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Review

Biomedical and biochemical applications of liquid chromatography–mass spectrometry

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Abstract

This review centres on the application of various LC–MS and LC–MS–MS techniques to the study and solution of practical problems in biomedical research. For this purpose it covers a selection of publications in this area included in the MEDLINE database for the period 1991–mid-1994. As shown herein, LC–MS is increasingly gaining in importance in the biomedical field, especially after the revolution brought about by the introduction of the new liquid-phase atmospheric pressure ionization (API) techniques, such as electrospray (ES) and ionspray. The information in this database shows that thermospray (TS), which clearly dominated LC–MS coupling in the 1980s, is on a downward trend relative to the more modern API techniques which will certainly dominate this application field in the present decade. Studies on drug metabolism, therapeutic drug monitoring and pharmacology have been traditionally carried out by GC–MS. However, LC–MS has lately been replacing classical GC–MS techniques in many of these applications. For instance, LC–ES–MS has greatly facilitated the application of both qualitative and quantitative LC–MS methods to highly polar molecules. This is possible without the need to resort to elaborate sample handling and derivatization procedures for relatively high-molecular-mass compounds such as drug conjugates, biosynthetic and natural peptides and therapeutic proteins obtained by recombinant DNA technology, all of them formerly totally inaccessible to the standard GC–MS or LC–MS methods. With regard to studies on metabolism and biochemical phenomena of endogenous compounds, LC–ES–MS is also becoming very strong in the analysis of structural biopolymers such as peptides, proteins, glycoproteins and glycolipids, and also lower molecular mass compounds such as fatty acids, vitamins, steroids and nucleic acids. For example, structural verification of post-translational modifications in proteins can be efficiently obtained in the time frame of an LC run and suitable MS methods for the location of glycopeptide-containing fractions in proteolytic digests of glycoproteins have been developed. Interesting examples are also shown of the use of LC–MS in clinical studies and the determination of biological markers of disease.

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1. Introduction

The growth of LC–MS within the past few years has been truly overwhelming and thus difficult to review in depth. With this limitation in mind, the present review will concentrate, as its title implies, on the application of the various LC–MS techniques available nowadays for the study and solution of practical problems in biomedical research. For this purpose, articles incorporated in the MEDLINE database have been searched. In fact, this work can be considered as an extension of the earlier and much more comprehensive general review covering biochemical and biomedical applications of mass spectrometry for the period 1988–mid-1991 [1]. In this case, the scope is reduced to LC–MS with coverage restricted to the period 1991–mid-1994. Even so, the ca. 500 articles listed in this database for this period and the realization that the database does not necessarily contain every paper published on this topic preclude a detailed and extensive coverage, so apologies are extended to all authors whose work is not cited here. The information contained in this review can be complemented with other recent reference publications such as those by Desiderio [2], Matsuo et al. [3], Niessen and van der Greef [4] and Burlingame et al. [5]. Much useful information can also be obtained from the monthly Current Awareness section in the new *Journal of Mass Spectrometry* (formerly *Biological Mass Spectrometry*).

One of the conclusions I was able to draw in the previous review [1] was that combined gas chromatography–mass spectrometry (GC–MS) was then, and most likely still is, the most frequently used technique in this field, but with LC–MS and MS–MS fast becoming increasingly important in biomedical research. For instance,

at that time, of a total of 3095 papers describing the application of mass spectrometric techniques in biomedicine, 67% were making use of GC–MS with selected-ion monitoring. Only 227 papers (7.3%) were clearly LC–MS applications and of those LC–MS thermospray was the technique of choice in more than half of the cases. No attempt is made here to evaluate the actual relative weights of GC–MS vs. LC–MS, although it can be inferred that the latter is increasingly gaining in importance, especially if we consider the revolution brought about by the new electrospray and ionspray techniques and their more recent high-flow possibilities.

As indicated in a recent review [5], over the past 20 years a total of about 25 different LC–MS interfaces have been described in the literature, but of those only a few have demonstrated their practical potential for real applications. As shown in Table 1, of a total of about 250 LC–MS-related papers selected from the BIOMED database for the period 1991–March 1994, 100 deal with applications of thermospray (TS) and 100 describe applications of atmospheric pressure ionization (API) with corona discharge [6]

Table 1
Use of LC–MS techniques in biomedical mass spectrometry (data from the MEDLINE database)

Technique ^a	1991	1992	1993	1994	Total
ES	6	6	24	8	44
IS	4	9	12	–	25
API	8	10	11	2	31
TS	42	27	28	3	100
FAB	10	4	7	–	21
PB	4	1	3	–	8

^a ES, electrospray; IS, ionspray; API, atmospheric pressure ionization; TS, thermospray; FAB, fast atom bombardment; PB, particle beam.

or in the electrospray (ES) [7] or ionspray modes (IS) [8]. The interesting point to highlight is the clear downward trend of TS in favour of the API techniques and especially ES. Continuous-flow fast atom bombardment (CF-FAB) shows a modest number of applications and it can be envisioned that it will effectively be replaced by the electrospray techniques, which appear capable of providing the same kind of information in a more routine way. CF-FAB is still restricted to extremely low microlitre flows (1–5 $\mu\text{l}/\text{min}$), whereas the modern so-called high-flow combined APCI–electrospray devices allow flows in the range 200–400 $\mu\text{l}/\text{min}$.

As also illustrated in Table 1, particle beam (PB) accounts for a very small number of papers within the same period and other interfaces show only a token presence in terms of practical applications in biomedical-oriented problems. For instance, only two references were found to the moving belt interface. As for other innovative techniques such as LC combined with time-of-flight (TOF) or laser desorption–Fourier transform ion cyclotron resonance (ICR) and capillary zone electrophoresis, practical applications are still scarce.

No mention will be made here of the general principles and instrumentation behind all of these techniques, as this aspect has been covered in some detail in the paper by Niessen in this volume. As the title of this review indicates, only applications will be considered and these, for the sake of discussion, will be approached in a similar manner to that in the earlier more general review, which included LC–MS up to March 1991 [1].

2. Exogenous compounds: drugs, metabolism, therapeutic drug monitoring and pharmacology

Mass spectrometry has been for a number of years a powerful tool for the study of the metabolism and biodisposition of drugs, both man-made and of natural origin, and their biotransformation products in living systems. These types of studies have been traditionally carried out by GC–MS techniques, which in most cases

required that the drugs or xenobiotics under study were rendered amenable to gas-phase analysis by suitable sample preparation and derivatization procedures. However, the use of LC–MS has been growing in importance, especially since the introduction of the thermospray interface, which works relatively well for compounds of moderate polarity. More recently, with the advent of modern liquid-phase ionization techniques, such as electrospray, it has become possible to apply LC–MS methods to highly polar molecules without the need to resort to elaborate sample handling procedures. These LC–MS techniques have been applied to a wide array of pharmaceutical products, such as anti-cancer drugs, anthelmintics, antibiotics, cyclosporins, anxiolytics, analgesics, antiepileptics, specific receptor antagonists, antioxidants and beta-blockers.

Further, relatively high-molecular-mass drug conjugates [9], biosynthetic [10] and natural peptides [11] and therapeutic proteins obtained by recombinant DNA technology [12], all of them formerly totally inaccessible to the standard GC–MS or LC–MS methods, can now be readily analysed by the new soft liquid-phase ionization techniques, which greatly facilitate the direct coupling of LC to MS. This has really expanded the scope of applications in terms of the molecular mass range of the compounds that can be studied. Nevertheless, now the understandable excitement over the tremendous field opened up to the study of relatively high-molecular-mass biopolymers and their significant roles in the understanding of structural biology is calming down, it is also becoming clear that the new electrospray, ionspray and atmospheric pressure chemical ionization APCI techniques can contribute significantly to the study of the biodisposition of low-molecular-mass pharmaceutical and therapeutic compounds [13,14].

2.1. LC–MS interfaces for drugs and metabolites: ionspray

The combination of LC with ionspray MS (LC–IS–MS) has been applied with success to the identification of different metabolites of

various drugs [14–23] in both humans and experimental animals. Several of these applications deal with anticancer drugs [14,16,17,19]. One of these [14] describes the first direct determination of tamoxifen (an antiestrogen routinely used for the treatment of breast cancer) glucuronide metabolites in human urine samples by on-line LC–tandem IS–MS. Also, a minor N-oxide metabolite was identified in patients' plasma extracts by collision-induced dissociation (CID) in the second quadrupole. For this work 1 mm I.D. columns were used at 0.2 and 0.1 ml/min. The metabolism of biantazole, another drug chemically related to mitoxantrone and under clinical investigation for the treatment of breast cancer, was also studied in human urine samples [16] using IS techniques. In this case, the authors developed a gradient system using a volatile trifluoroacetate–trifluoroacetic acid buffer with 10% dimethylformamide to improve peak shape, which they claimed to be compatible with ionspray ionization and also with thermospray and continuous-flow fast atom bombardment. The 1 ml/min HPLC solvent flow was split by the use of a needle valve coupled to a fused-silica capillary (30 cm \times 100 μ m I.D.) into a capillary flow of ca. 5 μ l/min. The ionspray mass spectra of the peaks from the parent biantazole drug (CI-941) and the two metabolites separated by the HPLC system are shown in Fig. 1. The addition of trifluoroacetic acid and dimethylformamide to the HPLC eluent led to the appearance of the corresponding cluster ions, as indicated in Fig. 1. This is useful in electrospray/ionspray-based techniques for the recognition of the quasi-molecular ion. Fragment ion mass spectra could be obtained after the injection of an extract of 200 ml of patient's urine and this allowed the identification of two polar metabolites.

