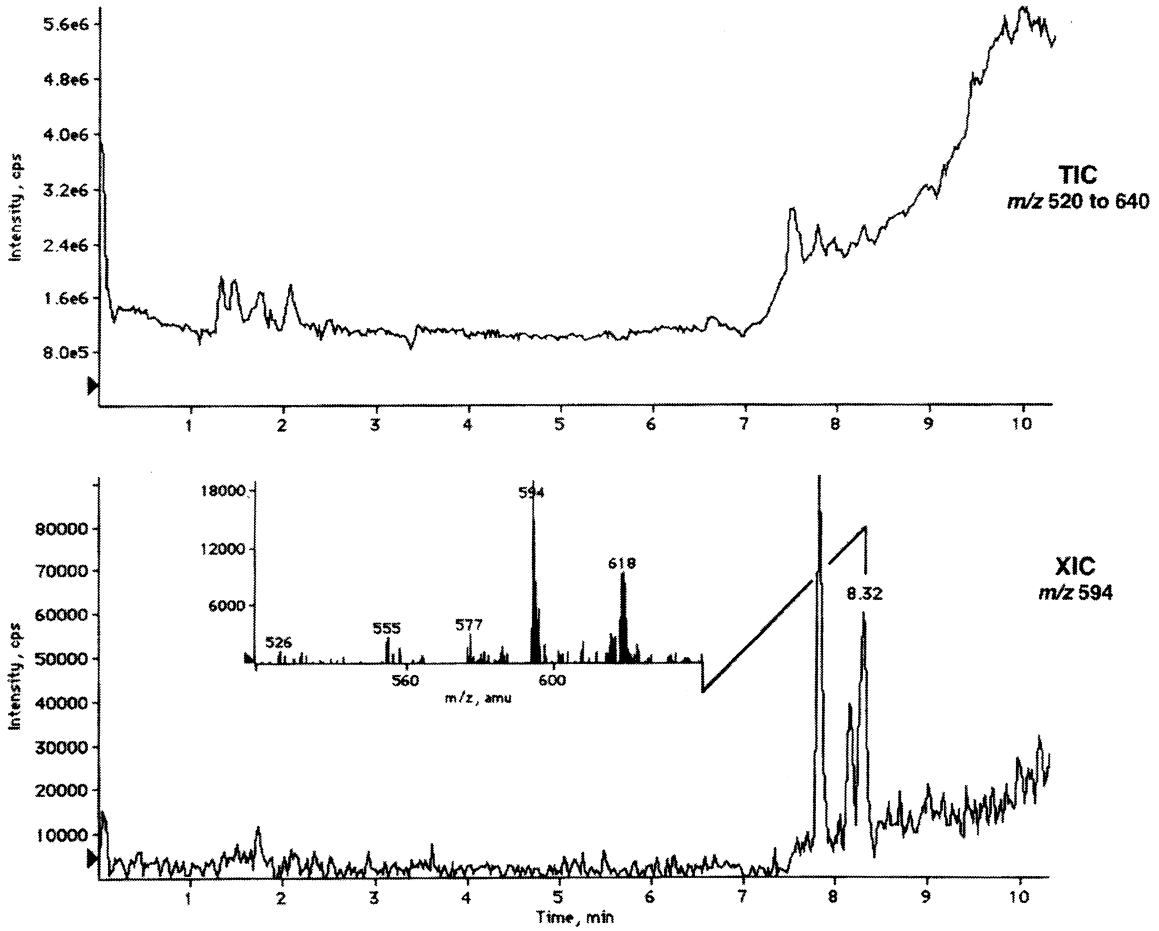

Fig. 2. Total ion chromatogram from a plasma extract of a patient suffering from an oleandrin intoxication (upper part). Reconstructed mass chromatogram of ion m/z 594 (ammonium adduct ion) indicating the presence of oleandrin (lower part). The inset shows the peak underlying ES mass spectrum (taken from Ref. [2]).

from parts of the toxic plant *Nerium oleander*. As the total ion chromatogram indicates, this case could not have been solved without mass spectral information. Using reconstructed mass chromatography with the ammonium adduct ion $[M+NH_4]^+$ ($C_{32}H_{48}O_9$= 576+18=594), the presence of oleandrin was indicated. The peak underlying ES mass spectrum (inset in Fig. 2) was compared for confirmation with the reference spectrum shown in the original paper [2].

