Journal of Chromatography, 394 (1987) 135–146
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 404

BIOMEDICAL APPLICATIONS OF THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Thermospray liquid chromatography–mass spectrometry has been applied in the solution of a number of problems of biological and biomedical interest. These include the analysis of phenazines from the Gram negative bacterium *Pseudomonas aeruginosa*, steroids released by rat adrenals and eicosanoids generated by human inflammatory cells. The application of the technique to leukotrienes in blood is discussed. Isotopic labelling prior to analysis, to facilitate identification and structure elucidation is outlined with reference to the steroids.

INTRODUCTION

Mass spectrometry (MS) has been used successfully for many years for the analysis and identification of a wide range of substances of biological importance. When coupled to a suitable high-resolution chromatographic technique such as gas chromatography (GC), it offers unrivalled specificity for both qualitative and quantitative analysis. For a GC-based analytical system, there is an absolute requirement for sample volatility; this is usually achieved by the formation of suitable derivatives. Derivatisation may, however, be accompanied by unexpected side-reactions, and is not always successful. In contrast, there are no such requirements for volatility if high-performance liquid chromatography (HPLC) is used as the inlet system for a mass spectrometer, and the recently developed thermospray liquid chromatography–mass spectrometry (LC–MS) interface[1,2] promises to be a useful addition to the range of MS methods applicable in biomedical research.

Thermospray MS is a soft form of ionisation and generates molecular ion species with little fragmentation directly post HPLC. It is comparable with other soft ionisation techniques such as fast atom bombardment mass spectrometry (FAB-MS), however, with thermospray LC–MS, data are obtained on-line, and handling losses are minimised. Thermospray MS data have already been obtained on a variety of polar and involatile substances, including underivatised steroid sulphates, drug metabolites and glucuronides[3–6]. We have now utilised a commercial thermospray interface to facilitate MS analyses of underivatised corticosteroid hormones from rat adrenals, phenazines from a pathogen which colonises the lungs of cystic fibrotics and the products of lipoxygenase metabolism of arachidonic acid.

0021-9673/87/$03.50

MATERIALS AND METHODS

In general, HPLC was undertaken on a Waters Assoc. (Harrow, U.K.) gradient system comprised of two Model 510 pumps, a Model 640 gradient controller and a Model U6K injector. A Model 440 UV absorbance detector was inserted prior to the mass spectrometer interface when required. Sample ionisation was achieved by thermospraying the HPLC eluent into a Finnigan (Sunnyvale, California) Model 4500 quadrupole mass spectrometer via an unmodified Finnigan thermospray interface. Data acquisition and processing were performed on a series 2000 Incos data system using Idos II software. All HPLC-grade solvents were purchased from Rathburn Chemicals (Walkerburn, U.K.), ammonium acetate was of AnalaR grade (BDH Chemicals, Poole, U.K.) and water for HPLC was of Milli-Q quality. In all the analyses performed the ion source block was held at 200°C; the vapouriser temperature and the repeller plate voltages were treated as ion source tuning parameters; *i.e.* they were optimised daily as required.

Steroid analysis

HPLC was performed on a C_{18} Nova-Pak reversed-phase column (15 cm × 0.46 cm I.D.). A linear gradient from 21% to 70% acetonitrile in 0.05 *M* aq. ammonium acetate over 20 min was used (1.2 ml/min). Each of ten steroids was injected to determine their retention times and positive ion thermospray spectra. The adrenals of fifteen female Wistar rats were decapsulated[8] to yield capsule fractions (glomerulosa cells) and inner zones (fasciculata/reticularis cells). Capsules and inner zones were incubated separately in Krebs Ringer bicarbonate for one hour and homogenised in 0.25 *M* sucrose solution. After ethyl acetate extraction, drying and reconstitution in 1 ml 0.05 *M* ammonium acetate, 100-μl aliquots were analysed by HPLC–MS.

