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Amino acid quantitation in aqueous matrices *via* trap and release membrane introduction mass spectrometry: homocysteine in human plasma

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Trap and release membrane introduction mass spectrometry (T&R-MIMS) using a removable direct insertion membrane probe (DIMP) is employed to determine the total homocysteine concentration (tHcy) directly from human plasma after derivatization with ethyl chloroformate. The method uses no chromatographic separation, is linear, reproducible, and displays limit of quantitation (2 μM) sufficiently below the threshold concentration of tHcy in plasma. It also combines chemical, membrane, and mass spectrometric discrimination, and can be used to determine selected amino acids in human plasma simultaneously. After derivatization with ethyl chloroformate, many amino acids in aqueous solution are observed to be efficiently detected; hence T&R-MIMS is promising as a simple and sensitive technique for simultaneous quantitation of selected amino acids in plasma and urine, and in other aqueous matrices.

Introduction

Hyperhomocysteinemia—increased total homocysteine concentration (tHcy) in plasma or serum—is a valuable marker of common diseases.[1] Numerous clinical studies and advances in Hcy quantitation have established hyperhomocysteinemia as a strong and independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular diseases. It is also a sensitive marker for deficiencies of folate and vitamins B_{12} and B_6, and is used to diagnose the inborn error in metabolism termed homocystinuria.

Several methods are applied to quantitate tHcy in plasma and other biological fluids.[2] Hcy is present in plasma mainly coupled *via* disulfide-bonds to another Hcy, to cysteine, or to proteins.[3] Hence to determine tHcy, chemical reduction of the disulfide bonds is commonly performed. Free Hcy is then most commonly determined *via* reverse-phase high performance liquid-chromatography (HPLC) with either fluorescence or UV detection after appropriate derivatization.[4] Capillary electrophoresis[5] and gas chromatography-mass spectrometry[6] (GC-MS) methods have also been proposed. A rapid enzyme conversion immunoassay for selective tHcy quantitation is also available.[7]

Membrane introduction mass spectrometry (MIMS)[8] first appeared for the direct quantitation of VOCs in air and aqueous matrices, showing outstanding speed and trace level detection limits. However, for semi-volatile (SVOCs) and more polar organic compounds, MIMS was shown to be unsatisfactory since detection limits were often too high to be useful. Trapping strategies,[9] ultrathin composite membranes,[10] indirect monitoring of a related VOC analyte,[11] and hyphenated MIMS techniques[12] have therefore been implemented to improve MIMS detection limits of SVOCs and to lower concurrently the detection limits of VOCs.

An efficient and generally applicable approach for trace SVOC analysis by MIMS is offered by the trap and release MIMS (T&R-MIMS) technique.[13] In T&R-MIMS, SVOCs are properly preconcentrated inside a capillary silicone membrane, and then thermally desorbed to the gas phase using the heat radiation from the ionization filament. T&R-MIMS has greatly expanded the applicability of MIMS by including detection of larger and more polar molecules so that now even compounds such as steroid hormones can be conveniently quantitated.[14]

We recently developed a simpler T&R-MIMS system using a removable, more versatile and interchangeable direct introduction membrane probe (DIMP).[15] Because of faster and more uniform membrane heating provided by our T&R-MIMS system, SVOC sensitivity improves and memory effects are minimized. We now report that T&R-MIMS can be used for simple and sensitive quantitation of selected amino acids in aqueous solutions, and tHcy quantitation in human plasma is described.

Experimental

Mass spectrometry was performed using 70 eV electron ionization (EI) and an Extrel (Pittsburgh, PA) mass spectrometer fitted with a high transmission quadrupole. The standard EI ion source was used with just a minor modification: the id of one of the two gas entrance lines was enlarged to 1.27 cm (Fig. 1). The analyte solutions at room temperature (23 ± 1 °C) were pumped through the system by an eight-roll peristaltic pump at a rate of 2 mL min^{-1}. The capillary membrane was provided by Dow Corning Co. (Silastic Medical-grade tubing) with a wall thickness of 0.056 cm, id of 0.063 cm, and od of 0.12 cm.