The mechanism of the cytotoxicity of mitoxantrone was also determined in a similar way by the same group [17]. Application of the same analytical procedure revealed seven biliary metabolites identified as thioether derivatives of the parent drug and its chain oxidation products. With the aid of HPLC–atmospheric pressure ionspray MS–MS, unambiguous evidence was obtained for the presence of haloperidol

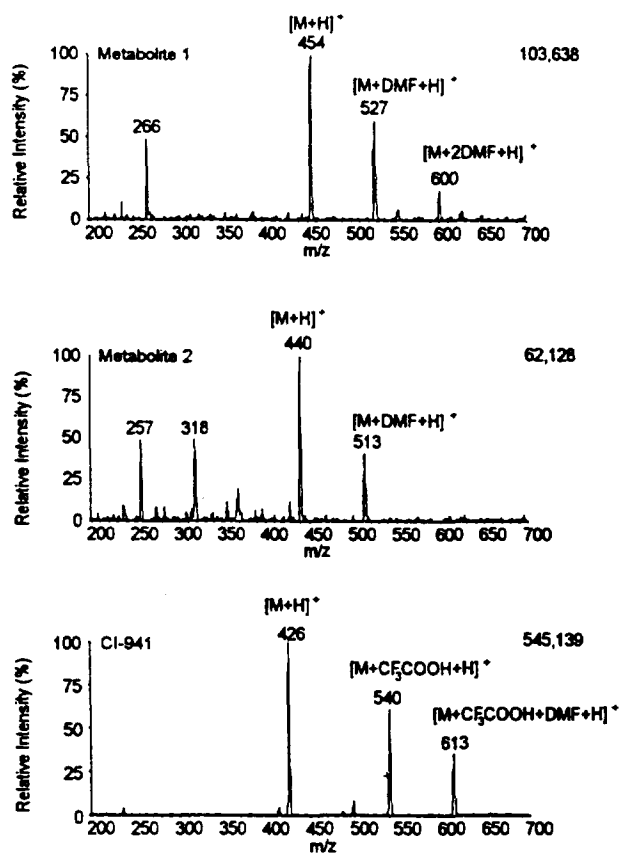


Fig. 1. Ionspray mass spectra of biantazole (CI-941) and its two metabolites (reprinted with permission from Ref. [16]).

pyridinium species in extracts of urine obtained from haloperidol-treated patients and in extracts of NADPH-supplemented human liver microsomal incubation mixtures containing haloperidol [24]. This neuroleptic agent is structurally related to the parkinsonian-inducing agent MPTP which, via its transformation into a pyridinium species, selectively destroys nigrostriatal neurons in humans and subhuman primates.

LC–tandem ionspray MS techniques on a triple quadrupole instrument have also been reported for the identification in bile and urine of glutathione and N-acetylcysteine conjugates of 2-chloroethyl isocyanate as metabolites of the antitumour agent N,N'-bis(2-chloroethyl)-N-nitrosourea [19]. Air was used as the nebulizing

gas for ionspray and direct injections of 50 μ l of crude rat bile or urine were made on to a narrow-bore HPLC column (150 \times 2.0 mm I.D.) coupled to the mass spectrometer via a splitting tee dividing the column effluent so that 50 μ l/min were delivered to the mass spectrometer (splitting ratio 3:1). Ionspray mass spectra are usually devoid of structural information since they mostly contain $[M + H]^+$ and cluster ions, as shown in Fig. 1. However, tandem MS affords product ion spectra that contain sufficient information to identify the analyte in question. This is illustrated in the tandem mass spectra shown in Fig. 2. As discussed by the authors [19], this is the tandem mass spectrum of one of the S-linked conjugates identified in this study, S-[N-(2[chloroethyl]carbamoyl)glutathione (SCG), which was obtained by collision-induced dissociation of the ^{35}Cl -containing $[M + H]^+$ ion at m/z 413. All of the different product ions shown in this mass spectrum can be attributed to fragments that allow the full characteriza-

tion of the SCG molecule, as discussed by the authors. In this way, specific detection of SCG can be readily achieved in crude bile samples by constant neutral loss scanning for 129 u (m/z 413 – m/z 284 in Fig. 2). By monitoring this loss in LC–MS–MS it is possible to obtain a single peak at the retention time of authentic SCG, with the correct response ratios in the channels for m/z 413 and 415, corresponding to the ^{35}Cl - and ^{37}Cl -containing $[M + H]^+$ species. The same analytical technique was investigated for the analysis of 21 antibacterial sulfonamide drugs [25]. Tandem ionspray mass spectrometry (IS–MS–MS) using collision-induced dissociation provided structural information, allowing for the identification of common fragmentation pathways and the differentiation of isomeric sulfonamides. The presence of sulfadimethoxine, a representative sulfonamide used in the aquaculture industry, was confirmed in cultured salmon flesh at levels as low as 25 ng/g.

As indicated above, one of the problems with

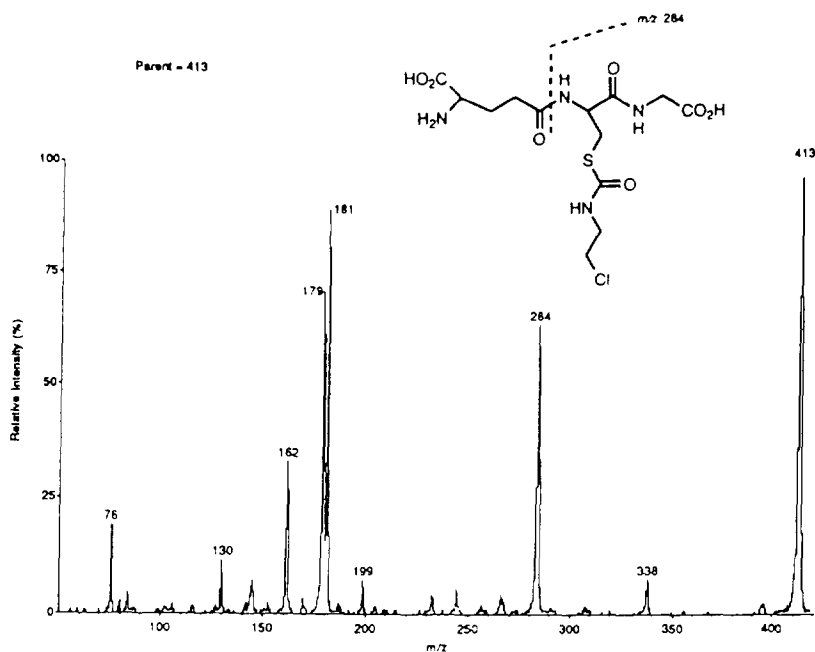


Fig. 2. Collision-induced dissociation mass spectrum of the $[M + H]^+$ ion of SCG at m/z 413. The origin of the ion at m/z 284 arising from neutral loss of m/z 125 is as shown (reprinted with permission from Ref. [19]).

most of the LC–MS interfaces is the variability of response factors, which are heavily dependent on the nature of the analyte compounds. In many cases this may severely handicap the sensitivity of the technique, although for some specific applications excellent sensitivities have been reported for ionspray. For instance, an instrumental limit of detection for cyclosporin of 500 fg (ca. 415 amol) has been attained, which according to the authors [15] is 1000 times better than that for HPLC with UV detection. However, the limit of quantification was 5 pg for single-ion monitoring and 100 pg when the mass range was scanned from m/z 1190 to 1250. The technique allowed for the determination of cyclosporin metabolite concentrations in 300- μ g biopsies from the kidney or liver. Detection of residues of danofloxacin in liver extracts down to 50 ppb was also made possible by LC–ionspray in the MS–MS mode [18]. In this case, the addition of 0.1% of trifluoroacetic acid to the mobile phase to enhance the preferential formation of $[M + H]^+$ ions gave a fourfold increase in danofloxacin response under selected-ion monitoring (SIM) conditions.

As ionspray is uniquely suited to produce ions from compounds that are ionic in the liquid phase, it can be very useful for the study of the metabolism of organic cations such as the quaternary ammonium derivatives of the enantiomeric drugs dextrorphan and levorphanol [20]. In this case, the drugs were labelled with a stable isotope and mixed with the unlabelled drugs to facilitate the recognition of the resulting unknown metabolites by LC–IS–MS–MS. The isotope cluster technique has also been effectively used for the identification of over 40 metabolites of omeprazole in the rat using LC–IS–MS [23]. Finally, a novel application of ionspray techniques involves the development of on-line immunoaffinity chromatography with coupled LC–MS. This technology is based on the use of antibodies immobilized on trapping columns for trace analyte extraction and enrichment directly from a complex biological matrix. Examples of its application to the determination of carbofuran in water and in a crude potato extract [26] and propranolol in urine [27] have been reported.

