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Review

# Liquid chromatography–mass spectrometry: potential in forensic and clinical toxicology

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**Abstract**

A relatively limited number of papers concerning applications of liquid chromatography–mass spectrometry (LC–MS) to forensic or clinical toxicology, or analytical methods directly applicable to these topics have been published so far, but their number have greatly increased in the past two years, probably due to technical improvements and to a decrease in the price of such instruments. After a brief presentation and exemplary applications of the interfaces and/or sources proposed in the past for coupling HPLC to mass spectrometry (direct liquid inlet, moving belt, fast atom bombardment and thermospray interfaces), this paper describes electrospray-type and atmospheric pressure chemical ionisation interfaces and their most recent applications in forensic or clinical toxicology. In a third section, the different LC–MS solutions proposed for typical applications in human toxicology, such as the determination of morphine metabolites, LSD and its metabolites and corticosteroids in blood or urine, are reviewed in detail in order to highlight the strengths and weaknesses of each ionisation device and/or analytical method. The last section envisages the new analytical fields opened up by LC–MS in toxicology, regarding mainly peptides, proteins and large molecules, as well as the possible use of LC–MS as a complement to GC–MS for ''general unknown'' screenings; it also deals with the perspectives concerning technical improvements in ionisation interfaces/sources or mass spectrometers, as well as in sample preparation and liquid chromatography techniques applied to this type of coupling. Though LC–MS is still a relatively new technique in toxicology, on taking into consideration its success so far and owing to the simplification of instruments and concept handling thanks to user-friendly software, it is the authors' opinion that it will become a major success in analytical toxicology in the next few years. 

*Keywords:* Reviews; Corticosteroids; LSD; Morphine

## Contents



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## 1. Introduction

Though drug analysis in biological matrices using LC–MS dates back to the early eighties [1,2], only a relatively limited number of papers really applicable to forensic or clinical toxicology have been published up till now. Indeed, the previous reviews of the subject, including ours [3–7], included a number of papers dealing with drugs or toxicants which were supposed to be concerned with human toxicology, but were dedicated in fact to pharmacokinetic, metabolic, environmental or biochemical studies. A reason why this hyphenated technology has seldom been used in analytical toxicology is probably because few laboratories could afford it; it was also not obvious, until recently, which type of LC–MS interface would bring about the greatest improvement (if any) over GC–MS in a toxicology laboratory. This situation has clearly evolved over the last two years, as the increasing number of studies using LC–MS show.

Forensic and clinical toxicology are very demanding applications of analytical chemistry: the toxicologist needs ideally to detect and identify any xenobiotic possibly responsible for an intoxication and to quantitate it, even at very low levels. These requirements of ubiquity, sensibility, specificity and linearity have more or less been met in the past, by the association of various immunochemical, radioimmunological and physicochemical techniques: the first two technologies are limited to a small number of therapeutic drugs or drugs of abuse, the analysis of which is frequent enough to be commercially profitable; separation techniques have long been used by the analytical toxicologist who is used to developing his own analytical techniques like a craftsman (and sometimes an artist), depending on the equipment at his disposal and the cases he has to face. Whatever the applications, he generally seeks for sensitive and specific techniques for the detection of the largest possible span of toxicants (''screening'' procedure) and for their quantitation. Over the last two decades, mass spectrometry coupled to gas chromatography (GC–MS) has offered such performances, but it is not applicable to polar, thermolabile or high mass molecules. Moreover, sample preparation for GC–MS is often time-consuming: biological samples often require tedious extraction procedures, while mildly polar compounds generally need to be derivatised to be amenable to GC. High-performance liquid chromatography (HPLC) is more ubiquitous than GC but, until recently, was hampered

by the detectors used, which evolved from single-wavelength UV, fluorimetric and electrochemical detectors to more specific ones, such as diode array UV detectors. Nevertheless, none of these possesses the same specificity and above all the same selectivity as mass spectrometers, so despite careful purification, interference may occur and induce false positive results or impede proper identification and quantitation of the compounds of interest. For these reasons, toxicologists have been waiting for reliable and efficient LC–MS coupling systems. The first tentative solution of LC–MS coupling was published in 1974, by Arpino and co-workers [8,9]. Since then, about 25 different coupling devices (interfaces or interfaces/ionisation sources) have been described in the literature [10], of which at least seven have had some success: the direct liquid introduction (DLI), moving belt (MB), particle beam (PB), continuous-flow fast atom bombardment (CF-FAB) and frit-FAB, thermospray (TS), electrospray (ES) and atmospheric pressure chemical ionisation (APCI) interfaces.

In this paper, the principle of the first five, ''historical'' LC–MS interfaces are briefly presented and a few illustrating examples given. The main focus is on the ''atmospheric pressure interfaces'', the use of which has considerably increased following recent technical improvements. Their advantages and drawbacks are illustrated with the most recent applications. Typical applications of LC–MS in forensic or clinical toxicology, including sports drug testing, are reviewed in more detail in Section 4 in order to point out the progress made following technical evolution as well as growing expertise. In the last part of this paper, we expose the future potential of these techniques, or of LC–MS techniques still in development, for the detection and quantitation of drugs of abuse, therapeutic drugs and other organic toxicants in human biofluids or solid tissues.

## 2. Principle of the "historical" LC–MS interfaces and applications in human toxicology

The LC–MS interface has always been the weak link in the coupling, as it is expected to solve two major problems: eliminate, before or into the evacuated sections of the mass spectrometer, a large volume of gases and vapours produced from the HPLC mobile phase and transform the molecules in solution in the mobile phase into ions in the gas phase, without thermal degradation. The different ionisation processes used by these interfaces depend on the volatility of the targeted analytes (Fig. 1). Volatile molecules can be converted into the gaseous state, either under vacuum where they can be ionised by electron impact (as with GC–MS, MB, PB) or chemical ionisation (GC–MS, DLI, MB, PB), or at atmospheric pressure where chemical ionisation can be used (APCI). Non-volatile molecules can be ionised: (i) by providing a burst of high energy, using laser desorption (compatible with MB), fast atom bombardment (FAB, compatible with MB, PB, CF-FAB), accelerated ions (secondary ions mass spectrometry, compatible with MB, PB), or naturally emitted particles, e.g., by $^{252}$Cf (plasma desorption, PD); (ii) or by using field desorption, under vacuum (proper field desorption, FD) or in the gas phase after nebulisation (TS, ES). Reciprocally, the different ionisation modes can accommodate different polarity ranges, the widest ones being covered by APCI and ES which, when associated in the same instrument, would theoretically be perfectly complementary to electron impact (Fig. 2).

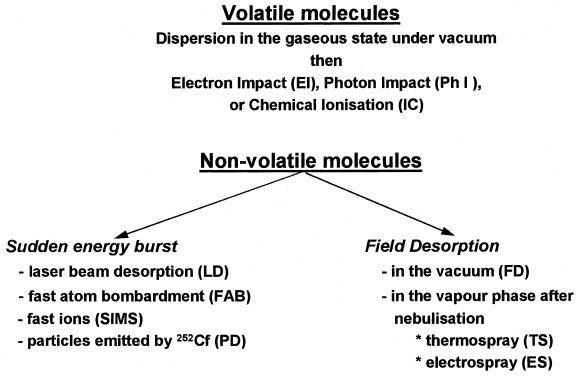


Fig. 1. Available mass spectrometric ionisation processes, as a function of the molecule volatility.

### 2.1. Direct liquid inlet (DLI)

The direct liquid inlet, or direct liquid introduction interface/ionisation source (Fig. 3) is a technique

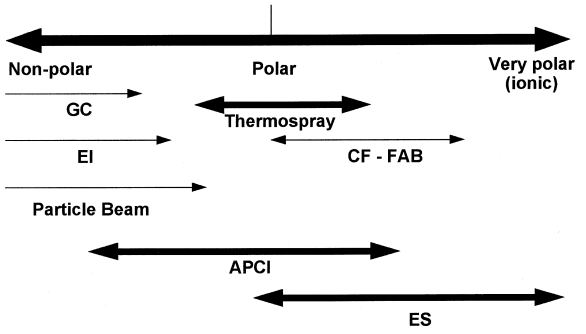


Fig. 2. Polarity range of the different LC–MS interfaces or sources, compared to that of gas chromatography and electron impact.

associated with the initial works of Arpino et al. [9] in 1974, which evolved until approximately 1985 [11]. The chromatographic column was connected to a direct introduction probe, with a 2–5 μm diameter diaphragm at the end [12,13]. The mobile phase was nebulised through the diaphragm into an evacuated, heated desolvation chamber, in which the droplets were desolvated [14,15]. Chemical ionisation (CI) of the compounds of interest was then performed, using the solvent molecules as reactant gas. Finally, ions were directed towards the mass spectrometer by an electric potential. This interface was relatively simple, and heating was moderate, generally respecting thermolabile compounds. On the other hand, the only ionisation process possible, i.e., CI with the solvent molecules was of low efficiency; the acceptable liquid input was low (10 to 50 μl/min), necessitating a post-column splitting (i.e., a loss in sensitivity, as DLI is sensitive to mass-flow) or a low chromatographic flow-rate; the nebulisation was poorly reproducible, above all when elution gradients were used, and the diaphragm suffered from frequent clogging. Very few applications readily transposable

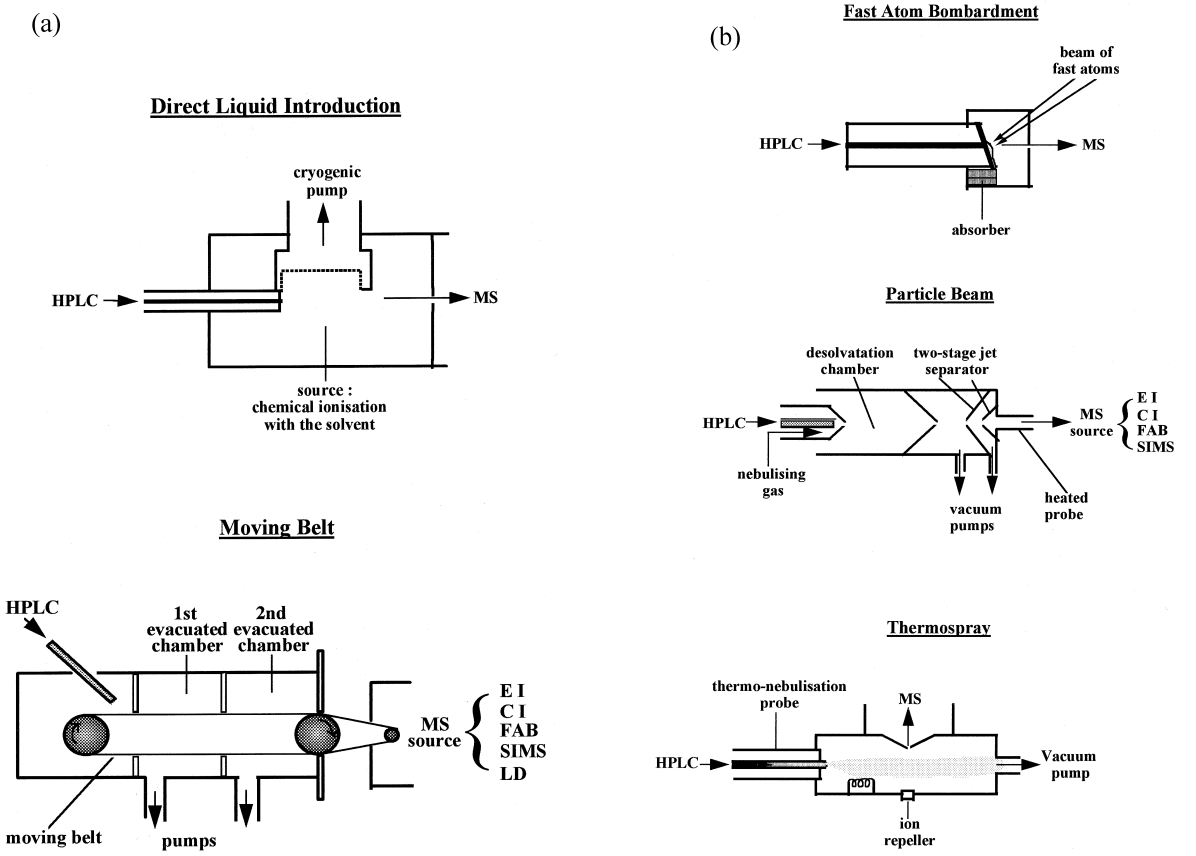


Fig. 3. Schematic diagrams of the ''historical'' LC–MS interfaces or ionisation sources (see Section 7).

to clinical or forensic toxicology were published. Eckers et al. [16] reported a micro-LC–DLI-MS technique that allowed the determination of three thiazide diuretics and three steroid drugs in equine urine, with limits of detection (LODs) in the low ng range in the positive ion mode; they demonstrated that this sensibility could be enhanced using negative ion detection. The use of flow-switching HPLC coupled to DLI-MS for the determination of LSD in human urine [17] will be detailed in Section 4.2.