2.3.2.6. *Cephem antibiotics*. Kobayashi et al. [3] developed a FAB-LC–MS procedure for the detection of cephem antibiotics. For example, they analyzed fatal forensic cases due to shock caused by intake of such antibiotics. A lot of mass spectral and retention data are published, but no validation data recorded in biosamples were given. Only ceftriaxone and cefazolin have already been successfully determined in serum of patients treated intravenously with the corresponding antibiotic.

2.3.2.7. *Corticosteroids*. Synthetic corticosteroids are illegally used as growth promotors in livestock breeding or as anti-inflammatory drugs for unfit race horses. An APCI-LC–MS–MS procedure was developed to control corticosteroid misuse in bovine

urine [62] and an ES-LC–MS–MS procedure to control triamcinolone doping in equine urine or serum [52].

2.3.2.8. *Immunosuppressants*. For detailed therapeutic drug monitoring of immunosupressant drugs and their metabolites in blood, PB-LC–MS [10] or ES-LC–MS–MS [11] assays were described for tacrolimus and an ES-LC–MS [8] assay for sirolimus. Surprisingly, the linearity range of the PB-LC–MS assay was the same as that of the ES assay combined with MS–MS detection.

2.3.2.9. *Neuroleptics* (*butyrophenones and phenothiazines*). Neuroleptics may lead to severe intoxications, so that fast diagnosis is required. Immunoassays are not available. Neuroleptics can easily be screened by GC–MS [1]. For quantification in blood, LC–MS procedures were described. Hoja et al. [44] described a precise ES assay for haloperidol and its metabolite. Verweij et al. [26] described a TS-LC–MS–MS assay for butyrophenone and bis-fluorophenyl neuroleptics (and opioids), but they have validated the assay in blood only for haloperidol. The second publication of these authors with quite similar contents was critically discussed in Section 2.3.2.3.

2.3.2.10. *Non-steroidal anti-inflammatory drugs* (*NSAIDs*). Non-steroidal anti-inflammatory drugs have been misused in horse doping. For doping control, PB- and ES-LC–MS procedures were described for 40 NSAIDs, but validated only for flunixin and its metabolites [21]. The authors stated that ES was more sensitive than PB. Since NSAIDs form anions or cations, the ES apparatus must of course be switched for the screening into the positive or negative mode.

2.3.2.11. *Opioids*. Opioids, often named narcotics, are potent analgesics especially used in a postoperative state or in the final stage of cancer diseases. Furthermore, they are abused (typically by medical staff) for their euphoriant and anxiolytic effects. Heroin addicts also take opioids, if heroin is not available. Finally, some opioids, such as methadone or buprenorphine, are used for substitution therapy of heroin addicts. For all these reasons, opioids must be analyzed in body samples. Most of the opioids can be analyzed by GC–MS [1]. Low-dosed buprenorphine and its nor metabolite can sensitively be detected in urine by GC–MS [109], but for determination in plasma, hair [40] or whole blood [39], ES-LC–MS has been proved to be the method of choice.

Verweij et al. [26] described a TS-LC–MS–MS assay for some opioids (and neuroleptics), but they have validated the assay in blood only for dextromoramide. The second publication of these authors with quite similar contents was critically discussed in Section 2.3.2.3.

2.3.2.12. *Quaternary ammonium drugs*. For determination of quaternary ammonium drugs in plasma a TS-LC–MS procedure using a resin-based stationary phase was reported [28]. Using such a column, ion-pairing reagents often incompatible with the LC–MS ionization are not necessary. The method covers eight drugs, but it was only validated for antrenyl.

2.3.2.13. *Xanthins*. The xanthin derivatives, theophylline, theobromine and caffeine, are ingredients of foods and beverages. Theophylline and caffeine are also used as therapeutic or doping agents and suicides occur. The xanthins can easily be detected in GC–MS drug screenings. A FAB-LC–MS assay was described for precise quantification in plasma [28]. This procedure using double-focussing MS may be a nice scientific application, but I cannot imagine that anyone would use it routinely.

