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Review

On-line high-performance liquid chromatography–fast atom bombardment mass spectrometry in forensic analysis

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Abstract

Various types of on-line high-performance liquid chromatography (HPLC)–mass spectrometry (MS) have been developed for polar, non-volatile or thermolabile compounds unsuitable for gas chromatography (GC)–mass spectrometry (MS). On-line systems of HPLC–fast atom bombardment (FAB) MS are now gaining popularity in forensic laboratories. In this paper, HPLC–FAB-MS systems are classified according to the inner diameter (I.D.) of the HPLC columns used, and each group is reviewed with respect to analyses for drugs, poisons or their metabolites. The coupling of capillary (I.D. 0.2–0.5 mm) HPLC with FAB-MS is especially recommended, because a high level of sensitivity can be achieved as splitting is not necessary. A special column-switching device with a large-volume (500 μl) injection loop before introduction to the capillary HPLC column is useful for obtaining even higher sensitivity. Such a combination has allowed the identification of very low (therapeutic) levels of drugs or their metabolites in serum or plasma. Octadecyl reversed-phase cartridges are used for clean-up of samples with high protein contents. In the FAB-MS system, both an internal quasi-molecular peak and adequate numbers of fragment peaks, which are useful for the determination of molecular mass or for the final identification of a compound, respectively, can be obtained from many polar compounds. This is one of the significant advantages of HPLC–FAB-MS analysis over most other HPLC–MS systems. In addition, this paper briefly mentions some other HPLC–MS interfaces, such as thermospray, monodisperse aerosol generation interface, atmospheric pressure chemical ionization and electrospray.

Contents

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1. Introduction

The use of fast atom bombardment (FAB) as an ionization technique to obtain the mass spectra of polar, involatile or thermolabile compounds has become universally accepted in the short time since its development in 1981 for a quadrupole mass spectrometer [1] and for a double-focusing magnetic mass spectrometer [2]. The combination of high-performance liquid chromatography (HPLC) with FAB mass spectrometry (MS) has been a very challenging and difficult task because FAB-MS can accept a rate of introduction of HPLC column effluent of not more than 10 μl/min. Off-line methods [3–10], *i.e.*, concentration of HPLC fractions followed by direct inlet FAB-MS, have been widely used in analyses for polar or thermolabile compounds in various fields. However, collecting and drying HPLC fractions in off-line measurements is time consuming and often results in a considerable loss of sample. On-line HPLC–FAB-MS of a compound provides significant advantages over off-line methods in that mass spectral analysis of a compound can be completed within the time of its HPLC separation, and intense peaks due to the matrix can be subtracted by the use of a computer system.

Many efforts have been made to establish on-line HPLC–FAB-MS. In particular, two forms of interfaces coupling them have been developed: "frit-FAB" [11–17] and "continuous-flow FAB" (CF-FAB) [18–27]. Both interfaces utilize a fixed silica capillary to transport the effluent from a HPLC column to its tip. In the former, the capillary end is attached to a stainless-steel mesh, or frit, used as a FAB target [11]; that of the latter terminates without any cap in the ionization chamber of the mass spectrometer with a special device [18].

Most applications of HPLC–FAB-MS using either interface have been made for analyses of biological materials such as peptides [15,16,18–21,23–25], bile acids [11,12] and oligosaccharides [13]. Recently some applications of the system have been reported for drugs [22,23,25], poisons [16,26] and their metabolites [17,27].

We have been establishing highly sensitive analyses for drugs and their metabolites in human sera or plasma by capillary HPLC–frit-FAB-MS utilizing a special column-switching device for injection and concentration of samples [28–32]. Hattori *et al.* [33] devised a similar system and applied it to the determination of quinolone antimicrobials in human plasma. Thus the application of HPLC–FAB-MS in forensic analysis has only recently started.

In this paper, we classify HPLC columns into five categories according to their inner diameters (I.D.) as described in the next section and review each category of HPLC coupled with FAB-MS using either a frit-FAB or CF-FAB interface,

especially for analyses for drugs, poisons and their metabolites. In addition, we briefly review some other forms of interfaces that directly couple HPLC with MS. The usefulness of various types of HPLC–MS systems in forensic analysis is also discussed.

2. Interfaces for high-performance liquid chromatography–fast atom bombardment mass spectrometry

2.1. Frit interface

In 1985, Ito *et al.* [11] first developed an HPLC to FAB-MS interface coupling, in which a porous stainless-steel frit was used as a FAB target. This form of interface was named "frit-FAB" [12–14].

Fig. 1 shows the tip parts of the frit-FAB interface used in a JEOL (Tokyo, Japan) HPLC–MS system [11–14]. The solvent of the HPLC column effluent (3–7 μl/min) is immediately vaporized on the surface of the frit where the solute and matrix are concentrated, and bombarded with energized Xe atoms [11–14]. In this way, a conventional FAB process takes place continuously in the thin liquid film formed on the target. The frit is warmed sufficiently to prevent freezing, and the ion source temperature is maintained at 50–60°C to produce stable ion currents. Glycerol is the most common matrix; thioglycerol or other viscous fluids are also used depending on the analyte compound.

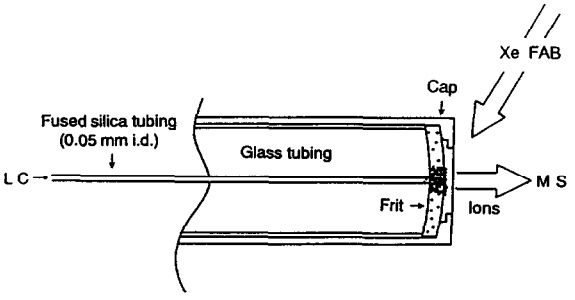

Fig. 1. Schematic illustration of ion generation in frit-FAB interface used in JEOL MS instruments (from Dr. K. Matsuura, with permission of JEOL).

2.2. Continuous-flow interface

In 1986, Caprioli *et al.* [18] developed a sample probe that allowed a continuous flow of solution to be introduced into a FAB ion source and applied it to combination of HPLC with FAB-MS [19]. Some improvements [20,21] were subsequently made and this form of interface was termed "CF-FAB".

Fig. 2 shows a schematic illustration of the CF-FAB probe used in a Shimadzu (Kyoto, Japan)–Kratos system, which is based on the probe of Caprioli *et al.* [18]. The effluent containing the solute and matrix does not evaporate in the capillary tube but forms a small droplet on the "target" at the end of the capillary in the ion source of the mass spectrometer (Fig. 2). The energized Xe atoms directly bombard the liquid droplet and a conventional FAB process takes place [19]. The source temperature is *ca.* 50°C.

