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Review

Fast atom bombardment mass spectrometry in the pharmaceutical analysis of drugs*

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Abstract: The principles, techniques and value of fast atom bombardment mass spectrometry are summarized in this review. Applications of the method in such areas as molecular weight determination, standard and metabolic studies, and peptide sequencing, as well as direct quantitative analysis, are surveyed, with examples from several classes of compounds with pharmacological activity.

Keywords: *Fast atom bombardment mass spectrometry; antibiotics; analgesics; peptides; peptide sequencing; nucleosides and nucleotides; steroids.*

Introduction

Fast Atom Bombardment (FAB) mass spectrometry has only recently been introduced into the pharmaceutical analysis of drugs. Since then, its advantages have been clear every time the technique is used to study another drug. In this report some recent applications resulting from the use of FAB-MS in the study of drugs, drug metabolites and biological samples will be presented.

Pharmaceutical analysis

Drugs are formulated in a wide variety of ways [1]. They are presented to the consumer under various forms, such as tablets, capsules, ointments, creams, lotions, etc. Different additives are also added to the drug to complement the active ingredient, or to enhance the physical appearance or simply to improve the stability of the formulation. These additives can also assist in the biological distribution of the drug [2, 3].

Since drugs are manufactured on a large scale, quality control is also an important aspect of pharmaceutical analysis. It is of prime importance to verify the presence of impurities in such formulations and to be able to detect, characterize and quantitate them. After the elimination of the drug from the system, studies of the drug metabolites are also an important step, as characterization of these metabolites will help to establish the biological pathway by which the drug was processed (Fig. 1).

Because (chemical) pharmaceutical analysis can embrace all of these aspects, it requires an analytical method of choice. Such a technique should bear qualities such as

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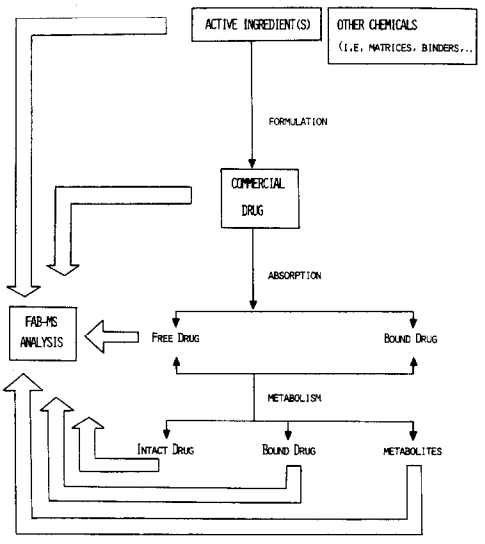


Figure 1
Metabolic pathways studies by FAB-MS.

being fast, specific, sensitive, easy to handle and give results that can be interpreted easily on a routine basis. Furthermore, since in clinical analysis the tendency is to reduce sample preparation work, selectivity becomes a major criterion.

Mass spectrometry has demonstrated its power and potential as an analytical tool. New developments in the field are heading towards bioanalytical applications [4–10]. New ways are being investigated to use the technique in the study of important biological compounds, polar drugs and their metabolites. Since biological chemistry comprises classes of compounds that are polar, have relatively high molecular weights, are complex, involatile and thermally degradable, the use of standard mass spectrometry (electron impact (EI) mode, for example) for such analysis has not always been conducive to success. But today, with the advent of FAB mass spectrometry, several of these problems can be resolved, at least for certain aspects, if not fully.

Fast atom bombardment mass spectrometry

The rapid and specific diagnostic technique for drug analysis discussed here is fast atom bombardment mass spectrometry (FAB-MS). Although not novel in principle [11–18], the technique has seen its bioorganic applications only in the last few years when adapted by Barber *et al.* [19] and by Surman and Vickerman [20]. It has now shown great potential in pharmaceutical analysis, as will be demonstrated below. FAB-MS has been applied to the analysis of a wide variety of drugs and biological samples. The technique can be used for qualitative or quantitative analysis of complex mixtures of compound classes or for specific compounds within a biological matrix. Some applications of FAB-MS in pharmaceutical analysis will be described and some of its strong points and potential as an analytical tool for the pharmaceutical community demonstrated. Reviews dealing with the technique itself are currently available [21–24].

General Concepts of Fast Atom Bombardment Mass Spectrometry FAB-MS

Technique

Fast atom bombardment mass spectrometry is a powerful structural tool. It has shown great potential for high molecular weight compounds, many of which are highly polar

and of generally low volatility. Molecular weight information [25], as well as structural information [26–28], has been obtained for various compounds.

The technique of FAB-MS is documented in the literature [21–24, 29, 30]. Its operational principle will therefore be described only briefly. FAB-MS is one of the youngest soft ionization techniques. It is based on the sputtering phenomenon, an old concept first reported by Grove in 1852 [22].

The phenomenon can be described very simply. An inert gas (xenon has been reported to be one of the most efficient gases for FAB [31, 32]) is ionized by a high potential and the resulting ion beam is accelerated through an electric field. The resulting (fast) ions enter a xenon gas chamber where they undergo charge-exchange reactions (see equation 1) that produce fast atoms that retain most of the original direction and kinetic energy of the fast ions.

$$Xe^{+\cdot}\ (fast) + Xe^{*} \rightarrow Xe\ (fast) + Xe^{+\cdot *} \qquad (1)$$

Residual fast xenon ions can be removed by a high voltage deflection plate leaving a beam of fast atoms. It is known that when such a fast atom beam bombardment process takes place (as illustrated in Fig. 2), some of the large amount of kinetic energy carried by the atoms is dissipated by volatilizing and ionizing part of the sample that was previously coated on the surface being bombarded. This is called the 'sputtering phenomenon'. Both positive and negative ions can be directed into the mass analyser by applying an appropriate electric gradient on the accelerating plates [33–38].

Information that can be obtained from FAB mass spectra

The samples used in FAB-MS are usually underivatized. Almost invariably true molecular ions are not observed in the spectra; the molecule forms a stable ion by addition or loss of a proton (depending on whether the instrument is operated in the

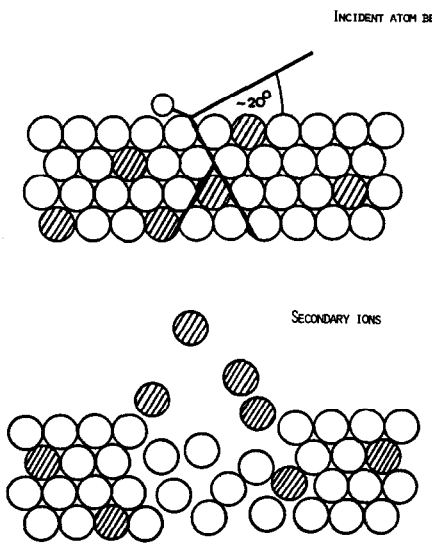


Figure 2
FAB-MS: the sputtering phenomenon.

*Indicates thermal ions.

positive or negative mode respectively). Alkali metal salts form similar stable ionic species, in relatively high abundance, by the addition or the loss of the alkali metal cation (e.g. Na^+ or K^+). Cationized species can also be formed from mixtures of organic molecules when salts are added or naturally present as impurities in the sample [22].

Fast atom bombardment mass spectra are basically always recorded with a support matrix. Glycerol is the preferred substance for that purpose [22, 23]; it has the advantage of providing enhanced sensitivity as compared to solid sample preparations. Background ions due to glycerol are found at *m/z* values corresponding to $(92n + 1)^+$ and $(92n - 1)^-$ where n is the number of glycerol molecules and has values up to 15. Other solvent matrices, such as thioglycerol, diethanolamine, etc., can also be used. For better sensitivity, the sample should form a perfect monolayer at the surface of a substrate having low volatility (although this is rarely encountered in practice; results have shown that it is not primordial [28]). It should be noted that one of the critical factors in FAB-MS is not the dissolution of the sample in the matrix solvent but that the matrix can actually bind itself to the molecule under study [28, 39]. Mixtures of solvent matrices can also be used with various degree of success, as well as judicious doping of the sample with alkali metal salts or protonating agents.

The experimental parameters that should be considered for optimal operation and results are the following [40, 41]:

(1) the nature of the collision gas;
(2) the angle of incidence of the atom beam;
(3) the target area;
(4) the matrix.

Also, FAB ionization efficiencies are affected not only by the chemical properties of the matrix but by those of the sample as well. Recently, it has been suggested that the degree of fragmentation could be increased by using larger sample concentrations and lower fast atom beam energies [42].

