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STUDIES OF COMBINED LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY WITH A MOVING-BELT INTERFACE

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SUMMARY

The advantages and limitations of a moving-belt interface for liquid chromatography–mass spectrometry are discussed using examples of combined liquid chromatography–mass spectrometry of natural coumarins, alkaloids, rotenoids, sugars, nucleosides, peptides and pesticides and drugs and their metabolites. Preliminary data obtained using a micro high-performance liquid chromatograph interfaced to the moving belt are reported.

INTRODUCTION

In recent years a large number of different approaches to coupling high-performance liquid chromatography (HPLC) with mass spectrometry (MS) have been reported. The various approaches, their advantages and disadvantages, and areas where the technique has been applied have been extensively reviewed[1–10].

We describe here some of the results which we have obtained over the past three years using a commercially available LC–MS interface of the moving-belt type[11,12]. A major advantage of this type of interface is that it allows both electron impact (EI) and conventional positive and negative chemical ionization (CI) spectra to be obtained, thus enabling the maximum versatility for both quantitative and qualitative studies. The disadvantages and current limitations of this approach to LC–MS will be discussed.

In addition to reviewing some of our published work in this area, new LC–MS data are presented which include negative CI studies of sugars and nucleosides, positive CI studies of glucuronide conjugates of the metabolites of CIPC and partially derivatized peptides. Preliminary data obtained using a Jasco micro HPLC system for LC–MS are also presented.

EXPERIMENTAL

LC–MS studies, unless stated otherwise, were performed using a Waters M600 pump and Cecil CE 272 UV detector, coupled to a Finnigan 4000 mass spectrometer equipped with a moving-belt interface. Kapton® belts were used for all the studies

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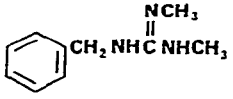
described. A temperature of 200°C (indicated) was used for the flash vapourizer with 200°C (indicated) for the clean-up heater. Source temperatures were 240°C for CI studies and 260°C for EI studies. On-column injection was used for all the studies, and solvents were redistilled prior to use. Some of the data described were obtained using an Incos data system interfaced to the Finnigan 4000, whilst other data were obtained using a potentiometric recorder and galvanometer paper.

RESULTS AND DISCUSSION

Initially studies were directed to evaluating the LC–MS interface as a form of automated probe. Spotting solutions of pure compounds onto the belt gave spectra in both the CI and EI modes which were in good agreement with spectra obtained using a direct introduction probe[13,14]. The only problems encountered were with polar compounds in the EI mode, when $(M+1)^+$ ions were obtained if the solutions were of high concentration (1 mg/ml). This effect was not observed at lower concentrations (10–100 μg/ml). Using the drug bethanidine (1) the system was evaluated quantitatively in this mode. Linear calibrations were obtained down to 1 ng spotted onto the interface with the detection limit being 50 pg (signal-to-noise ratio $> 2:1$)[12,14]. In general, good agreement is also obtained between the spectra obtained by spotting solutions onto the belt and those obtained when the system is used in the on-line mode. Recently we have found that this situation no longer pertains to thermally labile compounds of low volatility. This can be illustrated by our recent studies of mono- and di-saccharides[15]. The ammonia CI spectra obtained from xylose by on-line LC–MS and by spotting a solution onto the belt are given in Table I. As can be seen the ratios of the intensities of the *m/z* 168/150 ions are reversed, indicating that there is more thermal degradation in the on-line mode.



(1)

In general, good retention of chromatographic integrity is obtained when the system is used in the on-line mode. We have shown this in studies of steroids[8,14] and natural coumarins[16]. The percentage retention of chromatographic integrity depends on the dead volumes involved in the connection of the LC column to the interface. In both these studies, the connection was made via an ultraviolet detector. If this were omitted, even better retention of chromatographic integrity would be obtained.

Sensitivity of the system is difficult to define clearly since it is dependent on the classes of compounds being studied, the solvent systems being utilized, the sharpness of the LC peaks and the state of the mass spectrometer ion source. However, we find under routine operating conditions that full EI or CI spectra are obtainable from 200 ng upwards of sample injected onto the LC. For selected ion monitoring, detection limits range from the high picrogram and quantification is possible down to the low nanogram range[17]. These levels can be improved upon in favourable situations.

