Journal of Chromatography, 323 (1985) 67–79
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,471

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY OF DERIVATIZED AND UNDERIVATIZED AMINO ACIDS

DAVID E. GAMES* and EDWARD D. RAMSEY
Department of Chemistry, University College, P.O. Box 78, Cardiff CF1 1XL (U.K.)

SUMMARY

A mixture of six aromatic amino acids has been studied by moving belt liquid chromatography–mass spectrometry (LC–MS) using both conventional and microbore columns and by thermospray LC–MS using conventional columns. Microbore columns gave the best detection limits with the moving belt interface. Thermospray LC–MS provides better sensitivity, but one of the amino acids was not detected. Methodology for the direct use of a gradient system with thermospray LC–MS has been developed. Eleven phenylthiohydantoin-amino acids were also studied using a moving belt interface. With microbore columns, detection limits for full scan electron impact spectra ranged from 2 nmol to 20 pmol.

INTRODUCTION

Amino acids have been widely utilized to evaluate interfaces for liquid chromatography–mass spectrometry (LC–MS)[1]. However there are few reports of on-line HPLC–MS of amino acids or their derivatives. LC–MS of tryptophan using a vacuum nebulizing interface with microbore LC has been described[2] and a mixture of creatine, valine, leucine and isoleucine has been studied using ion evaporation LC–MS–MS[3]. Thermospray ionization shows considerable promise for the analysis of this class of compounds[4,5]. A procedure for peptide sequencing has been described[6], whereby underivatized peptide solutions are injected through a column containing immobilized carboxypeptidase Y and the amino acids released, starting from the C-terminus of the peptide chain are directly transported by a continuously flowing aqueous buffer into a thermospray mass spectrometer, where they are detected and quantified. Atmospheric pressure ionization LC–MS has been used for studies of methylthiohydantoin derivatives[7] and *tert.*-butylated derivatives of tryptophan have been characterized using direct liquid introduction LC–MS[8].

Identification of phenylthiohydantoin (PTH)-amino acids is a crucial step in protein sequence analysis by Edman degradation. The mass spectral behaviour of these compounds has been extensively studied[9,10] and LC has been used for their identification on the basis of retention time[11]. Use of LC–MS could have considerable advantages in this area both as a technique for the confirmation of identity of known PTH–amino acids and as an aid in the characterization of unusual amino acids.

0021-9673/85/$03.30 © 1985 Elsevier Science Publishers B.V.

We report here the results of studies aimed at evaluating the utility of LC–MS for the analysis of underivatized and PTH–amino acids. A mixture of six aromatic amino acids has been studied by LC–MS using a moving belt interface with conventional and microbore LC columns and a thermospray interface. Eleven PTH-amino acids were also studied using conventional and microbore LC–MS with a moving belt interface.

EXPERIMENTAL

Conventional LC and LC–MS studies were performed using a Waters 680 gradient former and two Waters 6000A pumps. A Cecil 212 UV detector set at 260 nm was used for the underivatized amino acids and a Waters 441 UV detector set at 254 nm for the PTH-amino acids. A Rheodyne 7125 valve loop injector was used together with a Spherisorb 5 μm ODS 250 × 4.6 mm column. For microbore LC–MS a Whatman Partisil 10 250 × 1 mm column was used and 0.5-μl solutions were injected using a Rheodyne 7410 valve loop injector.

LC–MS was performed on a Finnigan MAT 4500 quadrupole mass spectrometer fitted with a moving belt interface. Kapton belts were used and the vaporizer was at 225°C indicated. For the underivatized amino acids ammonia was used as the chemical ionization (CI) reagent gas at a source temperature of 150°C and pressure of $2.5 \cdot 10^{-5}$ Torr. With conventional columns 2% of the mobile phase was fed to the interface through a low dead volume splitter. Microbore LC–MS was performed with the column directly interfaced to the moving belt. LC–MS studies of the PTH-amino acids were performed in the electron impact (EI) mode, 70 eV, source temperature 150°C. Thermospray LC–MS was performed on a Finnigan MAT 4500 quadrupole mass spectrometer equipped with a Finnigan MAT thermospray source. Mass spectral data were collected and processed with an INCOS data system.

Standard solutions of the underivatized amino acids were made up in 0.1 *M* hydrochloric acid. The amino acids and PTH-derivatives were purchased from Sigma and used as received. All solvents used for LC were redistilled from glass before use.

RESULTS AND DISCUSSION

The liquid chromatogram obtained from a mixture of six aromatic amino acids using UV detection and a 250 × 4.6 mm LC column is shown in Fig. 1. Because of the high percentage of water in the mobile phase used at the commencement of the gradient less than 4% of the eluent from the column could be fed to the moving belt interface for LC–MS. Surprisingly we found that we were able to handle this high percentage aqueous mobile phase without the use of a co-solvent to improve wetting on the belt. Initially LC–MS was conducted using isobutane and methane CI. However, in the total ion current trace produced with both reagent gases, only three amino acids could be detected. This was because the trifluoroacetic acid used as a modifier for LC was ionized and produced a strong signal at m/z 115. By use of mass chromatography the other three amino acids could be detected, however $[M + 1]^+$ ions for the amino acids were absent or of low abundance in the spectra obtained. In order to overcome the former problem the use of sulphuric acid as a modifier for LC was investigated. Unfortunately with this approach we could no longer resolve