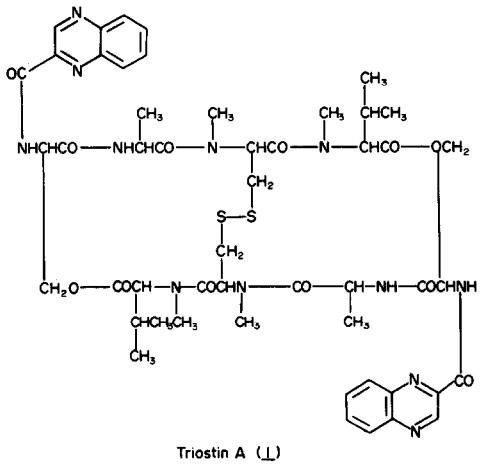
Applications of FAB-MS to Drug Analysis

The classes of drug compounds studied under FAB-MS conditions have been growing tremendously in the past few years. FAB spectra of substances ranging from biopolymers to antibiotics have now been successfully recorded. Various drugs will be discussed arranged into classes according to their structures, except for antibiotics that have been treated as a separate class. They were all studied by FAB recently or relate to some of the work under way in the present authors' laboratories.

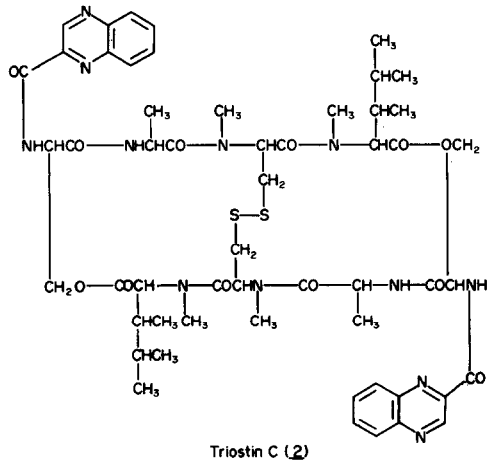
The antibiotics

Misra *et al.* [43] have reported successful characterization and structural elucidation studies of the antitumour antibiotic complex PR-1350 isolated from the fermentation broth of *Oidiodendron truncatum* Barron, using both FAB-MS and FAB-MS/MS techniques. These techniques were also used successfully in the studies of the anthraquinonoids of *Penicillium islandicum* Sopp. [43].

Negative FAB-MS was used by Williams and co-workers [44] in the identification of new quinoxaline antibiotics belonging to the triostin group (Scheme 1, Structures **1–7**) that had been prepared by directed biosynthesis. A mixture of alpha-thioglycerol and diglycerol was used for matrix since previous efforts to obtain FAB-MS of these substances with glycerol were unsuccessful.

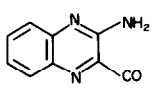


Triostin A (1)



Triostin C (2)

Analogs of triostin a were also disubstituted with the following chromophores, structures 3–7.



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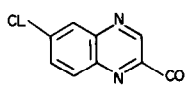

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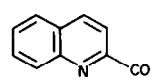

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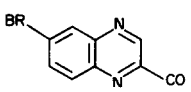

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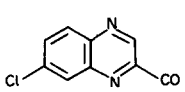
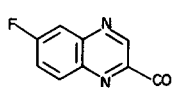


5B



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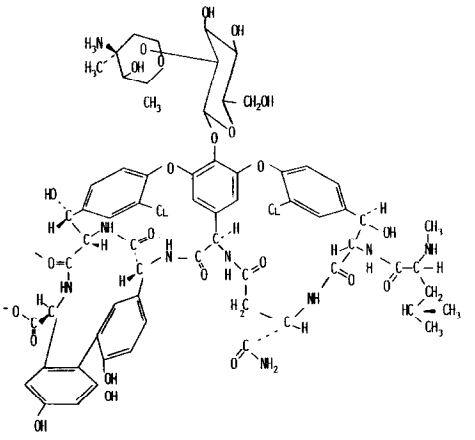
Scheme 1
Structures of the triostin group of antibiotics containing various chromophores (Structures **1–7**).

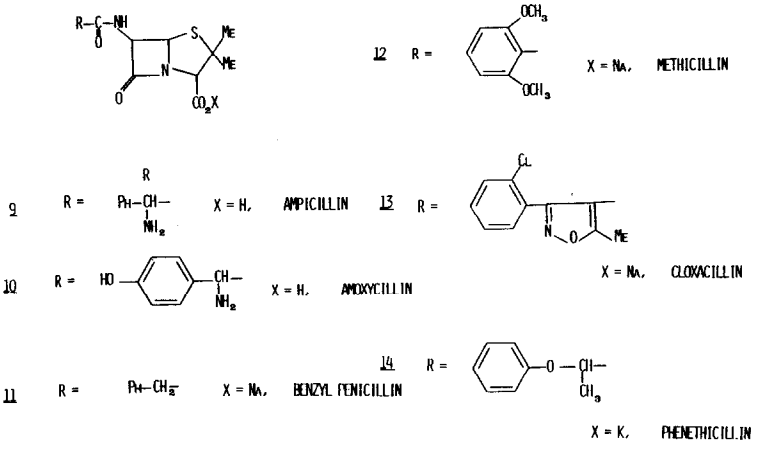
Barber *et al.* [45] reported FAB data for the glycopeptide antibiotic vancomycin (Scheme 2, Structure **8**). The structure of this antibiotic, which had been intractable until then, had been proved previously by X-ray diffraction analysis of its degradation product. The positive FAB-mass spectrum of vancomycin provided molecular weight information (which had been unobtainable by other methods, e.g. field desorption mass spectrometry (FD-MS)) as well as structurally useful fragment ions.

Positive and negative FAB spectra of commercially available penicillins, both as free acids and as alkali metal salts (Scheme 3, Structures **9–14**), were investigated by Barber *et al.* [46] using a glycerol matrix. Ampicillin and amoxycillin showed intense protonated molecular ions of the form $[M + H]^+$, while benzyl penicillin, methicillin, cloxacillin and phenethicillin showed intense alkali metal ions, in addition to the $[M + H]^+$ ion. The alkali metal salts species showed dimeric cluster ions of the form $[2M + X]^+$. Free acid species showed little tendency to form analogous $[2M + H]^+$ ions. A similar behaviour was also observed in the negative ion mode, i.e. $[M - H]^-$, $[M - X]^-$, etc. Fragmentation patterns were also proposed for both the positive and for the negative ion mode spectra.

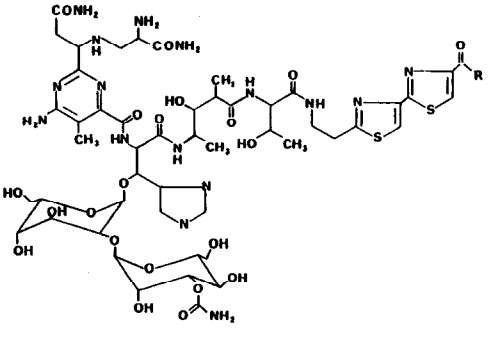
Barber *et al.* [47] have also investigated the complex glycopeptide antibiotic bleomycins, which are clinically useful antitumour agents. Bleomycin A_2 and B_2 (Scheme 4, Structures **15** and **16**) and some of their metal ion complexes were studied.



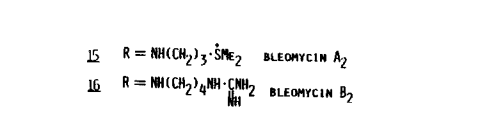
Scheme 2
Structure of vancomycin (Structure **8**).



Scheme 3
Structures of some penicillin analogues (Structures **9–14**)



Scheme 4
Structures of bleomycins A_2 and bleomycin B_2 (Structures **15** and **16**).



Characterization of these bleomycins, either separately or as mixtures, was achieved by FAB-MS, along with structurally significant fragmentation patterns. FAB-MS of a ferrous complex of bleomycin A_2 was used successfully in confirmation of the formulation. The FAB mass spectra of the Cu and other metal complexes were also in agreement with the proposed structures. The structures of the naturally occurring bleomycins A_2 and B_2 and some of their metal complexes were also studied by Dell *et al.* [48] in a complementary study using both FD-MS and FAB-MS. Although both techniques gave molecular weight information, FAB yielded spectra that were easier to interpret and is a technique more amenable to routine analysis. *In situ* derivatization of the bleomycins was also done experimentally during the course of these FAB studies and unexpected catalysis has been reported to occur in the glycerol matrix [48].