Recently, Iida [34] indicated that collection of the remainder of the matrix causing chromatographic broadening occurred at the tip of the customary CF-FAB probe without any cap (Fig. 2), and that altering the source temperature and solvent flow did not eliminate the problem of tailing. Hence she presented a modified probe, shown in Fig. 3. This modification, in which a metallic mesh with a stainless-steel cap was fixed at the tip of the capillary, minimized broadening of the peak significantly [34]. Some other researchers [25,26] also indicated the same problem for the customary CF-FAB interface and modified it for their own uses. Shimadzu–Kratos applied the new probe modified by Iida [34] to

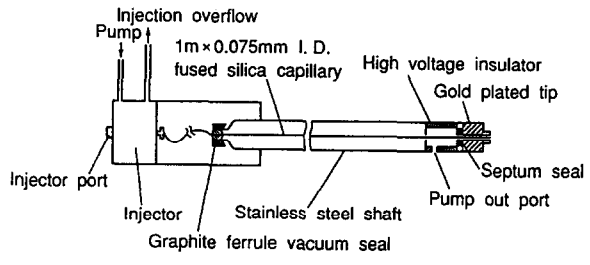

Fig. 2. CF-FAB probe used in Shimadzu–Kratos MS instruments (from Dr. J. Iida, with permission of Shimadzu and Kratos).

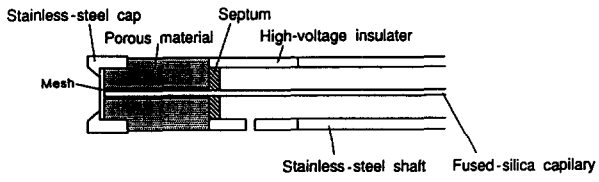

Fig. 3. New CF-FAB probe used in Shimadzu–Kratos MS instruments (from Dr. J. Iida, with permission of Shimadzu and Kratos).

their HPLC–CF-FAB-MS system. Interestingly, the new probe resembles the frit-FAB interface (Fig. 1) in that the capillary end is attached to a metallic mesh.

3. Classification of high-performance liquid chromatographic columns

In HPLC analysis, 4–6 mm I.D. columns packed with 3–30-μm materials have mostly been employed. However, research on miniaturization of the column dimensions in HPLC is continuing [35]. Thus, various dimensions of HPLC columns are now commercially available, and a suitable size for each purpose can be selected. Ishii *et al.* [35] classified HPLC columns into five categories according to their I.D., as listed in Table 1.

In recent reports [17,19,21,23,27], 1 mm I.D. columns have been called "microbore", although Ishii *et al.* [35] termed them "semi-micro". Similarly, 0.25–0.5 mm I.D. columns have been termed "capillary" [15,23,28–33]. Also, Moseley *et al.* [25] called their 0.05 mm I.D. column a "packed microcapillary" column. According to these papers, we classified HPLC systems into five groups to avoid confusion in this paper, and these are also listed in Table 1.

4. Conventional high-performance liquid chromatography–fast atom bombardment mass spectrometry

In analysis by on-line HPLC–FAB-MS, the flow-rate of the mobile phase introduced into the mass spectrometer is limited to 1–10 μl/min. When conventional HPLC is coupled with FAB-MS, most HPLC effluents must be split, and only a small fraction of the effluent can be introduced to the mass spectrometer, causing a marked sample loss. Kondo *et al.* [16] applied conventional HPLC–frit-FAB-MS to the separation and identification of microcystins in cyanobacteria. Microcystins RR, YR and LR were isolated and identified from lake surface blooms. The detection limits were reported to be 1 μg in injected amounts. This relatively low sensitivity originates in the postcolumn splitting (4:500); nevertheless, this application made it possible to shorten analysis times greatly.

5. Microbore high-performance liquid chromatography–fast atom bombardment mass spectrometry

Microbore (1 mm I.D.) HPLC–FAB-MS has been used mostly in molecular biology [19–21,23], with the CF-FAB interface. However, this does not mean that only the CF-FAB inter-

Table 1
Classification of HPLC columns according to their I.D.

Classification by Ishii *et al.* [35]	I.D. (mm)	Classification in this paper	I.D. (mm) cited here	Refs.
Densely packed columns:				
Conventional column	4–6	Conventional column	4	16
Semi-micro column	1–2	Microbore column	1	17, 19–21, 23, 27
Micro column	0.2–0.5	Capillary column	0.25–0.5	11–15, 23, 28–33
Loosely packed column	0.05–0.2	Microcapillary column	0.075	26
Open-tubular column	0.01–0.05	Open-tubular column	0.01	25

face is suitable for such analyses; the combination of microbore HPLC column with frit-FAB-MS is possible with postcolumn splitting. No reports dealing with forensic analysis by microbore HPLC–FAB-MS are available, to our knowledge. In other fields (pharmacological and environmental), a few studies have been conducted in recent years dealing with analyses of drugs or metabolites using HPLC–MS [17,23, 27].

Although Teffera *et al.* [5] succeeded in the FAB-MS of benzo[*a*]pyrene sulphate conjugates from benzo[*a*]pyrene-treated cells using the off-line method, they could not obtain good-quality full-scan mass spectra for the same compounds by on-line microbore HPLC–CF-FAB-MS using 1:6 splitting [27]. Fujiwara *et al.* [17] reported that some *in vitro* metabolites of chloroacetanilide by rat liver enzyme gave quasi-molecular peaks in microbore HPLC–frit-FAB-MS using a 1:10 split, but that structural analysis was not possible owing to the minimal fragmentation obtained. The postcolumn splitting results in a significant loss of sensitivity even in microbore HPLC–FAB-MS.

6. Capillary high-performance liquid chromatography–fast atom bombardment mass spectrometry

Until recently, capillary (0.25–0.5 mm I.D.) HPLC–frit-FAB-MS has been used mostly in molecular biology [11–13,15]. In this section, applications of capillary HPLC–frit-FAB-MS to drug analyses by our group [28–32] and Hattori *et al.* [33] are described in detail. Both groups utilize an on-line high-pressure precolumn concentration method and a special column-switching device for highly sensitive analysis.

6.1. Capillary high-performance liquid chromatographic system

An outline of the capillary HPLC system used by our group [28–32] is shown in Fig. 4. This system is a modification of the method of Takeuchi *et al.* [36].

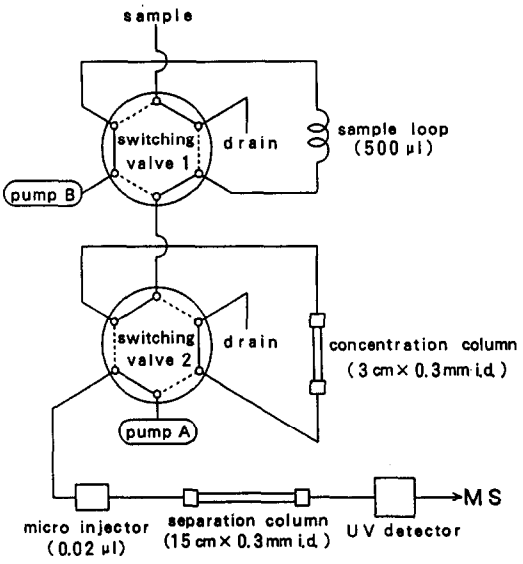

Fig. 4. Capillary HPLC system used in our research (from ref. 28, with permission of the Japanese Association of Forensic Toxicology).

Our system consists of two pumps, A and B. Pump A (micrometric pump; LDC/Milton Roy, Riviera Beach, FL, USA) is used to deliver mobile phase A at a rate of 4 µl/min to the capillary separation column. Pump B, a conventional pump (880-PU; Jasco, Tokyo, Japan), is used to send sample fluid to the concentration column with mobile phase B at a rate of 0.05–0.1 ml/min. A capillary separation column packed with 5-µm Develosil ODS or 5-µm Develosil PhA packing material and a concentration column packed with 15–30-µm Develosil ODS or 10-µm Develosil PhA packing material were purchased from Nomura Chemical (Seto, Aichi, Japan). The composition of mobile phases A and B varied from sample to sample [28–32]. Both mobile phases always contained 0.5% glycerol as matrix. This matrix concentration was much lower than that in most previous reports (5–20%).