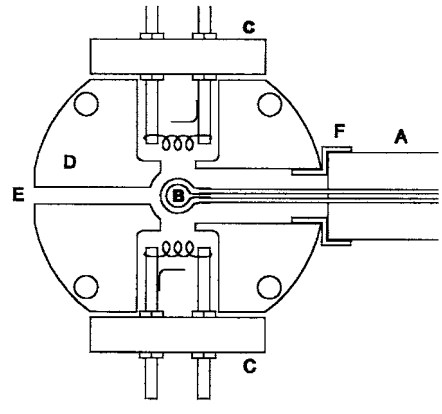

Fig. 1 Schematic of the T&R-MIMS system: (A) DIMP probe; (B) capillary membrane loop; (C) filaments; (D) ion source block; (E) CI gas entrance; and (F) ceramic probe adapter. Adapted from ref. 15.

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T&R-MIMS

Fig. 1 displays a schematic of the system: the removable DIMP probe (A) with a 10 mm long capillary membrane loop (B) is shown *in situ* in the ion source (D). The capillary membrane (B) is fixed into the DIMP probe (A) and a ceramic probe adapter (F) ensures proper sealing. By fine adjusting of the position of the DIMP probe, the membrane loop (B) can be placed exactly between the two filaments (C) so as to ensure more efficient analyte ionization but particularly faster and more uniform heating of the capillary membrane surface.

Sample preparation

Standard aqueous solutions of Hcy (D,L-homocysteine from Sigma Corp.) were prepared in de-ionized water by serial dilution of a 500 µM aqueous solution. For the plasma samples, 3 mL of human blood were collected in a Vacutainer EDTA-containing tube, and immediately centrifuged at 1000*g* for 5 min at 4 °C. To reduce disulfides and decouple them from plasma proteins so as to measure tHcy, 1 mL of the resulting plasma (or 1.5 mL of Hcy aqueous solutions) was treated with 250 µL of dithiothreitol (DTT) and incubated for 30 min at 36 °C. The proteins were then precipitated by adding 1 mL of 5% aqueous solution of trichloroacetic acid (TCA) and 100 µL of saturated EDTA solution under vigorous vortexing followed by centrifugation at 3000*g* for 15 min.

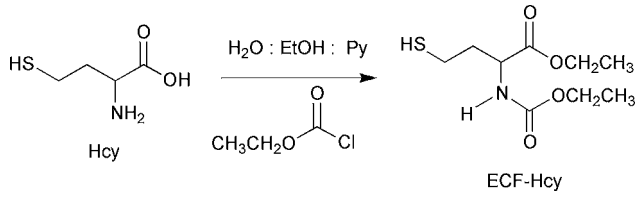
Derivatization

Alkyl chloroformate amino-acid derivatization[16] (Scheme 1) was employed: 40 µL of a 4 : 1 ethanol : pyridine solution and 25 µL of ethyl chloroformate (ECF) were added to the supernatant (1.5 mL) from the blood/plasma or standard sample treatment, the resulting mixture was Vortex-mixed for 1 min, its volume adjusted to 2.5 mL with deionized water, and then pumped continuously through the lines of the T&R-MIMS system for quantitation.

Results and discussion

Signal profile

Fig. 2 shows a typical signal profile for the T&R-MIMS analysis of ECF-derivatized Hcy (ECF-Hcy) in plasma using selected ion monitoring (SIM) of the $[M - H]^+$ ion of *m*/*z* 234.



Scheme 1

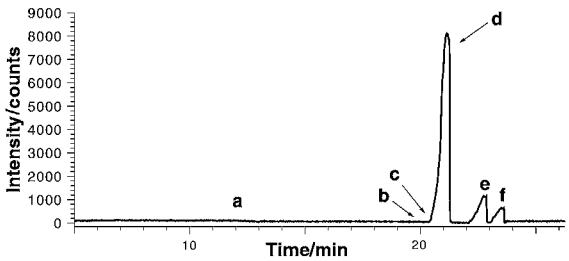


Fig. 2 Signal profile using SIM of *m*/*z* 234 for the T&R-MIMS analysis of a 20 µM aqueous Hcy solution after ECF derivatization.