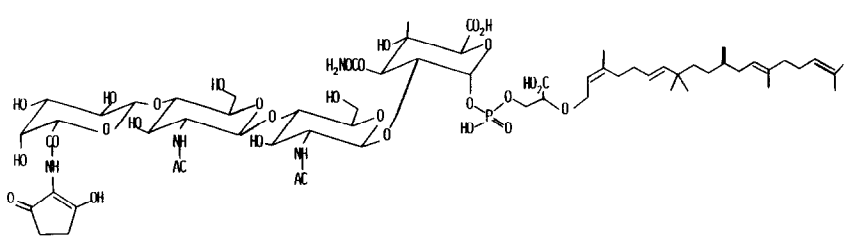
Rinehart *et al.* [49] used FAB-MS to study the structure of 11 zervamicin (Scheme 5, Structures **17–27**) and two emerimicin peptide antibiotics; the latter two proved to be identical with zervamicin IIA and IIB. These intact peptides that had long resisted, or at best had given poor results by FD-MS and EI-MS, provided meaningful results under FAB-MS conditions.

Scheme 5
The structures of some zervamicins (Structures **17–27**).

17	Ac-Trp-Ile-Glu-Iva-Val-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIA)
18	Ac-Trp-Val-Glu-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIB)
19	Ac-Trp-Ile-Glu-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIB)
20	Ac-Trp-Ile-Glu-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIC)
21	Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIIA)
22	Ac-Trp-Ile-Gln-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZIIB)
23	Ac-Trp-Ile-Gln-Aib-Val-Thr-Aib-Leu-Aib-HmGln-Aib-Hyp-Aib-Pro-Phol	(ZII-1)
24	Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Val-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZII-2)
25	Ac-Trp-Val-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZII-3)
26	Ac-Trp-Ile-Gln-Iva-Val-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZII-4)
27	Ac-Trp-Ile-Gln-Iva-Ile-Thr-Aib-Val-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol	(ZII-5)

The perhydro-derivative of pholipomycin (Scheme 6, Structure **28**), a phosphoglycolipid antibiotic, was studied under FAB-MS conditions by Takahashi *et al.* [50]. Molecular ion species are reported at *m/z* 1446 $[M + H]^+$; 1468 $[M + Na]^+$; 1484 $[M + K]^+$; and 1491 $[M + 2Na]^+$, thus indicating that the molecular weight of perhydropholipomycin is 1445. The corresponding intact natural species would have a molecular weight of 1435 ($C_{63}H_{96}O_{31}N_4P$).

In a recent paper [51] Brückner and Przybylski have used FAB-MS (both positive and negative) in conjunction with HPLC and FD-MS in order to demonstrate that the

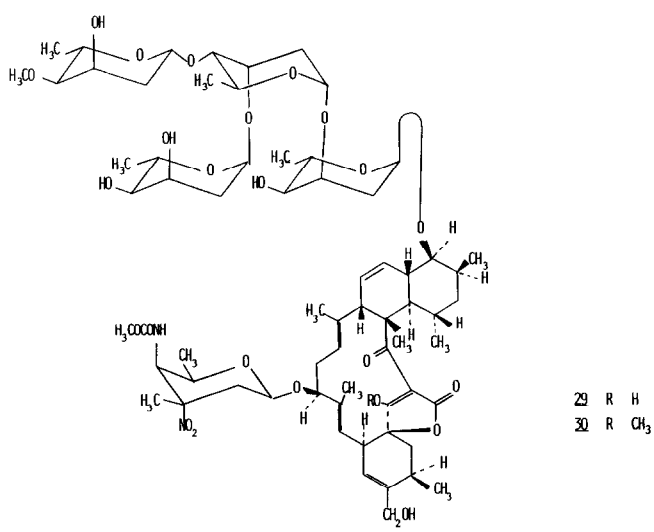


Scheme 6
The structure of pholipomycin (Structure **28**).

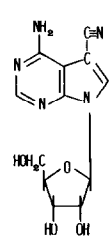
combination of these techniques could provide "a highly sensitive and universal method for the direct resolution and structural characterization of peptaibol components" [51]. Among the peptaibol class of compounds, they studied trichotoxin, alamethicin, suzukacillin, hypelcin and paracelsin. These polypeptide antibiotics have molecular weights *ca* 2000 daltons. Oligoethylene glycol (in particular TEG) was used as the matrix solvent for these substances, with a net improvement of the FAB spectra over the use of glycerol.

In a paper by Pramanik *et al.* [52] FAB-MS was used for structural elucidation problems of antibiotics containing oligosaccharides. These authors have recognized special fragmentation pathways for compounds containing nitro sugar moieties. Fragments such as $[M + Na - NO_2]^+$ and $[M + Na - 16]^+$ are observed as illustrated with the structure of kijanimicin (Scheme 7, Structures **29** and **30**), and its methyl ether derivative. Also observed is an ion corresponding to *m/z* $[M + Na - HNO_2]^+$.

Tondeur *et al.* [53] reported on the study of the nucleoside antitumor antibiotic toyocamycin (Scheme 8, Structure **31**) in a fermentation broth by a combination of positive and negative FAB-MS, High Resolution FAB-MS and MIKES. Best limits of detection in the whole broth were obtained by tandem MS and FAB-MS. Combination of HPLC and FAB were useful to monitor toyocamycin at all stages of strain development, fermentation and recovery.



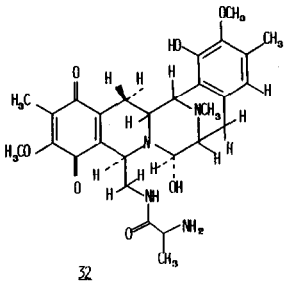
Scheme 7
The structure of kijanimicin and its methyl ether derivative (Structures **29** and **30**).



Scheme 8
The structure of toyocamycin (Structure **31**).

Another antibiotic with antitumor activity whose behaviour was studied under FAB-MS conditions, is the quinone EM 5519 (Scheme 9, Structure **32**) as reported by Cooper and Unger [54]. This compound was identical to safracin B by chemical and spectroscopic studies. Along with the protonated molecular species, $[M + H]^+$, ions corresponding to $[M + 2H]^{+\cdot}$ and $[M + 3H]^+$ were also observed. It appears that saframycin R undergoes an experimental condition-dependent reduction process under FAB ionization. The reduction of quinone and hydroquinone was also observed in positive and negative FAB-MS.

Scheme 9
The structure of quinone EM 5519 (Structure **32**).



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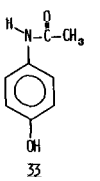
Analgesic

The major metabolites of the analgesic acetaminophen (APAP) (Scheme 10, Structure **33**) were studied under FAB conditions by Ackermann *et al.* [55]. Molecular information for these metabolites, both from synthetic and biological sources (urine extractions purified by HPLC) was obtained, as well as a spectrum of the sulfate conjugates which had until then resisted every other mass spectrometry technique. This paper also discusses more fundamental aspects such as background and S/B ratio and it describes a method of using FAB in the analysis of urine fractions containing metabolites at therapeutic levels after purification by reverse phase-HPLC.

Scheme 10
The structure of acetaminophen (APAP) (Structure **33**).



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Peptides

The peptides are amongst the classes of compounds that have been the most extensively studied by FAB-MS. This is due to the fact that this technique can provide accurate molecular weight measurements (for very high masses) as well as useful fragmentation information for these important biological molecules. This section will be devoted to a brief review of the application of FAB-MS to peptides from 1981 to 1985.

One of the very first papers in the literature dealing with FAB-MS was that by Barber *et al.* [30] involving the study of the pentapeptides enkephalins. Both methionine- and leucine-enkephalins were studied by positive and negative FAB-MS; the positive spectra being more complex. The spectra were recorded in glycerol and, along with protonated molecular ions, cationized species were observed (K^+ and Na^+; these salts were added to the samples). FAB-MS allowed complete sequencing of the two pentapeptides methionine (**34**) and leucine (**35**) enkephalins (Scheme 11).

Scheme 11
The structures of leucine and methionine enkephalins (Structures **34** and **35**).

Tyr–Gly–Gly–Phe–Met **(34)**
Tyr–Gly–Gly–Phe–Leu **(35)**

Peptide sequencing can occur by the loss of the neutral C-terminal, in which case the sequence found in the spectra of both peptides will be the same, i.e. after the loss of the terminal Met or Leu amino acid. It can also occur from the loss of the neutral N-terminal; the resulting sequence ions will vary evenly by the mass of their different amino acid, i.e. Met or Leu. Other fragment ions also occur in both peptides from side-chain cleavages of some of the amino acid residues, as well as 'diagnostic ions' due to particular amino acids. In negative FAB spectra, the sequence ion cleavages from the N-termini are generally dominant, as opposed to the positive spectra where the C-termini ions were generally more abundant. Either way, both positive and negative FAB spectra were complementary in establishing an unambiguous amino acid sequence for these peptides.

The opioid pentapeptide leucine enkephalin sequence of amino acids was also confirmed with FAB-CAD-linked field scanning by the group of Desiderio *et al.* [56]. These authors also used this technique to quantitate the sample and they reported finding 451 pmol g^{-1} tissue of leucine enkephalin in an extracted sample from a canine caudate nucleus tissue. In that experiment, labelled $^{18}O_2$ leucine enkephalin was used as internal standard.