TABLE I

MASS SPECTRA OBTAINED FROM MOVING-BELT INTERFACE

Compound	*Ionization mode*	*Method of obtaining spectrum*	*m/z (% rel. intensity)*
Xylose	ammonia CI	LC–MS	168(45), 150(100), 132(51), 114(25), 108(34)
	ammonia CI	spotting	168(100), 160(89), 132(16), 108(39)
	methylene chloride negative CI	spotting	187(32), 185(100), 149(6), 132(20), 114(13), 101(70)
Fructose	methylene chloride negative CI	spotting	217(31), 215(100), 179(17), 162(14), 131(17), 101(70)
Glucose	methylene chloride negative CI	spotting	217(32), 215(100), 179(28), 162(21), 131(17), 101(90)
Sucrose	methylene chloride negative CI	spotting	379(3), 377(100), 341(12), 217(12), 215(27), 199(4), 197(13), 179(14), 162(10), 126(27), 101(32)
Uridine	methylene chloride negative CI	LC–MS	281(33), 279(100), 243(14), 111(97)
Adenosine	methylene chloride negative CI	LC–MS	304(26), 302(78), 172(8), 170(24), 134(100)
Cytidine	methylene chloride negative CI	LC–MS	280(33), 278(100), 110(15)

Having established the viability of the system, its true test is its ability to assist in the solution of problems not readily amenable to solution by other techniques. We have utilized the moving-belt LC–MS system in a wide variety of areas some of which will now be briefly described.

Natural products

In the past we have encountered problems in the screening of crude plant extracts of members of the Guttiferae for new coumarins and biosynthetically related acids using combined gas chromatography (GC)–MS[18]. Many of the compounds undergo thermal decomposition in the GC and others are not sufficiently volatile for GC study. Combined LC–MS has proved to be very effective in this area, and extracts of *Calophyllum inophyllum*[14] and *Mammea africana*[16] have been studied. New compounds have been located and are currently being isolated by preparative HPLC for further structural study. Our methodology in this area consists of initially examining the crude or partially purified extract by field desorption (FD)–MS. This provides a molecular weight profile for the components of the extract and serves as a check for subsequent absorption or decomposition of the compounds under LC–MS study. The technique has also been applied in studies of coumarins in *Imperatoria ostruthium*[10,16], Amarylidaceae alkaloids from *Crinum glaucum*[16], and chinchona alkaloids[16]. These studies were performed using a wide variety of HPLC conditions, including

adsorption and reversed-phase conditions with volatile acid or base additives in the eluting systems.

A more challinging problem is the ability to study underivatized saccharides and glycosides. Using ammonia CI LC–MS we have obtained molecular weight information for the components of a mixture of the monosaccharides xylose, fructose and glucose, and the disaccharide sucrose[15]. The eluting system consisted of acetonitrile–water (85:15) with a trace of acetic acid and a Hypersil amide-bonded column was used. The spectrum obtained for xylose under these conditions is given in Table I. Similar data were obtained from a mixture of monoglycosides[15]. So far we have failed to obtain molecular weight information from trisaccharides using our LC–MS interface. Since desorption CI provides molecular weight data for trisaccharides[19], these results indicate that the belt LC–MS system falls between a conventional direct probe and desorption CI in terms of its ability to handle thermally labile compounds of low volatility. A recent report suggests that use of freons or methylene chloride as reagent gases in the negative CI mode yield good direct probe spectra of trisaccharides[20]. Although we have obtained good negative CI data for the mono- and disaccharides (see Table I) with a mixture of methane and methylene chloride as reagent gas. To date we have failed to obtain data containing molecular weight information from trisaccharides.

One of the problems of moving-belt LC–MS interface are the difficulties which are encountered when high-percentage aqueous systems are used as eluents. Droplets form on the belt causing pressure fluctuations in the ion source thus making the system unusable. The problem is less marked if stainless-steel belts are used. A number of solutions to the problem have been reported. Heaters have been placed in each of the differentially pumped chambers[21], a second water-miscible solvent has been added to the interface prior to the aqueous phase HPLC effluent[22], and a modified segment-flow extractor has been incorporated between the HPLC and the MS interface[23]. All of these approaches have resulted in improved ability to handle higher flow-rates of aqueous solvent systems. We have been studying the use of a micro HPLC system (Jasco) in this context. As yet our studies are in an early stage but the data obtained to date indicate that this approach should assist with the problem. The micro HPLC system gives less background interference, in that the total ion current traces are less noisy and enable better sensitivities to be obtained. However this is at the expense of loss of resolution. Fig. 1 shows the total ion current trace obtained from a chloroform extract of timbo powder. Comparison of this trace with that obtained with a conventional HPLC systems[24] shows that the first two components are no longer resolved. The methane CI spectra were in good agreement with those obtained previously[24] and no splitting of the column effluent was necessary since a flow-rate of 7 μl/min was employed.