### 2.2. Moving belt (MB)

The moving belt, or moving wire, was an interface introduced in 1974 [18] which did not evolve after 1988. The chromatographic effluent was deposited on a continuous ribbon or inox wire, passed through two successive differentially evacuated chambers ($10^{-1}$ and $10^{-3}$ Torr; 1 Torr=133.322 Pa) where the solvents were evaporated, then into the high vacuum of the source where the non-volatile compounds were vaporised by flash heating and ionised by EI (allowing library searching) or CI, or directly ionised on the ribbon by SIMS, FAB or laser (Fig. 3). This diversity of ionisation processes was a strength of this interface. The ribbon was further cleaned by heating on its way back to the deposition chamber. This interface could accommodate rather high chromatographic flow-rates (up to 1 ml/min) as well as gradients, but could not accept non-volatile buffers and was hampered by memory effects on the belt (above all when using highly aqueous solvents) and by the complexity of the transport mechanism. Moreover, the loss of volatile chemicals and above all the decomposition of thermally labile and low volatility compounds during the desolvatation step drastically limited its fields of interest, when compared to GC–MS. Limits of detection, down to the pg level at best, were very dependent on the molecular structure of the compounds analysed [18–22].

For the investigation of an attempted suicide in which the patient had taken about 200 mg bromazepam, 2.5 g clopenthixol and an unspecified amount of reserpine, Tas et al. in 1986 [23] used a MB interface to couple a liquid chromatograph (300×4 mm I.D. column) to a double focusing magnetic sector mass spectrometer equipped with an EI/CI source. The limits of detection obtained were about 100 pg on-column for the three drugs using low-resolution detection, and the concentrations found in the patient's serum were 5.82 mg/l, 0.9 mg/l and 1 μg/l for bromazepam, clopenthixol and reserpine, respectively. Though it is not possible to extrapolate a detection limit obtained with pure solutions to an LOD in a complex matrix such as serum, the sensitivity of this technique seems greater for bromazepam and clopenthixol than that generally obtained with usual methods, such as GC–MS or HPLC–DAD. However, all are sensitive enough for the diagnosis of intoxication.

### 2.3. Fast atom bombardment (FAB)

The FAB interface/ionisation source was first used in combination with an MB in 1983 [24], then became a proper interface itself under the form of frit-FAB in 1985 [25] and of continuous flow-FAB in 1986 [26]. With the frit-FAB interface, the mobile phase at a maximum flow-rate of 5–10 μl/min, containing 10% glycerol, was introduced into the high vacuum of the mass spectrometer by a fused-silica capillary (50–100 μm I.D.) with a frit on the end. The solvents were evaporated, leaving the compounds in solution in glycerol, in which they were ionised by fast atom bombardment and from which they were extracted by a high electric potential. In the CF-FAB interface, the chromatographic effluent flowed continuously towards a wick of filter paper in the lower part of the source, and the liquid film thus formed was bombarded by fast atoms. These interfaces were convenient for low-volatility or thermally labile molecules, but the very low admissible flow-rate was limiting and ionisation efficiency very dependent on the experimental conditions.

Sato et al. [27] reviewed the ''forensic'' applications of LC–FAB-MS, a good part of which were in fact papers dealing with therapeutic drugs tested in in vitro or animal experiments. More interestingly, the authors summarised their own previous works, performed using capillary HPLC coupled to a double-focusing mass spectrometer via a frit-FAB interface and applied to benzodiazepines, phenothiazines, cephalosporins and quinolones. Triazolam, chlordiazepoxide and its metabolites desmethylchlordiazepoxide, desmethyldiazepam, oxazepam and

demoxepam, are thermolabile benzodiazepines which were identified using full mass spectra and detected down to 2–10 ng/ml in clinical serum samples, after solid-phase extraction (SPE). Seventeen phenothiazines were detected as pure solutions down to 0.25–10 ng on-column, of which four (chlorpormazine, levomepromazine, promethazine and propericiazine) were identified in clinical serum samples; the detection limits were about one order higher for cephalosporins and quinolones in human serum. Another team reported a technique for the simultaneous determination of theophylline, theobromine and caffeine in human plasma and urine, using column-switching capillary HPLC also coupled to frit-FAB double-focusing sector mass spectrometry and yielding detection limits of 5 ng/ml [28]. These examples highlight the relatively good sensitivity of the systems used, thanks to very efficient chromatographic and mass spectrometric conditions which partially compensated for the low ionisation and ion-transmission efficiency of the FAB interfaces.

### *2.4. Particle beam (PB)*

The particle beam interface was developed in 1984 [29] and improved in 1988 [30]. It is probably still in use in a number of laboratories. The chromatographic mobile phase (0.1–2 ml/min) is nebulised at atmospheric pressure using helium, in a slightly heated desolvatation chamber. The solvent gases are eliminated in a two-stage momentum separator, comprised of two skimmers separating two differentially evacuated chambers, which only the high-mass molecules can cross without being deviated. The molecules are then transferred into the ionisation source by a heated probe, where those which are still in a condensed state can be vaporised. Several ionisation modes are compatible with this interface: EI (allowing library searching) and CI were in routine use, but FAB, LD and SIMS were also employed [31,32]. Compounds can be detected at the ng level, but the sensitivity depends on the volatility, as the jet separator is discriminating for both the highly volatile [33] and the non-volatile compounds. Non-volatile buffers, inducing skimmer clogging, should be avoided. The main advantages of this interface are its compatibility with EI and its suitability for thermolabile compounds when heating is adjusted. One of the most recently reported forensic applications concerned the determination of amitriptyline and nortriptyline in plasma using a triple liquid–liquid extraction and HPLC coupled to both diode array-UV and PB-MS detectors [34]. The authors compared EI, negative ion chemical ionisation (NICI) and positive ion chemical ionisation (PICI), using a quadrupolar mass spectrometer: in the EI mode, the predominating ions were of low mass (58 and 44 u), leading to a low signal-to-noise ratio, due to high chemical background; in the NICI mode, the major $m/z$ ratios were found at 276 and 262 u but were of low abundance; finally, PICI yielded intense $[M+H]^+$ ions at 278 and 264 u. At this last setting, LODs of 2 and 5 ng/ml were obtained in the selected ion monitoring (SIM) mode for amitriptyline and its metabolite, respectively, compared to 5 and 10 ng/ml for the UV detector. The method was linear from 10 to 1000 ng/ml with both detectors, but when applied to a forensic whole blood sample, quantitation was performed using the UV detector, while PB-MS recording was only used to verify the identity of the chromatographic peaks. Of course, a number of teams, including ours [35], have reported simpler and less expensive GC–MS techniques for the determination of these two antidepressants with similar quantitation limits (i.e., much lower than the therapeutic range) and often with quite similar sample preparation methods. This example is typical of the limitations of this interface, which of course allows the comparison of full mass spectra to those of commercially available libraries of EI spectra, but is limited to relatively non-polar compounds and is handicapped by low transmission of molecules from the liquid phase to the high vacuum of the ionisation source.

### *2.5. Thermospray (TS)*

Thermospray is an interface and ionisation source, developed as early as 1978 [36] and greatly improved over the next seven years [37]. The mobile phase (up to 2 ml) passes through a heated capillary in which it is almost completely vaporised and the molecules thermally ionised. The expanding gases induce a nebulisation of the residual liquid and the molecules are further desorbed in an evacuated

desolvatation chamber. A second ionisation step by electron impact produced in the gas phase by a filament (''filament on'' mode) or by a discharge electrode is frequently added at this stage. After complete desolvatation, the analyte ions are transferred to the MS by means of a repeller electrode which can be used to accelerate the ions produced and induce their fragmentation upon collision with residual solvent molecules. The main advantage of this technique is its compatibility with high flow-rates and non-volatile and even moderately thermolabile molecules. The limitation in the composition of the mobile phase, the poor reproducibility of the thermally-induced ionisation and the risks of pyrolysis are its major drawbacks. In 1999, it seems that TS has been superseded by APCI, as it is no longer available from manufacturers. A non-negligible number of papers have reported the use of TS in forensic or clinical toxicology and though this number has drastically decreased during the last two years, some teams are still doing a good job with this interface.

An interesting application of thermospray concerned glucuroconjugated metabolites of drugs, which could be determined directly, without hydrolysis, thanks to the compatibility of thermospray with polar compounds. Byrd et al. [38] reported a method for the direct determination of cotinine-*N*-glucuronide in human urine, using methyl-$d_3$-cotinine-*N*-glucuronide as I.S. The LOD was indirectly evaluated as 0.70 nmol/ml and the method was linear up to 30 nmol/ml. This method compared favourably with the indirect determination of this metabolite following enzymatic hydrolysis, but it was not sensitive enough to detect cotinine-*N*-glucuronide in the urine sample of one smoker out of four.

The exemplary work of Tatsuno et al. [39], who proposed the simultaneous determination of amphetamines, opiates, cocaine and metabolites in urine using LC–TS-MS, has recently been reviewed by Maurer [7]. The LODs reached were of the same order as those obtained using GC–MS for the determination of single families of drugs. This, and another LC–TS-MS technique [40] including the analysis of morphine metabolites, will be reviewed in detail in Section 4, as well as several other LC–TS-MS methods dealing with corticosteroids.

## 3. Principle of atmospheric pressure interfaces and recent applications in human toxicology

The first description of atmospheric pressure ionisation (API) sources for mass spectrometry was made in 1958, by Knewstubb and Sugden [41]. Further improvements arrived with the works of Dole et al. [42] and Horning et al. [43], giving rise to the first commercial system in 1974. Nevertheless, it was not until the late 1980s that these coupling techniques really began to spread to the various fields of science, while the first applications in toxicology appeared in the early 1990s. API interfaces are mainly comprised of different versions of electrospray and atmospheric pressure chemical ionisation interfaces/sources. The common features of these interfaces can be described as a combination of a liquid introduction device, an atmospheric pressure ion source, an ion sampling aperture, an interface between atmospheric pressure and high vacuum and an optical system, all of which meet different specifications, depending on the different manufacturers. For example, the transition from atmospheric pressure to high vacuum requires from one to four pumping stages, depending on the instrument. For a detailed history of API interfaces and detailed review of instrumental settings, see Niessen [44].

### *3.1. Electrospray (ES) and pneumatically-assisted electrospray (ionspray) interfaces*

#### *3.1.1. Principle*

In the real electrospray sources, the liquid flow is nebulised exclusively by means of an intense electric field between the tip of the capillary inlet and the aperture, which is only compatible with very low flow-rates (1–10 μl/min) [45]. In 1987, Bruins et al. [46] described a pneumatically-assisted electrospray interface, which they called ''ionspray'', that accepted flow-rates up to 200 μl/min with no loss in sensitivity, while most of the other high flow-rates solutions proposed previously resulted in an increase in the detection limits [44]. Ionspray (used in Sciex LC–MS instruments) combines the principles of ion evaporation [47] and electrospray, which revealed similar ionisation mechanisms (Fig. 4). Since the advent of ionspray, similar solutions have been produced by different firms under various names,

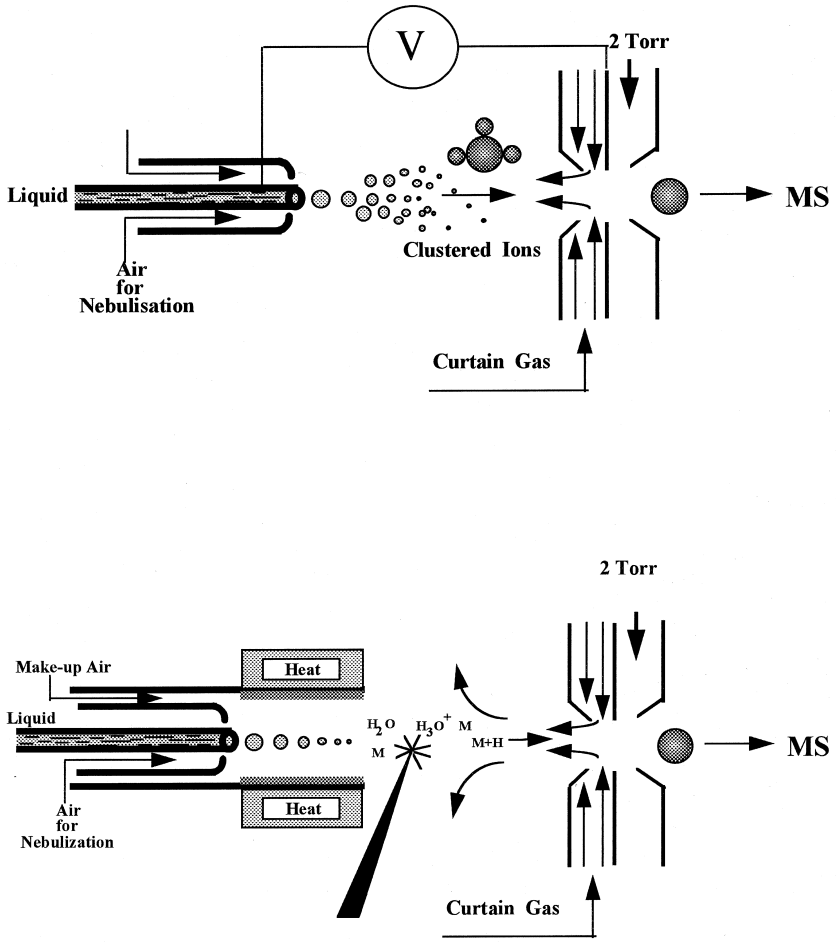


Fig. 4. Schematic diagrams of the ''atmospheric pressure ionisation'' interfaces: (a) pneumatically assisted electrospray and (b) atmospheric pressure chemical ionisation (APCI) sources.

e.g., high-flow or megaflow electrospray. Further steps in accommodating high flow-rates have consisted of: an additional drying of the spray by an orthogonal, heated nitrogen beam, used in the ''turbo-ionspray'' interface (Sciex); an orthogonal positioning of the electrospray with respect to the ion sampling orifice, using different technical solutions (Hewlett-Packard orthogonal electrospray system or Micromass Z-spray electrospray source).