2.3.2.14. *Miscellaneous drugs*. A number of LC–MS papers appeared dealing with the determination of single therapeutic drugs. As mentioned above they are not discussed in detail. However, procedures concerning single therapeutic drugs relevant to forensic or clinical toxicology are also summarized in Table 2, while those concerning future drugs will only be mentioned in Section 2.3.4.

2.3.3. Poisons

2.3.3.1. *Aconitum alkaloids*. *Aconitum* sp. alkaloids are toxic and may lead to severe intoxications. For diagnosis, a LC–UV procedure was recently published [12]. The authors stated that using FAB-LC–

MS the results could be confirmed and that they could detect 'therapeutic' levels of the alkaloids in blood and urine. Unfortunately, 'therapeutic' levels were not defined and authentic cases were not reported.

2.3.3.2. *Aflatoxins*. Aflatoxins are carcinogenic mycotoxins. They can be ingested via contaminated food or inhaled via dust generated by mould-infected products. Kussak at al. [13] developed an ES-LC–MS–MS procedure for the determination of aflatoxins in urine of feed factory workers. Unfortunately, no data are given on tolerable urine levels and so it cannot be concluded whether this procedure actually can be used for biomonitoring.

2.3.3.3. *Amanita toxins*. After ingestion of the toxic mushrooms of the *Amanita* species (e.g. *Amanita phalloides*) the amatoxins, α- and β-amanitin, may cause severe gastrointestinal disorders and fatal liver damage. Since diagnosis of an intoxication entails a large scale of invasive and expansive therapy, a highly specific detection of amanitins in body fluids is necessary. Determination of amanitins in urine by radioimmunoassay has several disadvantages, while ES-LC–MS was suitable for sensitive and specific detection of amatoxins in urine, as described by Maurer et al. [14]. In the meantime, this procedure has been improved [103,104,110]. In Fig. 3, a cutout of the ES spectra of the amanitins, the structures, the empirical formulae and the molecular masses are shown. As shown in Fig. 4, the analysis time could be shortened using gradient instead of isocratic elution, and the specificity could be improved using six instead of two selected ions. We have been developing an immunoaffinity extraction procedure

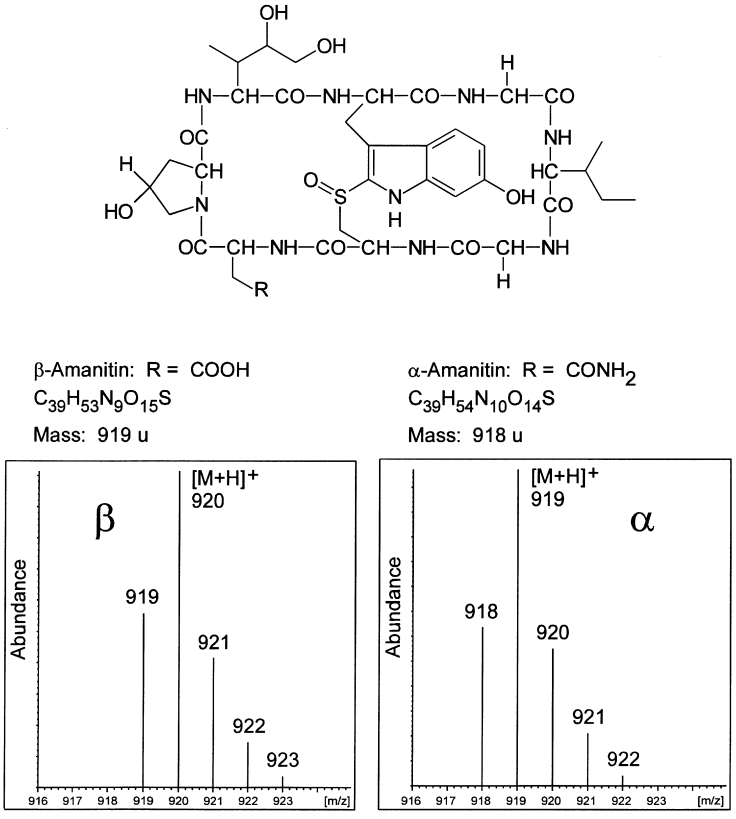

Fig. 3. Cutout of the ES spectra, structures, empirical formulae and molecular masses of α- and β-amanitin.