Nucleosides and peptides

Two groups[25,26] have shown the potential of LC–MS in studies of nucleosides and the technique should be particularly useful in the search for modified bases. Whilst we obtained good total ion current traces from the LC–MS of a mixture of uridine, adenosine and cytidine using methane CI, we did not obtain molecular weight data for all the components of the mixture. Use of ammonia CI rectified this situation providing ions characteristic of the intact molecule and for the base[15]. Negative CI

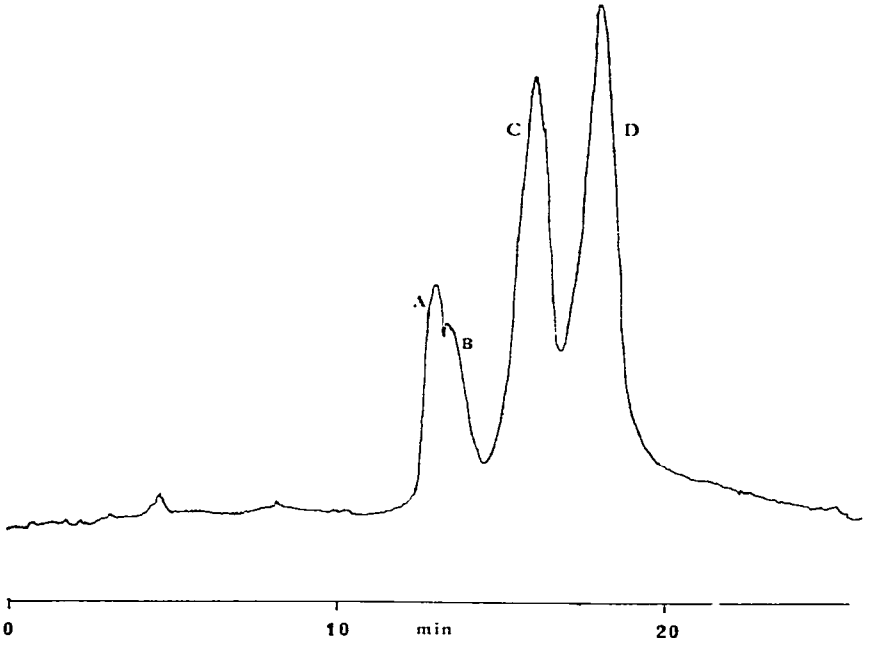


Fig. 1. Reconstructed total ion current trace of a chloroform extract of Timbo powder obtained during methane CI LC–MS. A. Jasco micro HPLC system was used with a 120 × 0.5 mm I.D. column packed with 10-μm ODS, methanol–water (7:3) was the eluting system and a flow-rate of 7 μl/min was used. Components A and B are unidentified. C is rotenone and D is deguelin.

using a mixture of methylene chloride and methane as reagent gas gave even better data, $(M+Cl)^-$ ions enabled molecular weight to be readily assigned, and ions characteristic of the base moiety were also present (see Table I).

Sequence studies of peptides is a further area of considerable potential for combined LC–MS. On-line LC–MS studies of mixtures of peptides with up to six amino acid residues, derivatized as acetyl methyl esters, have been reported[27]. Complete sequence information at the low nanomole level was obtained for all the components in many of the mixtures studied.

In our investigations in this area we have examined underivatized peptides by spotting solutions of them onto the interface under CI conditions. Although molecular weight data were readily obtainable from di- and tripeptides and for some

TABLE II

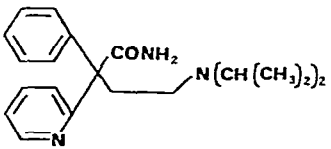
AMMONIA CI SPECTRA OBTAINED DURING LC–MS OF TETRAPEPTIDE METHYL ESTERS

Peptide	*m/z (% rel. intensity)*
Val-Leu-Val-Phe-OMe	491(20), 312(13), 294(15), 279(90), 213(22), 185(100), 180(84)
Leu-Leu-Val-Phe-OMe	505(5), 418(1), 392(1), 326(8), 308(20), 298(5), 279(80), 227(22), 199(89), 180(100)
Ile-Leu-Val-Phe-OMe	505(6), 418(1), 392(1), 326(10), 308(18), 298(5), 279(78), 227(22), 199(90), 180(100)
Tyr-Val-Leu-Leu-OMe	521(2), 384(3), 368(3), 358(8), 259(100), 235(40), 146(96), 136(36)

tetrapeptides, sequence ions were absent[8]. Hence we have centered our on-line studies on partially or fully derivatized peptides. Using ammonia CI, good sequence information was obtained from mixtures of tripeptides derivatized as acetylated methyl esters[8]. Peptides partially derivatized as their methyl esters also provide useful but not complete sequence information. Table II lists the ammonia CI spectra obtained during LC–MS of four tetrapeptide methyl esters.

Drugs and pesticides and their metabolites

This is a further area where combined LC–MS has considerable utility, both in qualitative and quantitative studies because of the polar nature of many of the compounds.