Phenazines

Aqueous ammonium acetate (1.8 ml/min) was pumped through the thermospray interface when making column bypass injections. With the HPLC column (Waters C_{18} Nova-Pak 15 cm × 0.46 cm I.D.) in place compounds were eluted using a 15-min gradient from 0 to 70% acetonitrile in 0.05 *M* aq. ammonium acetate. Mass spectra were recorded using 2-s scans across a mass range of 150–400. Gas chromatography was performed on a directly coupled 30 m × 0.25 mm I.D. SE-54 fused-silica capillary column (Jones Chromatography) with helium as carrier gas. Splitless injections were performed using a Grob type injector. Normally the injector temperature was held at 250°C, but for pyocyanin analyses a number of different injector temperatures were selected in order to observe patterns of thermal breakdown. All samples were injected in octane, and compounds were eluted by raising the column oven temperature from 100 to 280°C at 20°C min^{-1}. Electron impact spectra were recorded across a mass range of m/z 50–700.

Lipoxygenase products

Positive and negative ion thermospray mass spectra of hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) were recorded using 0.05 *M* aq. ammonium acetate (1.5 ml/min) to promote ionisation.

HETEs in human inflammatory cells. Arachidonic acid was converted to HETEs by mixed human inflammatory cells following ionophore stimulation. HETEs were extracted using C_{18} solid phase extraction cartridges (Bond Elut, Analytichem). The extracts were applied to a Waters C_{18} Nova-Pak column (15 cm × 0.46 cm I.D.) and eluted by a solvent gradient from 20% to 60% acetonitrile in 0.05 *M* aq. ammonium acetate over 30 min. The mass spectrometer monitored the eluent continuously at *m/z* 319 via the thermospray interface.

Leukotrienes. Extraction and HPLC analysis was carried out in one step on a Varian HPLC system with a column switching device[7]. Elution was undertaken with a 60-min solvent gradient from 57% to 83% methanol in 0.05 *M* aq. ammonium acetate (adjusted to pH 3.9 with acetic acid)[7]. LTs B_4, C_4, D_4 and E_4 (1.2 μg) were added to whole blood (5 ml) mixed with nordihydroguaritic acid–serine borate–cysteine in isotonic saline to inhibit LTs formation and metabolism, and samples (2.5 ml) were extracted and chromatographed. The mass spectrometer was scanned in negative ion mode from *m/z* 150–700 every 2 s as the eluent entered via the thermospray interface.

RESULTS AND DISCISSION

Rat adrenal steroids

Initially, ten synthetic steroids were examined by thermospray LC–MS to determine their suitability for analysis. Each steroid chromatographed as a single peak on HPLC and generated a positive ion mass spectrum (Table I). In most instances the base peak in the thermospray mass spectra corresponded to the protonated molecular ion species $[M+H]^+$. However, for the hydrogenated aldosterones and 18-hydroxycorticosterone, facile loss of water occurred to give $[M-H_2O+H]^+$ as the major ion (Fig. 1). Ammonium adduct ions $[M+NH_4]^+$ were sometimes observed which assisted molecular ion identification. In each case, full mass spectra were

TABLE I

POSITIVE ION THERMOSPRAY SPECTRA OF STEROIDS

Positive ion thermospray mass spectra (post HPLC) of ten steroids together with HPLC retention times (t_R). The major ion species are recorded, with the relative ion intensities (compared with the base peak at 100%) given in parentheses. Low intensity ions corresponding to dehydrogenations were also present.

Steroid	*Major ions*			t_R *(min)*
	$[M+H]^+$	$[M-H_2O+H]^+$	$[M+NH_4]^+$	
3α,5β-Tetrahydroaldosterone	365 (5)	347 (100)	382 (8)	7.5
18-Hydroxycorticosterone	363 (66)	345 (100)	380 (5)	7.6
Aldosterone	361 (100)	343 (35)	378 (18)	8.1
3α,5α-Tetrahydroaldosterone	365 (6)	347 (100)	382 (9)	8.6
5β-Dihydroaldosterone	363 (6)	345 (100)	–	9.5
5α-Dihydroaldosterone	363 (12)	345 (100)	380 (9)	10.0
11-Deoxy-18-hydroxycorticosterone	347 (100)	329 (12)	–	10.7
Corticosterone	347 (100)	329 (12)	364 (8)	11.4
11-Deoxycorticosterone	331 (80)	313 (100)	–	15.5
Progesterone	315 (100)	297 (10)	–	20.2

readily generated on 1 μg, with good quality data obtained on as little as 30 ng in favourable cases. This compares favorably with other soft ionisation techniques such as FAB-MS, where the requirement for HPLC purification prior to analysis (especially for samples present in complex biological matrices) limits the overall sensitivity to the μg level. Lower limits of detection are available with capillary GC–MS, but derivatisation is a prerequisite for analysis.