In the spray, the droplet size rapidly decreases, owing to a first desolvatation in the atmospheric pressure chamber, assisted in some instruments by a countercurrent of pure nitrogen. Therefore, the electrical field at the droplet surface increases until it explodes, giving smaller droplets that will undergo the same fate. The ions are extracted from the spray, which is frequently off-axis with respect to the orifice, by the electrical tension directed towards the intermediate or low-pressure chamber, where the residual ion-solvent clusters are broken down, due to an electrical acceleration. If this acceleration is further increased, dissociation of ions can occur through collision with the residual solvent and gas molecules, and the fragments produced by this collision-induced dissociation (CID) can be used as confirmation ions for quantitation methods in the SIM mode, or for structure elucidation. Indeed, in-source CID gives the same fragments as those produced by conventional CID in the collision cell of an MS–MS instrument. The former was even used in

addition to the latter in order to further elucidate the structure of compounds, mimicking an $MS^3$ instrument [48]. Nevertheless, contrary to CID in the collision cell of an MS–MS instrument where a precursor ion is selected before fragmentation, in-source CID first needs a thorough separation of compounds, as the fragmentation efficiency is dependent on the ion density in the transition zone [44]. This interface allows the analysis of moderately non-polar to highly polar compounds, even thermally labile or with a high molecular mass. In particular, it gives multi-charged ions from peptides and proteins, making it possible to analyse them using single quadrupole instruments, as the $m/z$ ratios resulting from molecules up to molecular mass 30 000–200 000 generally fall into their usual $m/z$ range. Electrospray is only surpassed in this application by the much more expensive matrix-assisted laser desorption ionisation (MALDI) source. Other advantages of ES is that it is compatible with chromatographic gradients and can be coupled with capillary electrophoresis, through a different introduction device. Its main drawbacks are a non-negligible chemical noise in the low mass range (though lower than with TS) and a low fragmentation, but which can be enhanced afterwards by CID.

### *3.1.2. Recent applications in human toxicology*

As we have previously reviewed the applications of LC–MS in forensic and clinical toxicology up to the beginning of 1995 [6] and Maurer has recently reviewed those in the period from January 1995 to April 1997 [7], we will mainly consider papers published between May 1997 and November 1998. Toxicological applications frequently found in the literature, for which different and successive LC–MS approaches can be compared, will be addressed in Section 4.

One particular interest of electrospray interfaces is their suitability for polar compounds, including conjugated metabolites of therapeutic or abused drugs. Such applications, of which two recently reported examples will be reviewed, are becoming increasingly frequent in the literature. The first example concerns the (unusual) analysis of ethylenediaminotetraacetic acid (EDTA) in dried bloodstains, to differentiate between genuine bloodstains and evidence tampering using blood stored in EDTA-containing tubes, in criminal cases [49]. A portion of the bloodstain was incubated with 25 μl water, EDTA was filtered out using a centrifugal filter commonly used for the determination of the free fraction of therapeutic drugs, then the filtrate was injected into a reversed-phase column. The mobile phase was a mixture of acetonitrile and diluted ammonium hydroxide. A pneumatically-assisted ES source was used in the positive then in the negative ion mode, and a triple-stage quadrupole MS used successively as a single quadrupole in the scan mode (limited range), then in the SRM mode. In the scan mode, adducts of EDTA with several metals and elements (Na, $Al^{III}$, $Fe^{III}$) were observed as both positive and negative ions. No efficient in-source CID was performed to break these adducts, nor to obtain confirmatory fragments, with the instrument used. Therefore, MS–MS in the SRM mode was used to obtain structural information and confirmation ions. In the positive ion mode, the intensity was about 80-times higher than in the negative mode, and three product ions were obtained compared to one. Of course, no real LOD or LOQ could be determined, as the exact quantity of blood extracted is unknown, but as little as 1 μl of blood containing EDTA as a preservative gave a satisfactory signal. This method proved to be sensitive and specific for confirming the presence or absence of EDTA in 42 samples. The second recent example of polar compound determination concerned sulphate and glucuronide conjugates of testosterone and epitestosterone, which were determined in human urine using microbore (1 mm I.D.) HPLC coupled to MS–MS via an ionspray interface [50]. Using deuterated testosterone-glucuronide and deuterated epitestosterone-sulphate as I.S., solid-phase extraction and SRM detection, limits of detection ''in the low nanomolar range'' were achieved, eliciting the detection of steroid conjugates, except that of testosterone-sulphate, in a normal male urine. LOQs were between 50 and 200 nmol/l, i.e., between 18 and 100 ng/ml. The LODs were of the same order as those reported in a previous study [51], when using more or less the same experimental conditions, but not as good as when using a 300 μm I.D. packed capillary HPLC column (LOD=25 pg for epitestosterone glucuronide). Nevertheless, this method was claimed to be

convenient for a direct and precise determination of the testosterone/epitestosterone metabolite ratio in doping control. However, this approach will probably need further physiological validation before it can replace the classical testosterone/epitestosterone ratio obtained by GC–MS after enzymatic hydrolysis of glucuroconjugated metabolites [51].

At the opposite end of the polarity range, but still in the field of doping control, Shackleton et al. [52] described an attempt to identify nine 17β-fatty acid esters of testosterone in plasma following administration of these drugs, using direct infusion-ES-MS for the underivatised esters and microbore HPLC–ES-MS for derivatised compounds. Using the latter conditions and SIM of the pseudomolecular ion and of two fragments produced by in-source CID for each compound, all nine could be separated and detected specifically; in addition, testosterone enanthate and testosterone undecanoate could be detected in human plasma after oral or intramuscular administration, but with poor sensitivity. Indeed, APCI is theoretically more convenient for non-polar compounds. Moody et al. [53] compared GC–MS in the PICI mode and LC–ES-MS–MS, for the determination of buprenorphine in human plasma, using buprenorphine-$d_4$ as I.S. After a single step liquid–liquid extraction from 1 ml plasma, buprenorphine was separated on a $C_8$ bonded stationary phase using an isocratic mobile phase delivered at 0.25 ml/min, and ionised in a pneumatically assisted electrospray source. The pseudomolecular ions selected in the first quadrupole were submitted to collision with argon in the collision cell, then these very same $[M+H^+]$ ions were selected in the second analyser. Under these conditions, the LOD achieved for buprenorphine was 0.1 ng/ml, better than the LOD of 0.5 ng/ml reached with GC–PICI-MS using a 2 ml sample volume; even the intra- and inter-assay precision and accuracy obtained by LC–ES-MS–MS were better than with GC–PICI-MS. However, we have previously described a LC–ES-MS technique for the simultaneous determination of buprenorphine and its desalkyl-metabolite norbuprenorphine in urine, serum [54] and, with an additional protein precipitation step, in whole blood [55], yielding better LODs (0.05 ng/ml) than this LC–ES-MS–MS technique, with similar precision and accuracy. This apparent absence of superiority of MS–MS over single-stage MS might be explained in at least two non-exclusive ways. First, the internal diameter of the chromatographic column used by Moody et al., though not indicated in their paper, is probably wider than the one we used (1 mm I.D.), as suggested by their higher flow-rate (250 μl/min versus 40 μl/min); this would result in a lower buprenorphine concentration in the chromatographic effluent and therefore a loss of sensitivity, as ES interfaces are sensitive to the concentration and not to the quantity of each compound entering the source. Secondly, the MS–MS conditions they chose necessarily resulted in a further loss of sensitivity, as the pseudomolecular ions had to travel through a first quadrupole, a collision cell (where some are broken down and others stopped) and a second quadrupole before being detected. Indeed, buprenorphine is not easy to dissociate, and we obtained only weak confirmation fragments using in-source CID, but it could be expected that the higher collision energy reached in a collision cell could produce measurable product ions, to be used as quantitation ions; if not, then the only usefulness of the second and third stages would be to eliminate eventual interferents with an $m/z$ ratio similar to that of buprenorphine and a much greater molecular fragility, which is probably not a frequent situation. Tracqui et al. [56] reported the case of a suicide by ingestion of the antipsychotic zuclopenthixol. The drug and its inactive isomer (*E*)-*trans*-clopenthixol were determined in post-mortem blood and tissues by HPLC–DAD, and their nature confirmed by submitting the same extracts to LC–ES-MS, using in-source CID for increasing the number and intensity of fragments. This case points out one more argument for the availability or need of a LC–MS system in a forensic toxicological laboratory, i.e., to be able to confirm the nature of a toxicant by a mass spectral technique for those molecules which are not suited to GC–MS or usually assayed by HPLC techniques. We have had several such opportunities in our laboratory, where we could directly and rapidly analyse extracts prepared for other chromatographic techniques by a standard LC–ES-MS technique, using a large gradient elution and scan acquisition (e.g., for benzodiazepines or antipsychotics). This was the basis of the ''general unknown procedure'' that will be evoked in Section 5. Finally, we recently published a LC–ES-MS

method for the determination of trimeprazine, metabolites trimepazine sulphoxide and its three main metabolites, including the potentially toxic and extremely thermolabile sulphoxide metabolite which we failed to analyse using GC–MS, whichever the injection and chromatographic temperature conditions [57]. The LOQ achieved was 0.5 ng/ml for all the analytes. Although this technique was intended for animal experiments, this is a good example of the usefulness of electrospray interfaces for assaying thermally labile molecules.

### *3.2. Atmospheric pressure chemical ionisation (APCI) interfaces*

#### *3.2.1. Principle*

Horning et al. [58] developed the APCI interface/source for LC–MS in the early 1970s. Since then, different technical solutions and designs have been proposed [44], and some are commercially available. Schematically, the chromatographic effluent flows through a heated nebuliser, either pneumatically assisted and housed in a quartz tube in which flows a make-up gas (Fig. 4), or assisted by a piezoelectric ultrasonic or a sonic spray device. Alternately, heat is not applied to the three concentric tubes (chromatographic effluent, nebulising and make-up gases) but in a ''vaporisation zone'' in the atmospheric pressure interface. However, the mixture of hot liquid and vapours expands into the atmospheric pressure interface where it is ionised by a corona discharge. The ions formed from the solvent molecules transfer a charge to the analytes, which are thus ionised by chemical ionisation. As in the ES source, ion clusters are formed, which can be broken down and further desolvated by a countercurrent stream of gas and/or by in-source CID. The pumping stages, intermediate pressure chamber, ion optic as well as in-source CID possibilities are the same as those of ES. This is either because or is the reason why both are generally available on the same instruments.

#### *3.2.2. Recent applications in human toxicology*

A method has recently been described for the determination if six sulphonylureas hypoglycaemic agents in serum by LC–APCI-MS [59]. As the second generation of these drugs is much more potent than the first one, low therapeutic doses are given, leading to low serum concentrations. Therefore, sensitive techniques are necessary for these drugs, both for therapeutic drug monitoring and toxicology. After a single-step liquid–liquid extraction in an acidic medium, using a chemical analogue of glibenclamid as I.S., the compounds were separated on an RP column, using a gradient of methanol in 0.05% acetic acid. After ionisation in the APCI interface and a slight CID, acquisition was performed in the SIM mode, using a single ion per analyte (either the molecular or the pseudo-molecular ion). Under these conditions, the detection limit of the method was 10 ng/ml serum for the six compounds, which is better than the LODs generally achieved using HPLC–UV [60] and allows the specific identification of these drugs in forensic cases, as well as in clinical cases were the abuse of sulphonylureas is often denied.

Bogusz et al. [61] described the determination of flunitrazepam (F) and its metabolites 7-aminoflunitrazepam (7-AF), *N*-desmethylflunitrazepam (N-DF) and 3-hydroxyflunitrazepam (3-OHF) in urine, serum and blood using deuterated analogues as I.S., SPE and macrobore RP-HPLC coupled to either ES-MS or APCI-MS. The comparison of ES and APCI in the positive ion SIM mode, following a single ion per compound, showed that APCI was seven-times more sensitive for F, about 20-times for N-DF and 40-times for 3-OHF than ES, but there was no difference for 7-AF. The LODs were 0.2 ng/ml for F and 7-AF, 1 ng/ml for N-DF and 3-OHF and the method was linear over a 1–500 ng/ml range. The qualities of this method are a simple sample preparation, a short chromatographic run (about 7 min) and a high sensitivity, while the use of a single ion for the identification of each compound limits its specificity. Bogusz also pointed out the risk of a contribution of a natural isotope of a highly concentrated compound to the mass peak of its deuterated I.S., when the latter is not more than 3 u heavier than the former (e.g., interference of the ''M+3'' isotope of morphine with the mass peak of morphine-$d_3$); this contribution at the M+3 peak for an injected amount of 500 ng was 0.4% for morphine, 0.3% for benzoylecgonine and 0.1% for MDEA and MDMA [62].

Kanasawa et al. [63] investigated the use of a column-switching system for desalting a mobile phase containing a non-volatile buffer and rendering

it compatible with an APCI interface, taking various sedative and anaesthetics drugs as examples (flumazenil, butorphanol, midazolam, lorazepam, phenobarbital and flunitrazepam). This heavy and cumbersome technique required the use of a UV detector to select the fractions containing the compounds of interest at the output of the chromatographic column and send them sequentially into the desalting column, where the 0.1 *M* phosphate buffer of the mobile phase was rinsed out with water during 17 s, then the selected compound back-flushed to the APCI-MS system over a period of 30s using pure methanol. This solution thus requires a delay of at least 47 s between chromatographic peaks, which was the case with the compounds selected. Indeed, the use of non-volatile buffers can be avoided in most cases, using different types of reversed-phase chromatographic columns, based on bound silica (end-capped or not) or on polymers, as demonstrated by numerous applications cited herein and elsewhere [6,7] where formate or acetate buffers were generally employed.