2.2. Electrospray; flow split

Applications of true electrospray in LC–MS at flow-rates of 1–10 μ l/min are scarce, which possibly reflects its lower practical convenience relative to the higher flow ionspray mode of operation (pneumatically assisted electrospray at flow-rates of 40–50 μ l/min). True electrospray and ionspray are sometimes confused in the literature, as for instance in Ref. [16], where the flow-rate of 5 μ l/min would be more in line with the electrospray mode of operation. However, the authors refer, erroneously in this instance, to ionspray.

Whereas ionspray coupled to 1–2 mm I.D. LC columns may require either no split or low splitting ratios such as the 3:1 mentioned above [19], true unassisted nebulization in electrospray requires large splitting ratios in order to achieve low enough flow-rates. For instance, a recent report describes the use of an HPLC–MS method with an electrospray interface for the study of β -lactam antibiotic residues in bovine milk [28]. In this case, the use of a 150 \times 2 mm I.D. LC column required a splitting ratio of 70:1 to reduce the inflow into the electrospray capillary needle to about 4 μ l/min. In contrast, when the separation was carried out on a 15 cm \times 320 μ m I.D. column no split was necessary as the mobile phase flow-rate through the column and into the electrospray interface was 4 μ l/min. CID fragmentation was controlled by variation of the potential difference (40–240 V) between the source skimmer and the end of the HPLC capillary nozzle (source CID). Also, the ES response in various mobile phase combinations and using various additives was tested under positive and negative ion detection. Whereas the response could be enhanced by the addition of formic or acetic acid to the methanol–water (1:1) mobile phase, it tended to be lower for negative ions.

Taxol and related diterpenoids from cell cultures of the yew tree have been determined by microbore HPLC–ES–MS (Fig. 3) with variation of the nozzle-to-skimmer bias value from 45 to 100 V. At the lowest value protonated molecular ions were dominant, whereas at 85 V increased

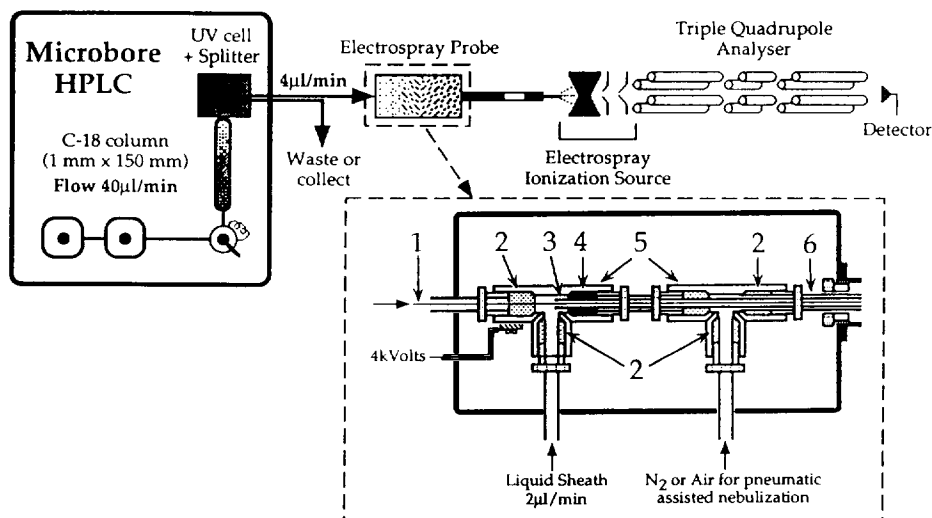


Fig. 3. Scheme of the microbore HPLC–ES–MS system designed for either pure electrospray or pneumatically assisted electrospray (ionspray). 1 = Fused-silica tubing, 150 μm O.D.; 50 μm I.D.; 2 = Vespel ferrule; 3 = stainless-steel tubing, 175 μm I.D.; 4 = graphite ferrule; 5 = stainless-steel tee; 6 = PTFE tubing (insulator) (reprinted with permission from Ref. [29]).

fragmentation provided structural information similar to that obtained from conventional tandem MS experiments were CID takes place in the middle quadrupole (Q_2) (Fig. 4). The flow-rate through the 150 mm \times 1 mm I.D. microbore column was set at 40 ml/min with 4 $\mu\text{l}/\text{min}$ entering the ES interface after postcolumn splitting, so that 90% of the sample was available for recovery through fraction collection [29].

The use of nanoscale capillary LC on 75 μm I.D. fused-silica capillaries packed with 10- μm particles for the determination of antibiotics by electrospray ionization has also been described [30]. Thus, direct coupling can be achieved with transfer of all of the injected sample to the mass spectrometer. Although low skimmer voltages were used (12 V), the authors claimed that significant fragmentation still occurred and that this can be useful for structure determination. This work was carried out using the Vestec electrospray source, which does not use nitrogen curtain gas but a heated block for spray desolvation. Under these conditions the fragmentations observed appear to be true fragmentations and not thermal degradations as it has been attributed to thermospray ionization.

2.3. Electrospray-based techniques for biopolymers

The preceding information is intended to provide an idea of the growing importance of electrospray-based techniques for the study of drugs or xenobiotics in living systems. However, in comparison not many data are available on studies of the biodisposition of high-molecular-mass biopolymers, e.g., synthesized by a biotechnology approach and administered for therapeutic reasons. In this regard, the role of synthetic peptides is of major importance in many fields of biomedical research. For example, a report has described the use of electrospray mass spectrometry for the study of the characterization of two circulating metabolites of des(64,65)-human proinsulin after subcutaneous administration in the rat [10]. The corresponding peptides were separated on a 2.1 mm I.D. column at 0.2 ml/min with a splitting ratio of 100:1, allowing about 2 $\mu\text{l}/\text{min}$ to enter the mass spectrometer. The peptide metabolites thus identified may mediate the pharmacological activity of the parent peptide.

The use of HPLC with electrospray MS has

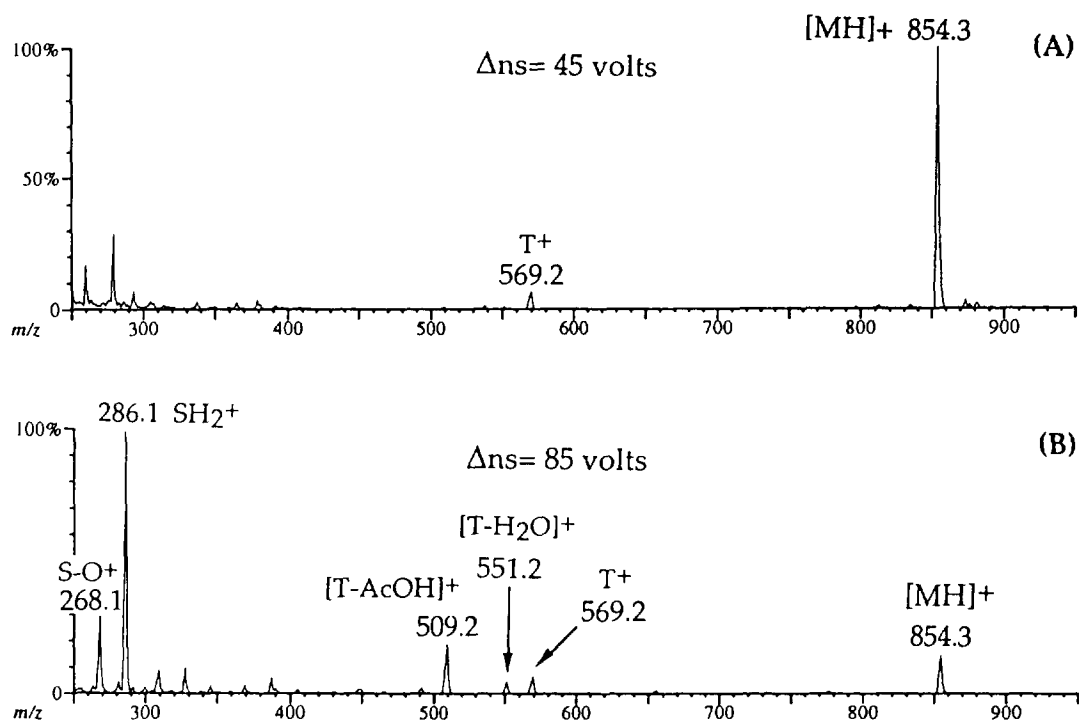


Fig. 4. Electrospray spectra of taxol recorded at a nozzle-to-skimmer (Δns) of (A) 45 V and (B) 85 V. Direct infusion of water-methanol containing 1% acetic acid. T denotes taxane ring fragments and S side-chain fragments (reprinted with permission from Ref. [29]).

also been reported for the study of the enzymatic degradation of therapeutic derivatives of gonadotropin-releasing hormone [31]. The authors attempted to investigate the feasibility of on-line LC-MS for this problem with a comparison of 2 and 0.32 mm I.D. LC columns using stream splitting. The ESI ion source was operated at a sample flow-rate of 2 $\mu\text{l}/\text{min}$. Using the 2 mm

I.D. column with postcolumn flow splitting, only 3.4 pmol of analyte were delivered to the mass spectrometer vs. 400 pmol injected. In contrast, using the 0.32 mm I.D. column with no split, the amounts injected were ca. 10 pmol of analyte and these were delivered in full to the mass spectrometer (Fig. 5).