The analyte solution (2.5 mL) is continuously pumped through the system, and during the 20 min of trapping, no signal is detected (a); although ECF-Hcy is efficiently absorbed by the membrane, its desorption to the gas phase is minor. But when the 60 s air plug (b) is introduced (simply by removing the pumping tube from the aqueous sample solution), and when it reaches the membrane (c), temperature is raised rapidly, and the preconcentrated ECF-Hcy is thermally and efficiently released. The signal rises and drops sharply producing a well-defined, relatively narrow, and intense desorption peak. When the air plug ends (d), and because room-temperature water is now flowing through the system, the membrane cools rapidly and signal drops sharply back to the baseline. Then, to clean the membrane from residual analyte, two additional 60 s air plugs at 1 min intervals (e and f) are intercalated into the water flow. The desorption peak in (f) is nearly as abundant as that continuously produced by heating the membrane after water pumping.[15]

Aquisition of full mass spectra

The time interval of the T&R-MIMS peak is considerably narrow, but long enough so as to allow the acquisition of several full mass spectra, which is particularly useful for secure analyte identification and mixture analysis. Fig. 3 shows the relevant portion of the mass spectrum collected close to the top of the elution peak during the T&R-MIMS analysis, whereas Fig. 4 displays the 70 eV EI mass spectrum of ECF-Hcy obtained by GC/MS analysis. Whereas the ion of *m*/*z* 207 is background from the membrane,[15] the EI-ions of ECF-Hcy ($[M - H]^+$ of *m*/*z* 234, 189, 188, and 175) are clearly detected in the spectrum. For SIM quantitation of ECF-Hcy, the highest mass fragment ion of *m*/*z* 234, $[M - H]^+$, was selected to minimize interferences in plasma analysis.

Linearity and reproducibility

Several T&R-MIMS calibration curves were plotted using SIM of *m*/*z* 234 to monitor aqueous solutions of ECF-Hcy at concentrations varying from 10 to 70 µM (normal tHcy in

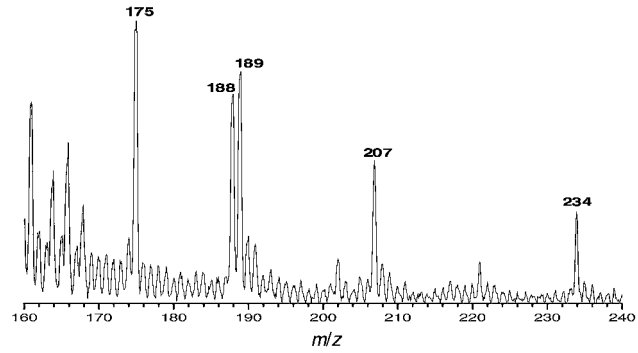


Fig. 3 70 eV EI mass spectrum collected close to the top of the elution peak during the T&R-MIMS analysis of Hcy in water after ECF derivatization.

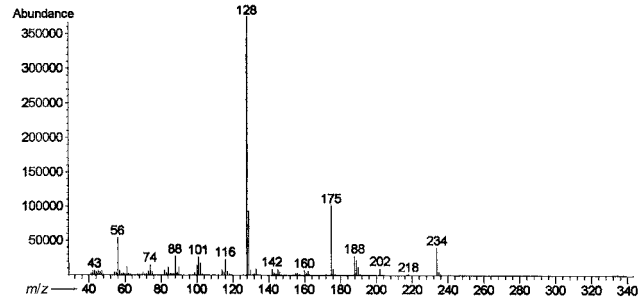


Fig. 4 70 eV EI mass spectrum of Hcy after ECF derivatization.