## 4. Typical applications of LC–MS in forensic or clinical toxicology

### *4.1. Morphine and metabolites*

If GC–MS is now the reference technique for the determination of morphine (M), codeine, codethyline, heroin and its first metabolite 6-monoacetylmorphine (6-MAM) in biological fluids, there is still a concern over the direct determination of morphine glucuronides. These metabolites can play an important role in the interpretation of toxic deaths involving heroin or morphine, for at least two reasons: first, morphine-6-glucuronide (M6G) is pharmacologically active and has even been advocated to have a slightly different, and maybe more respiratory depressant action than morphine, due to its binding to a different μ-receptor subtype [64]; secondly, the ratio of morphine over its metabolites can help evaluate the time elapsed between morphine (or heroin) intake and death, as well as the severity of the intoxication when death was delayed and most of the morphine metabolised. Several analytical methods for morphine and its glucuro-conjugated metabolites using LC–MS with different types of interfaces have been reported: at least two LC–MS procedures using a thermospray interface, three using an APCI interface and three using an ES interface (Table 1).

Polettini et al. [40] reported a qualitative LC–TS-MS–MS method for the confirmatory analysis of heroin metabolites in biological fluids. After a simple ultrafiltration of urine samples, the LODs ranged from 10 ng/ml (6-MAM, M and codeine) to 50 ng/ml [heroin, 6-MAM, 6-acetylcodeine (6AC), morphine-3-glucuronide (M3G) and M6G], while after SPE the LODs were lowered to 1 ng/ml for all except the glucuronides, the extraction recovery of which was low. Positive urine samples from heroin addicts were successfully analysed. The technique was also found suitable for serum and whole blood, using spiked specimens. The already cited technique of Tatsuno et al. [39], for the simultaneous determination of illicit drugs of various families in human urine, using a thermospray interface and a single-quad MS, included the analysis of 6-MAM, M, M3G and M6G. The authors reported LODs between 2 and 40 ng/ml in the SIM mode and between 50 and 400 ng/ml in the scan mode, without further precision.

The first paper concerned with LC–APCI-MS appeared in 1992 and was also from a Japanese team [65]. It dealt with M and M3G, whose LODs were respectively 1 and 3 ng/ml in the SIM mode and 80 and 350 ng/ml in the full-scan mode; linearity was verified between 30 and 2000 ng/ml for morphine, 30 and 1000 ng/ml for its metabolite. This technique was successfully applied to a urine sample from a heroin addict. Bogusz et al. [66] reported in 1997 the determination of 6-MAM, M, M3G, M6G, codeine and codeine-6-glucuronide (C6G) in biofluids, using LC–APCI-MS and deuterated I.S.: the limits of detection were between 0.5 and 2.5 ng/ml and the estimated LOQs twice as high for all compounds, except C6G (100 and 200 ng/ml for LOD and LOQ, respectively). The method was validated between 5 and 500 ng/ml in serum and revealed convenient also for post-mortem blood, urine, cerebrospinal fluid and vitreous humour samples in routine work. Zuccaro et al. [67] published the same year an LC–APCI-MS technique for the determination of heroin, 6-MAM, M, M3G, M6G and codeine in

Table 1
LC–API-MS procedures for the determination of morphine metabolites and other opiates in biological fluids (for abbreviations see Section 7)

| Ref. | Compounds analysed | Extraction type | Chromatographic conditions | | MS conditions | LOD (ng/ml) | LOQ (ng/ml) |
|---|---|---|---|---|---|---|---|
| | | | Column | Mobile phase | | | |
| [40] | Heroin, 6-MAM, 6-AC, M, C, M3G, M6G | Filtration (0.22 μm Millex-GS filters) or SPE (Bakerbond Diol) | Zorbax TMS 250×4.6 mm I.D. | Methanol gradient in (0.1 *M* ammonium acetate+0.17 *M* acetic acid+0.01 *M* triethylamine); 1.2 ml/min | TS, filament and discharge off, triple quadrupole MS (TSQ700, Finnigan) daughter ion scanning | Ultrafiltration All: 10–50[b] SPE Heroin, 6-MAM, 6-AC, M, C: 1.0[b] | NR[a] |
| [39] | 6-MAM, M, M3G, M6G | SPE (Sep-Pack $C_{18}$) | ODS 150×4.6 mm I.D. | Acetonitrile gradient in 100 m*M* ammonium acetate; 1 ml/min | TS, single quadrupole MS (QP-1100EX, Shimadzu) SIM and scan mode | Scan mode: 50–400 SIM mode: 2–40 | NR |
| [65] | M, M3G | SPE (Sep-Pack $C_{18}$) | ODS 150×4.6 mm I.D. | 50 m*M* Ammonium acetate–methanol (86:14, v/v); 1 ml/min | APCI, single quadrupole MS (brand?) SIM mode | M: 1.0 M3G: 3.0 | M: 30.0 M3G: 30.0 |
| [66] | 6-MAM, M, M3G, M6G, codeine, C6G | SPE (Bond Elut $C_{18}$) | Superspher RP18, 4 μm 125×3 mm I.D. | pH 3.0, 50 m*M* ammonium formate–acetonitrile (95:5, v/v); 0.6 to 1.1 ml/min | APCI, single quadrupole MS (SSQ 7000, Finnigan) SIM mode | 6-MAM: 1.0 M: 0.5 M3G: 2.5 M6G: 2.5 Codeine: 2.5 C6G: 100.0 | 6-MAM: 2[b] M: 1[b] M3G: 5[b] M6G: 5[b] codeine: 5[b] C6G: 200[b] |
| [67] | Heroin, 6-MAM, M, M3G, M6G, codeine | SPE (J.T. Baker ethyl phase) | Supelcosil LC-Si, 5 μm 250×2.1 mm I.D. | 1% Formic acid–methanol acetonitrile (35:59.8:5.2, v/v/v) 230 μl/min 46 μl/min post-split | APCI, single quadrupole MS (API 1 MS, Sciex) SIM mode | Heroin: 0.5 6-MAM: 4.0 M: 4.0 M3G: 1.0 M6G: 4.0 Codeine: 4.0 | NR |
| [68] | M, M3G, M6G | SPE (J.T. Baker ethyl phase) | Supelcosil ABZ, 5 μm 250×4.6 mm I.D. | Methanol gradient (15 to 60%)–water 0.8 ml/min 18 μl/min post-split | ES, single quadrupole (Trio 2, Fisons) SIM mode | M: 10.0 M3G: 100.0 M6G: 50.0 | M: 10.0 M3G: 100.0 M6G: 50.0 |
| [69] | M, M3G, M6G | SPE (Sep-Pak light $C_{18}$) | YMC ODS-AL $C_{18}$ 100×4.6 mm I.D. | Acetonitrile gradient (4 to 70%) 2 m*M* formic acid 1 ml/min 20 μl/min post-split | pneumatically assisted ES, single quadrupole (VG platform, Fisons) SIM mode | (20 pg on-column as pure solutions for all) | M: 0.8 M3G: 5.0 M6G: 2.0 |
| [70] | M, M3G, M6G, normorphine | SPE (Bond Elut $C_2$) | Zorbax SB-phenyl, 5 μm 250×4.6 mm I.D. | Methanol–0.1% formic acid (18:82, v/v) 0.7 ml/min 70 μl/min post-split | pneumatically assisted ES, triple quadrupole (VG Quattro, Fisons) MRM mode | M: 3.8 M3G: 5.5 M6G: 2.0 Normorphine: 5.0 | NR |

[a] NR=Not assessed or not reported.
[b] Estimated values.

mouse serum, using nalorphine as I.S. Using 1 ml samples, the LODs, corresponding to a signal-to-noise ratio of 3, were between 0.5 and 4 ng/ml, but no real quantitation limits were assessed. The method was linear up to 10 μg/ml. These three techniques thus achieved a very similar performance using different types of chromatographic separations and mass spectrometers of different brands, equipped with an APCI interface. On the other hand, the afore mentioned team reported two years earlier [68] a technique for the determination of M, M3G and M6G, using an electrospray interface and codeine or naltrexone as I.S., which yielded LOQs of 10, 50 and 100 ng/ml, respectively and was linear up to 1000 ng/ml. An explanation for this apparently lower sensitivity of ES with respect to APCI may be that the ES interface was fitted to a single-stage MS instrument which was not primarily designed for LC–MS coupling. The second method using LC–ES-MS dealt also with M, M3G and M6G in human serum, for pharmacokinetic studies [69]. The LODs obtained by injection of pure standards were 20 pg on-column for all the compounds and the validated LOQs from 1-ml human serum samples were between 0.84 and 5.0 ng/ml. Very recently, Zheng et al. [70] reported a third method using an ES interface coupling LC to a tandem mass spectrometer, for the determination of M, M3G, M6G and normorphine in rat plasma in view of a pharmacokinetic study. Nalorphine was used as I.S. The LODs were between 3.8 and 12 ng/ml, i.e., similar to those of the previous technique despite a 10-times lower sample volume, owing to the gain in signal-to-noise ratio provided by MS–MS.

This example of morphine metabolites is demonstrative of the importance of all the components of an analytical method, when a high sensitivity is required. Both types of API interfaces are apparently well suited for morphine and its metabolite, despite the theoretical advantage of ES for polar compounds. Moreover, while results obtained using APCI are homogenous, those obtained with non-pneumatically assisted ES [68] are not as good as those provided by pneumatically-assisted ES. On the other hand, the sensitivity of each mass spectrometer (using the same type but not necessarily the same model of interface) has probably also a non-negligible influence on the results. However, the extraction and chromatographic conditions chosen are important factors for the sensitivity of the whole procedure: in particular, Tyrefors et al. [69] pointed out the necessity of optimising the composition of the mobile phase, both for a good chromatographic separation and an optimal ionisation of analytes in the source (Fig. 5). Fig. 6 shows the chromatograms reconstructed from pseudo-molecular ions of morphine and its metabolites, using an LC–ES-MS method run on an API 100 Sciex Instrument in our laboratory (unpublished data).

Finally, MS–MS is known to yield better signal-to-noise ratio, selectivity and specificity than MS, which are important advantages for forensic analyses, as illustrated by the present comparative study. Another theoretical improvement of MS–MS over MS is the possibility of drastically reducing the chromatographic run-time, but this was not the case here [40,70] and this is probably quite impossible in as far as one would want to determine M3G and M6G independently, as these give rise to the same precursor and daughter ions and have therefore to be separated chromatographically.

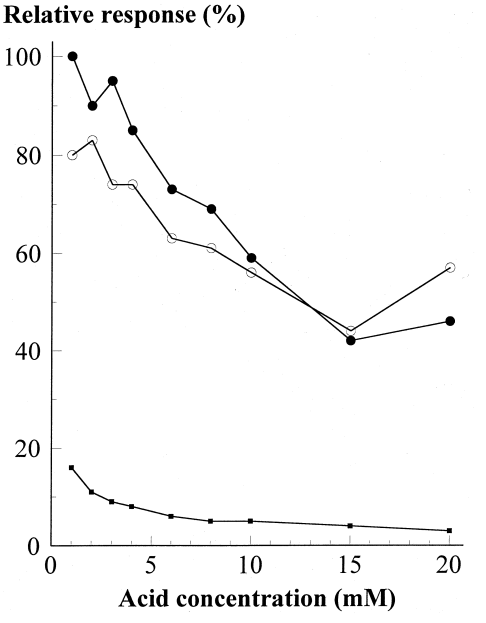


Fig. 5. Morphine signal intensity as a function of type and concentration of mobile-phase modifier; (●) formic acid, (○) acetic acid and (■) trifluoroacetic acid. Experiments were carried out in the flow-injection mode, without chromatographic separation (from Ref. [69], with permission).

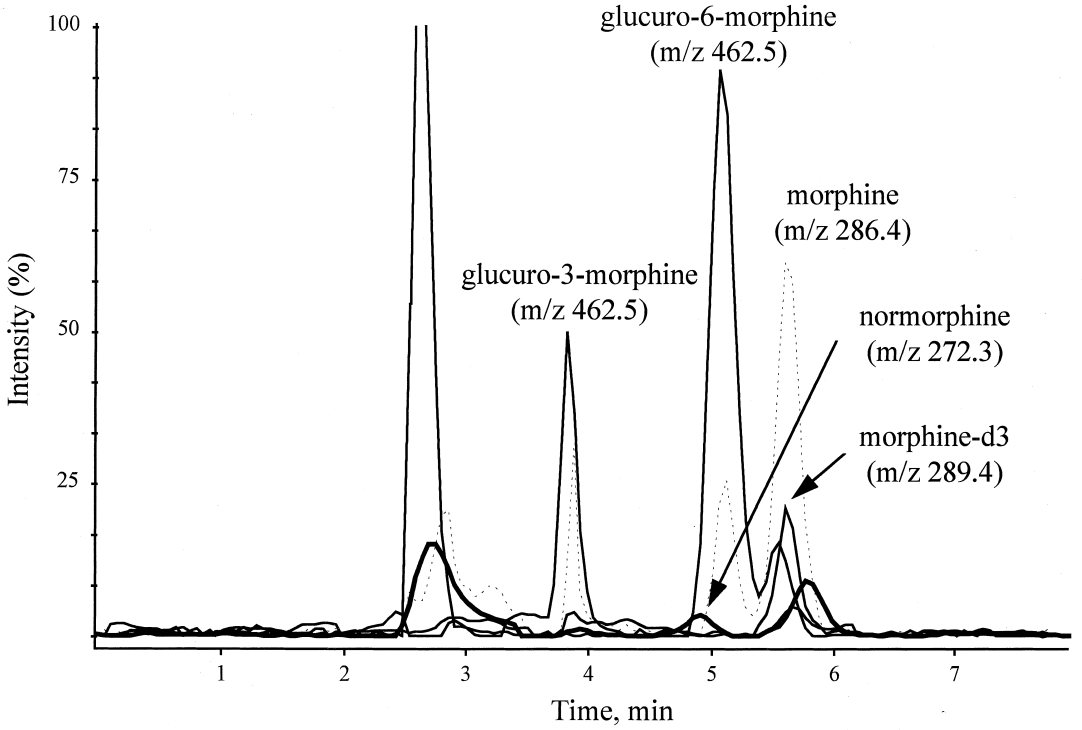


Fig. 6. LC–ES-MS chromatograms reconstructed from the respective quantitation ion of morphine-$d_3$ (I.S.), M, M3G, M6G and normorphine (unpublished data).