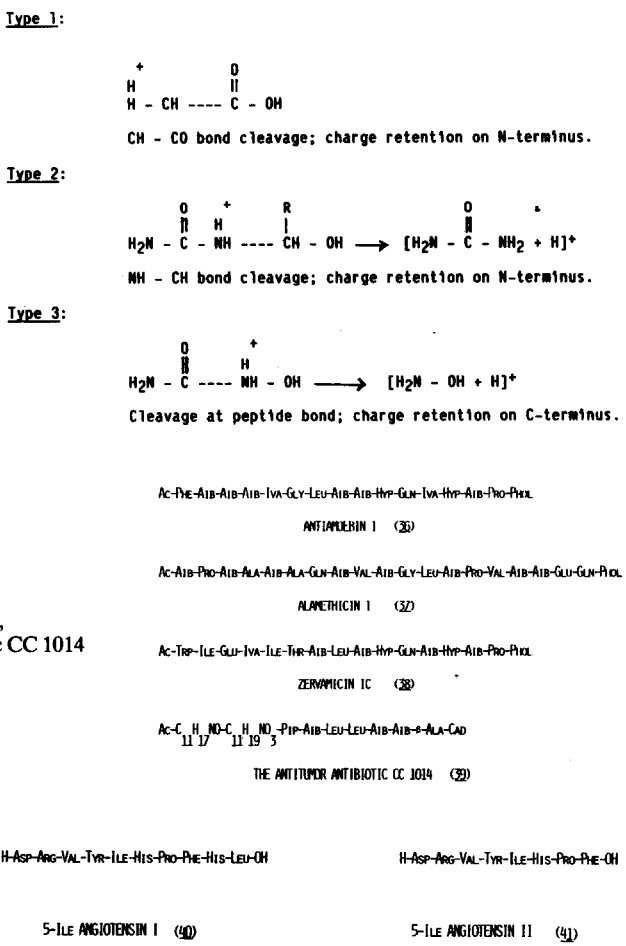
Williams *et al.* [57, 58] have used FAB-MS to determine the molecular weights and the sequences of what was termed 'difficult peptides'. These studies [57] involved two peptides from cytochrome C-550 from the bacterium *Paracoccus denitrificans* and one that was from the N-terminal portion of the dolphin (*Coryphaena hippurus*) cytochrome *c*, and were used successfully in conjunction with EI-MS of derivatized peptides. In another paper, the same group [59] also reported on the successful recording of FAB spectra for the neuropeptide substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; mol. wt = 1346), and the tridecapeptide Gly-Val-Val-Gly-Arg-Lys-Ile-Ala-Ser-Glu-Glu-Gly-Phe (mol. wt = 1347). These authors also note that sensitivity is improved when peptides bearing a net positive charge are run in the positive ion mode and those bearing a net negative charge are run in the negative ion mode. Suggestions are also made for the use of other matrix solvents; in cases where samples are not soluble or polar enough to dissolve in glycerol, they suggest using tetragol or teracol [59] (at the time, these spectra were recorded with argon as the bombarding gas. It has now been demonstrated that xenon gives better sensitivity [31]). The amino acid sequence in the undecapeptide amide substance P was also confirmed by fast atom bombardment — collision activated dissociation — linked field scanning experiments (B/E), performed by Desiderio and Katakuse [60].

Peptide antibiotics

With the advent of FAB-MS, Rinehart *et al.* [61] took advantage of the technique as a substitute to what they had previously termed a "General Procedure" [61] for the assignment of structures to peptides. The previous procedure involved six steps and relied on high resolution mass spectrometry (HRMS), field desorption mass spectrometry (FD-MS), gas chromatography electron ionization (GC-EIMS) and chemical ionization (CI-MS). In their new proposal, all these steps are replaced by a single recording of FAB spectra in the positive and negative ion modes, and were able to obtain both molecular weight and sequence information. Published work includes antiamoebin I, alamethicin, zervamicin I, C peptaibophol antibiotics, zervamicin II, the antitumor

antibiotic CC1014, the marine-derived didemnins A and B, gramicidin S, berninamycin A, polymyxins B, zorbamycin and its copper chelate, zorbonomycin B and its copper chelate, and saramycetin (Scheme 12, Structures **36–39**).

In their continuing account of peptide studies by FAB-MS, Barber *et al.* [62] characterized the decapeptide angiotensin I (mol. wt = 1295) and the octapeptide angiotensin II (mol. wt = 1045) (Scheme 13, Structures **40** and **41**). They observed molecular weight and sequence information and they used metastable scanning as a confirmation technique. The following three types of fragmentations were reported:



Scheme 12
The structures of antiamoebin, alamethicin, zervamicin 1C, and the antitumor antibiotic CC 1014 (Structures **36–39**).

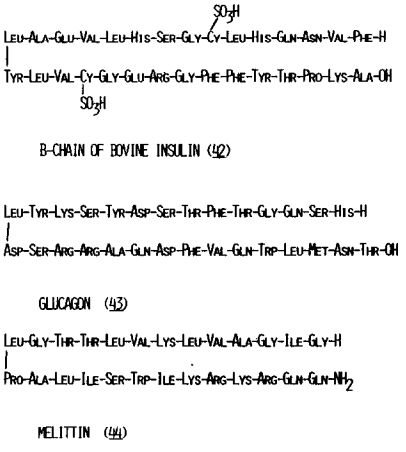
Scheme 13
The structures of 5-ILE-angiotensin I and 5-ILE angiotensin II (Structures **40** and **41**).

The spectra were run in both positive and negative ion modes in a glycerol/methanol matrix at levels of about 1 nmole per sample.

Heavier peptides

One of the great successes of FAB-MS has been the recording of spectra of very high molecular weight compounds.

In the early developments of the technique (1982), Barber *et al.* [63] and Morris *et al.* [64] reported recording with success the FAB-MS of large oligopeptides such as melithin (mol. wt = 2845.8), glucagon (mol. wt = 3481.5) and the oxidized B chain of bovine insulin (mol. wt = 3494.5) (Scheme 14, Structure **42–44**). Even though more sensitivity would have been required to obtain more structural information on these compounds, it was nevertheless a success in so far that these compounds were until then, considered to be mass spectrometrically intractable.



Scheme 14
The structures of the B-chain of bovine insulin, glucagon and melithin (Structures **42–44**).

The large natural biological molecule human proinsulin was investigated under FAB-MS conditions by Barber *et al.* [65]. An 'unresolved' protonated molecular ion was observed at *m/z* 9390 a.m.u. ($C_{410}H_{638}N_{114}O_{127}S_6$), in an acidified glycerol matrix using cesium iodide as a reference.

A sample of bovine proinsulin (mol. wt = 8679) was investigated under FAB-MS conditions by Cottrell and Frank [66]. These authors used α-monothioglycerol acidified with oxalic acid as their support matrix.

The FAB-MS of bovine insulin (mol. wt = 5731) was also studied by two other groups, Barber *et al.* [67] and Dell and Morris [68]. They observed partially resolved protonated molecular species $[M + H]^+$.

Samples of bovine, equine, ovine and porcine insulins were recorded in the positive FAB ionization mode [69]. These showed protonated $[M + H]^+$ species and isotope distribution for the protonated molecules, as well as significant structural fragments. Mass analysed ion kinetic energy spectra (MIKES) were used to indicate the fragmentation pathways of the $[M + H]^+$ and $[M - H]^-$ ions. Spectra were recorded in α-monothioglycerol as the matrix.

Desiderio and Katakuse [70] have reported on the FAB mass spectra of pure polypeptides such as bovine insulin (A and B chain; mol. wt = 5729.6, oxidized A-chain; mol. wt = 2529.9), porcine insulin (carboxymethylated B-chain; mol. wt = 3513.6) and glucagon (mol. wt = 3480.6). The samples were dissolved in methanol and glycerol was used as the matrix, except for glucagon, where thioglycerol was used and some trichloroacetic acid was added. For insulin A-chain (oxidized) and insulin B-chain (carboxymethylated), singly charged protonated molecular ions were observed in the

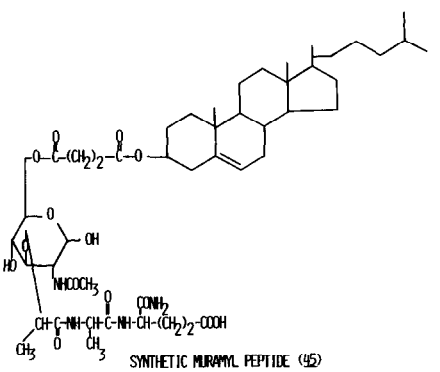
partial mass spectra, while doubly-charged molecular ions were observed for insulin and glucagon. In order to record these high mass ions the spectra were recorded with reduced accelerating voltage.