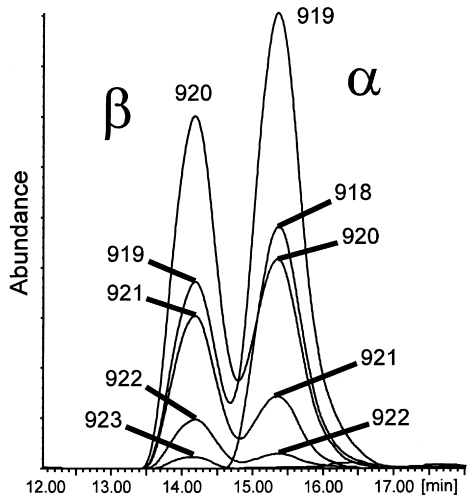

Fig. 4. Selected-ion chromatograms indicating α- and β-amanitin in urine (conc. 100 ng/ml).

to decrease the LOD and to make the procedure also suitable for other biosamples [103,104].

2.3.3.4. *Nicotiana alkaloids*. Ingestion of cigarettes may lead to life-threatening situations especially in children. Nicotine and its metabolites can easily be screened by GC–MS. In smoking-cessation therapies under nicotine substitution (e.g. via patches), nicotine plasma levels should be monitored to counteract the craving for cigarettes. Xu et al. [68] have described a sensitive, precise and fast APCI-LC–MS–MS procedure for quantification of nicotine and its metabolite cotinine in plasma, that was suitable for this purpose and that would also be suitable for clinical or forensic problems.

2.3.3.5. *Pesticides*. Pesticides must be analyzed in body fluids for diagnosis of an intoxication or for biological monitoring of occupationally exposed persons. Driskell et al. [67] applied APCI-LC–MS–MS for identification of metabolites of the herbicide alachlor in urine for biomonitoring purposes. The urine samples used for this study were collected from alachlor-exposed workers, but no data were given on tolerable urine levels.

Itoh et al. [69] described an APCI-LC–MS method for the detection of 30 pesticides, but only propoxur was studied in biosamples (serum). Unfortunately, no validation data recorded in biosamples were reported. On-column LODs of methanolic solutions are of minor value for toxicological analysis.

2.3.4. Applications in studies on pharmacokinetics or metabolism of future drugs

During the development of new drugs, studies on pharmacokinetics, including metabolism, must be performed. HPLC is a versatile method for such studies and is still commonly used. Classical detectors, such as UV, diode array (DAD) or fluorescence detectors, are selective and sensitive enough for many pharmacokinetic applications. However, for determination of low-dosed drugs (especially when they have low UV absorbance or no natural fluorescence), mass spectral detection is needed, especially in the MS–MS mode. Today, many pharmaceutical companies are using LC–MS and LC–MS–MS techniques for series of routine quantifications during pharmacokinetic studies [111]. Using LC–MS–MS, the sample preparation can be simple and fast, the (MS) separation very fast and the detection very specific and sensitive, so that this technique will become the *golden standard* in pharmacokinetics [111]. For the identification of new metabolites, especially of conjugates, LC–MS [93,96,112] or LC–MS–MS [29–31,113–115] are widely used today.