The thermospray HPLC–MS profile for rat adrenal capsular incubation medium is shown in Fig. 2. Selected ion profiles for *m/z* 347, 361 and 363 clearly show the presence of five species. The retention times and thermospray mass spectra of these species were consistent with the presence of 18-hydroxycorticosterone, aldosterone, 18-hydroxy-11-deoxycorticosterone, corticosterone and an unidentified steroid of the same mass as aldosterone[9]. The LC–MS profile for the capsular homogenate was similar to that obtained for the incubation medium. The absolute MS

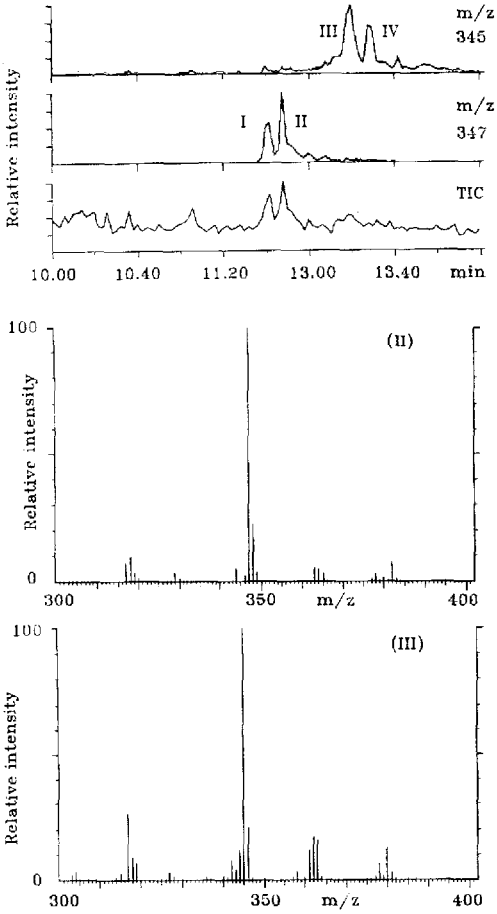

Fig. 1. Positive ion thermospray liquid chromatography–mass spectrometric profile of two isomeric dihydroaldosterones (*m/z* 345) and two isomeric tetrahydroaldosterones (*m/z* 347) together with their positive ion thermospray mass spectra showing the $[M-H_2O+H]^+$ ion as the base peak (30 ng of each were injected). The total ion current (TIC) is also shown; it is complicated by the high background from low mass solvent ions.

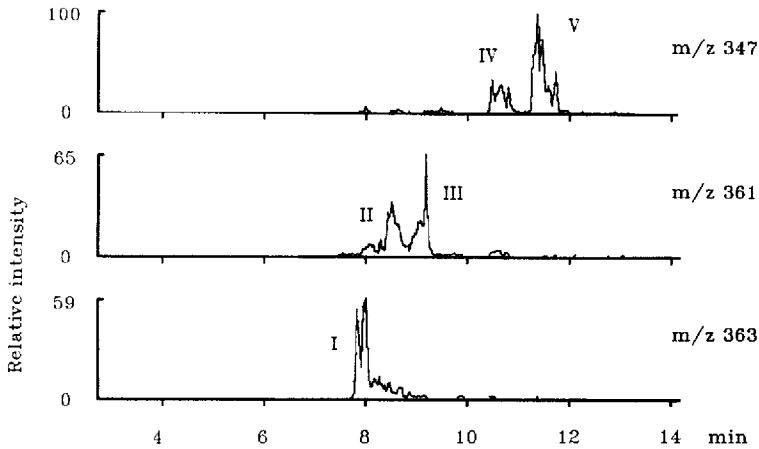

Fig. 2. Positive ion thermospray LC–MS profile of corticosteroids released from rat adrenal glands. Ion channels *m/z* 347, 361 and 363 were monitored (intensities are 100, 65 and 59%, respectively). 18-Hydroxycorticosterone (I), aldosterone (II), 11-deoxy-18-hydroxycorticosterone (IV) and corticosterone (V) were identified on the basis of their HPLC retention time and thermospray mass spectra. A fifth species (III), with the same mass as aldosterone was also observed.