Along these lines, the combination of micro-

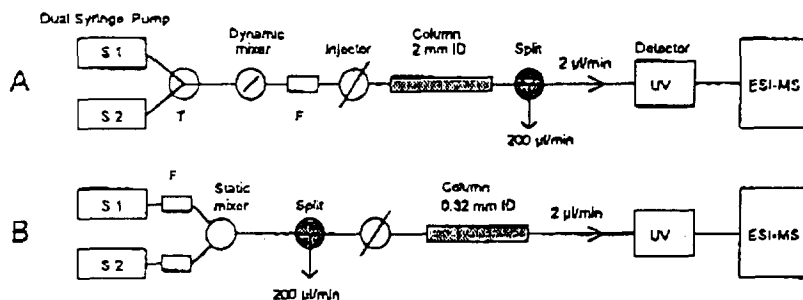


Fig. 5. On-line HPLC-ES-MS set-up for either (A) 2 mm I.D. or (B) 0.32 mm I.D. columns, both delivering a flow-rate of 2 $\mu\text{l}/\text{min}$ to the mass spectrometer. Sample injection of (A) 20 μl or (B) 0.5 μl (reprinted with permission from Ref. [31]).

LC–ESI–MS on a simple single-stage quadrupole mass spectrometer is highlighted as capable of providing information difficult or impossible to obtain by other methods in routine peptide mapping of therapeutic proteins produced by recombinant DNA technology. An illustrative description of the systematic use of a gradient microbore LC–ESI–MS procedure for verifying the primary structure of proteins at the micro-scale level, as applied to the confirmation of the structure of recombinant human growth hormone and tissue plasminogen activator glycoprotein with less than 100 pmol of material, is available [12]. For this purpose, 2 μ l/min of the effluent from a 1 mm I.D. column, corresponding to a 1:25 splitting ratio, were passed into the ES–MS system. Molecular mass associated ions are readily generated for each tryptic peptide and sequence information can be obtained from CID of the ions before they enter the single-stage mass spectrometer. It is claimed that MS with on-line LC rapidly facilitates information on the translational integrity of the recombinant protein and the degree of post-translational modification, constituting an ideal set-up for quality control of new therapeutic products.

2.4. Atmospheric pressure chemical ionization

Another of the atmospheric pressure nebulization techniques, where ionization is discharge assisted by a suitable electrode, known as atmospheric pressure chemical ionization (APCI), is clearly on the upsurge in terms of practical usage and it is probably destined to take the place of thermospray in many applications. Like the latter, APCI is run with conventional-bore LC columns at nominal flow-rates (1 ml/min). The technique has been used, for instance, in the determination of clenbuterol in human plasma with detection limits for SIM of the quasi-molecular ion of 0.1 ng on-column [32]. This is claimed to represent a 50-fold better sensitivity than in the TS mode. Nevertheless, as reported in the previous review [1], negative-ion chemical ionization GC–MS still provides femtomole-level sensitivity for this particular application.

APCI has recently been in the identification of the carbamoylated thiol conjugates released upon *in vivo* transformation of an antineoplastic nitrosourea derivative [9] related to that studied by ionspray methods [19]. The results provided evidence for the first time for the *in vivo* carbamoylating activity of these drugs. Another study involved the utilization of LC–APCI–MS–MS for monitoring patients' urinary extracts for the conjugated metabolites of 4-hydroxyandrost-4-ene-3,17-dione used as an anticancer drug [33] and provided the first evidence of the presence of the sulfate conjugates and analogues in patients' urine. The same technique of APCI-tandem MS has allowed the determination of a β -adrenergic blocker (timolol) in plasma after ocular administration to volunteers. The analyte and its deuterated internal standard were quantitatively detected by multiple reaction monitoring [34].

Some other routine applications of APCI techniques include the determination of a cysteine protease inhibitor and its ethyl ester in mouse serum and muscle samples [35], the simultaneous determination of medetomidine and other anaesthetic drugs in solutions and dog plasma [36], the characterization of metabolites of xylazine produced *in vivo* and *in vitro* [37] and the determination of L-654,066, a new 5α -reductase inhibitor in plasma [38]. The method has been reported to offer sufficient sensitivity, precision, accuracy and selectivity for the quantitative analysis of clinical samples containing the latter product at concentrations in the range 0.5–20 ng/ml.

In the past decade, the combination of HPLC–MS with thermospray ionization has been the mainstay of direct LC–MS coupling procedures in most analytical areas of application, including biomedical and biochemical [1]. However, whereas TS ionization was clearly the dominant LC–MS technique in the period 1988–March 1991, it is now losing ground to the more versatile LC–MS couplings via APCI and electrospray, as illustrated in Table 1. ESI experienced a fourfold increase in 1993 and this trend will no doubt continue with force if we consider the advantages of the new generation of

high-flow electrospray sources, which will allow routine operation of the LC–MS system at flow-rates of 200–500 $\mu\text{l}/\text{min}$, directly compatible with narrow-bore LC columns. As shown in Table 1, the combined power of API ionization techniques already equals that of thermospray in the biomedical literature with CF-FAB and PB representing only a small proportion of the total.

2.5. Thermospray

Despite the rapid and spectacular upsurge of the new API techniques, LC–thermospray MS continues to solve practical problems in everyday practice. For instance, although antibiotics, as indicated above, can now be efficiently studied by the new ESI methods [25,28,30], the HPLC–TS-MS of ten sulfonamide antibiotics in milk at low ng/ml levels by means of an on-line pre-concentration and clean-up system has been reported [39]. As expected for TS with these compounds, all of them exhibited simple mass spectra showing base peaks at the protonated molecular ions, except one which showed the ammonia adduct molecular ion. Collision-induced dissociation provided structural information. The same group also used LC–TS-MS techniques for the characterization of cyclic undecapeptides such as cyclosporin and its major hydroxylated and dealkylated metabolites in human blood samples [40].

More recently, filtered human urine samples were injected directly into an HPLC–TS-MS system for the determination of the metabolites of isbufylline, a bronchodilator, in healthy male volunteers after a single oral dose. The CID spectra allowed the identification of three metabolites [41]. This technique has been also used for the identification of five N-demethylated metabolites of a cholesterol acyltransferase inhibitor [42]. Discharge-assisted TS was used for the ionization of these compounds. Under these conditions, the TS mass spectra showed not only the protonated quasi-molecular ions but also characteristic fragments.

Filament-assisted thermospray has also been reported to generate characteristic fragments of

thromboxane B_2 in HPLC–TS-MS runs. These fragments are dependent on the interface temperature and solvent composition so that the adducts formed using different buffers can be used to confirm the structure of these fragments [43]. Along these lines, the usually limited structural information provided by TS ionization can also be enhanced by the use of appropriate derivatives, as shown in the HPLC–TS-MS determination of lipoxygenase metabolites of linoleic acid in maize embryos [44]. In this case, HPLC–TS-MS afforded the identification of two metabolites not previously detected by GC–MS.

Another interesting example of TS ionization is the study of the metabolic fate of a novel anticonvulsant containing a tetrahydropyridine moiety with a bulky substituent on the tertiary nitrogen, which was found to undergo biotransformation into a pyridinium derivative in man [45]. The authors claimed this to be the first report of such a metabolic pathway. In another study, the pharmacokinetics of an analgesic drug after its intravenous administration was established in human plasma using LC–MS via a thermospray interface [46].

Various examples of stable isotope dilution thermospray mass spectrometry can be found in the literature, such as those describing the evidence for the release of methyl isocyanate as a toxic *in vivo* intermediate following the *i.p.* administration of a dose of the investigational antitumour agent caracemide [47] or the determination of an anti-AIDS nucleoside in human plasma with a detection limit of 50 pg on-column [48]. Also, stable isotope pattern recognition techniques via thermospray ionization have been of use for the study of a specific dopamine agonist which may be a novel drug for the treatment of schizophrenia and/or Parkinson's disease. LC–thermospray MS allowed the direct confirmation of the conjugated O-glucuronide metabolite of the parent drug [49]. The technique was used to monitor urinary nicotine metabolic output for eleven smokers who smoked their regular cigarette brands *ad libitum*. Nicotine and eight metabolites, including glucuronide conjugates of nicotine, cotinine and trans-3'-hydroxycotinine, were identified [50].