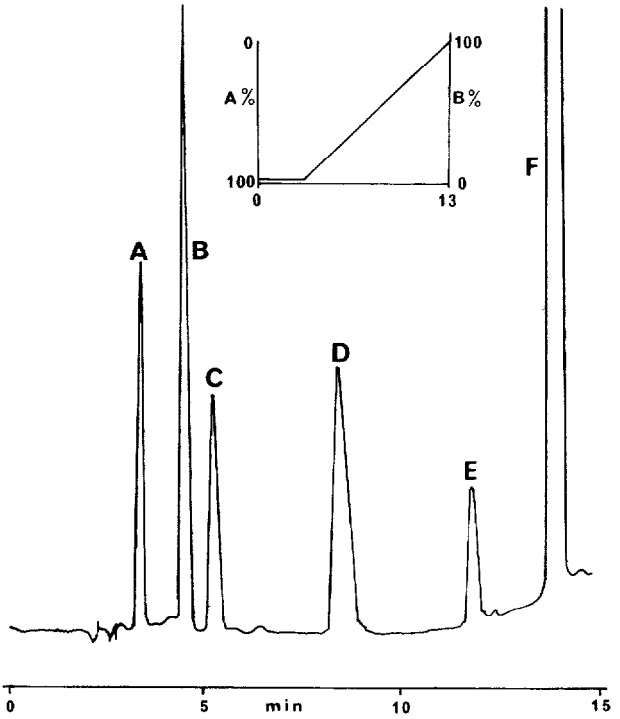

Fig. 1. UV trace obtained by LC of *p*-aminophenylalanine (A), *p*-hydroxyphenylglycine (B), phenylglycine (C), tyrosine (D), *β*-phenylalanine (E) and tryptophan (F). Column: Spherisorb ODS 5, 250 × 4.6 mm; flow-rate, 1 ml min^{-1}. Gradient as shown, solvent system A, methanol–water (7:93), pH 2.3 with ammonium hydroxide and trifluoracetic acid; solvent system B, methanol–water (90:10) pH 2.3 with ammonium hydroxide and trifluoroacetic acid.

p-hydroxyphenylglycine and phenylglycine. Use of ammonia CI with our original LC conditions overcame both these problems. Fig. 2 shows the computer reconstructed total ion current (TIC) trace obtained by LC–MS under these conditions. The spectra obtained (Table I) gave protonated molecular ions for all the amino acids, together with some characteristic fragment ions. In order to estimate detection limits, five experiments were conducted on solutions containing between 1 and 25 μg of each of the amino acids. Although acceptable TIC traces were obtained at the lowest concentration, only phenylglycine gave an $[M + 1]^+$ ion. The limits at which this point was reached with the other amino acids varied between 5 and 14 μg, with *p*-hydroxyphenylglycine proving to be the most labile compound. We attribute this behaviour to thermal decomposition of the amino acids on the belt at low concentrations.

Microbore LC–MS has been shown to provide improvements in detection limits with moving belt interfaces, because all of the effluent from the LC column can be handled by the interface[12,13]. This approach was investigated with the six amino acids using ammonia CI and the TIC trace obtained from injecting between 200 and 375 ng of each amino acid onto the LC column is shown in Fig. 3. Molecular weight information was obtained from *p*-aminophenylalanine, phenylglycine and phenylalanine, but not for the other amino acids. This failure to obtain molecular weight

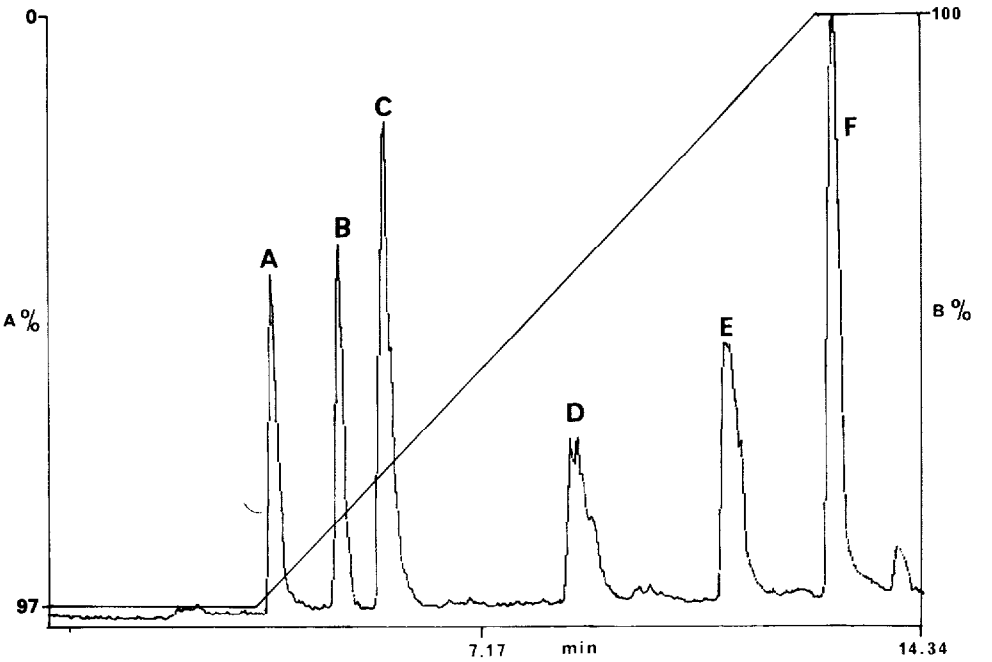

Fig. 2. Computer reconstructed TIC trace of mixture of six amino acids obtained using ammonia CILC–MS. Conditions as for Fig. 1.

information from all the amino acids at this level was disappointing and was probably due to the quality of the belt on the interface which had been used for some time for studies of a wide range of compounds. Deactivation of the belt using a silylation[14] or Carbowax 20M[15] may improve the situation.