The injection system (Model 7012; Rheodyne, Cotati, CA, USA) incorporates a 500-µl fixed load with a tubing dead volume of 50 µl. Accurate volumes of analyte (100–500 µl) are injected into the sample loop followed by deionized water (at least 50 µl) to fill the dead

volume. The compound to be analysed in the 100–500-μl sample is sent to the separation column without any loss after the purification and concentration process by our system, and the entire effluent is introduced to the frit-FAB interface without any splitting. All the tubing used to connect various sections of the HPLC system are of 0.05 mm I.D. A variable-wavelength UV detector (Jasco 875-UV) monitors the effluent prior to its introduction to the MS instrument. In this HPLC system, some cephalosporins could be determined with a tenfold increase in sensitivity [37] over our conventional HPLC procedure [38].

The HPLC system devised by Hattori *et al.* [33] is similar to ours except that it utilizes precolumn splitting for gradient formation. The details of the system have been described in their recent paper [33].

6.2. Mass spectrometric conditions

Both our group and Hattori *et al.* use a JMS-DX303 double focusing MS instrument fitted with a FAB ion source (MS-FAB 09A) and a JMA-5000 computer-controlled data analysis systems (JEOL, Tokyo, Japan). Xenon is used for generation of the fast atom beam with a gun current of 10 mA and a voltage of 3 keV. The mass spectrometers are scanned over the mass range m/z 50–800 for 5 s. Determination is made in the positive mode.

6.3. Application of capillary high-performance liquid chromatography–frit-fast atom bombardment mass spectrometry

6.3.1. Benzodiazepines

Benzodiazepines, which have hypnotic, tranquillizing and antiepileptic properties, are used worldwide, and are therefore very frequently encountered in forensic analysis.

Chlordiazepoxide is one of the typical and classical benzodiazepines, and is still used extensively. This drug, after its absorption is rapidly metabolized by demethylation and deamination to desmethylchlordiazepoxide and demoxepam, respectively. The latter is further metabolized to desmethyldiazepam, followed by hydroxylation to oxazepam [39]. Chlordiazepoxide and its metabolites were reported to be thermolabile and decomposed during capillary gas chromatography (GC) [40], and thus they have been mainly determined by HPLC [39].

We have reported that chlordiazepoxide and its metabolites could be identified in human serum after oral administration of chlordiazepoxide by capillary HPLC–frit-FAB-MS (Fig. 4) with high sensitivity [29]. A blood sample was drawn from a psychiatric in-patient receiving 30 mg of chlordiazepoxide and 500 mg of carbamazepine four times daily, in the morning before breakfast (12 h after the last administration of the drugs). Chlordiazepoxide and its metabolites were isolated from 0.5 ml of serum using a Bond-Elut C_{18} cartridge (100 mg/ml) (Varian, Harbor City, CA, USA) according to the method of Good and Andrews [41] with a minor modification [28]. The recoveries of chlordiazepoxide, desmethyldiazepam and oxazepam that had been added to blank sera were more than 90% with the Bond-Elut C_{18} isolation procedure.

Fig. 5 shows mass chromatograms with channels at m/z 271, 286, 287 and 300 for a patient's serum sample. The location of drug peaks is also indicated. The peaks due to both chlordiazepoxide and desmethylchlordiazepoxide appeared incompletely separated at a retention time of 17–18 min on the channel at m/z 286. A large peak

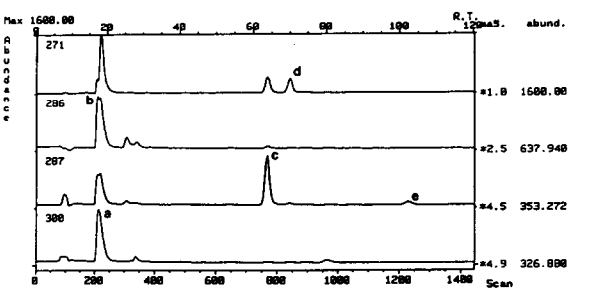

Fig. 5. FAB mass chromatograms for the extract from a 0.5-ml serum sample from a patient receiving 30 mg of chlordiazepoxide daily. (a) Chlordiazepoxide; (b) desmethylchlordiazepoxide; (c) demoxepam; (d) desmethyldiazepam; (e) oxazepam (from ref. 29, with permission of the Japanese Association of Forensic Toxicology). R.T. = Retention time in min.

appeared on the channel of m/z 271 at a similar retention time to that of chlordiazepoxide. By mass spectral measurements (data not shown), the peak was identified as a diol metabolite of epoxycarbamazepine, the major metabolite of carbamazepine [42]. Demoxepam, desmethyldiazepam and oxazepam were well separated under our conditions.

Fig. 6 shows the results of FAB-mass spectral measurements at each retention time, obtained from the serum of the patient, after appropriate subtraction of the background and interfering peaks appearing in the mass chromatograms (Fig. 5) by the use of a computer system. Chlordiazepoxide, desmethyldiazepam and oxazepam in the sample could finally be identified by comparison with the corresponding mass spectra of the authentic compounds [29]. For desmethylchlordiazepoxide and demoxepam, their identity was obvious because of the appearance of $[M+H]^+$ and $[MH-O]^+$ peaks together with each isotopic peak due to the chlorine group, although their authentic compounds were not available. The analysis of the fragmentation, shown in Fig. 7, also supports their identity in the sample.

The detection limits with respect to the mass spectral measurements were 2–5 ng on-column (corresponding to 4–10 ng/ml in serum) for chlordiazepoxide, 0.5–1 ng (2–4 ng/ml) for desmethyldiazepam and 1–2 ng (2–4 ng/ml) for oxazepam. Plasma concentrations of chlordiazepoxide and its metabolites in a patient receiving 30 mg of chlordiazepoxide daily were reported to be 100–200 ng/ml for chlordiazepoxide, desmethylchlordiazepoxide, demoxepam and desmethyldiazepam and 20–60 ng/ml for oxazepam [39]. Hence our system proved to be sufficiently sensitive to determine chlordiazepoxide and its metabolites in human serum or plasma.

Triazolam is a relatively new type of short-acting benzodiazepine hypnotic that is widely used. Its therapeutic doses are as low as 0.125–0.25 mg and hence its therapeutic concentration in serum was reported to be 2–20 ng/ml [43]. With our capillary HPLC–frit-FAB-MS system, triazolam could actually be identified by mass spectral measurements in the extracts from 0.5 ml of serum from two adults who had taken only one 0.25-mg tablet of the drug 1 h before sampling [28]. The recovery of triazolam, which had been added to blank serum, was more than 95% with the Bond-Elut C_{18} isolation procedure. The detection limit was 1–2.5 ng on-column, corresponding to 2–5 ng/ml in serum [28].