Buko *et al.* [71] investigated the negative FAB-MS spectra of human gastrin I, of human angiotensin I and of an equimolar mixture of the following four peptides: mastoparan, substance P, angiotensin and serum thymic factor, as well as another equimolar mixture of gastrin (an acidic peptide), angiotensin (a basic peptide) and serum thymic factor (a neutral peptide). All of these spectra of underivatized peptides showed prominent $[M - H]^-$ ions. The intensities of the latter varied for the above stated mixtures, depending on their acidic or basic properties. Characteristic fragmentation patterns were also reported (either amino terminal ions or carboxy terminal ions).

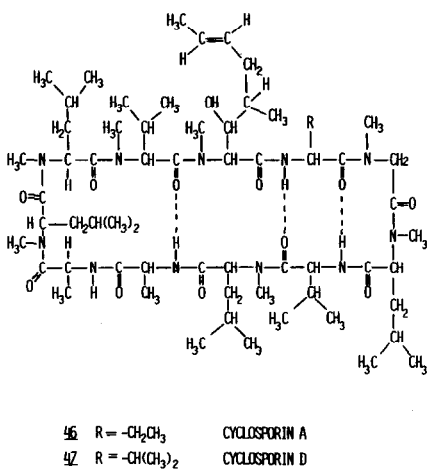
Other examples of peptides whose molecular weights were confirmed by FAB-MS include the synthetic β-melanocyte-stimulating hormone (β-MSH) [72], a synthetic immunostimulant muramyl peptide (Scheme 15, Structure **45**) [72] and Factor S, which is another muramyl peptide [72] that induces sleep in humans and mammals.

Cyclic peptides

The cyclic undecapeptide cyclosporin A (Scheme 16, Structure **46**), which is now routinely used as an anti-rejection drug for transplanted organs, was successfully recorded in our laboratories, using glycerol and glacial acetic acid as the matrix solvent.



Scheme 15
The structure of a synthetic muramyl peptide (Structure **45**).



Scheme 16
The structure of cyclosporin A and the structure of cyclosporin D (Structures **46** and **47**).

The high mass region of the FAB spectrum of a mixture of cyclosporin A and D, where cyclosporin D can be used as an internal standard for quantification experiments is presented in Fig. 3. More quantitative investigations on these compounds extracted from biological fluids and on their respective detection limits are currently under way in the present authors' laboratories (unpublished results). Jardine *et al.* [73] have presented recently FAB-MS data on some cyclosporin metabolites.

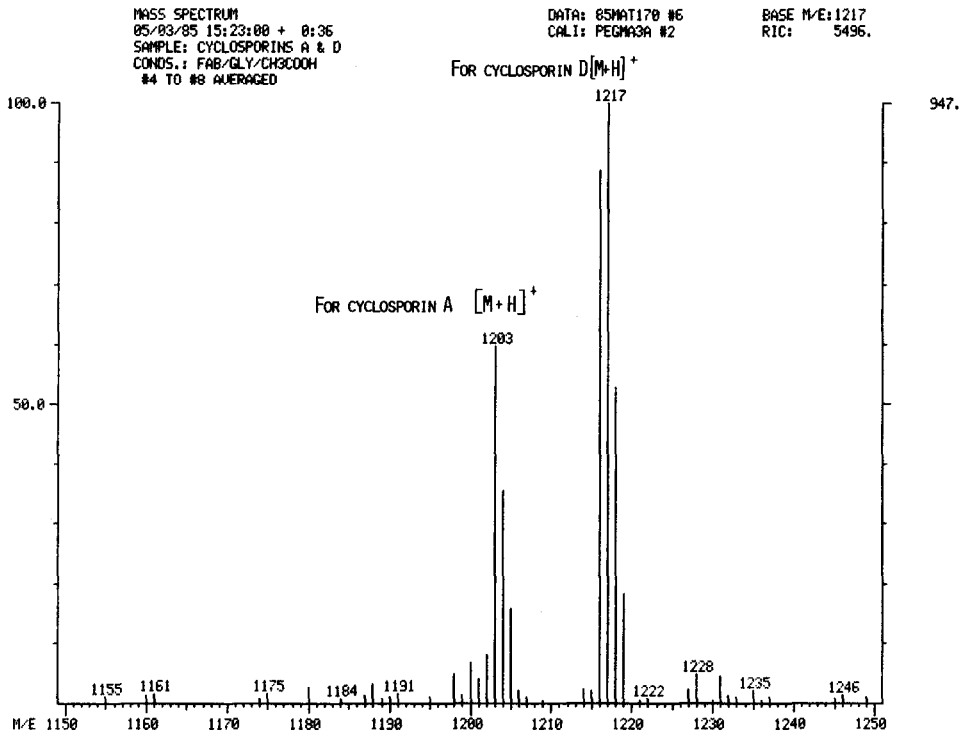
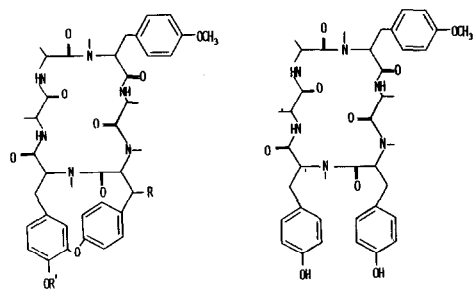


Figure 3
FAB-MS of cyclosporin A and D.

Other cyclic peptides have been investigated under FAB-MS conditions. For example, Slowikowski and Schram [74] have studied the fragmentation patterns of bouvardin, 6-*O*-methylbouvardin, deoxybouvardin and a synthetic analog (Scheme 17, Structures **48–51**). These cyclic hexapeptides (except the synthetic analog) are considered to be potential antitumor agents. Bouvardin possesses a phenolic bridged tyrosine moiety which influences the fragmentation pathway. This moiety is retained until the final stages of ion decomposition in the spectra, i.e. ring-directed cleavages. Since the synthetic analog does not possess this 14-membered phenolic ring it does not "cleave in a site-directed manner", rather it follows unusual sequential typical peptide bond cleavages.

Cyclic polypeptides with molecular masses ranging from 592 to 1352 a.m.u. were studied by FAB-MS and FAB-MS/MS by the group of Gross and co-workers [75]. These compounds gave protonated molecular ions. The protonation occurs on the nitrogen and cleavages occur between the protonated nitrogen and the adjacent carbonyl group. The following successive amino acid losses allow for the identification of the peptide

Scheme 17
The structure of bouvardin, 6-*O*-methylbouvardin, deoxybouvardin and a synthetic analog of bouvardin (Structures **48–51**).

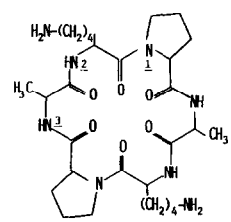


48 Bouvardin R = OH, R' = H
49 6-*O*-Methylbouvardin R = OH, R' = CH_3
50 Deoxybouvardin R = H, R' = H
51 A synthetic analog

sequence. The structure of cyclo-(Lys-Pro-D-Ala)$_2$ is presented as an example of one of these cyclic peptides (in Scheme 18, Structure **52**).

Both ceruletide, a decapeptide characterized by the presence of a tyrosyl-*O*-sulphate moiety, and [Tyr^4] ceruletide (Scheme 19, Structure **53**) display biological activities on the gastrointestinal and central nervous system. They were also successfully analysed by FAB-MS [76].

Scheme 18
The structure of a cyclic peptide cyclo-(Lys-Pro-D-Ala)$_2$ (Structure **52**).



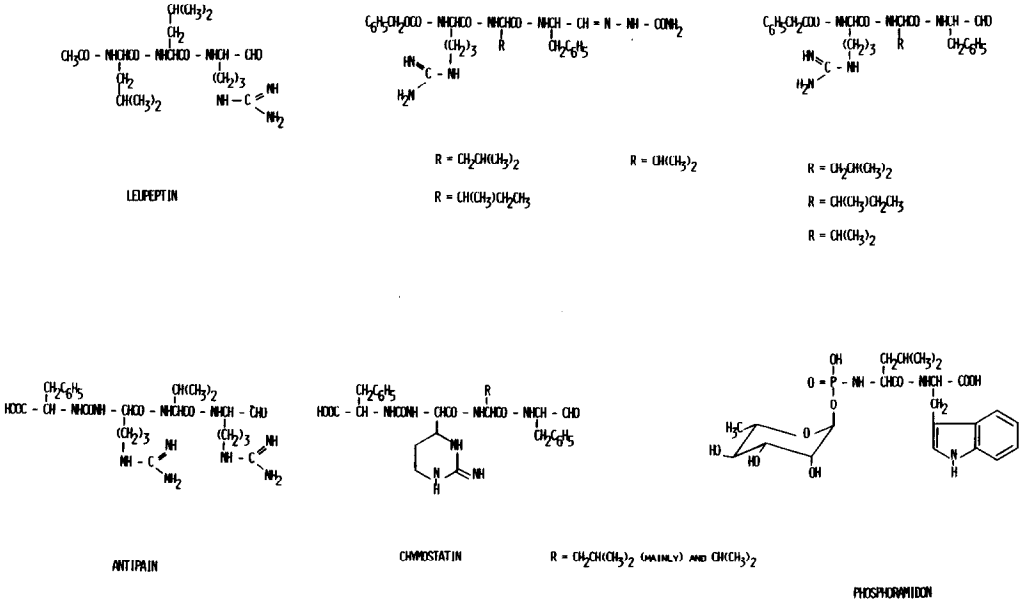
Scheme 19.
The structure of ceruletide (Structure **53**).