Since underivatized amino acids have been shown to be amenable to thermospray ionization[4,5] and the fact that the technique works best with mobile phase flow-rates in the region of 1.5 ml min^{-1}. We decided to study the LC–MS of our mixture of amino acids by this technique and also to investigate the possibility of performing gradient elution directly into the thermospray ion source using the temperature programming facility for the vaporizer. Recently gradient elution LC–MS

TABLE I

AMMONIA CI MASS SPECTRA OBTAINED BY LC–MS OF AMINO ACID MIXTURE

Amino acid	*Peak*	*Amount injected (μg)*	*Mol.wt.*	*m/z (% relative intensity)*
p-Aminophenylalanine	A	30	180	198(13), 181(100), 135(47), 106(10)
p-Hydroxyphenylglycine	B	35	167	185(11), 168(55), 122(100), 110(13)
Phenylglycine	C	28	151	169(35), 152(97), 106(100)
Tyrosine	D	40	181	199(10), 182(94), 136(100), 124(30)
Phenylalanine	E	30	165	183(14), 166(100), 120(37)
Tryptophan	F	25	204	222(7), 205(100), 159(52), 130(37)

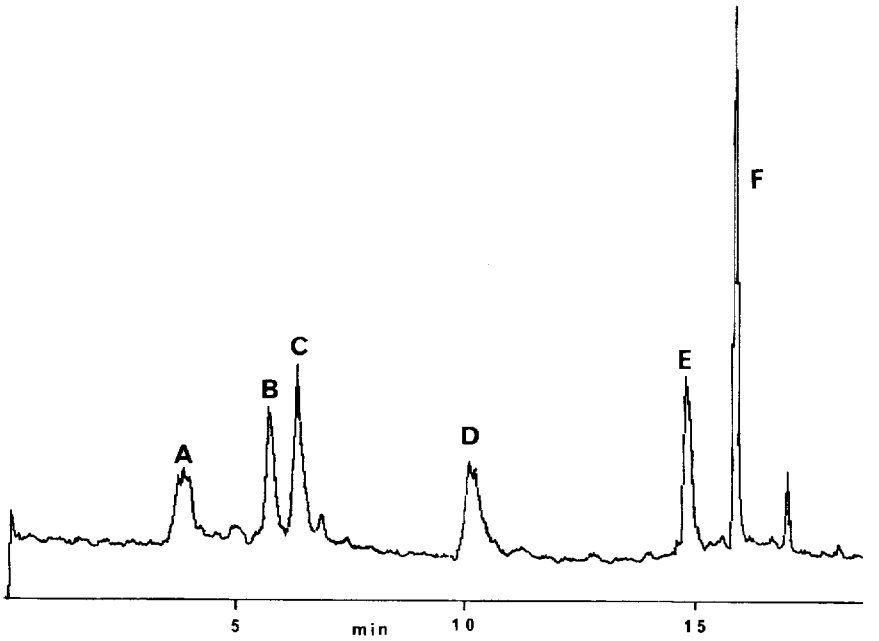

Fig. 3. Computer reconstructed TIC trace of mixture of six amino acids using microbore ammonia CI LC–MS. Whatman Partisil 10 ODS 3 250 × 1 mm column; flow-rate, 55 μl min^{-1}. Solvents and gradient as for Fig. 1.

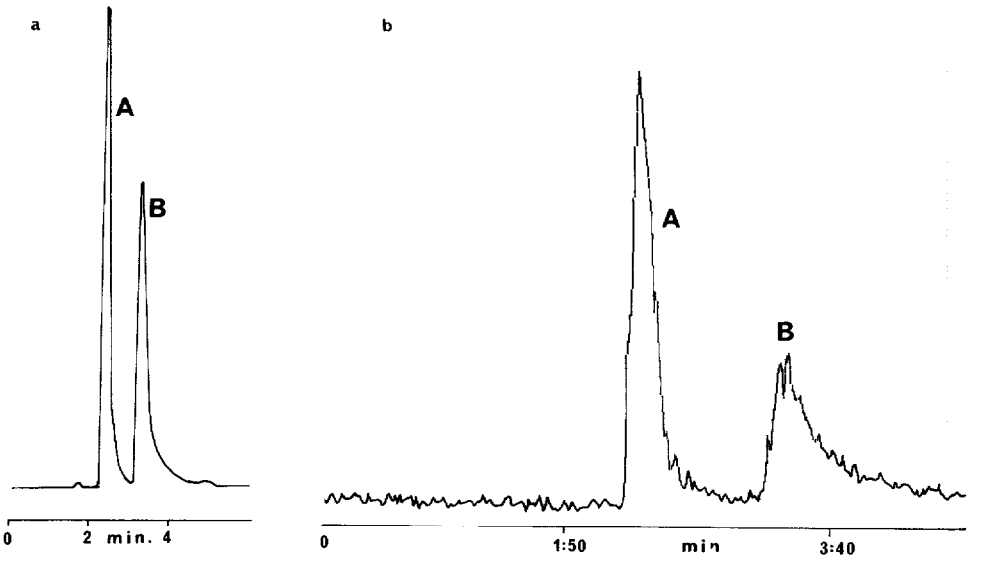

Fig. 4. UV (a) and thermospray TIC (b) traces obtained from a mixture of *p*-hydroxyphenylglycine (A) and *p*-aminophenylglycine (B). Spherisorb ODS 5, 250 × 4.6 mm, water–methanol–formic acid (95:5:0.1) made 0.1 *M* with respect to ammonium acetate. Flow-rate, 1.5 ml min^{-1}.