responses of the ion channels gave an indication of gross changes in steroid content between the medium and homogenate, although suitable internal standards will be required for accurate quantitation.

Of particular interest is the steroid eluting after aldosterone (8.7 min) but with a similar thermospray mass spectrum. Aldosterone is known to form the 11,18 hemiacetal and the 11,18 hemiacetal-18,21 hemiketal. When aldosterone was isomerised using methanolic sodium hydroxide two species with similar thermospray mass spectra to aldosterone were generated ($[M+H]^+$, *m/z* 361; $[M-H_2O+H]^+$, 343), however, both eluted earlier than aldosterone, indicating that the unknown was not a simple isomeric form of aldosterone. A less polar dehydrated aldosterone was also generated by base treatment.

Thermospray LC–MS yields molecular weight information on sub-μg levels of steroids. Without the use of external ionisation discharge/filament), little structural information, apart from the presence of labile hydroxyl groups, may be obtained. This may be partly overcome by derivatisation of suitable functional groups. Furthermore, the use of isotopically labelled reagents can facilitate identification of sample ions post HPLC. For example, if progesterone is methoximated with a 1:1 mixture of methoxyamine–[$^2H_3$]-methoxyamine hydrochloride in pyridine, two isomeric monomethoxyamine derivatives are generated, both with a 1:1 doublet protonated molecular ion $[M+H]^+$ at *m/z* 344/347 (Fig. 3). Similarly, aldosterone may be converted to its mono-methoxyamine labelled derivative at *m/z* 390/393; the dimethoxyamine derivative (generating a 1:2:1 triplet at *m/z* 419/422/425) is also formed. Isotopic labelling is especially valuable when dealing with complex matrices, when multiplets stand clearly above an otherwise complex background (see ref. 10).

Ciliostatic phenazines

Pyocyanin is a blue redox pigment generated by *Pseudomonas aeruginosa*, an

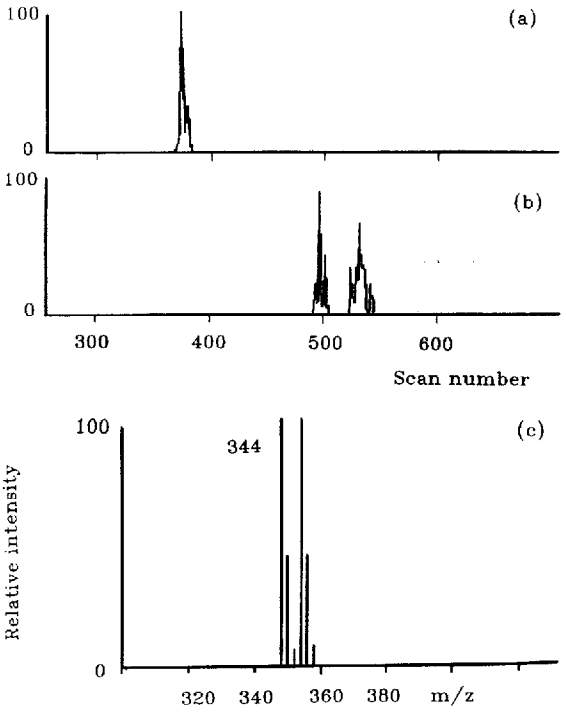

Fig. 3. Selected ion channels for positive ion thermospray LC–MS analysis of (a) progesterone, *m/z* 315; (b) its mono-methoxyamine derivative, *m/z* 344; (2 isomers); (c) the positive ion thermospray mass spectrum for the 1:1 methoxyamine-[$^2H_3$]-methoxyamine derivative of progesterone. The 1:1 doublet at *m/z* 344/347 clearly stands out.