Other interesting applications include the determination of human urinary metabolites of terfenadine after oral administration of terfenadine tablets [51] and the identification of the *in vivo* metabolites of the antimalarial arteether [52]. The thermospray mass spectra of arteether and sixteen of its potential metabolites all showed strong $[M + NH_4]^+$ ions. The metabolites were determined in plasma spiked with an internal standard (the propyl ether analogue of arteether), solid-phase extracted, then subjected to thermospray analysis using selected-ion monitoring and a C_{18} reversed-phase analytical column. Following the intravenous administration of arteether (11.6 mg/kg), the plasma was found to contain twelve metabolites in the 10–1000 ng/ml range 15 min post-injection, and within 60 min two of these metabolites attained concentrations higher than those of the parent compound, while several other metabolites attained concentrations similar to the parent compound. These data allowed a complex, multiple-step metabolic pathway leading to these twelve metabolites to be established.

The metabolism of 4-hydroxyandrost-4-ene-3,17-dione in hydrolysed urine samples from breast cancer patients has been studied using HPLC–MS [53]. Seven metabolites were identified from these samples, together with the parent drug, and these results can be compared with the more recent description by the same group of the use of APCI for the same application, as mentioned above [33].

2.6. Other interfaces

The application of other interfaces in this field in recent years has been mainly token relative to the preferred use of the TS and ES ionization interfaces, as indicated above. CF-FAB in pharmaceutical and drug monitoring has been applied to the determination of the resistance to chemical and enzymatic hydrolysis of a natural cyclic peptide antibiotic [11]. For this purpose, a double-focusing mass spectrometer with a CF-FAB source was used. The system was connected to a narrow-bore HPLC column run at a flow-rate of

100 μ l/min with a postcolumn splitting ratio of 1:20, so that only 5 μ l/min entered the ion source. In this manner the product lysobactin was found to resist hydrolysis by the digestive enzymes. This is an extension of earlier work from the same laboratory [54].

LC–particle beam MS has also been sparsely used, e.g., in applications involving confirmation of ivermectin residues in bovine milk and liver [55] and the determination nicotine metabolism in human serum and semen [56]. The characteristic problem of matrix effects in quantitative responses by PB-MS, limiting the useful linear range of the technique, was addressed in one of these publications, where the response and abundance pattern of the analyte were enhanced relative to controls by the co-eluting matrix interferences in a carrier effect like manner [55].

The so-called direct liquid introduction (DLI) type of coupling is not generally used nowadays. However, there are still a few applications in the literature such as a micro-HPLC–MS system recently described [57] for the separation and determination of PFB derivatives of vitamin A analogues and their oxo metabolites in human plasma using 0.32 mm I.D. microcolumns at flow-rates of 4–6 μ l/min. In this case the column was connected directly to the electron impact ion source of mass spectrometer via a 50 μ m I.D. fused-silica capillary ending with a commercially available SFC frit of porous water-glass. This kind of set-up with a frit restrictor at the end of the coupling capillary gave stable conditions at the low flow-rates used (1–10 μ l/min) without the need for external heating. A limit of quantification of 0.3 ng/ml was obtained.

HPLC has also been effectively interfaced with inductively coupled plasma mass spectrometry (LC–ICP–MS) for the analysis and pharmacokinetic studies of antineoplastic platinum-containing complexes [58,59]. Coupling can be accomplished through a long 0.1 mm I.D. capillary tube [59]. This technique has also been used for the study of the arsenic lipids in the digestive gland of the western rock lobster [60] and of metals in proteins [61] and for the elemental speciation of arsenicals and organotins by means of a direct injection nebulizer [62].

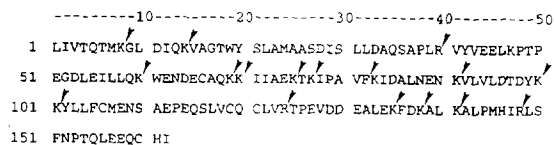


Fig. 6. Schematic representation of the cleavage points (arrows) in the digestion of β -lactoglobulin. The experimental conditions were 50 mM NH_4HCO_3 (pH 8.2) with 2% (w/w) trypsin TPCK, 3 h at 37°C [66].

3. Endogenous compounds: metabolism and biochemical phenomena

In the previous review [1], this classification was almost entirely dominated by GC–MS applications, with the exception of some relevant applications of HPLC–TS–MS. However, within the last 3 years many reports have appeared describing the use of LC–electrospray techniques in the determination of endogenous structural biopolymers such as peptides, proteins, glycoproteins and glycolipids, and also lower molecu-

lar mass compounds such as fatty acids, vitamins, steroids and nucleic acids.

3.1. Peptides and proteins

The significant advances in the application of mass spectrometry as a practical technique for obtaining structural information on peptides and proteins at the picomole level, especially by LC–ESI–MS, represent a significant contribution of this technique to the characterization of endogenous proteins at the primary structural level through peptide mapping strategies [12,63–65]. In this regard, the fragmentation of high-molecular-mass proteins into smaller peptides is first accomplished with suitable enzymes. For instance, the cleavage of β -lactoglobulin A by trypsin digestion is shown in Fig. 6. As shown, a total of eighteen fragments would be expected. The resulting mixture of peptides can then be resolved and analysed using HPLC–ESI–MS, as illustrated in Fig. 7 [66]. Each of these trypsin fragment peptides produces the corresponding

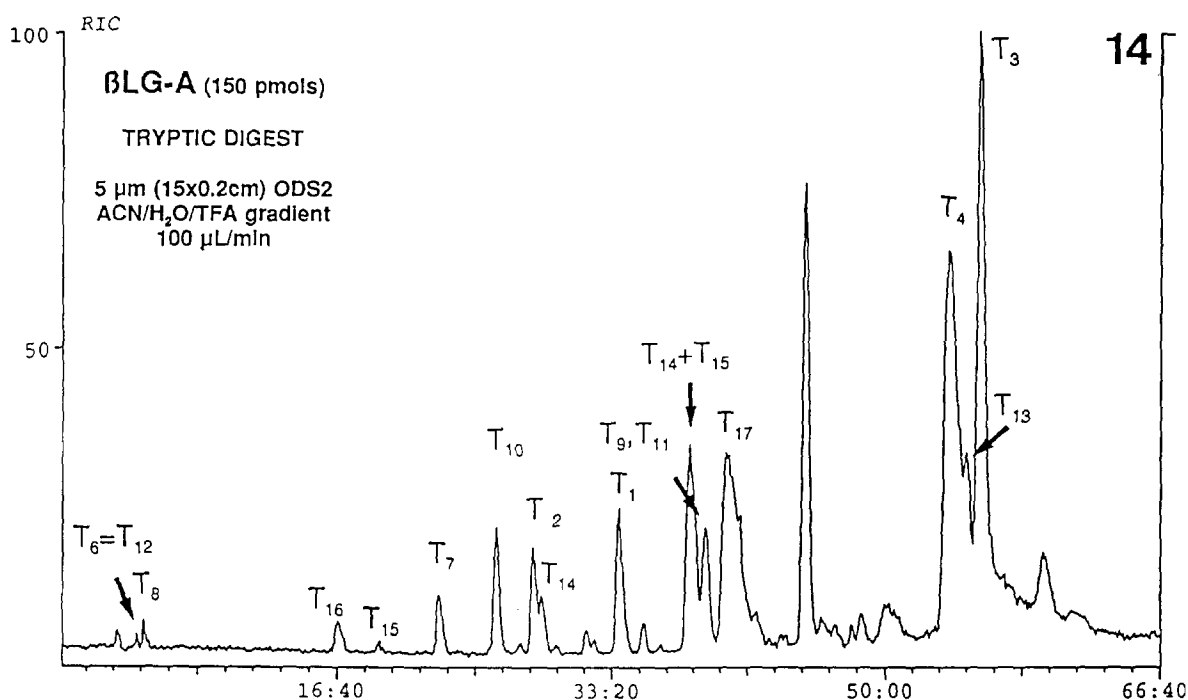


Fig. 7. On-line HPLC–ES–MS analysis of the tryptic digest from β -lactoglobulin (see Fig. 6).

Table 2
HPLC–ES-MS signals (m/z) detected from the peptides arising from the tryptic digestion of β -lactoglobulin, as shown in Fig. 7

Peptide	Sequence	[M + H] ⁺	[M + 2H] ²⁺	[M + 3H] ³⁺
1 1–8	LIVTQTMK	933.5 +	467.3 +	331.8 –
2 9–14	GLDIQK	673.4 +	337.2 +	225.1 –
3 15–40	VAGTWYSLAMAASDISLLDAQSAPLR	2707.4 +	1354.2 +	903.1 +
4 41–60	VYVEELKPTPEGDLEILLQK	2313.3 +	1157.1 +	771.8 +
5 61–69	WENDECAQK	1122.5 –	561.7 –	374.8 –
6 70	K	147.12 +	–	–
7 71–75	IIAEK	573.4 +	287.2 +	191.8 –
8 76–77	TK	248.16 +	–	–
9 78–83	IPAVFK	674.4 +	337.7 +	225.5 –
10 84–91	IDALNENK	916.5 +	458.7 +	306.3 –
11 92–100	VLVLDTDYK	1065.6 +	533.3 +	355.9 –
12 101	K	147.12 +	–	–
13 102–124	YLLFCMENSAPPEQSLVCQCLVR	2675.2 +	1338.1 +	892.4 +
14 125–135	TPEVDDEALEK	1245.6 +	623.3 +	415.9 –
15 136–138	FDK	409.2 +	205.1 –	–
16 139–141	ALK	331.2 +	166.1 –	–
17 142–148	ALPMHIR	837.5 +	419.2 +	279.8 –
18 149–162	LSFNPTQLLEEQCHI	1658.8 –	829.9 –	553.6 –

+ Signs indicate m/z values detected.

electrospray mass data signals, as shown in Table 2 [66].