Pyr-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2

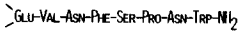
In the peptide series of what is called unusual peptides, some natural and synthetic enzyme inhibitors were studied [77]. These include leupeptin and six synthetic analogs, antipain, chymostatin and phosphoramidon (Scheme 20, Structure **54–63**). Positive and negative FAB-mass spectra were used, as one complements the information of the other, since each mode is sensitive to different structural aspects [77].

The structures of two cockroach neuropeptides (Scheme 21, Structures **64** and **65**) were also studied by FAB-MS and their amino acid sequence determined [78]. High resolution and linked scan (metastable) techniques were used. The matrix used was glycerol or a 5:1 mixture of dithiothreitol/dithioerythritol; this mixture being referred to as "Magic Bullet" by these authors since it increased production of ions and gave less background than glycerol.

A recent study by Roepstorff *et al.* [79] investigated the possibilities and limitations of FAB-MS for sequence determination of various unknown peptides. These authors report that FAB alone cannot give total sequence information but only partial sequence information. Molecular weight information is always obtained but sequence information varies with molecular weight of the peptides, the amount on the probe and the presence of alkali metal salt.

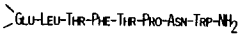


Scheme 20
The structures of leupeptin and six synthetic analogs, antipain, chymostatin, and phosphoramidon (Structures **54–63**).



64

Scheme 21
The structures of two cockroach neuropeptides (Structures **64** and **65**).



65

Takao *et al.* [80] used FAB-MS in the study of protein digests. They were able to demonstrate that over 90% of the protein sequence could be detected with this technique and that it only required very small amounts (~5 μg or less) of protein. The matrix used was glycerol and α-thioglycerol in a 1:2 ratio.

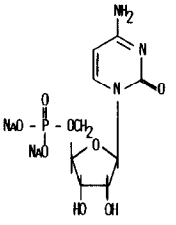
Recently, Gaudin *et al.* [81] reported the six fragmentation pathways occurring under positive FAB-MS, for sequence interpretation of peptides. A computer simulation of spectra for sequence analysis is also presented.

The ever-increasing amount of data obtained by FAB-MS brought about the need for a common nomenclature for sequence ions in mass spectra of peptides. Roepstorff and Fohlman [82] initiated a proposal that was extended by the present authors to make it truly universal [83].

Nucleosides and nucleotides

Self and co-workers [84] studied both positive and negative FAB spectra of two nucleosides, ten mononucleotides, two derivatized mononucleotides and eleven di-nucleosides monophosphates (Structure **66**, 5′-CMP, is presented as an example in Scheme 22). Spectra were recorded in glycerol and the principal ions observed were

Scheme 22
The structure of 5′-CMP (Structure **66**).



$[M + Na]^+$, $[M + H]^+$, $[M - Na + 2H]^+$, $[M - 2Na + 3H]^+$, $[B + Na]^+$ and $[B + H]^+$ and other structurally significant fragments.

Jankowski and Soler described a method to sequence unknown polynucleotides by FAB-MS. This method is based on "an analysis of two halves plus one nucleotide fragments from each end of the polynucleotide" [85].

The confirmation of synthetic nucleosides and nucleotides used in cancer research was made possible by the use of FAB mass spectrometry [86].

McCloskey and co-workers [87] studied the FAB and FAB-MS/MS of thirty nucleosides and two isomeric nucleotides. They included guanosine and related compounds, adenosine and related compounds, uridine and related compounds, substituted nebularines and substituted tubercidins; these are presented in Scheme 23, Structures **67–99**. Positive and negative FAB-MS and FAB-CAD-MS were recorded in glycerol and/or acidified glycerol. Both molecular weight information as well as structural information were found.

Other cyclic nucleotides, isolated from living systems [88], were identified under FAB and FAB-CID-MIKE spectra conditions by Beynon and co-workers. Among these, cytidine 3′,5′-cyclic monophosphate and guanosine 3′,5′-cyclic monophosphate were identified [88].

Brewer and Grisham [89] reported the positive FAB-spectra of the beta-gamma complexes of ATP and GTP with cobalt and chromium (Scheme 24, Structure **100**). Molecular weight information on these type of compounds is reported, with glycerol as the matrix.

In a very recent review, we report on various aspects of mass spectrometry as applied to nucleic acids; FAB-MS is, of course, extensively covered in its own right in that review [90].

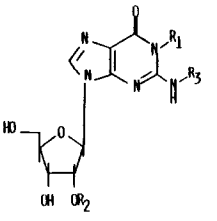
Finally, Grotjahn *et al.* [91] have recommended the use of the negative mode FAB-MS as a rapid routine identification technique for the monomeric and oligomeric nucleotide building blocks used in the phosphotriester synthesis of DNA fragments.

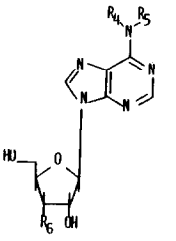
Steroids

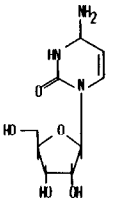
Caprioli and co-workers [92] have used FAB-MS in the study of a series of thirteen underivatized estrogen glucuronides and sulfates, such as estrone, estradiol, estriol and diethylstilbestrol conjugates. Protonated and cationized molecular ions were observed. Not much fragmentation occurred in these spectra, and the sensitivity was improved by doping the samples with sodium chloride salt.

Rose *et al.* [93] reported the negative FAB-mass spectra of digitonin (Scheme 25, Structure **101**) recorded in polyethylene glycol-200 (PEG-200). The $[M - H]^-$ ion was observed, as well as fragmentation characteristic of cleavages in the glycosidic side chain.

Digoxin (Scheme 26, Structure **102–107**) and a series of six other related cardenolide analogs, including digitonin, were recorded under positive FAB-MS conditions [94].







CYTIDINE

67 GUANOSINE $R_1 = R_2 = R_3 = H$

68 1-METHYLGUANOSINE $R_1 = CH_3$, $R_2 = R_3 = H$

69 2'-O-METHYLGUANOSINE $R_1 = R_3 = H$, $R_2 = CH_3$

70 N^2-METHYLGUANOSINE $R_1 = R_2 = H$, $R_3 = CH_3$

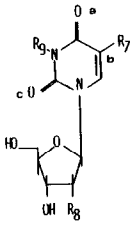
71 ADENOSINE $R_4 = R_5 = H$, $R_6 = OH$

72 N^6,N^6-DIMETHYLADENOSINE $R_4 = R_5 = CH_3$, $R_6 = OH$

73 PUROMYCIN NUCLEOSIDE $R_4 = R_5 = CH_3$, $R_6 = NH_2$

74 N^6-ISOPENTENYLADENOSINE $R_4 = H$, R_5 = ISOPENTENYL, $R_6 = OH$

75



76 URIDINE $R_7 = R_9 = H$, $R_8 = OH$

77 4-THIOURIDINE $R_7 = R_9 = H$, $R_8 = OH$ (OXYGEN A IS REPLACED WITH S)

78 2-THIOURIDINE $R_7 = R_9 = H$, $R_8 = OH$ (OXYGEN B IS REPLACED WITH S)

79 THYMINE RIBOSIDE $R_7 = CH_3$, $R_8 = OH$, $R_9 = H$

80 DIHYDROURIDINE $R_7 = R_9 = H$, $R_8 = OH$, (DOUBLE BOND B IS HYDROGENATED)

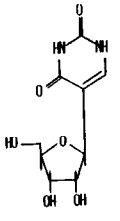
81 5-HYDROXYURIDINE $R_7 = R_8 = OH$, $R_9 = H$