### *4.2. LSD, metabolites and epimers*

Several immunoassay methods are now being proposed for LSD in urine [71,72], all of which more or less cross-react with various metabolites [72] as well as other chemicals or therapeutic drugs [73]. Moreover, LSD consumption is said to have increased in recent years, at least in Europe [74]. Therefore, there is a tremendous need for confirmation techniques for LSD and its main metabolite *N*-desmethyl-LSD (nor-LSD), as well as for their respective epimers iso-LSD and isonor-LSD. Indeed, iso-LSD appears as an impurity in LSD preparations, so that urine levels of iso-LSD can be higher than those of LSD. Moreover, LSD and iso-LSD are partly converted into each other in vitro, so as to achieve an equilibrium ratio of 9:1 in two weeks at pH levels over 7.0 and temperature over 37°C [75]. The positivity threshold proposed for the majority of immunoassays is 0.5 ng/ml [71,72], corresponding to a 12–24 h detection window after a typical dose of 50 μg. Until recently, the technique mainly used for LSD screening or confirmation in urine was GC–MS [76], or GC–MS–MS [77]. However, LSD being irreversibly adsorbed on GC columns, needs prior derivatisation (generally by trimethylsylilation or trifluoroacetylation). Even then, frequent column deactivation can be necessary [76]. For these reasons, LC–MS was proposed by different teams as the technique of choice for the determination of LSD in biological fluids, mainly urine (Table 2).

The first paper published concerning LSD urine analysis dates back to 1982 [17]. The authors took LSD as an application example for a proposed technique employing flow-switching chromatography coupled to a single quadrupole MS instrument via a DLI interface and requiring no sample preparation step. The compounds of interest were detected by a UV detector on-line with a switching valve and the DLI interface, so that only the portions of the chromatogram containing the compounds to be analysed were sent to the mass spectrometer. Using a 2

Table 2
LC–API-MS procedures for the determination of LSD, its metabolites and epimers in biological fluids (for abbreviations see Section 7)

| Ref. | Compounds analysed | Matrix | Extraction type | Chromatographic conditions | | MS conditions | LOD (ng/ml) | LOQ (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| | | | | Column | Mobile phase | | | |
| [17] | LSD | Urine | Flow-switching HPLC | RP 8, 10 μm 100×3.2 mm I.D. | 0.01 *M* ammonium formate acetonitrile (10:90, v/v) 2 ml/min | DLI, single quadrupole MS (5985 B MS Hewlett-Packard) SIM mode | LSD: ≥0.2 | NR[a] |
| [78] | LSD | Urine | HipPac protein G immunoaffinity column, 30 μm, 33×2.1 mm I.D., with rabbit LSD antiserum operated in the valve-switching mode | Trapping column: $C_{18}$ ISRP, 5 μm 10×3 mm I.D. Analytical column: Zorbax cyano, 5 μm 150×4.6 mm I.D. | 3 or 5 m*M* ammonium acetate (pH 5.0)–acetonitrile (50:50, v/v) 1.2 ml/min | Laboratory-made pneumatically assisted ES, single quadrupole MS (5985 B MS Hewlett-Packard) SIM mode | LSD: 0.5 | NR |
| [79] | LSD, nor-LSD, iso-LSD, isonor-LSD, methysergide | Urine | SPE (Bond-Elut Certify I) | Spherisorb ODS-2, 5 μm 100×1 mm I.D. | Gradient of acetonitrile and methanol in acetate buffer 60 μl/min | Pneumatically assisted ES, triple quadrupole MS (API III, Sciex) SRM mode | All: 0.05 | NR |
| [80] | LSD | Urine | SPE (Bond-Elut) | Hypersil $C_{18}$, 3 μm 125×3 mm I.D. | Ammonium acetate–triethyl-amine acetonitrile (74.75: 0.25:25, v/v/v) 0.5 ml/min | Pneumatically assisted electrospray, single quadrupole MS (SSQ7000, Finnigan) SIM mode | LSD: <0.3 | LSD: 0.5 |
| [81] | LSD, nor-LSD | Urine | SPE (Extrelut-3) | Nucleosil $C_{18}$, 5 μm 150×1 mm I.D. | 2 m*M* ammonium formate acetonitrile (70:30, v/v) 40 μl/min | Pneumatically assisted ES, single quadrupole MS (API 100, Sciex) SIM mode | LSD: 0.025 nor-LSD: 0.035 | LSD: 0.05 nor-LSD: 0.10 |
| [83] | LSD, nor-LSD, iso-LSD, isonor-LSD | Serum, blood | LLE at pH 9.5 using diethyl ether–toluene (60:40, v/v) | Nucleosil $C_{18}$, 5 μm 150×1 mm I.D. | 5 m*M* ammonium formate acetonitrile (65:35, v/v) 50 μl/min | Pneumatically assisted ES, single quadrupole MS (API 100, Sciex) SIM mode | LSD, nor-LSD, iso-LSD: 0.02 isonor-LSD: 0.05 | LSD, nor-LSD, iso-LSD: 0.05 isonor-LSD: 0.10 |
| [84] | LSD (qualitative confirmation of nor-LSD) | Urine, serum, plasma, blood | Automated SPE: RapidTrace (Zymark) (Bond-Elut Certify) | Zorbax SB-phenyl, 3.5 μm 75×4.6 mm I.D. | 2 m*M* ammonium acetate (pH 4.0)–acetonitrile–*n*-propanol (35:62:8, v/v/v) | Pneumatically assisted ES, triple quadrupole MS (Quattro II, Micromass) SRM mode | LSD: 0.025 nor-LSD: 0.025 | LSD: 0.05 |

[a] NR=Not assessed or not reported.

ng/ml spiked urine sample, an LOD of 0.2 ng/ml was advocated, probably by extrapolation from the height of the chromatographic peak acquired in the SIM mode. Similarly, the next paper published mainly considered urinary LSD detection as an example for a proposed column-switching immunoaffinity chromatography–ES-MS technique for the direct determination of drugs in urine, without extraction [78]. Using a laboratory-made pneumatically assisted electrospray source to couple this rather complicated chromatographic system to a now outdated single quadrupole instrument, the authors could obtained an LOD as low as 0.5 ng/ml and indicated that they thought possible to lower it using chromatographic columns of smaller internal diameter. Four years later, the same team proposed both a LC–ES-MS–MS and a capillary electrophoresis–ES-MS–MS technique for the elucidation of LSD metabolism in vitro and identification of metabolic products in positive human urine samples [79]. Using this time a microbore RP-HPLC separation and the SRM detection mode, they could reach LODs 10-times lower (50 pg/ml) for LSD, nor-LSD, iso-LSD, isonor-LSD and methysergide in human urine. Additionally, this method and capillary electrophoresis allowed respectively the identification of 11 and nine (total 13) other LSD metabolites. Using a single quadrupole MS instrument, White et al. [80] designed a simple technique for the determination of LSD in urine. LOQ was 0.5 ng/ml and linearity was verified up to 20 ng/ml. We reported the first fully validated, quantitative technique for the sensitive determination of LSD and nor-LSD in human urine, using LC–ES-MS [81]. The LODs reached were 25 and 35 pg/ml, respectively, with LOQs twice as high. Using deuterated LSD as I.S., linearity was excellent up to 20 ng/ml and the technique was found precise and accurate, both on an intra-day and inter-day basis. It was retained as a recommended method in the United Nations International Drug Control Programme [82]. It was later adapted to whole blood samples and extended to the determination of iso-LSD and isonor-LSD (Fig. 7), with equivalent and sometimes better LODs and LOQs [83]. De Kanel et al. [84] obtained the same LODs for LSD and nor-LSD in urine, serum, plasma and blood, and the same LOQ for LSD alone (while only a qualitative confirmation of nor-LSD was claimed), using MS–MS in the SRM mode.

In summary, in the last three years LC–ES-MS has been progressively recognised as a technique at least equivalent to, and probably more convenient than GC–MS for the confirmation of LSD abuse.

### *4.3. Corticosteroids*

The determination of corticosteroids in biological fluids, mainly urine, is necessary for identifying the abuse of such agents by athletes, due to their anti-inflammatory and analgesics properties. GC–MS methods have been developed over the last 20 years, requiring an initial hydrolysis of the conjugated metabolites and a derivatisation of functional groups of the parent compounds. Therefore, several LC–MS methods were proposed for the analysis of natural or exogenous corticosteroids in human plasma or urine, as this coupling technique generally allows the direct analysis of conjugated metabolites and needs no derivatisation for most compounds (Table 3).

Girault et al. [85] reported a LC–PB-MS technique for the determination of beclomethasone and two of its esters in human plasma and urine, yielding a LOD of 1 ng/ml for the three compounds studied in the NICI and SIM mode.

Five teams at least proposed qualitative or quantitative methods using LC–TS-MS or LC–TS-MS–MS. Liberato et al. [86] studied the ionisation conditions of endogenous and exogenous corticosteroids, of sex steroids, and of their respective metabolites following their separation on a macrobore reversed-phase column and noted that, for unconjugated steroids, the sensitivity in the positive-ion SIM mode was excellent, provided thermal decomposition was avoided by a careful optimisation. Additionally, the LC–TS-MS full mass spectrum of the 18-hydroxylated corticosteroids were found much more informative and reproducible than the EI mass spectra obtained with GC–MS after derivatisation and chemical modification of the molecules, due to the variety of forms produced by these chemical manipulations. Moreover, the glucuroconjugated metabolites could also be readily and sensitively determined by LC–TS-MS in the negative-ion SIM mode, while they were extensively

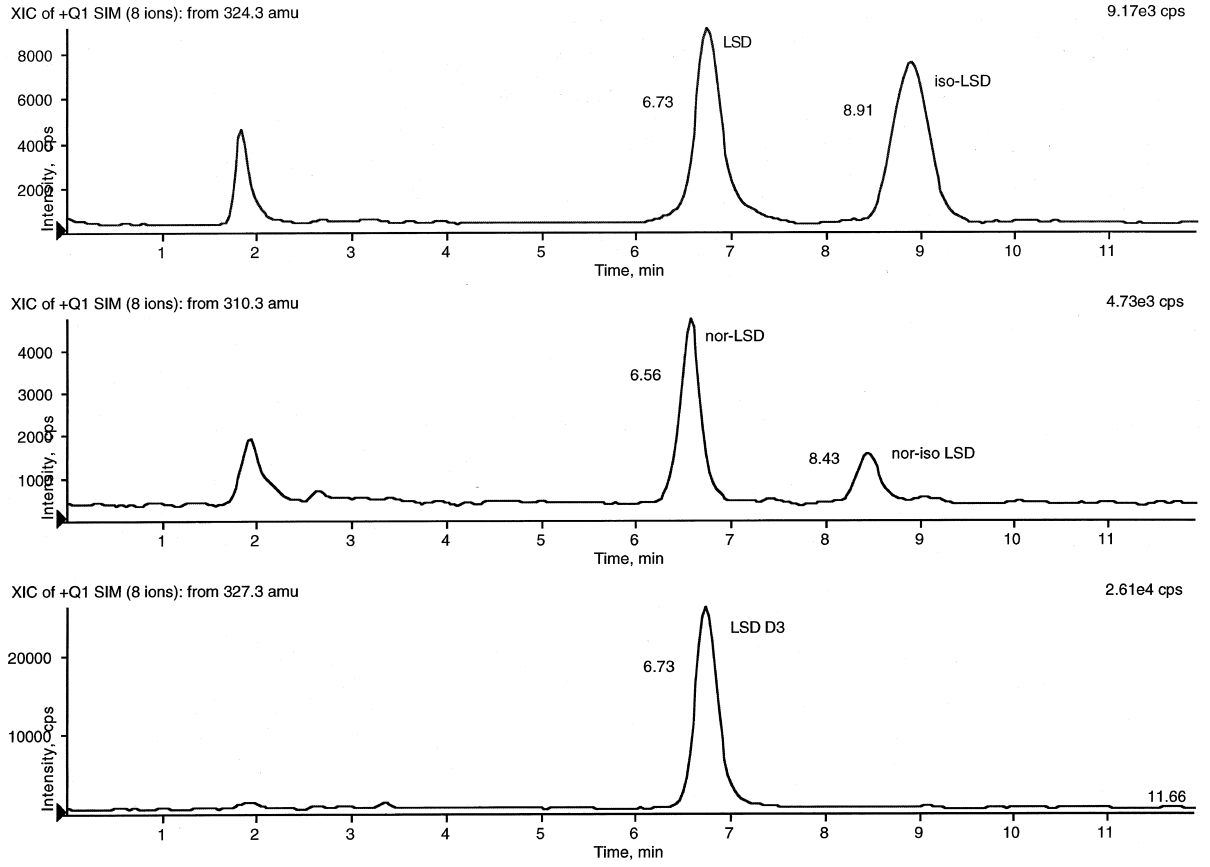


Fig. 7. LC–ES-MS reconstructed chromatograms from a whole blood sample spiked with LSD and iso-LSD at 1 ng/ml, and with nor-LSD and isonor-LSD at 0.8 and 0.2 ng/ml, respectively (from Ref. [83], with permission).