opportunist infecting organism often found in the lungs of patients with cystic fibrosis. Pyocyanin has recently been shown to possess potent inhibitory action on human nasal ciliary beat and has been implicated as a mediator of reduced mucociliary clearance in cystic fibrosis and other diseases associated with severe bronchiectasis in man[11]. Pyocyanin was extracted from agar plate cultures of *Ps. aeruginosa*, recrystallised and purified by HPLC. No conclusive data could be obtained on this species by GC–MS or desorption chemical ionisation MS. However a protonated molecular ion species $[M+H]^+$ at *m/z* 211 was obtained by thermospray MS in the presence of a low (0.005 *M*) concentration of aq. ammonium acetate, defining the molecular mass as 210 (Fig. 4c)[12]. In the presence of 0.05 *M* ammonium acetate buffer or acetic or trifluoroacetic acid, the molecular ion species shifts to *m/z* 212, $[M+2H]^+$ (Fig. 4d). This probably arises as a result of the unusual zwitterionic and aromatic nature of pyocyanin, with a one-electron redox capture mechanism (allowing addition of e^-/H^+, ref. 13) followed by thermospray ionisation to generate the diprotonated, singly charged species. We have recently observed similar behaviour with FAB ionisation. On treatment with base pyocyanin was converted to 1-hydroxyphenazine, which generates an intense protonated molecular ion on thermospray ionisation $[M+H]^+$, 197; Fig. 4a); neither this species, nor its methyl ether, 1-methoxyphenazine, an isomer of pyocyanin (Fig. 4b), exhibited concentration-dependent thermospray mass spectra.

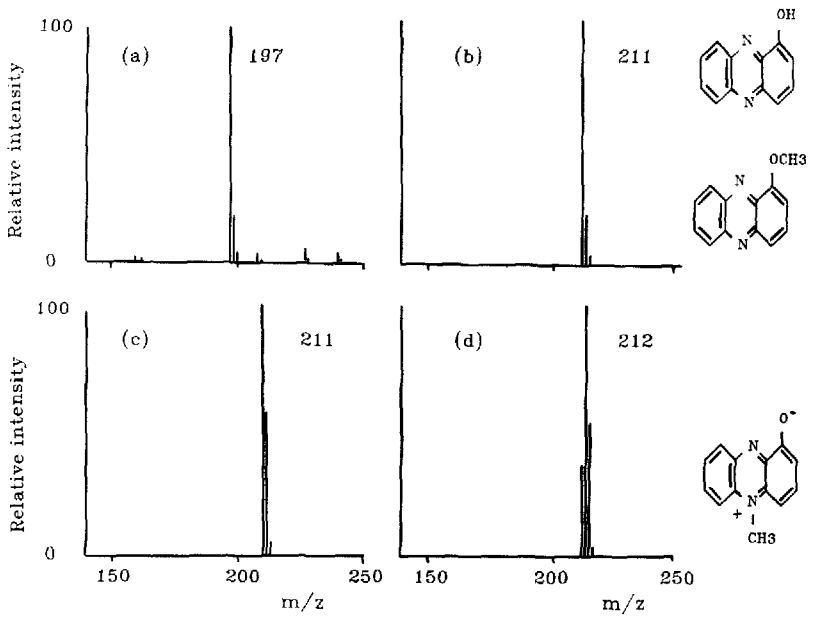

Fig. 4. Positive ion thermospray mass spectra of: (a) 1-hydroxyphenazine $[M+H]^+$, *m/z* 197; (b) 1-methoxyphenazine $[M+H]^+$, *m/z* 211; (c) pyocyanin in low salt buffer; the base peak at *m/z* 211 corresponds to $[M+H]^+$, however, the ion at *m/z* 212 is too intense to arise solely from isotope content, and represents $[M+H_2]^+$ species; (d) pyocyanin in higher ammonium acetate concentrations, *m/z* 212 becomes the base peak in the spectrum. Each species elutes as a single peak on HPLC, and generates the mass spectra shown. The structures of these three phenazines are shown next to their thermospray mass spectra.