82 THYMIDINE $R_7 = CH_3$, $R_8 = R_9 = H$

83 3-METHYLURIDINE $R_7 = R_8 = H$, $R_9 = CH_3$

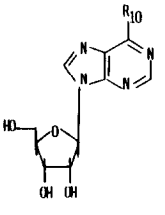
84 2'-O-METHYLURIDINE $R_7 = R_9 = H$, $R_8 = OCH_3$

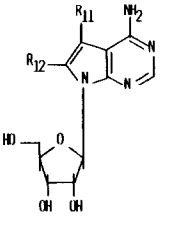
85 URIDINE-5-OXYACETIC ACID $R_7 = OCH_2CO_2H$, $R_8 = R_9 = H$



PSEUDOURIDINE

86





87 NEBULARINE $R_{10} = H$

88 SUBSTITUTED NEBULARINE R_{10} = 3-PHENYLPROPYL

89 R_{10} = PHENYL

90 R_{10} = CYCLOHEXYL

91 R_{10} = ETHYL

92 R_{10} = 2-PHENYLETHYL

93 R_{10} = ISOPENTENYL

94 TUBERCIDIN $R_{11} = R_{12} = H$

95 SUBSTITUTED TUBERCIDIN $R_{11} = Br$, $R_{12} = H$

96 $R_{11} = -CO_2-CH_3$, $R_{12} = H$

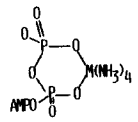
97 R_{11} = -2-BUTENYL, $R_{12} = H$

98 $R_{11} = H$, $R_{12} = Br$

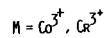
99 R_{11} = 2-PHENYLETHENYL, $R_{12} = H$

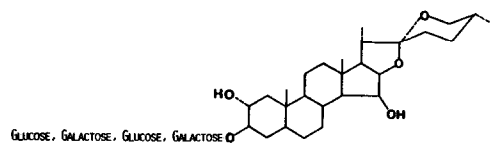
Scheme 23

The structures of guanosine and related compounds, adenosine and related compounds, uridine and related compounds, substituted nebularines and substituted tubercidins (Structures **67–99**).

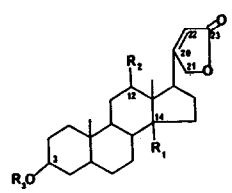


Scheme 24
The structure of a metal nucleotide complex (Structure **100**).





Scheme 25
The structure of digitonin (Structure **101**).



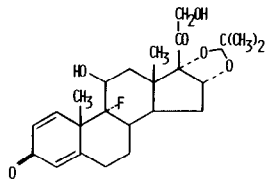
	Compound	R_1	R_2	R_3
102	Digitoxin	OH	H	2,6-Dideoxy-ribo-hexose (3 moles)
103	Digoxin	OH	OH	2,6-Dideoxy-ribo-hexose (3moles)
104	Dihydrodigoxin (No Δ 20,22)	OH	OH	2,6-Dideoxy-ribo-hexose (3moles)
105	Acetodigoxin			
106	Digitoxigenin	OH	H	H
107	Digoxigenin	OH	OH	H

Scheme 26
The structures of digoxin and some related cardenolides (Structures **102–107**).

Various matrices were used and thioglycerol proved very useful for digoxin. Very low levels (11 ng ml^{-1}) were detected with this technique in samples of human urine spiked with digoxin.

A series of corticosteroids was also studied by FAB-MS [39]. This gave very interesting results when thioglycerol was used as the solvent. Triamcinolone acetonide (Scheme 27, Structure **108**) is presented as a general example of this series of steroids.

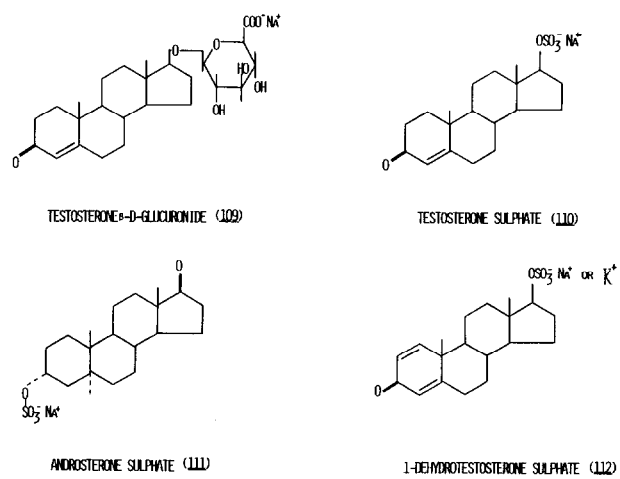
Shackleton [95] used negative SIMS to study steroids in biological samples. No attempts were made to purify or isolate these steroids from their complex mixtures or any derivatization. Good results were obtained in using the technique to produce profiles of conjugated human urinary steroids and to delineate some inborn errors of steroid biosynthesis.



Scheme 27
The structure of triamcinolone acetonide (Structure **108**).

Other steroidal conjugates were studied under FAB-MS conditions by Williams and co-workers [96]. The samples studied under positive and negative ionization modes in glycerol are: testosterone-β-D-glucuronide, testosterone sulphate, androsterone sulphate and 1-dehydrotestosterone sulphate (Scheme 28, Structures **109–112**), isolated from horse urine. The technique allows determination of molecular weight as well as the type of cation present in these steroid conjugates.

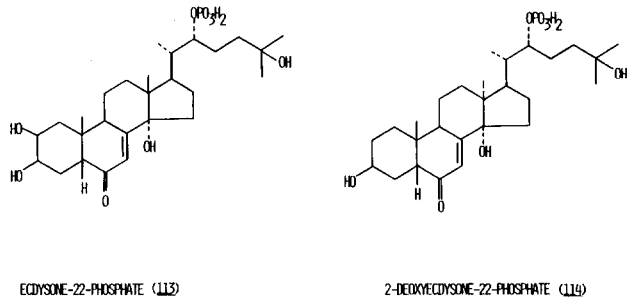
The study of some steroid conjugates by FAB-MS was also reported by Shackleton and Straub [97]. In fact, these authors report studying these steroid sulphates and glucuronides by SIMS with Cs^+ as the primary ion beam. The SIMS and FAB spectra of these compounds are similar. Both positive and negative spectra were recorded in a glycerol matrix, but the negative spectra gave more significant results, consisting of pseudomolecular ion $[M - H]^-$ and little, if any, fragmentation. Steroid metabolites extracted from urine and plasma were also recorded under SIMS conditions and therefore show great potential for clinical analysis.



Scheme 28
The structures of testosterone β-D-glucuronide, testosterone sulphate, androsterone sulphate, and 1-dehydrotestosterone sulphate (Structures **109–112**).

FAB-MS has been used in conjunction with NMR spectroscopy by Rose and co-workers [98] in the identification of two ecdysteroid conjugates, i.e. 2-deoxyecdysone-22-phosphate and ecdysone-22-phosphate (Scheme 29, Structures **113** and **114**). These types of ecdysteroids were further confirmed under FAB-MS analysis performed by the research group of Tabet and co-workers [99].

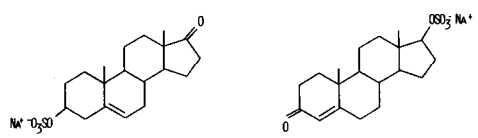
Steroid sulphates (among which were the following: dehydroepiandrosterone (DHA) sodium sulphate (Scheme 30, Structure **115**), dehydroepiandrosterone potassium sulphate, androsterone sulphate, epiandrosterone sulphate, testosterone sulphate,



ECDYSONE-22-PHOSPHATE (113) 2-DEOXYECDYSONE-22-PHOSPHATE (114)

Scheme 29
The structures of ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate (Structures **113** and **114**).

Scheme 30
The structures of dehydroepiandrosterone sodium sulphate and testosterone sodium sulphate (Structures **115** and **116**).



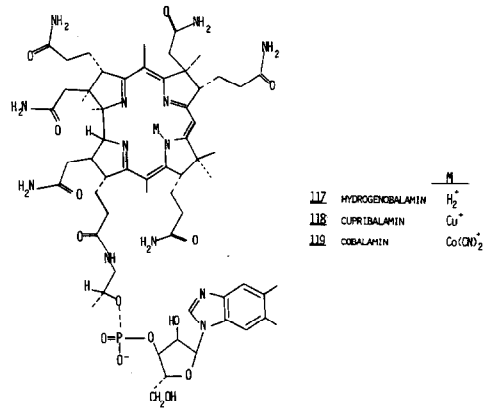
DEHYDROEPIANDROSTERONE SODIUM SULPHATE (115) TESTOSTERONE SODIUM SULPHATE (116)

epitestosterone sulphate, oestradiol-17β 3-sulphate, oestradiol-17α 3-sulphate, pregnenolone sulphate and 17-hydroxypregnenolone 3-sulphate, testosterone sodium sulphate (Scheme 30, Structure **116**)) were investigated by Gaskell *et al.* [100, 101] with success reported mostly in the negative mode. Attempts at quantification analysis of these steroid sulphates [100], as well as distinction between isomeric steroids [101] by deuterium exchange, are also reported, along with low levels of detection. Jardine *et al.* [102] also investigated the use of FAB-MS for the analysis of underivatized steroid and vitamin D glucuronides and sulphates. Glycerol and/or thioglycerol were used as the matrix, and the technique has proved useful for samples isolated from biological sources. Molecular weight and structural information were gained from both positive and negative FAB-mass spectra.