fragmented in the positive mode. Park et al. [87] reported the simultaneous determination of nine corticosteroids in urine by diode-array detection (DAD) and TS-MS, following their liquid–liquid or solid-phase extraction and their chromatographic separation on a macrobore $C_{18}$ column using a gradient elution. The vaporiser probe temperature was programmed according to the composition of the mobile phase and ionisation was performed in the filament-on, positive ion mode. The detection limits, determined from standard solutions, ranged from 10 to 50 ng on-column in the scan mode and from 1 to 5 ng in the SIM mode, but these results cannot be extrapolated to concentrations in biological specimens. On the contrary, LODs achieved by DAD were measured using 5 ml spiked urine samples, and were found to be 10 ng/ml for each compound. Therefore, it seems likely that the only advantage of TS-MS upon DAD in this application was its higher selectivity, which rendered possible the simultaneous determination of prednisolone and prednisone, which were chromatographically unresolved and had the same UV maximum absorbency. Indeed, the diode array detector, but not the mass spectrometric system, was used by the authors during the 24th Seoul Olympic Games to determine corticosteroids in athletes' urine samples. Steffenrud and Maylin [88] proposed a confirmation technique for the identifica-

Table 3
LC–MS procedures for the determination of corticosteroids in biological fluids (for abbreviations see Section 7)

| Ref. | Compounds analysed | Matrix | Extraction type | Chromatographic conditions | | MS conditions | LOD (ng/ml) | LOQ (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| | | | | Column | Mobile phase | | | |
| [85] | Beclomethasone (B)<br>B-monopropionate (B-monoP),<br>B-dipropionate<br>(B-diP) | Human plasma and urine | LLE at pH 7 with dichloromethane | Microsphere $C_{18}$, 3 μm<br>100×4.6 mm I.D. | Methanol–water (75:25, v/v)<br>0.4 ml/min | PB (59980 A, H.P.), single quadrupole (5988, H.P.)<br>NICI, SIM mode | NR[a] | B, B-monoP and B-diP: 1 |
| [86] | Corticosterone, cortisol, cortisone, prednisone, prednisolone, unconjugated and glucuronidated metabolites | Urine | SPE<br>parent and conjugated steroids: Sep-Pak $C_{18}$ unconjugated metabolites: Sephadex LH-20 | Altex Ultrasphere $C_{18}$<br>250×4.6 mm I.D.<br>(ambient temp.) | Methanol–0.1 *M* ammonium acetate pH 3.5 (unconjugated steroids: 30:70, v/v; conjugated metabolites: 55:45, v/v) 1.25 ml/min | TS (Vestek), single quadrupole MSs (Finnigan and Biospect)<br>positive and negative scan and SIM modes | Qualitative study only | Qualitative study only |
| [87] | Betamethasone, corticosterone, cortisone, deoxycorticosterone, hydrocortisone, 11-α-OH-progesterone, prednisolone, prednisone, triamcinolone, trimacinolone acetonide | Urine | LLE at pH 9 with diethyl ether<br>SPE<br>(Sep-Pak $C_{18}$) | Hypersil-ODS, 3 μm<br>60×4.6 mm I.D. (40°C) | Gradient methanol in 0.15 *M* ammonium acetate<br>0.8 ml/min | TS (temperature programmed, filament-on), single quadrupole (5988, H. P.)<br>positive ion, scan and SIM modes | scan mode:<br>10–50 ng on-column<br>SIM mode:<br>1–5 ng on-column | NR |
| [88] | Cortisone, hydrocortisone, dexamethasone, prednisone, prednisolone | Urine (equine) | Enzymatic hydrolysis and LLE at pH 10 with ethyl acetate | Nova-Pak $C_{18}$,<br>4 μm<br>100×8 mm I.D. | Acetonitrile–25 m*M* ammonium formate (70:30, v/v)<br>1.3 or 1.5 ml/min | TS (Vestek Model 201)<br>positive and negative scan and SIM modes | Qualitative study only | Qualitative study only |
| [89] | Cortisol, cortisone, prednisolone, prednisone | Plasma | SPE<br>(Sep-Pak $C_{18}$)<br>I.S.: cortisol-$d_5$ and cortisone-$d_5$ | LiChroCART<br>Supersupher | Acetonitrile–0.05 *M* ammonium formate (65:35, v/v) | TS (Vestec Model 750B), single quad.<br>MS (QP 1000 EX, Shimadzu)<br>positive ion, SIM mode | Cortisol: 250 pg<br>Cortisone: 500 pg<br>Prednisone and prednisolone: 1 ng on-column | NR |
| [90] | Betamethasone | Urine | adjustment to pH 6–6.5<br>(±enzymatic hydrolysis)<br>filtration<br>(0.22 mm Millex-GS filters) | LC–LC:<br>Microspher $C_{18}$, 3 μm<br>50×4.6 mm I.D.<br>Zorbax TMS,<br>5 μm<br>250×4.6 mm I.D. | Methanol–0.1 *M* ammonium acetate (50:50, v/v)<br>Acetonitrile–0.15 *M* ammonium acetate (37:63, v/v)<br>1.3 ml/min | TS (TSP-2, Finnigan)<br>MS–MS<br>(TSQ 700, Finnigan)<br>positive ion, SRM mode | Betamethasone: 0.2 | Betamethasone: 1.0 |
| [91] | Betamethasone, cortisone, dexamethasone, flumethasone, flunisonide, hydrocortisone, prednisone, prednisolone, 6α-methyl-prednisolone, triamcinolone, trimacinolone acetonide | Urine (bovine) | Enzymatic digestion<br>SPE<br>(Bond-Elut $C_{18}$) | Spherisorb ODS $C_{18}$,<br>5 μm 250×4.6 mm I.D. | Acetonitrile–0.1 *M* ammonium acetate (pH 6.8)<br>(40:60, v/v)<br>1 ml/min | APCI, MS–MS<br>(API III-Plus, Sciex)<br>negative ion, daughter ion scan and SRM modes | All except triamcinolone: 0.05 to 1.0 | All except triamcinolone: 1.0 |
| [92] | Hydrocortisone, (testosterone) | Plasma | SPE<br>(Specplus $C_8$) | Supelcosil LC-18-DB,<br>5 μm 33×4.6 mm I.D. | Methanol–5% acetic acid<br>(60:40, v/v)<br>0.5 ml/min | APCI, ion-trap MS–MS<br>(LCQ, Finnigan)<br>positive ion, daughter ion scan and SRM modes | NR | NR |
| [93] | Budesonide, cortisone, deoxycorticosterone, dexamethasone estriol-3-sulphate, estrone-3-sulphate, fludrocortisone, flumethasone, hydrocortisone, 6-methylprednisolone, prednisone, prednisolone, triamcinolone | Urine | SPME<br>(50 μm CW/TPR fibers) | YMC/3-4-5 ODS-AQ,<br>3 μm 50×4.0 mm I.D. | Gradient of acetonitrile=methanol (50:50, v/v) in 0.1 *M* ammonium acetate<br>1 ml/min,<br>60 μl/min post-split | ES, MS or MS–MS<br>(API 300, Sciex)<br>negative ion, SIM mode using a single quad., daughter ion scan and neutral loss modes, using the triple quad. | All: 4–30 | NR |

[a] NR=Not assessed or not reported.

tion of four corticosteroids in equine urine, after hydrolysis of glucuronidated metabolites, using LC–TS-MS in the positive and negative ion modes. They found that the sensitivity was similar or slightly improved in the negative, as compared to the positive ion mode, giving full-scan spectra for 10 ng injected. More recently, Shibasaki et al. [89] reported the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in human plasma, using deuterated analogues as internal standards and SPE. The authors could not find a compromise between a mobile phase yielding a good chromatographic resolution but a poor sensitivity, and a more eluent phase giving the opposite results. Therefore, the LODs reported (250 pg to 1 ng on-column) using LC–TS-MS were not as good as those obtained using GC–MS (10–250 pg) as a comparative technique. Polettini et al. [90] compared LC–MS–MS, LC–LC–MS and LC–LC–MS–MS for the determination of betamethasone in urine and found that column-switching HPLC coupled to tandem mass spectrometry gave the best signal-to-noise ratio, leading to a LOD of 0.2 ng/ml with no pre-concentration step.

Two teams at least reported techniques using an APCI interface. Rizea Savu et al. [91], using SPE and LC–APCI-MS–MS, reported LODs in the range 0.05–1 ng/ml and LOQs of 1 ng/ml for nine of the most common synthetic corticosteroids in the negative ion SRM mode. In order to demonstrate the ability of LC–APCI-ion-trap MS–MS to provide reliable and validated quantitative data, Tiller et al. [92] developed a method for the determination of testosterone and hydrocortisone using deuterated analogues as I.S. After SPE of a blank serum sample, addition of the standards to the dry extract and separation on a macrobore, reversed-phase column, the compounds were detected in the positive ion SRM mode, after selection and fragmentation of the $[M+H^+]$ ions in the trap. The linearity of the method, between 94 pg/ml and 12 ng/ml, was satisfactory (but took no account of the extraction procedure), while no LOD or LOQ was given.

Finally, a single paper reported the use of LC–ES-MS (and MS–MS) for the determination of 11 corticosteroids and two steroid conjugates in urine, after solid-phase microextraction [93]. Using single MS in the negative ion SIM mode, LODs were no better than 4 and 30 ng/ml, despite satisfactory extraction recovery. Moreover, MS–MS was used for identification and not for quantitation purposes.

In summary, thermospray and APCI interfaces seem to perform better than electrospray for the analysis of corticosteroids, while MS–MS apparently gives a better specificity but not higher sensitivity over single MS. Whichever the instrument, careful optimisation of the extraction, chromatographic, ionisation, fragmentation and detection steps were necessary to achieve very good results. In the best cases, LC–MS revealed more sensitive, and above all was less time-consuming for sample preparation and chromatographic separation than GC–MS.

## 5. Perspectives

### *5.1. New applications*

#### *5.1.1. "General unknown" screening procedures*

One of the main challenges faced by forensic and clinical toxicologists is the identification of the xenobiotics responsible for an intoxication, when there are no clues to guide the search. Moreover, the volume of blood or urine samples available is generally low and the need for accurate information may be urgent, especially when living patients are involved. This problem is generally handled by using a panel of automated immunological screenings for the most common drugs and, after a non-selective extraction from the matrix, chromatographic techniques, ideally coupled to specific detectors. GC–MS is still the gold standard in this field, and very powerful procedures, both for sample preparation and for data analysis, have been developed by Maurer and co-workers [94,95] and others [96,97]. Nevertheless, this technology usually shows poor results for some polar, above all acidic, compounds, even when a derivatisation is performed. For this reason, and probably also because not all laboratories are equipped with GC–MS systems, complementary or alternate procedures were proposed using in particular HPLC–DAD [98–102]. In this case, the limitations are mostly the limited specificity of UV spectra and their variability as a function of pH (which limits the use of this technology to a given pH value for a given library of UV spectra). This is

probably the reason why few UV spectrum libraries are commercially available. Moreover, compounds with little or no UV absorbency are poorly or not detected.

However, a complete screening strategy using different immunological and chromatographic techniques takes a long time, which may decrease its usefulness in clinical toxicology as far as a living patient's outcome is concerned. Moreover, it costs a lot, which is a concern in both forensic and clinical toxicology. As no universal and instantaneous ''general unknown'' screening technique seems available so far, an improvement might be the association of LC–MS (that uses a more universal and specific detection principle than HPLC–DAD) to GC–MS, in order to form a reliable association of screening techniques to use in first instance.

To the best of our knowledge, no such LC–MS ''general unknown'' screening procedure other than ours have been proposed. We have designed a procedure using in-source CID at various orifice voltages in an ionspray interface, both in the positive and negative ion modes [103]. A reversed-phase chromatographic separation of extracted chemicals was performed on a Nucleosil $C_{18}$, 5 μm (150×1 mm I.D.) column, using a gradient of acetonitrile (5–95% in 50 min) in 5 m*M* ammonium formate, pH 3 as mobile phase (flow-rate: 40 μl/min). Four ionisation conditions were continuously alternated, with a run-over time of 5.8 s: positive ion mode with low CID energy (20 eV); positive ion mode with high CID energy (80 eV); negative ion mode with low CID energy (−20 eV); negative ion mode with high CID energy (−80 eV). Acquisition was performed in the full-scan mode, from 100 to 1100 u, with a step of 0.2 u. Then, weakly and highly fragmented positive spectra were added within each given acquisition time, to give a single, richer mass spectrum, as were negative spectra. The result was a pair of full mass spectra acquired every 2.7 s. These reconstructed spectra compared favourably with LC–MS–MS spectra, as they were as or more informative, due to pseudomolecular ions of higher intensity and sometimes to the presence of high mass adducts. Moreover, the use of both positive and negative ionisation in the same run obviously provides significantly more information than either of them alone for those compounds giving both positive and negative ions, and allows the detection of compounds (mainly acids) giving only negative ions. In November 1998, the pairs of spectra of more than a thousand compounds of interest (therapeutic or abused drugs, environmental and industrial toxicants) were already stored in parallel positive ion and negative ion mass spectrum libraries, respectively. Additionally, software was developed to automatically reconstruct such spectra and compare each pair of positive and negative spectra, together with their retention time [in standardised conditions (optional)] to those in the libraries. The reconstructed spectra were shown to be reproducible over a wide range of compound concentrations, as well as over time. The whole procedure was evaluated by comparison with GC–MS and HPLC–DAD on clinical and forensic samples, after non-selective solid-phase extraction, showing it was remarkably powerful at detecting most compounds present, even at very low levels (as verified afterwards by dedicated quantitative methods) in such complex matrices as hemolised whole blood. In particular, these preliminary results suggested that the proposed LC–ES-MS procedure was complementary to GC–MS for the detection of ''general unknowns'' [103].