Our HPLC–frit-FAB-MS system, with a capillary column of 0.3 mm I.D. and a large injection capacity (Fig. 4), has permitted mass spectral measurements of as little as 0.5–5 ng (on-column) of a benzodiazepine and/or its metabolites. The sensitivity of HPLC–FAB-MS was almost comparable to that of the GC–MS with selected-ion monitoring (SIM) procedure of Hattori *et al.* [44], in which a wide-bore capillary column was used instead of a medium-bore capillary column to prevent thermolabile benzodiazepines from decomposing.

6.3.2. Cephalosporins

Medical accidents due to shock following the use of cephalosporin antibiotics have been reported [45], although such cases are becoming less frequent nowadays. Cephalosporins are polar, involatile, thermolabile and thus not suitable for analysis by conventional GC–MS in their underivatized forms. FAB-MS analyses of cephalosporins using direct inlet methods have been reported [46,47].

We have reported that cefaclor, one of the most popular cephalosporins, can be identified in human serum after oral administration by capillary HPLC–frit-FAB-MS (Fig. 4) [31]. Blood samples were taken from two adults 2 h after oral administration (with food) of only one 250-mg capsule of the drug. Cefaclor was isolated from the 0.5 ml of serum using Bond-Elut C_{18} cartridges (200 mg per 3 ml) (Varian) according to the manufacturer's manual with a minor modification [31]. The recovery of the drug, which had been added to blank serum, was $101.4 \pm 5.2\%$ for the isolation procedure. Both mass spectra obtained from sera from two different subjects were almost identical with that of authentic cefaclor. In another paper, we reported that cephalexin and cephaloridine, two of the most common cephalosporins, could also be

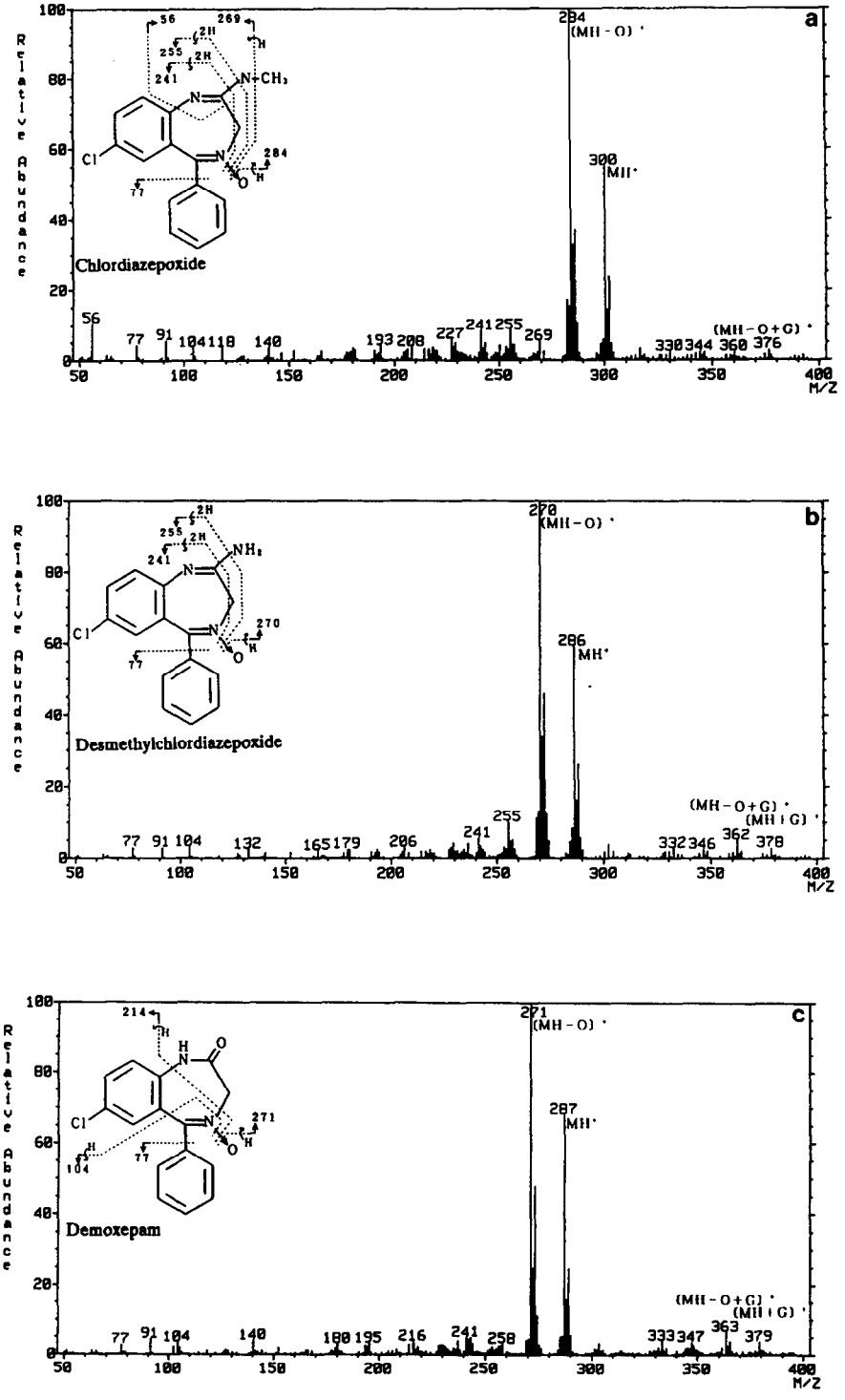
a
Chlordiazepoxide
284 (MH-O)·
300 MH·
(MH-O+G)·
b
Desmethylchlordiazepoxide
270 (MH-O)·
286 MH·
(MH-O+G)·
(MH+G)·
c
Demoxepam
271 (MH-O)·
287 MH·
(MH-O+G)·
(MH+G)·
Relative Abundance
M/Z

Fig. 6.