Interestingly, pyocyanin is converted to 1-hydroxyphenazine when injected into a heated GC inlet and when chemical derivatisation is attempted. Since 1-hydroxyphenazine is present in cultures from *Ps. aeruginosa* and exhibits a similar ciliostatic aetion, GC–MS analysis alone will not suffice to elucidate the roles of these two phenazines in human bronchial disease states. Thermospray LC–MS, on the other hand readily distinguishes between the two in non-derivatised form and is compatible with direct on-line analysis of untreated bacterial culture filtrates, provided that an HPLC guard column is included before the analytical column.

Hydroxyeicosatetraenoic acids

There are various lipoxygenase enzymes present in different cells, which catalyse the formation of a number of isomeric HETEs. Some isomers (for example 15-HETE), have been implicated in the control of the important 5-lipoxygenase pathway which generates the leukotriene family. Although GC–MS methodology has been applied to the analysis of these species (see *e.g.* ref. 14), in our hands the technique has not proved entirely suitable, notably because of problems of thermal instability and poor GC. The thermospray behaviour of the HETEs were examined[15]. Each HETE generates an intense positive ion mass spectrum corresponding to water loss from the protonated molecular ion ($[M+H-H_2O]^+$; *m/z* 303). The deprotonated molecular ion is the base peak in the negative ion mass spectrum ($[M-H]^-$; *m/z* 319, Fig. 5a); the absolute MS response of *m/z* 319 was proportional to the amount of

sample loaded onto the HPLC (Fig. 5b and c), although the system may only considered to be semi-quantitative, as suitable (deuterated) internal standards were not available. The negative ion mode was used to monitor the HPLC eluant of HETEs generated by ionophore A23187 stimulated human inflammatory cells (Fig. 6); 5-, 9- and 11-HETEs were observed. Thermospray LC–MS was used to monitor HETE production from these cells in the presence of cyclosporin. Anecdotal evidence had linked cyclosporin treatment with a remission of the symptoms of psoriasis[16]. As HETEs had previously been observed in psoriatic scale[17], it was considered that cyclosporin could act via inhibition of lipoxygenase. In fact, using the semi-quantitative thermospray MS assay, no significant change in the ionophore-stimulated release of HETEs was observed.

Leukotrienes

The LTs are perhaps the most interesting lipoxygenase metabolites, arising via peroxidation at the 5 position of arachidonic acid[18]. All the LTs have been analysed as derivatives by GC–MS[19,20] although in all cases extensive sample preparation procedures are involved, including complete cleavage of the peptide moities for LTC_4, LTD_4 and LTE_4; thus the routine GC–MS analysis of peptidoleukotrienes of biological origin remains unestablished. In contrast, the underivatised LTs all generate intense negative ion thermospray HPLC–MS at sub-μg levels. In each case, the deprotonated molecular ions $[M-H]^-$ were generated (LTC_4, m/z 624; LTE_4, m/z 495; LTE_4, m/z 438; LTB_4, m/z 335). A representative background-subtracted spectrum of LTD_4 is shown in Fig. 7. Some fragmentation is observed; in the case of LTD_4, this includes loss of water (m/z 477), loss of the peptide side chain (m/z 317),