TABLE II

TUNING SOLUTION COMPOSITIONS AND THEIR VAPORIZER PROGRAMMES FOR DEVELOPMENT OF THERMOSPRAY LC-MS OF THE MIXTURES OF AMINO ACIDS

Jet = 200°C for each compound.

Tuning solution number	*Ammonium acetate stock solution (ml)*	*Water (ml)*	*Methanol (ml)*	*Amino acid stock solution* (ml)*	*Vaporiser program*		
					Initial temp. (°C)	*Final temp. (°C)*	*Rate (°C/min)*
1	50	400	50	0.75	145	110	4.5
2	50	300	150	0.75	140	110	4.5
3	50	200	250	0.75	140	95	4.5
4	50	100	350	0.75	130	85	4.5
5	50	–	450	0.75	125	80	4.5

* At a flow-rate of 1.2 ml/min this level of "spiking" should result in approximately 40 ng/sec of each amino acid being thermosprayed.

has been reported with thermospray ionization, which involved post-column addition of buffer using a low linear gradient profile with manual control of the capillary heater[16].

Initial studies were performed on a prototype Finnigan MAT thermospray interface which had the capillary in a heated copper block and no facilities for precise control and programming of the vaporizer temperature. A mixture of *p*-hydroxyphenylglycine and *p*-aminophenylglycine was studied under isocratic conditions. The UV and TIC traces obtained from an injection of 20 μg of each amino acid are shown in Fig. 4. Simple spectra containing exclusively $[M + 1]^+$ ions for the two amino acids were obtained. Response was best for the *p*-hydroxyphenylglycine and using selected ion monitoring it was readily detectable at the 20-ng injected on-column level. At this concentration *p*-aminophenylalanine was only just detected.

In order to effect most efficient thermospray ionization, the presence of ammonium acetate is required in the mobile phase. Hence, we redeveloped our LC system for gradient elution using 0.05 *M* ammonium acetate. In order to optimize the conditions for gradient elution study solutions of the six amino acids were pumped into the thermospray ion source under the conditions shown in Table II. Reconstructed total ion current profiles were obtained for each solution by programming the vaporizer over a range of temperatures. The profile obtained for the tuning solution 5 is shown in Fig. 5 and indicates an optimal vaporizer temperature of 102°C. Plotting of the optimal vaporizer temperature for each solution against the percentage methanol in the solution gave a linear relationship. Thus we were able to select the optimal vaporizer temperatures for the start (134°C) and end (102°C) of

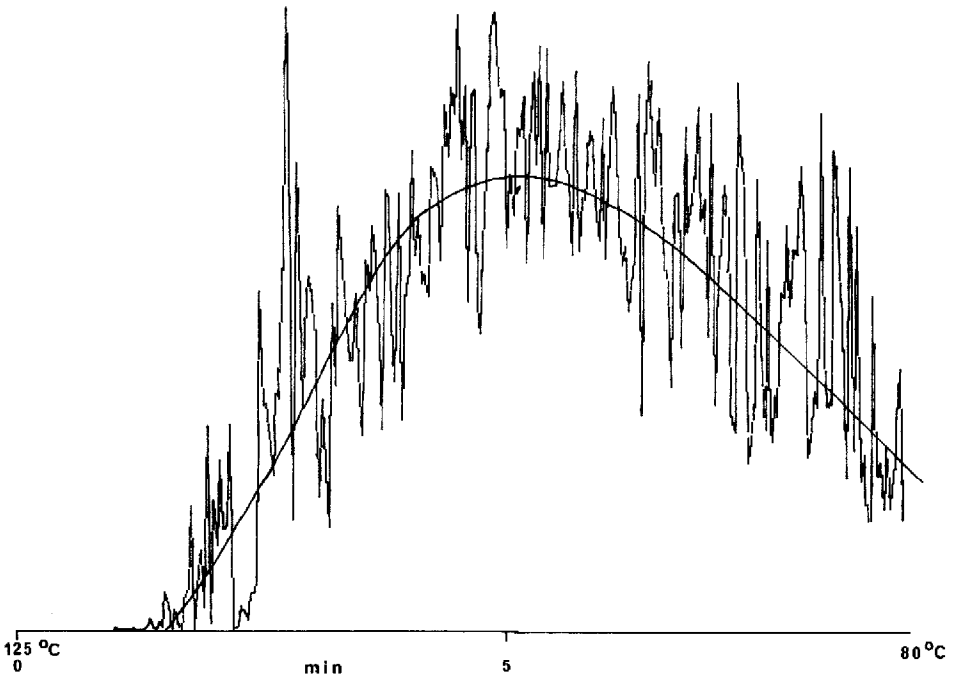

Fig. 5. Reconstructed total ion current trace obtained with tuning solution 5.