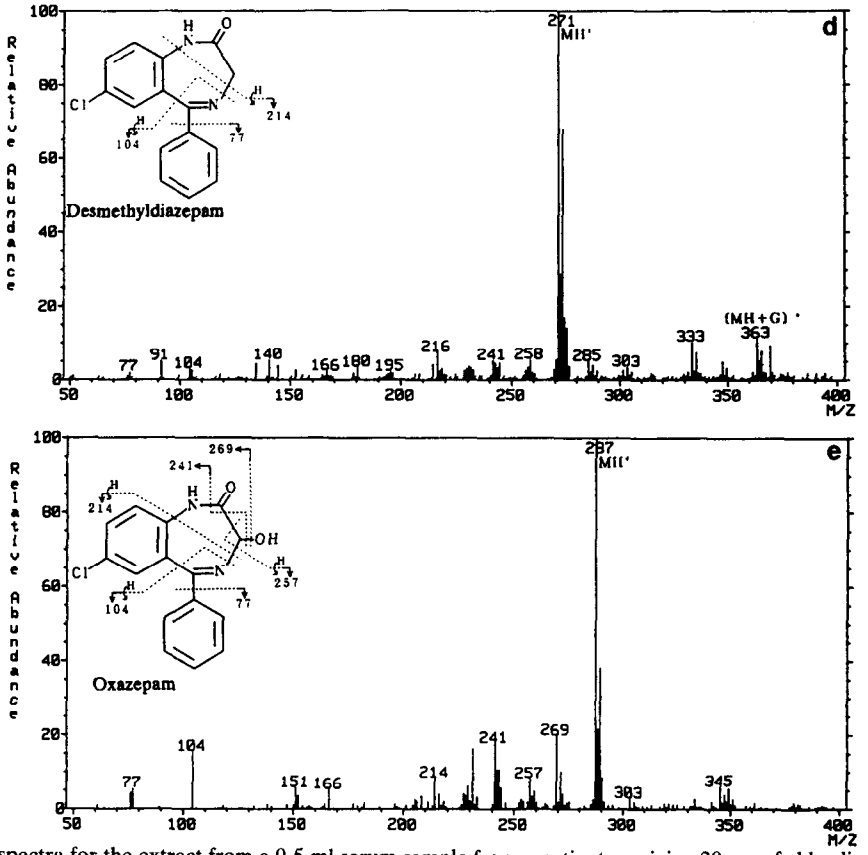

Fig. 6. FAB mass spectra for the extract from a 0.5-ml serum sample from a patient receiving 30 mg of chlordiazepoxide daily. (a) Chlordiazepoxide (M_r 299); (b) desmethylchlordiazepoxide (M_r 285); (c) demoxepam (M_r 286); (d) desmethyldiazepam (M_r 270); (e) oxazepam (M_r 286) (from ref. 29, with permission of the Japanese Association of Forensic Toxicology).

identified in human plasma spiked with 2 μg/ml of each drug by the same system [30]. For the three compounds examined in both studies [30,31], the quasi-molecular $[M+H]^+$ ions appeared as intense peaks; cations due to cleavage of the side-chains in the α-position to the carbonyl group gave the base peaks (m/z 106 for cefaclor and cephalexin and m/z 97 for cephaloridine). For cephaloridine, an intense peak due to liberation of the pyridinyl ring was also observed.

The detection limits for their mass spectral measurements were 10–20 ng on-column for cephalexin [30], 25–50 ng on-column for cefaclor [31] and 50–100 ng for cephaloridine [30]. The sensitivity for the cephalosporins (10–100 ng on-column detection limits) is almost comparable to that for quinolone antimicrobials (10–20 ng on-column) reported by Hattori *et al.* [33], but is one order of magnitude lower than that for the benzodiazepines (0.5–5 ng on-column) [28,29] or that for seventeen phenothiazines (0.25–10 ng on-column) [32]. This may be due to the difference in cation formation between basic and acidic compounds [30].

6.3.3. *Phenothiazines*

Many phenothiazine derivatives have been synthesized and are widely used as antipsychotics, antiparkinsonian drugs or antihistaminics. These drugs are also often encountered in forensic analyses. Recently, Ishikawa *et al.* [48] reported determinations of nineteen phenothiazines by GC–MS utilizing a wide-bore capillary column, and found that six of the compounds were not detectable. Most of the ther-

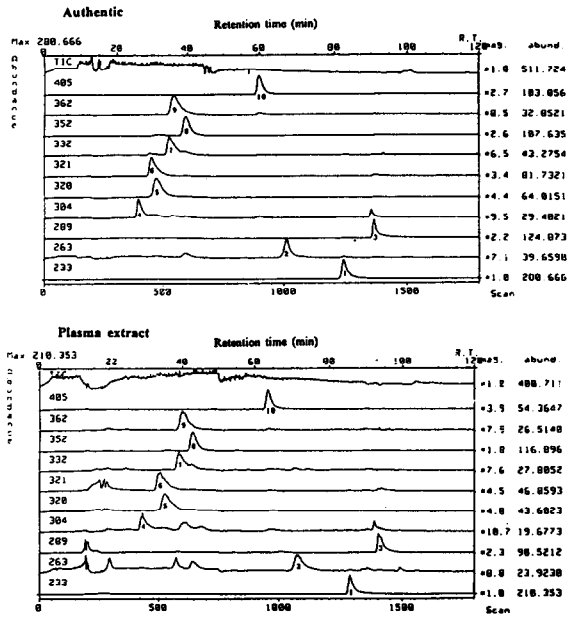

Fig. 7. FAB mass chromatograms for the authentic quinolone antimicrobials (top) and for spiked plasma (bottom). 1 = Nalidixic acid; 2 = cinoxacin; 3 = piromidic acid; 4 = pipemidic acid; 5 = norfloxacin; 6 = enoxacin; 7 = ciprofloxacin; 8 = lomefloxacin; 9 = ofloxacin; 10 = tosufloxacin. The mixture of the ten quinolone antimicrobials (1 μg of each) was added to 1 ml of plasma; a 300-μl aliquot including about 100 ng each drug of the final supernatant was injected (from ref. 33, with permission of the Japanese Association of Forensic Toxicology).

molabile phenothiazines possess long piperazinyl side-chains and thus seem unsuitable for GC–MS analysis.

We have reported a preliminary study of the determination of seventeen phenothiazines using our system [32]. Quasi-molecular peaks $[M+H]^+$ along with adequate fragment peaks were detected for all compounds with detection limits of 0.25–10 ng on-column.

Human sera obtained from four different subjects receiving chlorpromazine, levomepromazine, promethazine and propericiazine, four of the most common phenothiazines, were also analysed with our system [32]. Bond-Elut C_2 cartridges (100 mg/ml) (Varian) were effective for the isolation of the drugs and their metabolites from the sera. For all four drugs including propericiazine, having long piperazinyl side-chains, the parent drugs and their oxide compounds could be identified in the extracts from 0.5 ml of sera after oral administration of therapeutic amounts of the drugs.

6.3.4. Quinolones

Quinolone carboxylic acid antimicrobials are one of the most popular drug groups for the treatment of bacterial infection. They are usually determined by HPLC because of their high polarity and thermolability [49,50]. It has been reported that concomitant administration of enoxacin and fenbufen induced severe convulsions in several cases [51]. This may be true for other quinolones if they are administered together with fenbufen.

Hattori *et al.* [33] presented a detailed procedure for the detection of ten quinolone antimicrobials in human plasma by capillary HPLC–frit-FAB-MS, in which the capillary HPLC system utilized preinjection splitting for gradient formation. For extraction of the drugs from spiked plasma (1 μg/ml), perchloric acid precipitation was used.

When FAB mass spectra of the ten antimicrobials were determined, the $[M+H]^+$ quasi-molecular ions constituted the base peaks for all compounds. The $[M+2]^+$ peaks were also relatively intense for all compounds. The peaks at m/z M − 17 and M − 43, which probably correspond to MH − H_2O and MH − CO_2, respectively, were also observed for most compounds [33].

Fig. 7 shows mass chromatograms obtained with their capillary HPLC–frit-FAB-MS system for the authentic quinolone drugs (1 μg each) and a plasma sample spiked with the same amount of drugs, with use of each quasi-molecular base peak. With their gradient programme, all drugs could be detected within 100 min.

To check for the quantitative recovery of the drugs by the method, peak-area intensities were plotted against the amounts of drugs on-column for nalidixic acid, tosufloxacin and lomefloxacin as shown in Fig. 8. The calibration graphs were not sufficiently linear, but semi-quantitative. The detection limits for ten drugs were 0.1–0.2 μg/ml in plasma (*ca.* 10–20 ng on-column).