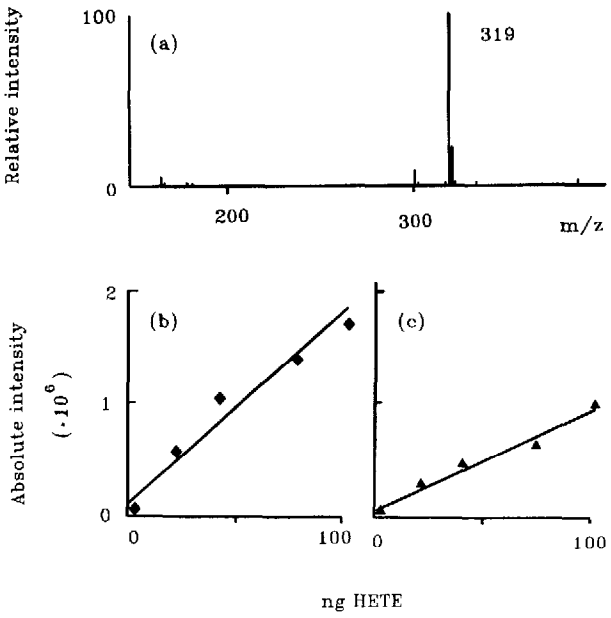

Fig. 5. (a) Negative ion thermospray mass spectrum of 5-HETE showing $[M-H]^-$ at m/z 319; (b) and (c) extracted standard curves for 9- and 5-HETEs, respectively based on absolute mass spectrometric responses.

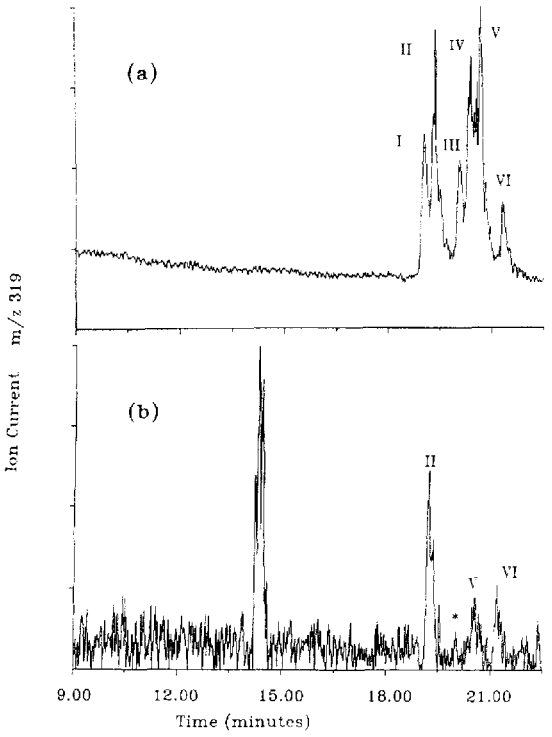

Fig. 6. (a) Thermospray LC–MS profile, monitoring in the negative ion mode at *m/z* 319, of a mixture of 75 ng each of 7 standard HETE isomers: I = 14/15- (unresolved); II = 11-; III = 8-; IV = 9-; V = 12-; VI = 5-HETE. (b) Profile of HETEs generated from human inflammatory cells by action of calcium ionophore A23187. The 5-, 9- and 11- isomers were present, with trace amounts of 8-HETE.

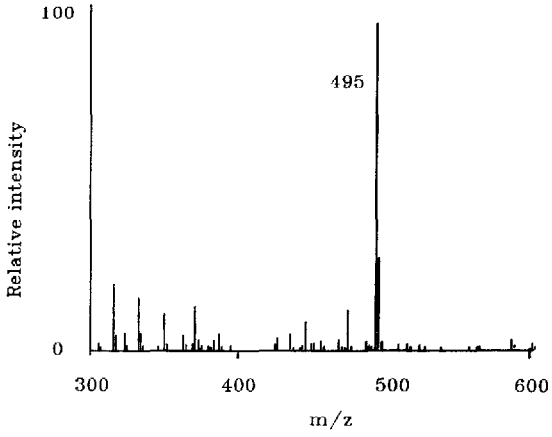

Fig. 7. Negative ion thermospray spectrum of LTD_4 generated in 0.05 *M* ammonium acetate showing the deprotonated molecular ion $[M-H]^-$ at *m/z* 495. The ion at *m/z* 317 (corresponding to loss of the peptide side chain) may be more intense, notably when the source is contaminated.

and some evidence for N-terminal amino acid loss with proton transfer at *m/z* 438 (possibly via a FAB–MS type mechanism). In buffer, the LTs may be detected at the 10–20 ng level on column, although HPLC and MS conditions must be favorable[7]. The positive ion spectra $[M+H]^+$ are also generated.