The role of peptide mapping in the analysis of isoforms of the high-molecular-mass enzyme acetyl-CoA carboxylase isolated from rat liver was discussed in a recent paper on analytical and micropreparative peptide mapping by microbore HPLC–ESI-MS of low-picomole amounts of proteins purified by gel electrophoresis [63]. Briefly, a sample of the protein to be studied was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted on to Immobolon CD membranes and cleaved on the matrix with trypsin. The resulting peptide fragments were released into the supernatant and separated by microbore reversed-phase HPLC coupled to a tandem triple-quadrupole mass spectrometer through an ionspray interface. The masses of some of the tryptic fragments thus generated are given in Table 3. The 50 μ l/min flow through the column was split in a splitting tee so that 5 μ l/min entered the mass spectrometer and the rest was available for fraction collection (Fig. 8). Optimization for maximum MS sensitivity indicated that

the response was determined by the concentration of the sample in the eluate entering the mass spectrometer and not by the absolute amount of the material.

In general, in the last few years several reports

Table 3
Masses of tryptic fragments of bovine carbonic anhydrase observed by HPLC–MS compared with calculated masses (reproduced in part from Ref. [63])

Peptide No.	Theoretical MH ⁺ (average)	Observed MH ⁺
25	448.5	448.2
16	974.1	973.2
9	980.0	979.0
4	1002.1	1002.7
15	1013.1	1013.6
20	1347.6	n.d. ^a
14	2254.6	2253.6
10	2583.8	2583.4
22	2854.3	2853.7

These signals were derived from 350 pmol of protein applied to the gel and 10% of the column eluate was diverted to the mass spectrometer and analyzed as described in Fig. 8.

^a Not detected.

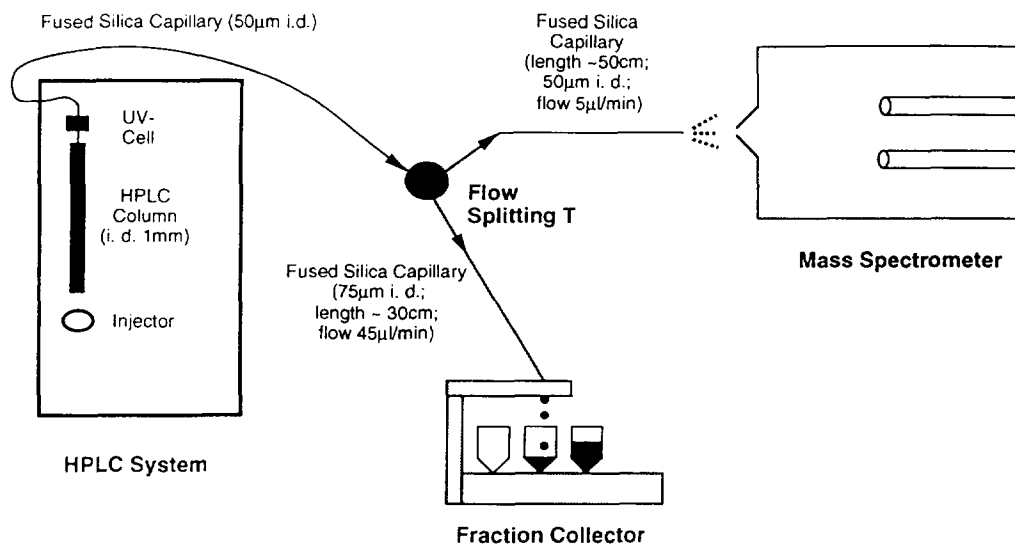


Fig. 8. Schematic diagram of microbore HPLC-MS system (reprinted with permission from Ref. [63]).

have clearly demonstrated the feasibility of using mass data generated from the proteolytic digestion of peptides or proteins to search protein databases in order to locate protein or gene sequences, or alternatively to identify proteins through the similarity of sequences of short stretches of amino acids. The first analytical approaches in this direction made use of FAB techniques but the scope of peptide mapping has been very much improved by the recent introduction of electrospray ionization, which facilitates the coupling of micro-HPLC and capillary electrophoresis techniques to mass spectrometers. Using these approaches it has recently been demonstrated that a set of experimentally determined masses which is less than 50% of the total number of predicted masses can be used to identify a protein sequence in the database [64].

In many cases, semipreparative LC is used to isolate individual fractions from proteolytic digests which are then studied by MS techniques, as has been described, for instance, in a study of the scrapie prion protein [67]. Fig. 9 illustrates a reversed-phase HPLC trace for an endoproteinase Lys-C digest of a truncated form of the prion protein. The application of different MS approaches allowed the identification of the pep-

tide fragments which are indicated in the figure. For instance, the peptide eluting around 34 min was expected to be glycosylated at the Asn residue from its MS signal at m/z 1153.5, MS-MS data and Edman sequencing. The N-linked oligosaccharide was removed with peptide-N-glycosidase, which converts Asn to Asp, resulting in a 1 u increase in the mass of the peptide to m/z 1154.5. The position of this residue was established by MS-MS analysis.

The application of semipreparative LC techniques reflects the experimental simplicity of off-line LC purification of protein or peptide cell components and their subsequent infusion into the electrospray or ionspray source via fused-silica capillary tubing connected to an infusion pump and a suitable LC injector. However, on account of the special nature of this special volume on coupled techniques, on-line LC-MS applications have been preferentially considered in this review.

The application of LC-ESI-MS in conjunction with off-line infusion MS and CID-MS-MS techniques is illustrated in a recent publication describing the differentiation of minor sequence variations in a family of related antibacterial proteins/peptides extracted from bovine neutro-

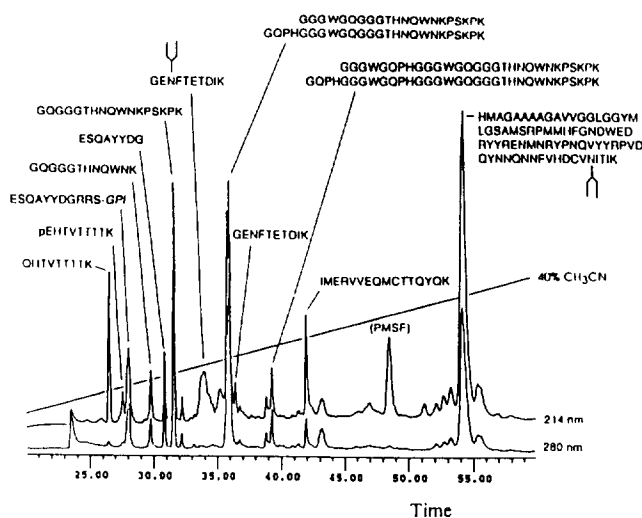


Fig. 9. Reversed-phase HPLC of a Lys-C digest from a truncated form of a prion protein (reprinted with permission from Ref. [67]).

phile granules [68]. In this instance LC–ionspray MS was instrumental in the characterization of a proline- and arginine-rich peptide of similar molecular mass and amino-acid composition to other known members of this family. Another example of the power of these techniques for the characterization of peptide modifications is the report describing a rapid method for the identification of phosphorylation in peptides and proteins [69] by HPLC–ESI–MS. The authors described the relative complexity of the classical approach involving pretreatment of cells with radioactive [^{32}P]ATP and isolation of the phosphoproteins by immunoprecipitation. This is followed by immunoblotting and enzymatic digestion on PVDF membranes with gas-phase Edman degradation of the radiolabelled peptide fragments. A number of major disadvantages are associated with this procedure so that alternative methods have been evaluated. Thus, negative-ion LC–ESI–MS with high nozzle-to-skimmer orifice potential CID in the low mass region of the spectrum to enhance the formation of diagnostic m/z 79 ions has been found to provide a method for identifying phosphorylated peptides from protein digests. In this case, peptide separations were carried out on a 320- μm capillary

LC column at a flow-rate of 5 $\mu\text{l}/\text{min}$ adjusted with the aid of a 50:1 precolumn split. The end of the column was connected directly to a piece of 75 μm I.D. fused-silica tubing terminated at the ionspray needle assembly.