Table 1 Total homocysteine (tHcy) quantitation in human plasma using both HPLC with fluorescence detection and T&R-MIMS after ECF derivatization

Sample	HPLC/μM	T&R-MIMS/μM	Deviation (%)
01	9.5	10.5	+11
02	11.2	11.0	−2
03	12.5	11.6	−7
04	13.6	12.5	−8
05	10.9	12.7	+17
06	11.3	12.5	+11
07	13.2	11.8	−11
08	15.0	13.4	−11
09	11.6	10.8	−7
10	9.7	11.4	+18
11	12.7	11.7	−8
12	13.0	14.4	+11
13	11.6	10.7	−8
14	10.5	11.3	+8
15	10.2	9.5	−7
16	11.6	9.5	−18
17	10.8	9.1	−16
18	12.6	13.7	+9

plasma varies from 5 to 15 μM).[1] The curves demonstrate the good linearity of the technique (even up to 500 μM), and correlation coefficients typically of 0.998 were obtained. Within this concentration range, the variance of the SIM signal, for four consecutive analyses using the height of the SIM peak, was always below 5%.

Quantitation limit

For standard aqueous solutions of Hcy, SIM of the most intense *m*/*z* 128 ion of ECF-Hcy can be performed, and a quantitation limit of 0.2 μM was easily attained. For tHcy quantitation in human plasma, however, SIM monitoring of the *m*/*z* 234 ion was performed so as to minimize interferences, and then the quantitation limit was 2 μM. This limit is sufficiently below the 5 μM threshold of tHcy in human plasma.

Recovery

Plasma samples were spiked with known amounts of Hcy, treated as described, and tHcy determined by the T&R-MIMS technique using SIM of *m*/*z* 234. High recoveries, typically of 97–98%, were obtained for concentrations ranging from 10 to 100 μM.

Comparison with HPLC quantitation

A total of 18 plasma samples were analyzed concurrently by T&R-MIMS and HPLC with fluorescence detection.[3] Table 1 compares the results, and shows that the agreement is satisfactory, within 2–18%.

Simultaneous quantitation of Hcy and Cys

When plasma is treated with dithiothreitol (DTT), disulfide bonds are reduced and both the sulfur amino acids Hcy and cysteine (Cys) are released. After ECF derivatization, we attempted simultaneous quantitation of these two sulfur amino acids using T&R-MIMS. $[M - H]^+$ ions of Hcy (*m*/*z* 234) and Cys (*m*/*z* 220) were both clearly detected (nearly 10 times more abundant for Cys) in the mass spectra, and SIM monitoring was shown to allow for their simultaneous quantitation. The linearity, recovery, reproducibility, and detection limit for tCys quantitation in plasma by T&R-MIMS is currently being evaluated, with similar to better results when compared to tHcy quantitation.

Other amino acids

ECF derivatization of amino acids in aqueous matrices is generally applicable, fast, and gives high yields, and most amino and other organic acids can be efficiently ECF-derivatized for GC analysis.[16,17] We tested many amino acids,[18] and as for the two sulfur amino acids Hcy and Cys, their ECF-derivatized forms were efficiently detected in water by T&R-MIMS using the DIMP probe.[15]

Conclusion

The total concentration of Hcy in plasma can be determined directly by T&R-MIMS after ECF derivatization. The method uses no chromatographic separation, is linear, reproducible, and has a quantitation limit sufficiently below the threshold concentration of Hcy in plasma. Since the method combines chemical, membrane, and mass spectrometric discrimination, and since most amino acids are efficiently detected after ECF derivatization, T&R-MIMS can be used to simultaneously determine selected amino acids in plasma. To gain accuracy, selectivity and to minimize interferences specially during the direct SIM monitoring in human plasma samples, improved procedures are currently being tested such as the use of internal standards, refined clean-up procedures, and desorption chemical ionization.[14] The T&R-MIMS technique is therefore promising as a simple, sensitive and selective method for simultaneous amino-acid quantitation in plasma and urine, and in most aqueous matrices.