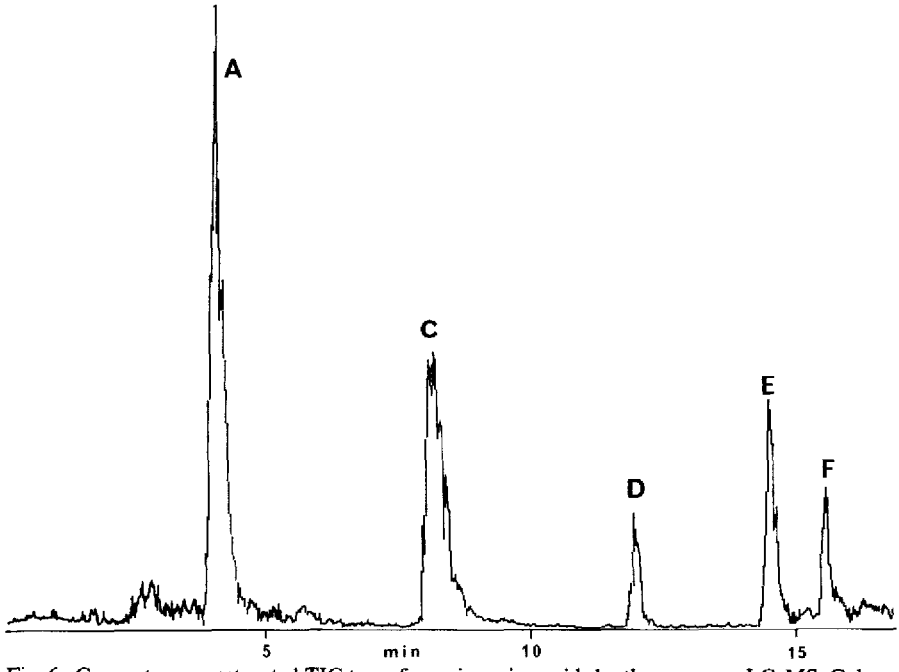

Fig. 6. Computer reconstructed TIC trace from six amino acids by thermospray LC–MS. Column, Spherisorb 5μ ODS 250 × 4.6 mm; flow-rate, 1.2 ml min^{-1}. Solvent system A: methanol–water (10:90) made 0.05 *M* with ammonium acetate and to pH 2.3 with trifluoracetic acid; solvent system B: methanol–water (90:10) made 0.05 *M* with ammonium acetate and to pH 2.3 with trifluoracetic acid. Gradient: 90% A isocratic for 4 min, then linear gradient over 14.6 min to 100% B.

the gradient and programme it linearly using the microprocessor control, taking into account the t_0 of 1.6 min for the LC column.

Fig. 6 shows the computer reconstructed TIC trace obtained from the mixture of six amino acids by thermospray LC–MS. The signal for *p*-hydroxyphenylglycine is absent and it was not observed when the test mixtures of amino acids were studied. Its absence may be due to thermal decomposition in the interface or an insufficiently high source temperature. The interface used in this study results in a longer residence time of the sample in the capillary than with the earlier version, where we were able to obtain spectra of this amino acid. The spectra obtained for the other amino acids are given in Table III. In all cases the $[M + 1]^+$ ion was the base peak in spectrum,

TABLE III

THERMOSPRAY MASS SPECTRA OBTAINED BY LC–MS OF AMINO ACIDS

Compound	*Peak*	*Mol.wt.*	*m/z (% relative intensity)*
p-Aminophenylalanine	A	180	181(100), 195(4), 203(10)
Phenylglycine	C	151	152(100), 303(17)
Tyrosine	D	181	182(100), 196(6), 363(5)
Phenylalanine	E	165	166(100), 180(5), 331(68)
Tryptophan	F	204	205(100), 219(22), 409(25)

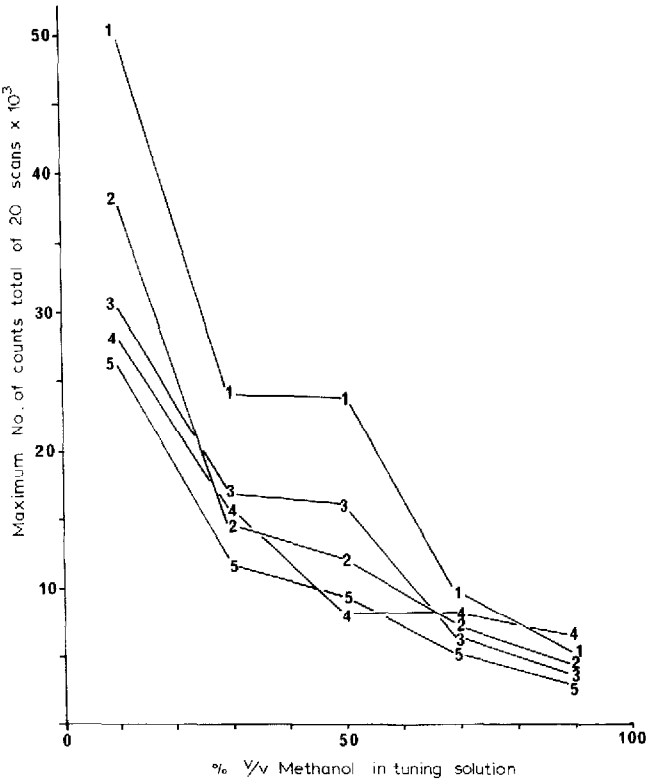

Fig. 7. Relative thermospray responses of different amino acids with different mobile phase compositions. 1 = β-Phenylalanine; 2 = *p*-aminophenylalanine; 3 = phenylglycine; 4 = tryptophan; 5 = tyrosine.