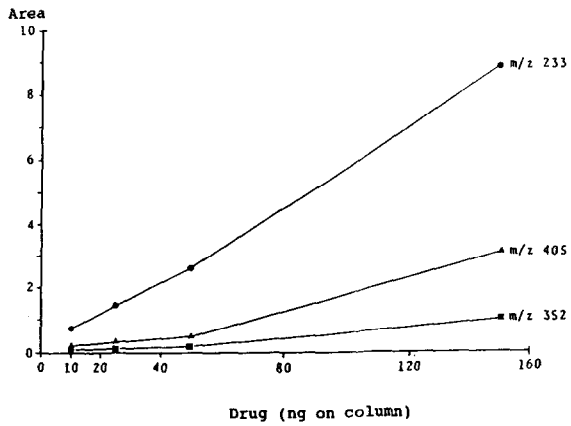

Fig. 8. Calibration graphs for (●) nalidixic acid, (▲) tosufloxacin and (■) lomefloxacin of peak-area intensity *versus* amount of drug on-column obtained by mass chromatography (from ref. 33, with permission of the Japanese Association of Forensic Toxicology).

7. Microcapillary high-performance liquid chromatography–fast atom bombardment mass spectrometry

A microcapillary (0.0075 mm I.D.) HPLC column is loosely packed with 10–30-μm diameter particles embedded inside the column of 0.05–0.2 μm I.D. [35]. Only a few reports [26,52] dealing with coupling of microcapillary HPLC with MS have been found in recent years.

Pawlosky and Mirocha [26] reported the mass spectral analysis and fragment ion structure of fusarochromanone, a toxin produced by the fungus *Fusarium equiseti*, by microcapillary HPLC–CF-FAB-MS. The detection limit of the toxin was 500 pg on-column in the SIM mode; the calibration graph was not sufficiently linear, but semi-quantitative. A relatively good-quality FAB mass spectrum with a thioglycerol matrix was also obtained although no mention was made of the detection limit in the full-scan mode [26].

As this microcapillary HPLC is operated at a flow-rate of 5 μl/min, similar to that of the capillary HPLC system, the coupling of the microcapillary column with our column-switching device (Fig. 4) is possible. This coupling will give even higher sensitivity than that of our own capillary system, because the resolution capability increases with decreasing HPLC column I.D. [35]. However, difficulty in achieving a smooth flow through such narrow columns is to be expected.

8. Open-tubular liquid chromatography–fast atom bombardment mass spectrometry

Open-tubular (OT) columns were originally developed for GC analysis and are now widely used. Similar columns have been tested for HPLC [35]; an OT-LC column is coated with a stationary phase, such as a liquid or finely dispersed solid, on the inside wall without any packing material. Efficient separation requires not only long columns (1–10 m) and very narrow I.D.s (1–20 μm), but also operation at very low flow-rates (5–250 nl/min) [53] with a special device such as a gas pressure regulator connected with mobile phase reservoirs [54]. The low flow-rate permits direct liquid introduction of the effluent into the ionization chamber [53,55]. The direct coupling of OT-LC with MS could be made in the conventional electron impact (EI) and chemical ionization (CI) modes. However, the applicability of these approaches was limited to volatile or less polar compounds [53–56].

In 1988, De Wit *et al.* [24] developed a method to couple OT-LC with CF-FAB using a coaxial CF-FAB probe. This coaxial "capillary within a capillary" interface utilized two fused-silica capillary tubes to deliver the LC effluent and the FAB matrix independently to the CF-FAB probe tip. They applied this interface to couple OT-LC (1.5 m $\times$ 10 μm I.D. column) with CF-FAB-MS for analyses of biomolecules, and reported that full-scan mass spectra of corticosterone and glutathione were obtained from 2.2 to 4.4 ng of the authentic compounds [25]; this OT-LC system was operated at a flow-rate of 53 nl/min with an injection volume of 4.4 nl.

As OT-LC should be performed at very low flow-rates (5–250 nl/min), extremely small volumes of samples can be applied. To our knowledge, no forensic application of this method has

been reported; further studies are required to assess its utility.

9. Other high-performance liquid chromatography–mass spectrometry systems

To date, many forms of interfaces besides frit-FAB and CF-FAB have been developed for directly coupling HPLC with MS. In this section, we briefly review some of them which are widely used, or expected to be useful, in forensic analysis. Excellent reviews [53,57,58] dealing with on-line HPLC–MS (not from forensic viewpoints) have been published recently, and details of other interfaces can be found in them.

9.1. Thermospray

In the early 1980s, Blakley *et al.* [59–61] developed and improved the thermospray (TSP) interface for coupling conventional HPLC with MS. In the TSP interface probe, the HPLC column effluent passes through a heated vaporizer, forming a supersonic jet of vapour with entrained fine droplets. These droplets are electrically charged while travelling through the heated source. As the size of the droplets decreases, the electric field at the liquid surface increases until the ions present in the droplet are ejected. The ions formed in this way subsequently undergo ion–molecule reactions in a similar way to conventional CI [57]. For the TSP process to be efficient, the mobile phase should contain a high percentage of water and electrolyte.

Because TSP is capable of handling flow-rates of HPLC column effluent of 1–2 ml/min, it became the first popular HPLC–MS interface. In spite of the rapid heating in the interface, it usually yields molecular ion adducts with little or no fragmentation even for some of thermolabile compounds [59–61].

Subsequently, the combined use of a discharge electrode and a repeller electrode in the ion source was devised to produce collision-induced dissociation (CID) [62]. In this device, only molecular ion adducts were observed at low repeller voltages (0–65 V), but significant fragmentation could be produced at higher repeller voltages (65–190 V). With such improvements, the TSP interface is still most popular among various HPLC–MS interfaces. Many reports dealing with analyses of relatively high-molecular-mass compounds, such as peptides and phospholipids, by HPLC–TSP-MS have been published in the molecular biology field. Several reports [17,63–65] on analyses of drugs, poisons or their metabolites by the system are also available.

However, some disadvantages of the TSP technique have been indicated [53,57,58]. Careful physical optimization of many parameters in the interface is required to achieve maximum sensitivity. The sensitivity of the technique is compound dependent even when careful optimization is made. Fujiwara *et al.* [17], in analyses of metabolites of a fluorinated herbicide, reported that the amount of the metabolites required for their HPLC–TSP-MS was normally 10–100 times more than that for HPLC–FAB-MS, and that polar compounds often degraded during analysis by HPLC–TSP-MS. In addition, it is not straightforward to use TSP with gradient elution because of the dependence of ion production on solvent composition [58], although this has been eased by the use of either the discharge electrode or a filament in the isocratic mode [53,57,58].

9.2. Monodisperse aerosol generation interface

In 1984, Willoughby and Browner [66] reported the design of a new type of monodisperse aerosol generation interface for chromatography (MAGIC). In this interface, the HPLC effluent passes into the desolvation chamber through a glass orifice to form a liquid jet. This jet is broken up into small droplets due to an orthogonal gas flow, formed by the monodisperse aerosol generation, at atmospheric pressure, at which heat transfer is more efficient. As the drops pass through the desolvation chamber, the solvent rapidly evaporates from the drops. In this way, a high-velocity particle beam having uniform size is formed; this is the basis of the alternative term for the technique, HPLC–par-

ticle beam MS. An aerosol beam separator connects the desolvation chamber to the mass spectrometer ion source. The analyte particles strike the heated source and are then vaporized. The resultant source pressure is approximately 10^{-6} Torr (1 Torr = 133.322 Pa). This type of interface is capable of handling HPLC-flow-rates of up to 1 ml/min, following their improvement [67].