In another interesting application of combined LC–MS in the field of immunology, microcapillary HPLC–ES–MS–MS was used for the characterization of peptides bound to HLA-A2.1, one of the most widely distributed Class I MHC molecules in humans [70]. For this purpose, these Class I molecules were immunoprecipitated from a human lymphoblastoid cell line and the associated peptides were released by acid extraction and separated by filtration. The extracted peptides were fractionated on a 10 cm \times 75 μm I.D. C_{18} microcapillary column and eluted into the ESI source of the tandem mass spectrometer at 1–2 $\mu\text{l}/\text{min}$ with a sheath flow of 2–4 $\mu\text{l}/\text{min}$ of methanol–0.5% acetic acid (3:1). About 200 peptides were detected at levels down to 30–150 fmol. Eight of these were sequenced as nonamers and four were found in cellular proteins. Recently, the same group reported on a system in which flow splitting of the effluent from the HPLC microcapillary column allows simultaneous immunological and mass spec-

trometric analysis [71] of individual HPLC fractions. Using this system, the authors identified a peptide epitope that is recognized by high-affinity human cytotoxic T cell lines from five melanoma patients.

Structural verification of post-translational modifications in recombinant proteins can be efficiently obtained in the time frame of an LC run, as shown recently using as an example an LC–MS set-up with postcolumn (narrow-bore) or precolumn (capillary column) flow splitting for the analysis of the primary structure of a recombinant *Schistosoma mansoni* antigen (Fig. 10). Complete primary structure verification of this 210 amino acid protein was obtained from 7 pmol of an in-gel tryptic digest [72]. In this interesting application, 4 nmol of the recombinant protein were subjected to SDS-PAGE, digested in the gel slice and 7 pmol of the tryptic digest were analysed by on-line LC–MS using a 250- μm capillary column. As discussed by the authors, this technique should facilitate precise mass analyses of more proteins in the future and may help in identifying a protein band from the

mass values of a few peptide fragments by appropriate screening of protein sequence databases, as indicated above [64].

Another example of the power of on-line capillary LC–MS analysis of peptides containing modified amino acid residues involves the identification of a lipoic acid modified lysine residue in a purified extract of a subunit of a mitochondria glycine cleavage complex. This work was carried out by using both LC–CF-FAB-MS and LC–ESI-MS, whereas tandem LC–MS allowed the determination of the exact location of this cofactor [73]. The authors concluded that electrospray would be a more suitable ionization technique for the characterization of limited amounts of samples from complex mixtures. For instance, under similar conditions of flow-rate, gradient elution and scan time, the detection limits for CF-FAB and ESI were 60 and 20 pmol, respectively. Also, ESI is more efficient in the ionization of peptides of $M_r > 2000$.

The combined use of these two LC–MS techniques was also described in other recent publications and has been compared [74]. For in-

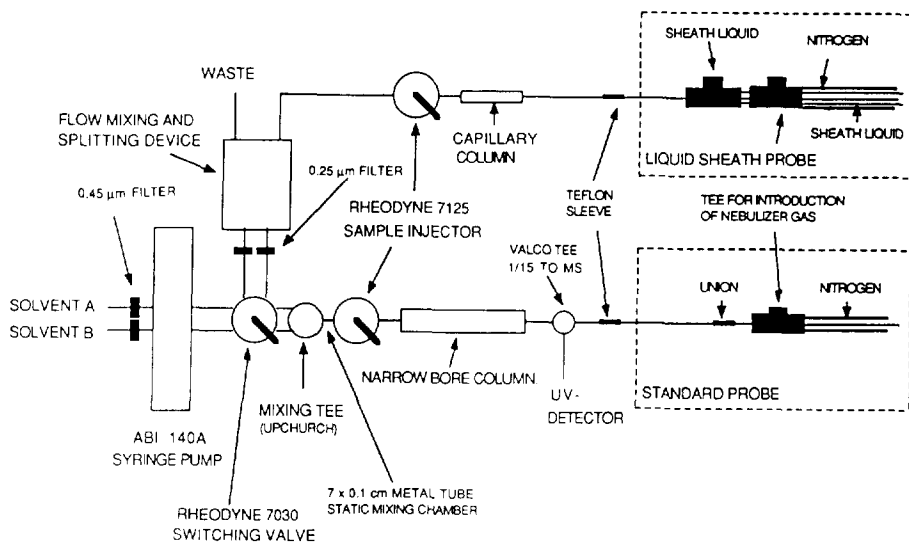


Fig. 10. HPLC–MS set-up for narrow-bore LC with postcolumn flow splitting or capillary LC with precolumn flow splitting (reprinted with permission from Ref. [72]).

stance, the interaction between lysozyme and its inhibitor tri-N-acetylglucosamine has been shown to involve the Asp 101 and Trp 62 residues in the protein [75]. For this purpose, the functional groups on the surface of the lysozyme were chemically modified, then the modified and unmodified lysozyme was reduced, carboxy-methylated and digested with chymotrypsin. The molecular masses of the peptides thus generated were determined by LC–continuous flow frit-FAB-MS at 5 $\mu\text{l}/\text{min}$ and ESI was used to establish the degree of modification. The rationale behind this approach was that if a functional group is modified in the absence of the inhibitor but unmodified in its presence, this group must be involved in the interaction with the inhibitor.

Although more limited in scope than the electrospray techniques for the elucidation of protein structure, CF-FAB is still being efficiently used. This is illustrated by two recent papers describing the application of this LC–MS technique to the determination of the rates of amide hydrogen exchange as a general tool for investigating protein structure and dynamics [76] and to the analysis of enzymatic digests of a bacterial protease [77].

3.2. Oligosaccharides and glycoproteins

Glycoproteins are implicated in a wide variety of biological processes and therefore a detailed knowledge of their structure is of prime importance for the understanding of complex functional roles. The carbohydrate chains of glycoproteins can be linked through and asparagine (N-linked) or a serine or threonine (O-linked) side-chain. To date, the determination of the carbohydrate content has involved their chemical or enzymatic release, leading to the loss of the sequence context for each family of glycoforms. Hence, suitable mass spectrometric methods for the location of glycopeptide-containing fractions in proteolytic digests of glycoproteins have been developed. In this regard, since the relatively high masses of the tryptic glycopeptides (2000–5000) together with their relatively high hydro-

philic character limit the use of flow-FAB techniques, electrospray ionization MS has mostly been used in this context. Again, there are many examples in the literature of the use of off-line semipreparative LC methods with subsequent MS study of purified fractions. However, as for peptides and proteins, these types of studies can also be carried out by on-line LC–ESI-MS techniques capable both of detecting the location of glycopeptide-containing fractions and of providing structural information while retaining at the same time the necessary information on the attachment site. For instance, two research groups at Genentech in California and SmithKline Beecham in Pennsylvania have been instrumental in the development of LC–MS methods for the identification of N- and O-linked oligosaccharides in glycoproteins [65,78–80].

Basically, one of these methods, which has been claimed to be the first integrated LC–MS strategy for glycoproteins [78], involves selective detection at low-picomole levels of diagnostic sugar oxonium ions (HexNAc^+ at m/z 204) from collisionally excited glycopeptides, either in single- or triple-stage quadrupoles, and differentiation of N- from O-linked glycopeptides by LC–ES-MS analysis of the digested glycoproteins prior to and after selective removal of N-linked carbohydrates by peptide N-glycosidase F. The method was successfully applied to a soluble complement receptor type I glycoprotein of M_r 240 000 containing 25 potential sites for N-glycosylation. The other method also uses LC–electrospray MS for the characterization of protein digest peptides separated by reversed-phase HPLC on narrow-bore 2.1 mm I.D. columns [80]. The authors illustrated the power of the method using trypsin-digested recombinant tissue plasminogen activator. The glycopeptides were initially observed as a cluster of negatively sloping ions in a contour plot of data from the LC–MS run (m/z vs retention time) or as a characteristic series of masses at different elution times. The search for a particular glycopeptide can be based on previously known carbohydrate structures and on consensus glycosylation sites. Further structural information is obtainable with

neuraminidase digestion and LC–MS analysis. The mass shifts following the specific glycosidase digestion allow further confirmation of the structure.

3.3. Nucleosides and nucleotides

True on-line LC–MS applications in this field are scarce compared with peptides and proteins and are mostly carried out by thermospray techniques. For example, an anti-AIDS nucleoside, 2',3'-dideoxycytidine (DDC), was determined in plasma samples by LC–thermospray MS. A stable isotope analogue of DDC ($[^{13}\text{N}_2, ^2\text{H}_2]$ DDC) was used as an internal standard. Selected-ion monitoring of the protonated molecular ions for DDC and the internal standard was used to record mass chromatograms. The detection limit was 50 pg on-column and the calibration graph was linear over the desired range, 0.25–20 ng/ml [81]. Calf thymus DNA adduct formation with an environmental contaminant such as 3-nitrobenzo[*a*]pyrene (3-nitro-B[*a*]P) was studied through the reaction of N-hydroxy-3-amino-B[*a*]P. After enzymatic digestion of the DNA, the resulting modified nucleosides were analysed by HPLC–thermospray MS and high-resolution proton NMR spectroscopy. The major adduct was identified as 6-(deoxyguanosin-N²-yl)-3-amino-B[*a*]P [82].