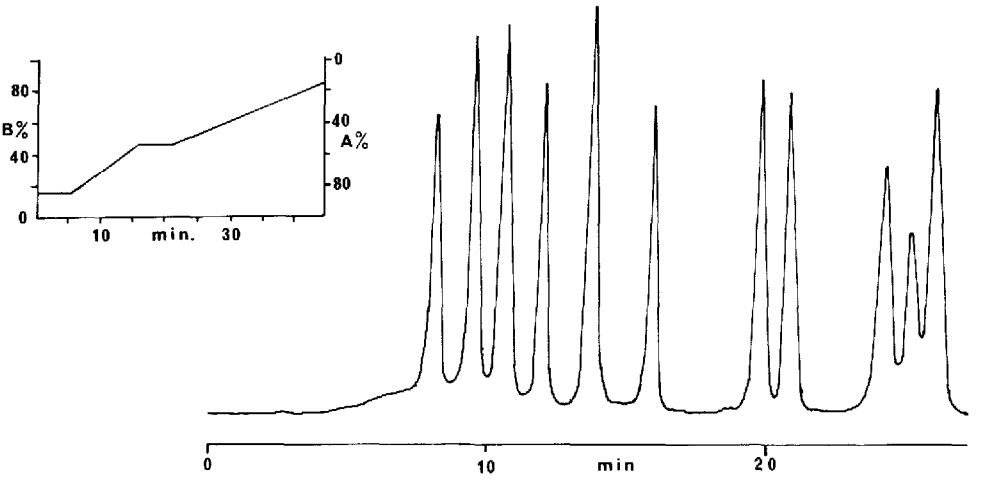

Fig. 8. UV trace obtained by LC of mixture of eleven PTH-amino acids. Column: 250 × 4.6 mm 5μ Spherisorb ODS; flow-rate, 1 ml min^{-1}. Gradient as shown. Solvent A: acetonitrile–water–formic acid (15:85:0.3); Solvent B, acetonitrile water–formic acid (72:28:0.3). Order of elution as in Table IV.

TABLE IV

EI MASS SPECTRA OBTAINED FROM PTH-AMINO ACIDS BY LC–MS USING A CONVENTIONAL COLUMN

PTH-amino acid	*Mol.wt.*	*m/z (% relative intensity)*
Serine	222	222(18), 204(51), 192(58), 135(87), 77(100)
Aspartic acid	250	250(41), 204(100), 175(15), 135(60), 119(16), 104(19), 77(86)
Glycine	192	192(100), 163(14), 135(72), 120(27), 77(58)
Glutamic acid	264	264(36), 246(34), 218(50), 135(100), 77(93)
Alanine	206	206(88), 177(20), 135(100), 77(49)
Tyrosine	298	298(22), 192(54), 135(21), 107(100), 77(43)
Methionine	266	266(85), 205(100), 192(57), 135(55), 77(90)
Proline	232	232(51), 203(6), 135(100), 77(35)
Tryptophan	321	321(20), 192(13), 130(100), 102(31), 77(29)
Phenylalanine	282	282(27), 191(5), 135(15), 117(13), 91(100), 77(26)
Leucine	248	248(59), 219(11), 205(20), 192(38), 135(100), 77(64)

and with the exception of *p*-aminophenylalanine $[2M + 1]^+$ ions were observed. A further feature was the presence of ions corresponding to protonated methyl esters. Some sensitivity measurements were conducted and solutions containing 1 μg and 500 ng of each of the amino acids were subjected to LC–MS under the same conditions. At the 500-ng level the TIC trace was very poor although acceptable spectra could be obtained. The sensitivity situation could probably be considerably improved by use of small bore columns with lower flow-rates and post column addition of

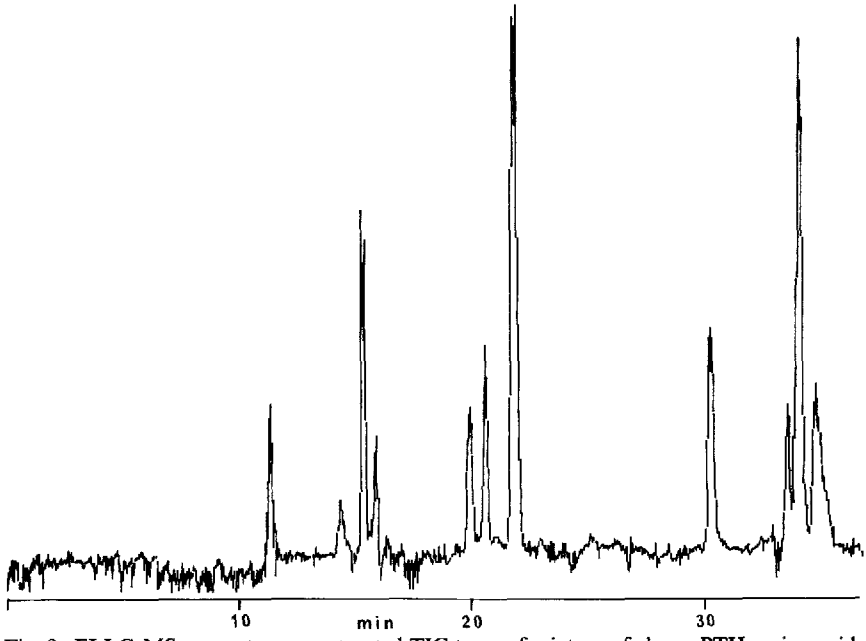

Fig. 9. EI LC–MS computer reconstructed TIC trace of mixture of eleven PTH-amino acids using a 250 × 1 mm Whatman Partisil 10 ODS 3 column with a flow-rate of 80 μl min^{-1}. Gradient as for Fig. 8. Order of elution as in Table V.