The low source pressure enables the analyst to obtain standard EI spectra which can be compared with spectra in the library of the National Bureau of Standards. CI spectra can also be obtained by standard CI procedures. In these respects, MAGIC will be useful for analyses for volatile compounds in forensic laboratories. Winkler *et al.* [67] reported that the detection limits were 10 ng on-column in the full-scan EI mode and 1 ng for SIM even for a relatively involatile compound (retinol acetate) with their improved HPLC–MAGIC-MS system. However, flash vaporization of the analyte in the source is part of the ion formation process, and hence its sensitivity appears to be low for non-volatile compounds at present [53,57]. As coupling of MAGIC with FAB-MS is now being tried, increased sensitivity even for non-volatile compounds may be realized in the near future.

9.3. *Atmospheric pressure ionization*

As the name indicates, the source of atmospheric pressure ionization (API) instruments is maintained at atmospheric pressure. Although various interfaces utilizing API-MS have been developed, they are divided into two groups in this paper: atmospheric pressure chemical ionization (APCI) and electrospray (ESP).

9.3.1. *Atmospheric pressure chemical ionization*

Pioneering work by Horning *et al.* [68], demonstrating a kind of HPLC–APCI-MS, was first described in 1973. This method utilized ion–molecule reactions which occur in pure nitrogen irradiated by beta particles from ^{63}Ni foil in the ion source, but yielded significant amounts of cluster ions, which interfered with MS analysis.

In 1982, Thomson *et al.* [69] devised HPLC–liquid ion evaporation MS in which a curtain of nitrogen gas prevented non-ionized interferents from entering the mass spectrometer and also disrupted cluster ions. In this device, nebulization of the HPLC effluent without heating is used to produce a fine mist of solvent droplets. When these droplets pass through a needle held at a high voltage, a corona discharge occurs and produces charged droplets, which evaporate and emit ions from the surface. These are then electrically focused through a 100-μm orifice into the high-vacuum analyser region, where the orthogonal curtain of nitrogen gas disrupts cluster ions prior to mass analysis. As ionization occurs in a high-pressure region, CI spectra are obtained. All preionized species appear as protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions, with essentially no fragmentation. Many neutral compounds, such as the anabolic steroid dianabol [70], also appear as protonated ions. This interface is capable of handling the liquid flow-rate of 1 ml/min at which a conventional HPLC system is operated. The sensitivity of the device is reflected by the fact that a single dose of dianabol could be detected as the epi-dianabol metabolite for 16 days [70]. However, this device seems unsuitable for forensic identification because no fragmentation is obtained.

In 1988, Sakairi and Kambara [71] reported a modification of HPLC–APCI-MS that permitted CID analysis in a relatively high-pressure region of a mass spectrometer. In this device, a drift voltage between two apertures in the intermediate vacuum region was applied to increase the efficiency of transmission of ions into the ion source housing and to dissociate cluster ions into quasi-molecular ions. At a low applied voltage (60–100 V), abundant pseudo-molecular ions with a little fragmentation were observed, but significant fragmentation resulting from CID could be produced at a high voltage (130–200 V).

Instead of the pneumatic nebulizer without heating, a heated nebulizer, the temperature of which was controlled up to 400°C, was also used to treat a wide range of HPLC effluent flows (0.1–2.0 ml/min). This system could produce

pseudo-molecular ions of many non-volatile compounds including amines, peptides, antibiotics, steroids, vitamins and alkaloids. The detection limit of the system in the SIM mode was 5 pg for some compounds such as theophylline and caffeine. In addition, the isomers of kanamycin could be differentiated from each other on the basis of their mass spectra obtained at relatively higher drift voltages [71].

Kawasaki *et al.* [72] established the screening and identification of 21 organophosphorus pesticides in blood from patients suffering from acute agricultural chemical toxicity, using a further improved version of the HPLC–APCI-MS system developed by Sakairi and Kambara [71]. In comparison with a GC–MS method, the chemicals indicated a similar specificity and were within equivalent detection limits (100–1000 ng in the full-scan mode, 2–50 ng in the SIM mode).

From the results of Kawasaki *et al.* [72] and Sakairi and Kambara [71], the sensitivity of HPLC–APCI-MS also seems to be compound dependent. This may be partly due to the heating of a sample by the heated nebulizer, which is usually operated around 250°C [73]. In addition, a recent excellent review [73] describing HPLC–APCI-MS in detail, has indicated that APCI-MS still has the propensity to form low-molecular-mass cluster ion adducts even with the use of the CID technique, and that they can interfere with the trace detection of low-molecular-mass compounds ($M_r < 300$). Further studies are needed to show the applicability of HPLC–APCI-MS to forensic analyses, although the usefulness of the system can be expected in the near future.

9.3.2. Electrospray

The production of a fine mist of charged droplets by ESP was first described by Zeleny [74] in 1917. Dole and co-workers described the usefulness of ESP as an HPLC–MS interface in patents [73,75], but the first literature report was published by Yamashita and Fenn in 1984 [75].

The sample is injected into nitrogen gas at atmospheric pressure through a metal capillary tube that is at a potential of several kilovolts relative to the surrounding chamber walls. Charge is deposited on the surface of the emerging liquid, resulting in the production of Coulomb repulsion forces that are sufficient to overcome surface tension. Hence the liquid is dispersed in a fine spray. Ions emitted from charged droplets are transferred into the vacuum chamber of a mass spectrometer and are mass analysed.

As ESP operates without the input of heat into the spray-ionization step, polar and thermolabile compounds are ionized without thermal degradation [76]. The technique works best with flow-rates in the range of 5–10 μl/min [75,76]. In 1987, Bruins *et al.* [77] successfully combined the electrostatic charging atomization of ESP and the pneumatic nebulization used in liquid ion evaporation [69] to produce the ionspray (ISP) interface. In this approach, the capillary itself is charged to several kilovolts and a coaxial nitrogen gas flow is used to assist atomization. This allows the use of flow-rates as great as 100 μl/min, which make the technique compatible with microbore HPLC.

The absence of heat in these interfaces makes possible the detection of thermolabile species, such as sulphate conjugates of drugs. For example, the ISP interface was used to detect picogram amounts of the thermolabile anabolic steroid metabolite boldenone sulphate in the SIM mode. This allowed the detection in urine of the metabolites from a single dose of boldenone in a horse 45 days after administration [78]. The sensitivity of the system shown by these results was almost in agreement with that given by Bruins *et al.* [77], who reported that the full-scan detection limit appeared to be about 10 ng for ionic or selected neutral compounds and about 10 pg under SIM conditions with their HPLC–ISP-MS system. In addition, CID reactions generated fragment ions corresponding to structural units with their system [77].

On the other hand, there is a report [79] that very few fragment ions due to CID were observed in the analysis on glycine conjugates of carboxylic acids by HPLC–ESP-MS even when the drift voltage was varied. Further studies are also needed for applying HPLC–ESP (or ISP)-MS in forensic analysis.