Likewise, mutations in human mitochondrial DNA have also been studied by quantitative micro-LC–MS determination of 8-hydroxydeoxyguanosine (8-OH-dG), a hydroxyl-radical adduct of deoxyguanosine, in mtDNA [83]. Thermospray and electrospray techniques have been reported to be of use for the determination of post-transcriptional modified nucleotides in RNA [84]. The method is based on the accurate measurement of the individual molecular mass of the various oligonucleotides generated by enzymatic cleavage. In a similar way as was done for tryptic peptides, the base composition of the different fragments can then be derived from these masses and placement of the modification at a specific site can be made by comparison with the molecular mass value predicted from the gene sequence. LC–CF–FAB–MS has been used

for the detection of a growth-promoting adenosine–cytidine dinucleotide and neurofibroma extracts [85].

3.4. Clinical studies, diagnosis, biological markers of disease

Although the use of LC–MS techniques cannot be considered to be widespread in the clinical setting, as is the case with their use in academic and industrial biochemistry and biology laboratories, interesting examples have already been presented in the literature. In this regard, cost and efficiency of new methods for the specific detection and identification of disease-specific biological markers, especially in rare cases, is of paramount interest if these methods are to be accepted by clinicians.

Along these lines, there is an interesting publication addressing the experimental expediting of rare variant haemoglobin characterization by introducing combined HPLC–ESI–MS in the haemoglobinopathy laboratory of the Children's Hospital in Oakland [86]. In this case, the authors were able simultaneously to separate and identify intact globins and tryptic peptides, thus eliminating the preparative-scale HPLC of globin chains and the semi-preparative HPLC of proteolytic digests which had been used prior to mass spectrometry. The use of microbore 1 mm I.D. HPLC columns at a flow-rate of 40 $\mu\text{l}/\text{min}$ reduced the time required for analysis substantially and solvent usage 100-fold. The eluates from these columns were split 10:1 so that about 4 $\mu\text{l}/\text{min}$ together with 2 $\mu\text{l}/\text{min}$ of 0.2% TFA in methanol, added as a sheath liquid, were introduced into the electrospray probe. Molecular masses of intact globins and masses of and sequence information on tryptic peptides could be obtained without collecting and separately analysing chromatographic fractions. As an example of the use of these methods, the authors reported the characterization of an unknown haemoglobinopathy case that was finally authenticated as Hb P-Galveston [β 117(G19)His \rightarrow Arg], using the following sequence of analyses: (1) ES–MS of the complete haemolysate, (2) analytical HPLC of globin chains, (3) combined

microbore HPLC–ES-MS of globin chains to determine their molecular masses, (4) cysteine derivatization and tryptic digestion of the mixture of all globins, followed by microbore separation of the peptides, molecular mass determination and generation of fragmentation patterns, allowing confirmation of amino acid sequences. This four-part strategy should allow the characterization of almost all variant Hbs.

The same group also reported on the use of a LC–thermospray MS interface to demonstrate that HIV infection is associated with anomalous hepatic metabolism in the form of accelerated *de novo* lipogenesis [87]. Electrospray has also been used in a clinical context for the detection and identification of urinary metabolites of an anti-tumour drug in the urine of a patient with advanced breast cancer, as discussed before [16].

Thermospray MS detection has been reported for the determination of an oral cephalosporin antibiotic, exhibiting antibacterial activity, in samples of human sputum. In this case a column-switching LC–LC–TS-MS system was operated in the TS positive-ion mode with the filament off. The method was applied in a clinical study protocol with a detection limit of 500 ng/ml and linear calibration graphs in the working concentration range, for instance, in the case of patients undergoing tracheostomy or laryngectomy, where the method allowed pharmacokinetic applications [88].

On account of the relatively low molecular masses and nature of many of the compounds monitored in patients' plasma or urine samples, one of the most promising LC–MS interfaces in this field is APCI. Its relatively high sensitivity and the possibility of implementing it in inexpensive automated benchtop systems will make it very useful in the clinical laboratory, as illustrated by several recent reports. For instance, APCI techniques were instrumental in the determination of serum 1,5-anhydroglucitol in uraemic and diabetic patients [89] and in the identification of novel cystathionine mono-oxo acids, S-(3-oxo-3-carboxy-*n*-propyl)cysteine, S-(2-oxo-2-carboxyethyl)homocysteine and cystathionine ketimine, in the urine of a patient with cystathioninuria [90]. The cystathioninuric

patient excreted twenty times more cystathionine mono-oxo acids in the urine than healthy subjects.

A similar experimental LC–MS approach has been reported also for the identification and determination of urinary iminodipeptides in prolidase deficiency, since the urine of patients with this deficiency contains various iminodipeptides with a carboxyl-terminal proline (hydroxyproline). Patients were found to excrete much more Pro–Hyp than normal volunteers [91]. In another clinical case study, a 3.5-year-old Japanese boy with a mild speech disturbance excreted a large amount of α -amino adipic acid into the urine, as identified using LC–API-MS [92].

Micro-HPLC–CF-FAB-MS has been demonstrated to be of use for the detection of inborn errors of bile acid metabolism. This was achieved through the clinical analysis of urinary bile acids and their conjugates [93].

3.5. General conclusions on the use of modern LC–MS techniques

Of all the currently available interfaces, which are described in some detail in the paper by Niesser in this volume, those based on the ionization of the sample at atmospheric pressure seem to offer today the best possibilities for a wide range of compound types. Each can be characterized by its inherent advantages and limitations, which have also been discussed in some detail in the literature, often from a theoretical point of view. It is evident that one can list a number of characteristics for an ideal LC detector and that these would apply to the mass spectrometer when coupled to the LC system. However, from a more practical point of view it would be more interesting to gain an insight into the positive and negative characteristics of LC–MS interfaces that can be inferred from the literature as workers use them in their everyday or special applications. Thus, an evaluation of the references included in this review with regards to electrospray and ionspray clearly shows what workers in their laboratories often perceive as distinct advantages or shortcomings.

Amongst the advantages and practical possibilities of these techniques one could list the following, for instance.

(a) The many possibilities for direct analysis without prior derivative formation of the analytes.

(b) The mildness of the ionization process since the spray is generated by high voltage rather than heat. This often translates into fewer fragments than in thermospray [13].

(c) Their suitability for very polar compounds.

(d) The potential for extremely high sensitivity, as recently demonstrated with the optimization of a micro-electrospray ionization source with detection limits at the zeptomole/attomole per microlitre level for neuropeptides at submicrolitre flows [94].

(e) The possibilities for the study of covalent or non-covalent interactions between analytes and structurally relevant biopolymers (conjugates, peptides, proteins, oligonucleotides).

(f) When the quasi-molecular ion $[M + H]^+$ is not evident in the ESI spectrum, the addition of mobile phase buffers may facilitate its recognition through cluster ion formation [16].

(g) Even though fragmentation is suppressed by the mild ionization process, structurally relevant product ions can be induced by increasing the potential difference between the capillary end and the source skimmer. This results in CID fragment ions and the extent of fragmentation can be controlled by the voltage difference applied.

(h) For the routine chromatographer, the ion-spray mode of operation is more practical because of the higher flow-rates and thus lower splitting ratios or no splitting at all in micro-HPLC. In this regard, it is expected that the new high-flow interfaces now commercially available will enhance the application of LC-MS to biomedical problems.

The main shortcomings of spray ionization techniques could be summarized as follows.

(a) Mobile phases with volumetric flow-rates exceeding $5 \mu\text{l}/\text{min}$ and with high conductivity or surface tension cannot be used [91]. Accordingly, to extend the mobile phase composition range, an ultrasonic nebulizer has recently been

introduced [95]. Nevertheless, there are ways to avoid these limitations. For instance, flow-rates of 50–400 can be efficiently used in the new commercial designs of electrospray interfaces where desolvation is mostly aided by heat, although in principle this could result in some thermal degradation of very labile compounds. The conductivity restriction prevents the use of ion exchange and mobile phase modifiers such as TFA because mobile phase additives would compete with sample ionization. Nevertheless, in practice all of the studies on peptide and protein separations discussed above were carried out in the presence of small amounts of TFA. Finally, with regard to the surface tension restriction, it has been shown that when the mobile phase composition is very high in water content or the analytes are extremely hydrophilic, good results can be obtained with the addition of a sheath liquid, as indicated above [70] and shown in Figs. 3 and 10. For instance, a very hydrophilic peptide was missing from the HPLC trace of a tryptic digest, because by eluting early it was in the zone of a high water content of the mobile phase, which resulted in a loss of sensitivity. Addition of a coaxial sheath flow of 2-methoxy-ethanol–2-propanol (2:1) containing 1% formic acid at $4 \mu\text{l}/\text{min}$ allowed the detection of this peptide [72]. Another example where the relatively high water content of the mobile phase called for a sheath flow of 0.2% TFA in methanol is the separation of tryptic peptides from haemoglobin [86].

(b) Despite all of these seemingly effective approaches to the minimization of the above shortcomings, there is still the limitation of using volatile mobile phase buffers only.

(c) The response can vary depending on the compound type and LC mobile phase composition, although determination is often possible by using the proper standards and calibration graphs.

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