TABLE V

EI MASS SPECTRA OBTAINED FROM PTH-AMINO ACIDS BY LC–MS USING A MICROBORE COLUMN

PTH-amino acid	*Mol.wt.*	*m/z (% relative intensity)*
Serine	222	222(11), 204(58), 192(46), 135(70), 77(100)
Aspartic acid	250	250(3), 204(80), 175(13), 135(49), 119(8), 104(22), 77(100)
Glycine	192	192(44), 163(9), 135(51), 120(35), 77(100)
Glutamic acid	264	264(4), 246(17), 218(56), 135(70), 77(100)
Methionine sulphone	298	298(39), 218(42), 205(32), 189(11), 135(60, 77(100)
Alanine	206	206(100), 177(15), 135(64), 77(55)
Tyrosine	298	298(7), 192(50), 135(9), 107(100), 77(24)
Proline	232	232(71), 203(8), 135(100), 77(51)
Tryptophan	321	321(2), 192(4), 130(100), 77(15)
Phenylalanine	282	282(22), 191(4), 135(9), 91(100), 77(23)
Leucine	248	248(76), 219(13), 205(21), 192(36), 135(100), 77(69)

water to provide solutions with a higher percentage of water entering the thermospray ion source. The experiments conducted to determine the gradient conditions showed that much higher responses were obtained for the amino acids with systems with a high percentage of water (see Fig. 7). This observation is in agreement with studies reported by another group on other classes of organic molecule[17].

Derivatized amino acids should be more amenable to LC–MS study than their underivatized counterparts and this was confirmed in our studies of PTH-amino acids. The UV trace obtained by LC of a mixture of eleven of these compounds is shown in Fig. 8. Because of the high percentage of water in the mobile phase used at the commencement of the gradient only 5% of the eluent from the liquid chromatograph could be fed to the LC–MS interface. A satisfactory total ion current trace was obtained under EI–LC–MS conditions and the EI spectra obtained are summarized in Table IV. With this approach sensitivity measured in terms of sample injected on-column is going to be limited and in order to explore detection limits four experiments were conducted using microbore LC–MS. The mixture of PTH-

TABLE VI

DETECTION LIMITS FOR PTH-AMINO ACIDS USING MICROBORE LC–MS SYSTEM

PTH-amino acid	*On-column detection limit (nmol for full EI spectrum)*
Serine	0.4–2
Aspartic acid	0.5–2.5
Glycine	0.05–0.5
Glutamic acid	0.4–2
Methionine sulphone	0.2–1.5
Alanine	$\geqslant$0.02
Tyrosine	$\geqslant$0.08
Proline	$\geqslant$0.05
Tryptophan	0.3–1.5
Phenylalanine	$\geqslant$0.05
Leucine	$\geqslant$0.04

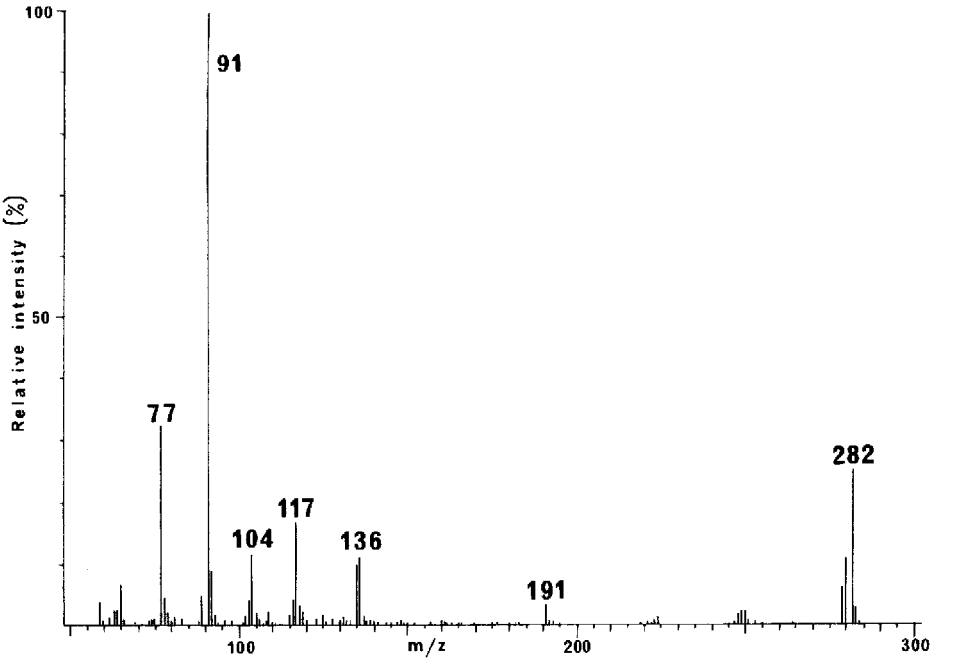

Fig. 10. EI mass spectrum of PTH-phenylalanine obtained by LC–MS from 13 ng injected on-column. LC conditions as for Fig. 9.