In addition, it should be noted that multiply

charged ions appear in the corresponding mass spectra obtained with HPLC–ESP (or ISP)-MS. The ability to produce multiply charged molecular ions for biomolecules allows mass spectrometers to measure compounds with molecular masses exceeding the mass-to-charge (m/z) range of the instrument by a factor equal to the molecule's charge state [73]. In fact, proteins with molecular masses in excess of 130 000 were successfully determined by HPLC–ESP-MS with a quadrupole mass spectrometer of limited m/z range (1700) [80,81]. Although multiply charged ions produced in HPLC–ESP (or ISP)-MS are useful in the molecular biology field, they seem not always helpful in forensic analyses because of their complicated mass spectra.

10. Conclusions

HPLC–MS techniques have recently been introduced in forensic toxicology. In this review, we have mainly presented HPLC–FAB-MS systems, in which we are engaged, together with brief mentions of other types of HPLC–MS. Examples of analyses for compounds of forensic interest by HPLC–FAB-MS, arranged according to HPLC column types, are given in Table 2.

The interfaces utilizing heat for the ionization of liquid compounds are usually capable of handling liquid flow-rates of 0.5–2 ml/min, at which a conventional HPLC system is operated, but sometimes cause decomposition of thermolabile compounds [53,73]; TSP, MAGIC and APCI with a heated nebulizer have such disadvantages, although each interface has its own individual advantages, as described before. APCI with a pneumatic nebulizer has a stronger propensity to form low-molecular-mass cluster ion adducts than that of the heated nebulizer [73]. The application of ESP or ISP is limited to highly polar or ionic compounds.

Frit-FAB and CF-FAB require no heating during analysis of a sample and can handle low- to high-polarity compounds by selection of a suitable matrix [82]. HPLC–FAB-MS is also suitable for non-volatile and thermolabile compounds that are unsuitable for GC–MS analysis. On-line HPLC–FAB-MS gives clean mass spectra free of dirty peaks due to the matrix and gives intense quasi-molecular peaks together with an adequate fragment peaks (Fig. 6), which are useful for both screening and identification of drugs or poisons.

Capillary HPLC without any splitting of effluents, and the special column-switching device allowing a 500-μl injection volume (Fig. 4), contributed greatly to enhancing the sensitivity of HPLC–FAB-MS. Capillary HPLC–FAB-MS combined with the special column-switching device seems highly recommendable for forensic analysis by HPLC–MS for the above reasons, although it gives semi-quantitative results without stable isotope internal standards.

Tandem mass spectrometry (MS–MS) was developed to obtain structural information of molecules mainly in organic chemistry [26,52, 83]. As the first MS instrument can be utilized for the separation of a target compound from impurities, crude samples can usually be analysed by the direct-inlet method on the second mass spectrometer. Trial use of MS–MS in forensic chemistry has begun. Recently, even the combination of HPLC with MS–MS has been tried [84–86]. The balance between the utility and the high cost of MS–MS instruments should be evaluated.

11. Abbreviations

APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
CF	Continuous-flow
CI	Chemical ionization
CID	Collision-induced dissociation
EI	Electron impact
ESP	Electrospray
FAB	Fast atom bombardment
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ISP	Ionspray
LC	Liquid chromatography

Table 2
Analyses of drugs, poisons or their metabolites by HPLC–FAB-MS

Analytical system	Compound	Sample	Work-up	Analytical column packing material and dimensions	Injection volume	Splitting ratio	Detection limits[a]		Refs.
							Injected amount	Sample concentration	
Conventional HPLC–frit-FAB-MS	Microcystins (RR, YR and LR)	Lake surface blooms	ODS cartridge (Baker)	Chromatorex ODS-5, 5 μm (25 cm × 4.0 mm I.D.)	–[b]	4:500	1 μg	–[b]	16
Microbore HPLC–frit-FAB-MS	Metabolites of chloroacetanilide	Incubation mixture	Methanol	Brownlee RP5, C_{18} (25 cm × 1.0 mm I.D.)	3 μl	1:10	5–100 ng	1.7–33 ng/ml	17
Microbore HPLC–CF-FAB-MS	Teicoplanin (A2-2)	Authentic	–	Spheri S5, ODS2 (25 cm × 1.0 mm I.D.)	0.5 μl	3:100	28 μg	56 mg/ml	23
	Benzo[*a*]pyrene (BP), BP conjugates, BP sulphate and BP glucuronide	Cell culture medium	Chloroform–methanol Sep-Pak C_{18}	C_{18}, 5 μm (25 cm × 1.0 mm I.D.)	20 μl	1:6	0.45–18 ng[c]	22.5–900 ng/ml[c]	27
Capillary HPLC–frit FAB-MS	Benzodiazepines (chlordiazepoxide, desmethyldiazepam, oxazepam and triazolam)	Serum	Bond-Elut C_{18} (100 mg/ml)	Develosil ODS, 5 μm (15 cm × 0.3 mm I.D.)	100–500 μl	1	0.5–5 ng	1–10 ng/ml	28, 29
	Cephalosporins (cephalexin, cefaclor and cephaloridine)	Serum or plasma	Bond-Elut C_{18} (200 mg per 3 ml)	Develosil ODS, 5 μm (15 cm × 0.3 mm I.D.)	100–500 μl	1	10–100 ng	20–200 ng/ml	30, 31

	Phenothiazines (chlorpromazine levomepromazine, promethazine and propericiazine)	Serum	Bond-Elut C_2 (100 mg/ml)	Develosil PhA, 5 μm (15 cm × 0.5 mm I.D.)	100–500 μl	1	0.25–2.5 ng	0.5–5 ng/ml	32
Capillary HPLC–frit-FAB-MS (precolumn splitting system)	Quinolones (cinoxacin, ciprofloxacin, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, pipemidic acid, piromidic acid and tosufloxacin)	Plasma	Perchloric acid	Develosil PhA, 5 μm (25 cm × 0.5 mm I.D.)	300 μl	1	10–20 ng	0.1–0.2 ng/ml	33
Capillary HPLC–CF-FAB-MS	Teicoplanin (A2-2)	Authentic	–	Spherisorb ODS, 5 μm (25 cm × 0.32 mm I.D.)	0.5 μl	1	940 ng	1.9 mg/ml	23
Microcapillary HPLC–CF-FAB-MS	Fusarochromanone	Ground corn samples	Sep-Pak silica cartridge	C_8-bonded micro capillary column (3 m × 75 μm I.D.)	0.5 μl	1	500 pg[d]	1 μg/ml[d]	26
OT-LC–CF-FAB-MS	Corticosterone and glutathione	Authentic	–	OTLC column without any coating (1.5 m × 10 μm I.D.)	4.4 nl	1	2.2–4.4 ng	0.5–1.0 mg/ml	25

[a] Detection limits described here are for full-scan mass spectra unless specified otherwise.
[b] No mention made.
[c] Detection limits in SIM mode were described, but full-scan mass spectra of extracts of cell culture medium were of low quality.
[d] Detection limits in SIM mode were described, but no mention was made for those for the full-scan mode.

MAGIC	Monodisperse aerosol generation interface for chromatography
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
OT-LC	Open-tubular liquid chromatography
SIM	Selected-ion monitoring
TSP	Thermospray
UV	Ultraviolet

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