Negative ion thermospray spectra were obtained directly post HPLC for each LT added to whole blood using the one step extraction/purification protocol developed for assay of these species[7]. The selected-ion channel LC profile for LTB_4, together with the thermospray mass spectrum is shown in Fig. 8. Although the spectrum is not clean, the protonated molecular ion at *m/z* 335 is clearly present. Further the ion current for *m/z* 335 corresponds with the known elution position of LTB_4, with no evidence for such a peak in control samples. Based on the known recoveries of exogenous LTs in blood, it was estimated that 300 ng of each were presented to HPLC–MS[21]. Thus molecular weight data are generated on relatively small amounts of these species in complex matrices such as blood. The data compare favorably with the other soft ionisation techniques (such as FAB–MS, where extensive purification is required prior to analysis) and point the way for structural studies on putative LT metabolites such as glucuronides.

Problems were however encountered in routine detection of peptidoleukotrienes by thermospray MS, as the generation of molecular ions does not always occur. Often the mass spectra are dominated by ions corresponding to facile water losses or cleavage of the peptide moiety (to form *m/z* 317 as the base peak), particularly when the vapouriser and ion source block become contaminated. Rigorously clean source conditions (achieved, for example, by electropolishing) may offer a means of overcoming these problems.

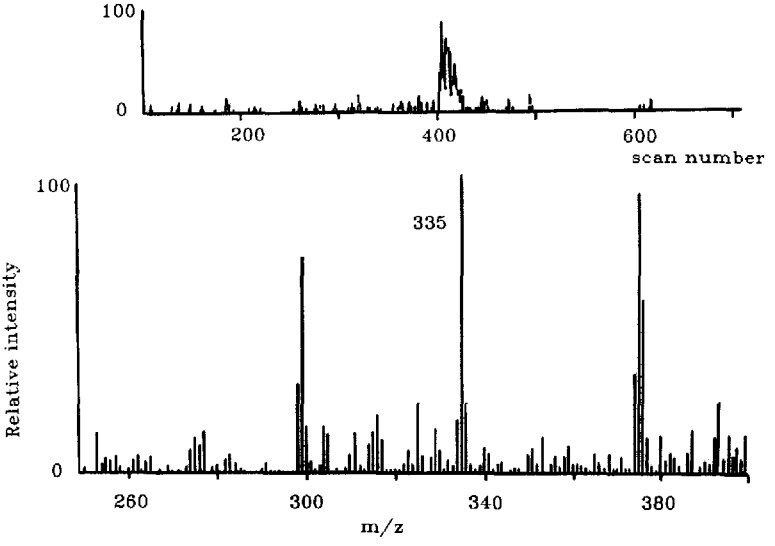

Fig. 8. Ion chromatogram (*m/z* 335) and negative ion thermospray spectrum of exogenous LTB_4, added to whole blood, after on-line column switching purification and analytical HPLC. A single peak was observed in the ion channel for *m/z* 335, with a retention time identical to that for authentic LTB_4[21]. The deprotonated molecular ion ($[M-H]^-$, *m/z* 335) is clearly visible in the thermospray mass spectrum. Similar data were obtained for the other leukotrienes.

CONCLUSIONS

Thermospray MS, although commercially available for only three years, is showing considerable promise as an analytical tool. We have demonstrated the importance of thermospray MS in obtaining molecular weight data in circumstances beyond the scope of GC–MS, or indeed in some cases, that of FAB–MS. The use of 1:1 isotopically labelled derivatives may be a useful technique for providing additional structural information in direct ionisation thermospray MS.

The chief benefit of the technique for biomedical research is the ability to generate molecular weight data on polar, thermally labile substances in complex biological matrices without the need for derivatisation, and with minimal handling. The major limitation at present is that of sensitivity: although full spectra can be obtained at the sub-μg level, the limits of detection for assay purposes are insufficient (at 1–5 ng) for thermospray to compete effectively with HPLC–radioimmunoassay or GC–MS. However, as thermospray hardware and methodology improve, the technique should become an important and widespread tool for both qualitative and quantitative analysis.

ACKNOWLEDGEMENTS

We thank the Medical Research Council and the Wellcome Trust for financial support.

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