Acknowledgments

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References

1 (*a*) H. Refsum, P. M. Ueland, O. Nygard and S. E. Vollset, *Annu. Rev. Med.*, 1998, **49**, 31; (*b*) L. J. Langman and D. E. C. Cole, *Clin. Chem. Acta*, 1999, **286**, 63; (*c*) D. W. Jacobsen, *Clin. Chem.*, 1998, **44**, 1833; (*d*) R. van der Griend, D. H. Biesma and J.-D. Banga, *Neth. J. Med.*, 2000, **56**, 119; and references cited therein.
2 P. M. Ueland, H. Refsum, S. P. Stabler, M. R. Malinow, A. Andersson and R. H. Allen, *Clin. Chem.*, 1993, **39**, 1764.
3 J. Selhub, *Annu. Rev. Nutr.*, 1999, **19**, 217.
4 (*a*) A. Araki and Y. Sako, *J. Chromatogr.*, 1987, **422**, 43; (*b*) B. Vester and K. Rasmussen, *Eur. J. Clin. Biochem.*, 1991, **29**, 549; (*c*) L. Campanella, G. Crescentini and P. Avino, *J. Chromatogr. A*, 1999, **833**, 137; (*d*) E. Bald, E. Kaniowska, G. Chwatko and R. Glowacki, *Talanta*, 2000, **50**, 1233.
5 E. Caussé, R. Tierrier, S. Champagne, M. Nertz, P. Valdiguié and R. Salvayre, *J. Chromatogr. A*, 1998, **817**, 181.
6 J. O. Sass and W. Endres, *J. Chromatogr. A*, 1997, **776**, 342.
7 F. Frantzen, A. L. Faaren, I. Alfheim and A. K. Nordhei, *Clin. Chem.*, 1998, **44**, 311.

8 (*a*) T. Kotiaho, F. R. Lauritsen, T. K. Choudhury and R. G. Cooks, *Anal. Chem.*, 1991, **63**, 875A; (*b*) F. R. Lauritsen and T. Kotiaho, *Rev. Anal. Chem.*, 1996, **15**, 237; (*c*) R. C. Johnson, R. G. Cooks, T. M. Allen, M. E. Cisper and P. H. Hemberger, *Mass Spectrom. Rev.*, 2000, **19**, 1.
9 G. L. Kok, M. E. Cisper and P. H. Hemberger, *J. Am. Soc. Mass Spectrom.*, 1996, **7**, 1172.
10 R. Alberici, R. Sparrapan, M. N. Eberlin, D. Windmöller and R. Augusti, *Anal. Commun.*, 1999, **36**, 221.
11 L. A. B. Moraes, M. N. Eberlin, J. R. Cagnon and L. H. Urbano, *Analyst*, 2000, **125**, 1529.
12 (*a*) M. A. Mendes, R. S. Pimpim, T. Kotiaho and M. N. Eberlin, *Anal. Chem.*, 1996, **68**, 3502; (*b*) R. Kostiainen, T. Kotiaho, I. Mattila, T. Mansikka, M. Ojala and R. A. Ketola, *Anal. Chem.*, 1998, **70**, 3028; (*c*) M. A. Mendes, R. Sparrapan and M. N. Eberlin, *Anal. Chem.*, 2000, **9**, 2166; (*d*) R. M. Alberici, R. Sparrapan, W. F. Jardim and M. N. Eberlin, *Environ. Sci. Technol.*, 2001, **35**, 2084.
13 (*a*) F. R. Lauritsen and R. A. Ketola, *Anal. Chem.*, 1997, **69**, 4917; (*b*) A comparable thermal desorption MIMS technique has also been reported, see:; G. Matz and F. Lennemann, *J. Chromatogr. A*, 1996, **750**, 141.
14 (*a*) F. R. Lauritsen, M. A. Mendes and T. Aggerholm, *Analyst*, 2000, **125**, 211; (*b*) F. R. Lauritsen and J. Rose, *Analyst*, 2000, **125**, 1577.
15 M. A. Mendes and M. N. Eberlin, *Analyst*, 2000, **125**, 21.
16 (*a*) P. Husek, *J. Chromatogr.*, 1991, **552**, 289; (*b*) P. Husek, *J. Chromatogr. B*, 1998, **717**, 57.
17 P. Husek, *J. Chromatogr. B*, 1995, **669**, 352.
18 R. Haddad, A. P. Velasco, M. A. Mendes, N. F. Höehr and M. N. Eberlin, to be submitted.