(2)

(3)

Our studies of the drug disopyramide (2) using both EI and CI in combination with LC–MS and selected-ion monitoring show that quantification is possible in plasma samples down to the low nanogram level[17].

We have also made considerable use of LC–MS in our studies of the metabolism in rats of the carbamate herbicide CIPC (3)[7,8,16]. LC–MS examination of extracts of hydrolysed and non-hydrolysed urine of rats fed with CIPC has enabled a number of new metabolites to be located. Subsequent identification has been based on comparisons of HPLC retention times and mass spectral data with synthetic material.

Of particular interest in the metabolic field is the ability to assist in the identification of conjugated materials. Our LC–MS studies of underivatized conjugated bile acids[8] show the potential for investigations of this type. More recently we have been able to obtain good LC–MS data from underivatized glucuronides[15]. We have attempted to apply the method in our studies of CIPC metabolites. Although a good reconstructed total ion current trace (Fig. 2) was obtained from a partially purified glucuronide containing extract of the urine of a rat fed with CIPC, we have so far failed to obtain on-line mass spectra which provide molecular weight information for the underivatized glucuronides. The MS data obtained from the peaks containing CIPC metabolites are summarized in Table III.

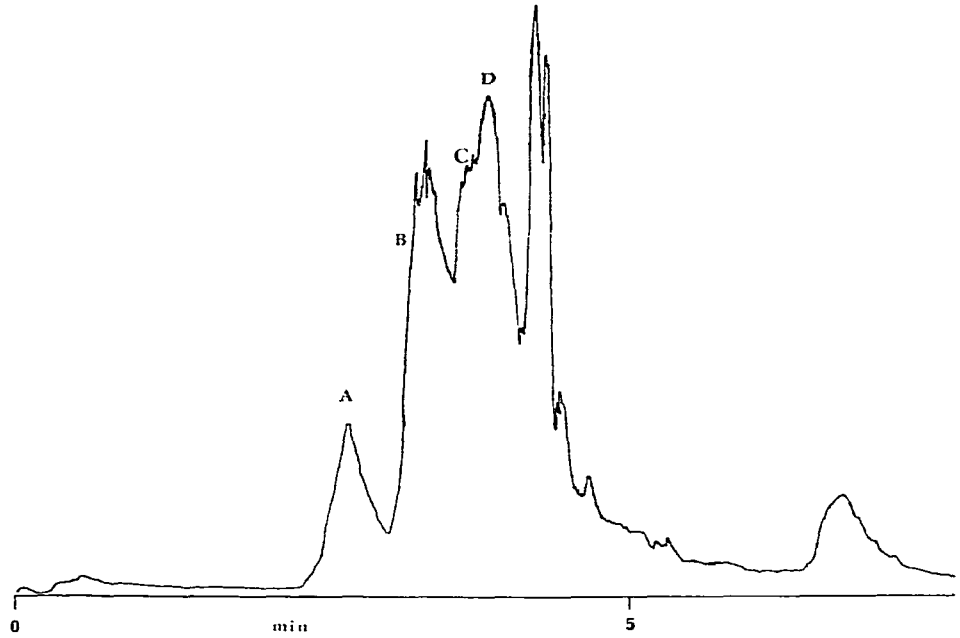


Fig. 2. Reconstructed total ion current trace obtained during ammonia CI of a partially purified glucuronide containing extract from the urine of a rat fed with CIPC. A 100 × 5 mm I.D. column packed with Spherisorb ODS was used and the eluting system was acetonitrile–water (85:15) buffered to pH 5 with sodium acetate and acetic acid at a flow-rate of 0.5 ml/min.

TABLE III

AMMONIA CI SPECTRA OBTAINED DURING LC–MS OF GLUCURONIDE FRACTION (FIG. 2)

Only spectra of CIPC metabolite-containing peaks are given.

Peak	*m/z (% rel. intensity)*
A	249(5), 247(19), 232(16), 230(59), 205(5), 203(16), 188(43), 186(100), 146(10), 144(29), 143(14)
B	249(9), 247(29), 232(27), 230(93), 205(6), 203(20), 196(4), 194(14), 188(51), 186(100), 146(12), 144(28), 143(16)
C*	249(2), 247(8), 232(6), 230(27), 188(25), 186(62), 146(14), 144(27), 143(11)
D*	249(2), 247(7), 232(5), 230(18), 188(19), 186(53), 146(31), 144(25), 143(9)

* Only ions due to metabolites are given.

CONCLUSIONS

The moving-belt LC–MS system is a viable approach to combined LC–MS and can be applied to a wide variety of areas in its present form. Future developments should include improvements in sensitivity and an extension of the current range of compounds from which molecular weight data are obtainable. In the latter context, techniques such as laser desorption mass spectrometry may prove useful.

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