For reasons of space, papers concerning the quantification of future therapeutic drugs as part of pharmacokinetic or metabolic studies are not reviewed in detail, but they are mentioned here. They were not omitted for two reasons: firstly such therapeutic drugs may also occur in future toxicological cases and secondly they may give hints for developing new procedures for similar compounds of toxicological interest. Only ES or APCI procedures are mentioned, since these techniques have become standard. ES-LC–MS quantification was described for the HIV protease inhibitor BMS-186318 [88] and the platelet inhibitor Ro 44-3888 [89]. APCI-LC–MS quantification was described for the antianginal drug ranolazine [90], the antibiotic azithromycin and the epimers of the glucocorticoid budesonide [91]. ES-LC–MS–MS quantification was described for the antiarrhythmic MK-0499 [92], the antimigraine drugs rizatriptan [75] and GR-151004 [76], a col-

lagenase-inhibiting antirheumatic [78], the endothelin receptor antagonist bosetan [77], the HIV protease inhibitor saquinavir [79], mevinolinic acid, a metabolite of the antihypercholesterolemic lovastatin [80], and the muscarinic agent LY-297802 [81]. APCI-LC–MS–MS quantification was described for the the antihypercholesterolemic 447C88 [82], the antifungal fenticonazole [83,84], the M_3 blocker darifenacin [85], and the muscarinic agent xanomeline [86].

In drug metabolism, LC–MS or LC–MS–MS provide new aspects in the identification and quantification of phase II metabolites, such as glucuronide [9,23,29–31,37,38,55,72,93–98], glycine [73] or especially sulfate conjugates [9,30,93].

3. Conclusions and perspectives

In the last 2 years an increasing number of papers appeared concerning LC–MS identification and/or quantification of drugs, poisons and/or their metabolites in biosamples relevant to forensic and clinical toxicology. Sample preparation was performed either by classical off-line liquid–liquid or solid-phase extraction, or by modern on-line extraction on reversed-phase or immunoaffinity columns. The chromatographic systems used were rather similar and classical: isocratic or gradient elution with C_{18} columns and mixtures of volatile buffers, such as ammonium acetate, with variable pH and organic modifiers, such as methanol or acetonitrile. Pre- or post-column addition of volatile acids, such as formic, acetic or trifluoroacetic acid, improved the ionization of the analyte. Different types of LC–MS interfaces, mass analyzers and detection modes were used. However, the more recent papers on analytical toxicology focus on ES and APCI in combination with MS or MS–MS detection. It can be concluded, that the two relatively robust atmospheric-pressure ionization techniques ES and APCI will become the *golden standard* in LC–MS. This review documents that LC–MS has become a powerful tool in analytical toxicological science and practice. However, at least at present, LC–MS is a complementary but not an alternative technique to GC–MS, still the *golden standard* in analytical toxicology. For example, LC–MS is not suitable for a broad screening for unknown drugs or poisons, because neither its separation power nor the mass spectral information are comparable to that of capillary GC and electron impact full-scan MS. However, LC–MS will 'play the first violin' in analyzing peptide or nucleotide drugs, interesting drugs of the future.

4. List of abbreviations

ACN	acetonitrile
AM	amphetamine
APCI	atmospheric-pressure chemical ionization
API	atmospheric-pressure ionization
B	blood
CID	collision-induced dissociation
CNLS	constant neutral-loss scanning
DAD	diode array detection
DIS	daughter-ion scanning
EHY	enzymatic hydrolysis for cleavage of conjugates
ES	(atmospheric-pressure) electrospray ionization
FAB	fast atom bombardment ionization
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
IA(E)	immunoaffinity (extraction)
ID	identification
INN	international non-proprietary name (WHO)
LC	liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
LIN	linearity
LLE	liquid–liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LSD	lysergic acid diethylamide (INN, lysergide)
M	mol/l
M	metabolite
MA	methamphetamine
MAM	6-monoacetylmorphine
MDA	methylenedioxyamphetamine
MDE	methylenedioxyethylamphetamine
MDMA	methylenedioxymethamphetamine
MeOH	methanol
M3G	morphine-3-glucuronide

M6G	morphine-6-glucuronide
MRM	multiple-reaction monitoring=SRM with more than one ion
MS-MS	tandem mass spectrometry
P	plasma
PB	particle beam ionization
QU	quantification
REC	recovery
S	serum
SIM	selected-ion monitoring
SPE	solid-phase extraction
SRM	selected-reaction monitoring
th. conc.	therapeutic concentrations detectable
t_R	retention time
TS	thermospray ionization
U	Urine
UV	ultraviolet spectrophotometry

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