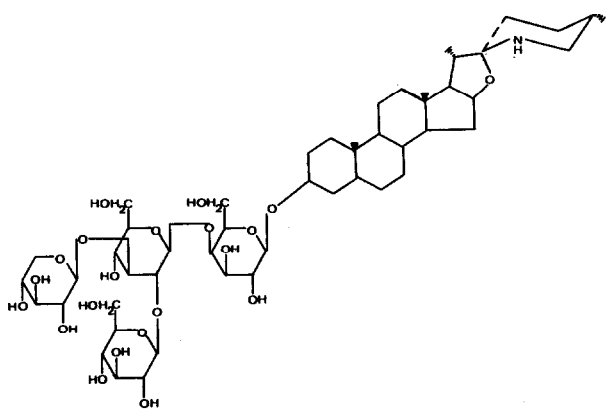
Other compounds of biological interest

Cyanocobalamin (vitamin B_{12}, mol. wt = 1354), methyl- and hydroxocobalamin and coenzyme B_{12} were first reported to be studied under FAB-MS conditions by Barber *et al.* [21, 103]. Grotjahn *et al.* [104] reported on the positive and negative FAB-MS of hydrogenobalamin (the metal-free vitamin B_{12}) and of cupribalamin (Scheme 31, Structures **117–119**). They observed pseudomolecular ion and some characteristic ions and demonstrated by high-resolution positive ion FAB-MS that, for vitamin B_{12}, $(M + H)^+ - CN - 59$ resulted from the loss of acetamide and not of the central cobalt atom.

Fast Atom Bombardment Mass Spectrometry was employed by Self and co-workers [105] for the rapid analysis of a series of ten glycoalkaloids extracted from the solanaceae species. The three major fragmentation processes observed for the sugar linkages are illustrated here, with tomatine as an example (Scheme 32, Structure **120**).



Scheme 31
The structures of hydrogenobalamin (metal-free vitamin B_{12}), cupribalamin and cobalamin (Structures **117–119**).



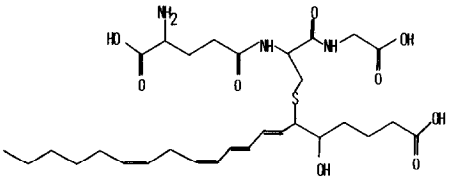
Scheme 32
The fragmentation pattern of tomatine (Structure **120**).

The following types of cleavage are observed along with abundant $[M + H]^+$ species:
(1) cleavage between aglycone and the first carbohydrate residue,
(2) cleavage of the various glycosidic bond, and
(3) cleavage of C_1, C_2 and C_5, O bond (within a carbohydrate residue).

FAB-MS was used in conjunction with NMR spectroscopy in a study by Egge *et al.* [106] concerning structural elucidation of complex glycosphingolipids (GSL). Negative FAB mass spectra were recorded on native GSL, while positive FAB spectra of their permethylated derivatives were recorded and yielded molecular weight and sequence information.

Morris *et al.* [107] reported definite molecular weight data for leukotriene-D and C (Scheme 33, Structure **121**) at the microgramme levels. Pseudomolecular species such as $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ were observed with no fragmentation.

Self and co-workers [108] have reported on the FAB spectra of a class of hexaiodinated compounds, mainly iodoxamic acid and iodoxamic acid dimethyl ester. They observe a weak molecular ion and subsequential ions of iodine atoms with H substitution. The completely deiodinated species appears to give a fragmentation pattern usually observed in EI/MS of crown ethers.

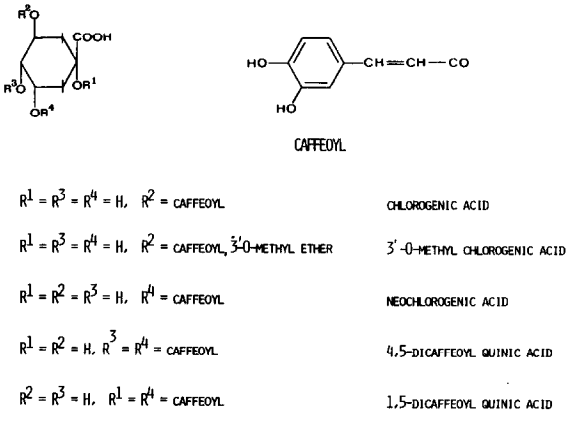


Scheme 33
The structure of leukotriene C (Structure **121**).

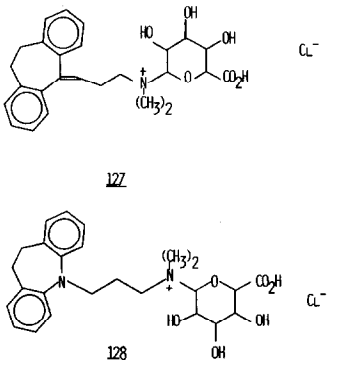
FAB-MS was also used as an analytical tool in the characterization of iopamidol [109], a substance used as a medium in myelography and angiography. It was also used in the analysis of six by-products of the iopamidol synthesis [110]. The main breakthrough in the use of FAB for Iopamidol was again to be able to record the mass spectra of this relatively high molecular weight (777 a.m.u.), low volatility polar compound in its underivatized form. Characteristic fragmentation ions were also observed for iopamidol, giving structural information not available before by conventional mass spectrometry unless under derivatization conditions.

Recently, the use of FAB-MS (in conjunction with HPLC-MS) was used in the characterization of acyl carnitines compound at physiological levels by Millington *et al.* [111]. The fact that FAB (B/E linked scan) could not distinguish between valproyl-carnitine and its isomer octanoylcarnitine was overcome by combining this technique with HPLC-MS which distinguished them by retention times.

The compounds of some pharmaceutically important chlorogenic acids (Scheme 34), i.e. chlorogenic acid, 31-*O*-methyl-chlorogenic acid, neochlorogenic acid, 4,5-dicaffeoyl quinic acid and 1,5-dicaffeoyl quinic acid, were studied under both positive and negative FAB-MS conditions by Sakashima *et al.* [112]. EI-MS does not allow for molecular weight determination of these compounds, but presents some information for structural elucidation. Therefore, combined with positive and negative FAB-MS (who then allow for molecular weight determination), the study of this class of compounds becomes feasible by MS.



Scheme 34
The structures of chlorogenic acid, 3'-*O*-methylchlorogenic acid, neochlorogenic acid, 4,5-dicaffeoyl quinic acid and 1,5-dicaffeoyl quinic acid (Structures **122–126**).



Scheme 35
The structures of quaternary ammonium-linked glucuronide of amitriptyline and quaternary-linked glucuronide of imipramine (Structures **127** and **128**).

In the line of metabolite studies, the tricyclic antidepressants, the quarternary ammonium-linked glucuronides of amitriptyline and imipramine (Scheme 35) were studied by FAB-MS. Lehman *et al.* [113] used this technique for direct characterization of these involatile, thermally labile metabolites from a urine sample from a patient.

Conclusion

This brief review has brought together a general overview of the recent applications of FAB-MS in the pharmaceutical analysis of drugs. Obviously the technique has a lot to offer and a huge amount of work remains to be done before it can be said that all applications have been exhausted. FAB-MS does not suffer from limitations inherent to several systems of interest to the pharmaceutical field, such as high molecular weight and low volatility. Furthermore, it rarely requires extensive separatory and isolatory procedures as it is very versatile in terms of the wide range of matrices that can be used to insert the sample into the ion source and in terms of its inherent sample-related specificity.

FAB-MS is a new ionization mode and not a clean-up procedure or a separatory step, such as GC-MS, for example. This fact alone implies that all the various scanning techniques in MS/MS experiments can be used directly in FAB-MS. A recent review by Boyd [114] covers such scan modes; it shows their large breadth of applicability and clearly demonstrates how little has been done this far with FAB-MS. In principle every application of EI-MS (see Harvey's review, for example [115]) should have its counterpart in FAB-MS. In fact, at first sight it almost seems appropriate to claim that the only apparent real limitation of FAB-MS, in terms of applications in pharmaceutical analysis, is the worker's originality.

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