#### *5.1.2. Toxic peptides, proteins and other high-molecular-mass molecules*

As a particularity of electrospray-type interfaces is to allow the detection of high-molecular-mass compounds due to the formation of multi-charged species, LC–ES-MS is potentially the method of choice for the screening and quantitation of peptides and proteins with therapeutic or toxic properties. In this class, one can include toxins from marine, plant or animal origin (venoms), hormones, cytokines, etc., as well as therapeutic peptides and proteins, produced by genetic engineering. Up till now, few papers have been published on this matter, in comparison to the large number of papers dealing with the identification of endogenous peptides, proteins, glycoproteins, nucleosides and nucleotides. Indeed, the feasibility of using mass data generated from the proteolytic digestion of peptides or proteins to create and search protein databases was demonstrated several years ago [4] and this technique is now routinely used in the field of biochemistry and molecular biology.

Papers on the LC–MS detection and/or quantitation of various mycotoxins (in plant and animal samples) and marine toxins (in shellfish or rodents' samples) were previously reviewed by Hoja et al. [6], but none concerned human intoxication. Maurer et al. [104] proposed a sensitive and specific LC–ES-MS technique for the detection, in human urine, of the amatoxins α- and β-amanitin, responsible for the toxicity of Amanita mushrooms.

Bowers proposed that the presence of peptide hormones used in sports doping [adrenocorticotropin, human growth factor (hGH), chorionic gonadotropin, erythropoietin and their releasing factors] could be confirmed by mass spectrometry, after ionisation in an electrospray source [105]. An HPLC separation was applied for the detection of IGF-1 as a pure solution, and the overall sensitivity was found to be satisfactory for the detection of doping (LOD better than 10 fmol on column). The same study demonstrated that a recombinant hGH could be differentiated from the native one, due to a difference in a single amino acid. Liu and Bowers [106] developed an immunoaffinity trapping of human chorionic gonadotropin (hCG) in urine prior to its LC–ES-MS detection, and recommended this extraction and enrichment technique for the trace determination of peptide hormones, as it increases both the sensitivity and the selectivity of LC–ES-MS. As the heterogeneity of the oligosaccharides moieties of glycoproteins, such as hCG and EPO, precludes the direct monitoring of the intact peptide, a tryptic digestion, similar to that performed for endogenous proteins, was proposed by these authors [106,107] and others [108]: the selected ion monitoring of a few tryptic fragments, whose distribution is unique, provided the opportunity of identifying the parent hormone.

The different variants of human hemoglobins could be detected in mixtures of intact globins and characterised using ES-MS. Their molecular masses could be determined with an accuracy of 1–2, when the minimum precision required was 14 [109]. Though we could not find any LC–MS methods applied to animal or recombinant insulins, which may be involved in murders or suicides, or even to high- or low-molecular-mass heparins, sometimes involved in medical responsibility cases, the determination of these proteins would benefit from such a specific technique for detection and identification.

The identification and quantitative determination of toxic proteins, peptides or other large molecules, from natural or synthetic origin, is thus a new field that is being opened up to clinical or forensic toxicology, thanks to LC–MS with atmospheric interfaces, that will need the merging of the experience and requirements of analytical toxicology and biochemistry.

### *5.2. New systems*

Though one cannot exclude the emergence of new ionisation sources or interfaces, atmospheric pressure interfaces will probably have a longer career and a larger success in toxicological laboratories than any other previous LC–MS interface, due to their sensitivity and versatility. ES-MS for instance can now be regarded as a detector for LC. Sakairi and Kato [110] recently presented an API interface including five, interchangeable ionisation modes (electron impact, chemical ionisation, spray ionisation, electrospray ionisation and sonic spray ionisation), which they called a ''multi-atmospheric pressure ionisation interface''. The combination of these modes would broaden the polarity range of API interfaces. In particular, small and volatile compounds with low polarity such as hydrocarbons and aromatic compounds would be rendered detectable owing to the electron impact ionisation device. Other improvements to come will probably include API sources specifically designed for ion traps [111], magnetic sectors or other types of mass spectrometers [44]. Time-of-flight and quadrupole/time-of-flight analysers (Q-TOF), with a better mass resolution and wider mass range than single or triple quadrupoles, are now available for coupling with LC-ES. Even such expensive technologies as magnetic sector spectrometers will soon be proposed as benchtop instruments, hopefully at a more affordable price than the bigger ones. Based on these considerations and on the simplification of instrument and concept handling, owing to user-friendly softwares, the authors predict that LC–MS will be a major success in analytical toxicology.

Other sources of improvement in LC–MS will come from sample preparation and chromatographic techniques. LC–MS–MS is now widely used in the pharmaceutical industry because of its high through-

put (e.g., one sample injected every 2 to 5 min), but this throughput is still limited by the sample preparation procedures, which either require several technicians to ''feed'' each LC–MS–MS instrument or need to be automated. Automated SPE extraction procedures are now routinely used for HPLC, either on-line (compounds eluted from the SPE cartridge with the chromatographic mobile phase, directly into the HPLC column) or off-line, using standard SPE cartridges, with a sorbent bed volume of 50–500 mg. However, specific techniques and procedures have been proposed for LC–MS–MS, such as automated SPE extraction using reduced sorbent bed cartridges (10–35 mg) or thin disk membranes adapted to 96-well format plates and compatible with micro-samples [112–115]. This strategy would be of limited use in forensic or clinical toxicology (except maybe in sports doping control), because of the generally limited number of samples being analysed in the same run. Alternatively, several teams proposed a chromatographic purification of the samples as single preparation: coupled-column LC techniques with purification and enrichment of the sample on the first, restricted access media column, intended for the direct injection of plasma or urine, are already classical in HPLC, and were proposed for LC–MS as well [116–118], reducing the total analytical time to 5–10 min; turbulent flow chromatography, achieved by the use of high mobile-phase flow-rates and large particle size stationary phases on a single column, was recently proposed by Ayrton et al. [119] for the direct analysis of plasma or serum samples, with a very short turnover time (ca. 3 min). This latter technique, using an initial water or buffer wash during which the effluent is directed to waste, and a fast gradient elution into the mass spectrometer, offers the advantages of simplicity, speed and increased chromatographic resolution, as turbulent flow decreases the theoretical plate height. Another, simpler track for improvement is the use of narrow-bore, microbore [120] or even nanoscale chromatographic columns [121], avoiding the need for a splitting of the chromatographic effluent and increasing the concentration of the analytes in the chromatographic effluent, the concentration to which the response is proportional when using API interfaces. Though this solution, for which micro-flow-rate chromatographic pumps are now available at a minor additional price, has been around for a long time and is systematically used by different teams, ours included, it is still not widely accepted nor employed. More generally, the liquid chromatographic part of LC–MS is rather different from HPLC, in that it benefits from using mobile phases with low salt content and avoiding non-volatile salts or counterions.

In summary, in the near future, the improvements in LC–MS as applied to analytical toxicology will probably concern the preparation procedures and chromatographic techniques and the software interfacing the instrument and the operator, rather than the interface/source devices.

## 6. Conclusion

The potential of LC–MS in human toxicology has been demonstrated over the past few years, mainly as far as atmospheric pressure interfaces are concerned. Electrospray-type and APCI interfaces represent, at the moment, the sources of choice for a wide range of organic compounds, from the slightly to the highly polar. In most fields where they have been used, the limits of detection or quantitation obtained generally compared favourably with the other chromatographic techniques. Moreover, they allowed the mass spectrometric determination of different classes of molecules that were not suited to GC–MS, such as conjugated metabolites, thermolabile compounds, peptides and proteins, etc. We think LC–API-MS can even be used for broad screenings of a priori unknown compounds, in complement to GC–MS.

Nevertheless, LC–MS is still a relatively new technique that will probably mature thanks to adapted sample preparation and chromatographic techniques, or to higher resolution MS, and spread to a much larger number of laboratories owing to lower prices. Moreover, if LC–MS does not replace GC–MS in most of its toxicological applications because of limited advantages, LC–MS–MS could well do as it allows simple sample preparation procedures, if any, and very short analytical times; moreover, it yields high sensitivity and specificity and will probably be more affordable in the near future. It is worth noting that LC–MS–MS has now superseded all other analytical techniques in the pharmaceutical industry.

## 7. Abbreviations

| | |
|---|---|
| 6-MAM | 6-Monoacetylmorphine |
| amu | Atomic mass unit |
| API | Atmospheric pressure ionisation |
| APCI | Atmospheric pressure chemical ionisation |
| C6G | Codein-6-glucuronide |
| CF-FAB | Continuous-flow fast atom bombardment interface |
| CI | Chemical ionisation |
| CID | Collision induced dissociation |
| CV | Coefficient of variation |
| DAD | Diode array detection |
| DLI | Direct liquid introduction |
| EI | Electron impact |
| ES | Electrospray |
| FAB | Fast atom bombardment |
| FD | Field desorption |
| FIA | Flow-injection analysis |
| GC | Gas chromatography |
| GC–MS | Gas chromatography–mass spectrometry |
| HPLC | High-performance liquid chromatography |
| I.D. | Internal diameter |
| I.S. | Internal standard |
| LD | Laser desorption |
| LOD(s) | Limit(s) of detection |
| LOQ(s) | Limit(s) of quantitation |
| M | Morphine |
| M3G | Morphine-3-glucuronide |
| M6G | Morphine-6-glucuronide |
| MALDI | Matrix-assisted laser desorption ionisation |
| MB | Moving belt |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS–MS | Tandem mass spectrometry |
| $m/z$ | Mass-to-charge ratio |
| PB | Particle beam |
| PD | Plasma desorption |
| RP-HPLC | Reversed-phase high-performance liquid chromatography |
| SIM | Selected ion monitoring |
| SPE | Solid-phase extraction |
| SRM | Selected reaction monitoring |
| TOF | Time of flight |
| TIC | Total ion current |
| TS | Thermospray |
| UV | Ultraviolet |

## References

[1] J.D. Henion, G.A. Maylin, Biomed. Mass Spectrom. 7 (1980) 115.
[2] C. Eckers, D.S. Skrabalack, J. Henion, Clin. Chem. 28 (1982) 1882.
[3] M. Linscheid, Int. J. Environ. Anal. Chem. 49 (1992) 1.
[4] E. Gelpi, J. Chromatogr. A 703 (1995) 59.
[5] M. Careri, A. Mangia, M. Musci, J. Chromatogr. A 727 (1996) 153.
[6] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, B. Pénicaut, G. Lachâtre, J. Anal. Toxicol. 21 (1997) 116.
[7] H. Maurer, J. Chromatogr. B 713 (1998) 3.
[8] P.J. Arpino, B.G. Dawkins, F.W. McLafferty, J. Chromatogr. Sci. 1 (1974) 80.
[9] P.J. Arpino, M.A. Baldwin, F.W. McLafferty, Biomed. Mass Spectrom. 1 (1974) 80.
[10] A.L. Burlingame, R.K. Boyd, S.J. Gaskell, Anal. Chem. 66 (1994) 634R–683R.
[11] J.A. Appfel, U.A. Brinkman, R.W. Frei, J. Chromatogr. 312 (1984) 153.
[12] A. Melera, Adv. Mass Spectrom. 8 (1980) 1597.
[13] P.J. Arpino, P. Krien, S. Vajta, G. Devant, J. Chromatogr. 203 (1981) 117.
[14] M. Dedieu, C. Juin, P.J. Arpino, J.P. Bounine, G. Guillochon, J. Chromatogr. 251 (1982) 203.
[15] R.G. Christensen, V.E. White, S. Maiselman, H.S. Hertz, J. Chromatogr. 271 (1983) 61.
[16] C. Eckers, D.S. Skrabalak, J. Henion, Clin. Chem. 28 (1982) 1882.
[17] F. Erni, J. Chromatogr. 251 (1982) 141.
[18] R.P.W. Scott, G.C. Scott, M. Munroe, J.J. Hess, J. Chromatogr. 99 (1974) 395.
[19] W.H. McFadden, H.L. Schwartz, S. Evans, J. Chromatogr. 122 (1976) 389.
[20] R.D. Smith, A.L. Johnson, Anal. Chem. 53 (1981) 739.
[21] M.J. Hayes, E.P. Lankmayer, P. Vouros, B.L. Kearger, Anal. Chem. 55 (1983) 1745.
[22] J. Games, P. Hirter, W. Kuhnz, E. Lewis, N.C.A. Weerashinge, S.A. Westwood, Anal. Chem. 203 (1984) 131.
[23] A.C. Tas, J. van der Greef, M.C. ten Noever de Brauw, T.A. Plomp, R.A.A. Maes, M. Hohn, U. Rapp, J. Anal. Toxicol. 10 (1986) 46.
[24] P. Dobberstein, E. Korte, G. Meyerhoff, R. Pesch, Int. J. Mass Spectrom. Ion Phys. 46 (1983) 185.
[25] K. Ito, T. Takeuchi, D. Idhii, J. Chromatogr. 346 (1985) 161.
[26] R.M. Capprioli, T. Fan, J.S. Cottrel, Anal. Chem. 58 (1986) 2949.
[27] K. Sato, T. Kumazawa, Y. Katsumata, J. Chromatogr. A 674 (1994) 127.