amino acids was slightly changed in that methionine sulphone PTH was substituted for methionine. The reconstructed total ion current trace obtained from this mixture is shown in Fig. 9 and the spectra obtained are given in Table V. The elution order of some of the derivatives has changed. There is molecular weight information for all the derivatives but the abundance of the molecular ions of the more labile compounds *e.g.* PTH-aspartic acid, PTH-glutamic acid, PTH-tyrosine and PTH-tryptophan are of lower abundance. The on-column detection limits to obtain a full EI spectrum with molecular weight information is given in Table VI and the EI spectrum obtained from PTH-phenylalanine with 13 ng injected on-column is shown in Fig. 10.

CONCLUSIONS

Moving belt LC–MS systems are capable of handling underivatized aromatic amino acids. However, for full spectra high ng amounts of sample are required even if microbore columns are used. Use of spray deposition devices[18] will improve detection limits for conventional columns and the most recently developed moving belt interface should also improve detection limits, since there should be less thermal decomposition[19]. Thermospray LC–MS enables molecular weight information to be obtained at lower levels with these compounds, however total ion current traces are not good below the 1-μg-injected level and mass chromatography is necessary to obtain satisfactory spectra when small amounts of sample are being investigated. The absence of one of the amino acids in the thermospray LC–MS is of concern. It may

well be that conditions were not fully optimized and further studies are in progress to see if this compound can be handled with our current interface.

PTH-amino acids acids work well with moving belt systems. With detection limits varying for full spectra between low nmol and low pmol depending on the amino acid. Use of CI together with spray deposition may well improve detection limits.

ACKNOWLEDGEMENTS

One of us, E.D.R. thanks the SERC and ICI Pharmaceuticals for financial support. We thank the SERC and Royal Society for assistance in the purchase of some of the MS equipment used in these studies and thank Finnigan MAT for assistance in the thermospray studies. Finally we are indebted to Dr. B. J. Woodhall and Mr. D. A. Catlow, ICI Pharmaceuticals Division for their continuing advice and support in this study.

REFERENCES

1 C. G. Edmonds, J. A. McCloskey and V. A. Edmonds, *Biomed. Mass Spectrom.*, 10 (1983) 237.
2 H. Yoshida, K. Matsumoto, K. Itoh, S. Tsuge, Y. Hirata, K. Mochizuki, N. Kokubun and Y. Yoshida, *Fresenius Z. Anal. Chem.*, 311 (1982) 674.
3 B. Sushan, J. E. Fulford, B. A. Thomson, W. R. Davidson, L. M. Danylewych, A. Ngo, S. Nacson and S. D. Tanner, *Int. J. Mass Spectrom. Ion Phys.*, 46 (1983) 225.
4 C. R. Blakley, J. C. Carmody and M. L. Vestal, *Clin. Chem.*., 26 (1980) 1467.
5 C. R. Blakley, J. J. Carmody and M. L. Vestal, *Anal. Chem.*, 52 (1980) 1636.
6 D. Pilosof, H.-Y. Kim, M. L. Vestal and D. F. Dyckes, *Biomed. Mass Spectrom.*, 11 (1984) 403.
7 E. C. Horning, D. I. Carroll, I. Dzidic, K. D. Haegele, M. G. Horning and R. N. Stillwell, *J. Chromatogr.*, 99 (1974) 13.
8 D. J. Dixon, *Analusis*, 10 (1982) 343.
9 W. Vetter, in G. R. Waller and O. C. Dermer (Editors),*Biochemical Applications of Mass Spectrometry*, Wiley, New York, 1980, 1st Suppl., p. 439.
10 K. Biemann, in G. R. Waller and O. C. Dermer (Editors), *Biochemical Applications of Mass Spectrometry*, Wiley, New York, 1980, 1st Suppl., p. 469.
11 C. T. Wehr and R. L. Cunico, *Biotechnology Laboratory*, (1983) 62.
12 D. E. Games, M. S. Lant, S. A. Westwood, M. J. Cocksedge, N. Evans, J. Williamson and B. J. Woodhall, *Biomed. Mass Spectrom.*, 9 (1982) 215.
13 N. J. Alcock, L. Corbelli, D. E. Games, M. S. Lant and S. A. Westwood, *Biomed. Mass Spectrom.*, 9 (1982) 499.
14 I. G. Beattie, D. E. Games, J. Gilbert and J. R. Startin, *14th Meeting of the British Mass Spectrometry Society*, Heriot-Watt University, Edinburgh, 1984, in press.
15 J. van der Greef, personal communication.
16 R. D. Voyksner, J. T. Bursey and E. D. Pellizzari, *Anal. Chem.*, 56 (1984) 1507.
17 R. D. Voyksner, C. A. Haney and J. R. Hass, *Anal. Chem.*, in press.
18 M. J. Hayes, E. P. Lankmeyer, P. Vouros, B. L. Karger and J. M. McGuire, *Anal. Chem.*, 55 (1983) 1745.
19 D. E. Games, M. A. McDowall, K. Levsen, K. H. Schafer, P. Dobberstein and J. L. Gower, *Biomed. Mass Spectrom.*, 11 (1984) 87.