[28] Y. Hieda, S. Kashimura, K. Hara, M. Kageura, J. Chromatogr. B 667 (1995) 241.
[29] R.C. Willoughby, R.F. Browner, Anal. Chem. 56 (1984) 2626.
[30] P.C. Winckler, D.D. Perkins, W.K. Williams, R.F. Browner, Anal. Chem. 60 (1988) 489.
[31] C.S. Creaser, J.W. Stygall, Analyst 118 (1993) 1467.
[32] C.S. Creaser, J.W. Stygall, Instrum. Sci. Technol. 22 (1994) 185.
[33] P. Arpino, J. Anal. Chem. 337 (1990) 667.
[34] K. Kudo, N. Jitsufuchi, T. Imamura, J. Anal. Toxicol. 21 (1997) 185.
[35] E. Lacassie, S. Ragot, J.M. Gaulier, P. Marquet, G. Lachâtre, Acta Clin. Belg., in press.
[36] C.R. Blakeley, M.J. McAdams, M.L. Vestal, J. Chromatogr. 58 (1978) 261.
[37] M.L. Vestal, G.J. Fergusson, Anal. Chem. 57 (1985) 2373.
[38] G.D. Byrd, M.S. Uhrig, J.D. de Bethizy, W.S. Caldwell, P.A. Crooks, A. Ravard, R.M. Riggs, Biol. Mass Spectrom. 23 (1994) 103.
[39] M. Tatsuno, M. Nishikawa, M. Katagi, H. Tsuchihashi, J. Anal. Toxicol. 20 (1996) 281.
[40] A. Polettini, A. Groppi, M. Montagna, Adv. Forens. Sci., Proc. Meet. Int. Assoc. Forensic Sci. 13 (1995) 197.
[41] P.F. Knewstubb, T.M. Sugden, Nature 181 (1958) 474.
[42] M. Dole, R.L. Hines, L.L. Mack, R.C. Mobley, L.D. Ferguson, M.B. Alice, J. Chem. Phys. 49 (1968) 2240.
[43] E.C. Horning, M.G. Horning, D.I. Carroll, I. Dzidic, R.N. Stillwell, Anal. Chem. 45 (1973) 936.
[44] W.M.A. Niessen, J. Chromatogr. A 794 (1998) 407.
[45] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[46] A.P. Bruins, T.R. Covey, J.D. Henion, Anal. Chem. 58 (1987) 2642.
[47] B.A. Thomson, J.V. Iribarne, P.J. Dziedick, Anal. Chem. 54 (1982) 2219.
[48] F. Bitsch, C.H.L. Shackleton, W. Ma, G. Park, M. Nieder, Rapid, Commun. Mass Spectrom. 7 (1993) 891.
[49] M.L. Miller, B.R. McCord, R. Martz, B. Budowle, J. Anal. Toxicol. 21 (1997) 521.
[50] K.A. Bean, J.D. Henion, J. Chromatogr. B 690 (1997) 65.
[51] L.D. Bowers, Sanaullah, J. Chromatogr. B 687 (1996) 61.
[52] C.H.L. Shackleton, H. Chuang, J. Kim, X. De La Torre, J. Segura, Steroids 62 (1997) 523.
[53] D.E. Moody, J.D. Laycock, A.C. Spanbauer, D.J. Crouch, R.L. Foltz, J. Anal. Toxicol. 21 (1997) 406.
[54] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, J.L. Dupuy, M.F. Dreyfuss, G. Lachâtre, Analusis 24 (1996) 104.
[55] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, J.L. Dupuy, G. Lachâtre, J. Anal. Toxicol. 21 (1997) 160.
[56] A. Tracqui, P. Kintz, V. Cirimele, F. Berthault, P. Mangin, B. Ludes, J. Anal. Toxicol. 21 (1997) 314.
[57] M.F. Sauvage, P. Marquet, A. Rousseau, J. Buxeraud, C. Raby, G. Lachâtre, J. Liq. Chromatogr. 21 (1998) 3173–3185.
[58] E.C. Horning, D.I. Carroll, I. Dzidic, K.D. Haegele, M.G. Horning, R.N. Stillwell, J. Chromatogr. 99 (1974) 13.
[59] F. Susanto, H. Reinauer, Fresenius J. Anal. Chem. 357 (1997) 1202.
[60] G.M. Shenfield, J.S. Boutagy, C. Webb, Ther. Drug Monit. 12 (1990) 393.
[61] M.J. Bogusz, R.D. Maier, K.D. Kruger, W. Fruchtnicht, J. Chromatogr. B 713 (1998) 361.
[62] M.J. Bogusz, J. Anal. Toxicol. 21 (1997) 246.
[63] H. Kanazawa, Y. Konishi, Y. Matsushima, T. Takahashi, J. Chromatogr. A 797 (1998) 227.
[64] G.C. Rossi, G.P. Brown, L. Leventhal, K. Yang, G.W. Pasternak, Neurosci. Lett. 216 (1996) 1.
[65] M. Nishikawa, K. Nakajima, K. Igarashi, F. Kasuya, M. Fukui, H. Tsuchihahshi, Jpn. J. Toxicol. Environ. Health 2 (1992) 121.
[66] M.J. Bogusz, R.D. Maier, M. Erkens and S. Driessen. J. Chromatogr. B 703 (1997) 115.
[67] P. Zuccaro, R. Ricciarello, S. Picini, R. Pacifici, I. Altieri, M. Pellegrini, G. D'Ascenzo, J. Anal. Toxicol. 21 (1997) 268.
[68] R. Pacifici, S. Pichini, I. Altieri, A. Caronna, A.R. Passa, P. Zuccaro, J. Chromatogr. B 664 (1995) 329–334.
[69] N. Tyrefors, B. Hyllbrant, L. Ekman, M. Johansson, B. Langström, J. Chromatogr. A 729 (1996) 279.
[70] M. Zheng, K.M. McErlane, M.C. Ong, J. Pharm. Biomed. Anal. 16 (1998) 971.
[71] A.H.B. Wu, Y.J. Feng, A. Pajor, T.G. Gornet, S.S. Wong, E. Forte, J. Brown, J. Anal. Toxicol. 21 (1997) 181.
[72] J.T. Cody, S. Valtier, J. Anal. Toxicol. 21 (1997) 459.
[73] J. Röhrich, S. Zörntlein, J. Lotz, J. Becker, T. Kern, C. Rittner, J. Anal. Toxicol. 22 (1998) 393.
[74] A. Verstraete, Toxicorama 10 (1998) 122–125.
[75] S.J. Salamone, Z. Li, A.J. McNally, S. Vitone, R.S. Wu, J. Anal. Toxicol. 21 (1997) 492.
[76] H.K. Lim, D. Andreyak, P. Francom, R.L. Foltz, Anal. Chem. 60 (1988) 1420.
[77] C.C. Nelson, R.L. Foltz, Anal. Chem. 64 (1992) 1578.
[78] G.S. Rule, J.D. Henion, J. Chromatogr. 582 (1992) 103.
[79] J. Cai, J. Henion, J. Anal. Toxicol. 20 (1996) 27.
[80] S.A. White, T. Catterick, M.E. Harrison, D.E. Johnston, G.D. Reed, K.S. Webb, J. Chromatogr. B 689 (1997) 335.
[81] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, J.L. Dupuy, G. Lachâtre, J. Anal. Toxicol. 692 (1997) 329.
[82] United Nations International Drug Control Programme, United Nations, Vienna, 1998.
[83] M.F. Sauvage, P. Marquet, S. Ragot, F. Lachâtre, J.L. Dupuy, G. Lachâtre, Toxicorama 10 (1998) 73.
[84] J. de Kanel, W.E. Vickery, B. Waldner, R.M. Monahan, F.X. Diamond, J. Forens. Sci. 43 (1998) 622.
[85] J. Girault, B. Istin, J.M. Malgouyat, A.M. Brisson, J.B. Fourtillan, J. Chromatogr. 564 (1991) 43.
[86] D.J. Liberato, A.L. Yergey, N. Esteban, C.E. Gomez-Sanchez, C.H.L. Shackleton, J. Steroid Biochem. 27 (1987) 61.
[87] S.-J. Park, Y.-J. Kim, H.-S. Pyo, J. Park, J. Anal. Toxicol. 14 (1990) 102.
[88] S. Steffenrud, G. Maylin, J. Chromatogr. 577 (1992) 221.
[89] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. B 692 (1997) 7.
[90] A. Polettini, G. Marrubini-Bouland, M. Montagna, J. Chromatogr. B 713 (1998) 339.

[91] S. RizeaSavu, L. Silvestro, A. Haag, F. Sörgel, J. Mass Spectrom. 31 (1996) 1351.
[92] P.R. Tiller, J. Cunniff, A.P. Land, J. Schwartz, I. Jardine, M. Wakefield, L. Lopez, J.F. Newton, R.D. Burton, B.M. Folk, D.L. Buhrman, P. Price, D. Wu, J. Chromatogr. A 771 (1997) 119.
[93] D.A. Volmer, J.P.M. Hui, Rapid Commun. Mass Spectrom. 11 (1997) 1926.
[94] H.H. Maurer, J. Chromatogr. 580 (1992) 3–41.
[95] K. Pfleger, H.H. Maurer, A. Weber, Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites, 2nd ed, VCH Cerlagsgesellsschaft, Weinheim, 1992.
[96] C. Köppel, J. Tenczer, J. Am. Soc. Mass Spectrom. 6 (1995) 995–1003.
[97] A. Polettini, A. Groppi, C. Vignali, M. Montagna, J. Chromatogr. B 713 (1998) 265–279.
[98] W.E. Lambert, E. Meyer, A.P. De Leenheer, J. Anal. Toxicol. 19 (1995) 73–78.
[99] R.D. Maier, M. Bogusz, J. Anal. Toxicol. 19 (1995) 79–83.
[100] Y. Gaillard, G. Pépin, J. Chromatogr. A 763 (1997) 149–163.
[101] C.K. Lai, T. Lee, K.M. Au, A.Y.W. Chan, Clin. Chem. 43 (1997) 312–325.
[102] S.P. Elliott, K.A. Hale, J. Anal. Toxicol. 22 (1998) 279–289.
[103] P. Marquet, J.L. Dupuy, G. Lachâtre, B. Shushan, E. Duchoslav, C. Monasterios, P. Ilisieu, J. Anacleto, in: Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando, FL, 31 May–4 June, 1998.
[104] H.H. Maurer, T. Kraemer, O. Levinka, C.J. Schmitt, A.A. Weber, J. Chromatogr. B 689 (1997) 81–89.
[105] L.D. Bowers, Clin. Chem. 43 (1997) 1299–1304.
[106] C. Liu, L.D. Bowers, J. Chromatogr. B 687 (1996) 213–220.
[107] C. Liu, L.D. Bowers, J. Mass Spectrom. 32 (1997) 33–42.
[108] P. Laidler, D.A. Cowan, R.C. Hider, A. Keane, A.T. Kicman, Rapid Commun. Mass Spectrom. 9 (1995) 1021–1026.
[109] C.H.L. Shackleton, A.M. Falick, B.N. Green, H.E. Witkowska, J. Chromatogr. 562 (1991) 175–190.
[110] M. Sakairi, Y. Kato, J. Chromatogr. A 794 (1998) 391–406.
[111] P.R. Tiller, J. Cunniff, A.P. Land, J. Schwartz, I. Jardine, M. Wakefield, L. Lopez, J.F. Newton, R.D. Burton, B.M. Folk, D.L. Buhrman, P. Price, D. Wu, J. Chromatogr. A 771 (1997) 119–125.
[112] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811–816.
[113] R.S. Plumb, R.D.M. Gray, C.M. Jones, J. Chromatogr. B 694 (1997) 123–133.
[114] S.L. Callejas, R.A. Biddlecombe, A.E. Jones, K.B. Joyce, A.I. Pereira, S. Pleasance, J. Chromatogr. B 718 (1998) 243–250.
[115] H. Simpson, A. Berthemy, D. Buhrman, R. Burton, J. Newton, M. Kealy, D. Wells, D. Wu, Rapid Commun. Mass Spectrom. 12 (1998) 75–82.
[116] R.A.M. van der Hoeven, A.J.P. Hofte, M. Frenay, H. Irth, U.R. Tjaden, J. van des Greef, A. Rudolphi, K.S. Boos, G. Marko Varga, L.E. Edholm, J. Chromatogr. A 762 (1997) 193–200.
[117] E.A. Hogendoorn, P. van Zoonen, A. Polettini, G.M. Bouland, M. Montagna, Anal. Chem. 70 (1998) 1362–1368.
[118] S.R. Needham, M.J. Cole, H.G. Fouda, J. Chromatogr. B 718 (1998) 87–94.
[119] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallet, R.S. Plumb, Rapid Commun. Mass Spectrom. 11 (1997) 1953–1958.
[120] P. Krien, G. Devant, M. Hardy, J. Chromatogr. 251 (1982) 129–139.
[121] L.J. Deterding, M.A. Moseley, K.B. Tomer, J.W. Jorgenson, J. Chromatogr. 554 (1991) 73–82.