Journal of Chromatography B, 877 (2009) 3267–3269

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Letter to the Editor

Liquid chromatography: Is it essential for the determination of arginine and methylated arginines by tandem mass spectrometry?

Keywords:
Asymmetric dimethylarginine
Mass spectrometry
Chromatography
Internal standards

1. Introduction

We welcome the discussion initiated by Martens-Lobenhoffer et al. [1]. They raise a number of points, all significant and valid, even though many are not related to their central discussion of the necessity for the use of chromatography. This discussion is particularly pertinent and timely, not only to the analysis of methylated arginines, but also to the discussion of the analysis of routine clinical samples in general. Advances and improvements in technology have led to reductions in the cost of mass spectrometers, bringing them within the reach of routine clinical laboratories. This has led to the development of methods for routine clinical use.

Martens-Lobenhoffer et al. [1] ask the question, with respect to the quantification of arginine and the methylated arginines (NMMA, ADMA, SDMA), "is LC superfluous?" The question arose following the publication by Weaving et al. [2] where we described a method which did not employ either derivatisation or liquid chromatography pre-MS/MS determination.

In contrast to previous reports we demonstrated for the first time that by use of specific daughter ions (ADMA $m/z = 46$ and SDMA $m/z = 172$) the underivatised methylarginines could be measured without the need of LC. We also described procedures for the synthesis of SDMA-d_2 and MMA-d_2 which allowed the development of an MS/MS method with stable isotopically labelled internal standards for each analyte.

2. Background

The methylated arginines (MAs) are analogues of L-arginine, the substrate of nitric oxide synthase (NOS), and it is thought that they may play a role in the regulation of nitric oxide (NO). This has created interested in the medical areas of cardiovascular disease and in renal medicine, and stimulated a drive towards more suitable analytical methods.

From an analytical point of view determination of the isomers ADMA and SDMA has been particularly challenging. Long analysis times, up to 45 min, are necessary to separate them using HPLC (see list of methods in [2]). Many of the methods involving HPLC–MS/MS are analytically elaborate involving several time and labour consuming stages of sample pre-treatment. For example, the method described by Schwedhelm et al. [3] involves protein precipitation, centrifugation, drying (85 °C, 10 min), preparation of derivatives (65 °C, 15 min), drying (85 °C, 30 min), dissolving the derivatives in mobile phase, adjusting pH followed by LC–MS/MS. Although this method gave good precision and appears to give accurate measurement of low concentration of MAs this, and other multi-stage methods, are not best suited for use in a busy hospital laboratory.

Our primary aim was to develop a faster, simpler procedure for estimating MAs in samples from patients with chronic kidney diseases as part of an on-going clinical study. In this group of patients' concentrations of MAs are known to be significantly higher than in healthy individuals. For example mean concentrations of SDMA were reported to be 5.5 times higher in patients with renal disease than in a healthy control group [4]. In haemodialysis patients the concentrations have been reported to be further increased [5]. We required a relatively simple method involving as few stages as possible to measure in particular ADMA and SDMA at the higher concentrations found in renal patients. To this end a compromise was reached accepting less precise measurements at low "normal" concentrations in favour of a faster less complex method suitable for routine use in a hospital laboratory.

3. Method validation, selectivity and matrix effects

We used linearity and recovery studies to substantiate the selectivity of our method [2]. For example, calibration standards for ADMA and SDMA (0.5, 1.0, 2.0, and 5.0 μM) gave a straight line calibration plot. The higher than normal concentrations were to enable us to measure the raised values to be encountered in patients with kidney disease. For ADMA and SDMA we tested analyte recovery at 1 and 5 μM. Corresponding information on the other analytes is to be found in the original publication [2]. Given that we had adequate sensitivity and the linearity and recovery studies indicated no significant interferences we did not feel it necessary to report on the degree of ion suppression or indeed to investigate possible problems from potential/hypothetical isobaric daughter ions. However, given the critical comments made about this work [1] we provide, below, additional discussion and experimental evidence to help readers assess the value of the work.

3.1. Matrix effects

One factor that can affect the performance of electrospray ionisation MS/MS techniques is ion suppression. The many organic substances and the high salt content of biological fluids, such as blood serum, cause ion suppression leading to a reduction in detection signal. This type of interference although, well known, is poorly understood. Many of the unwanted constituents of samples can be removed prior to analysis by a variety of methods including protein precipitation, filtration, solvent extraction, SPE and different types of chromatography. None of these techniques, either alone or in combination, have proved to be totally effective in preventing ion

1570-0232/$ – see front matter
doi:10.1016/j.jchromb.2009.07.005

suppression resulting from a blood sample matrix. There have been few detailed studies comparing the effectiveness of these clean-up procedures. Mallet et al. [6] reported that for certain basic drugs SPE offered a better pre-MS/MS treatment procedure than liquid chromatography.

3.2. Internal standards

The use of carefully matched internal standards offers a means of adjusting for certain analytical deficiencies including ion suppression. Stable isotopes that offer a near match to the analyte are often employed. These are not always commercially available and, as in our case, experimentalists sometimes resort to synthesising their own [2]. With regard to the internal standards we synthesised, each was analysed individually, following the same extraction procedure as for plasma samples, and only the labelled products were detected.

3.3. Selective extraction

Martens-Lobenhoffer et al. [1] have suggested that the following substances might produce isobaric interferences in our method: tryptophan, homoarginine, tyrosine, acetyl glutamine, Nα-acetyl lysine and Nε-acetyl lysine. To investigate this 200 μM solutions of each substance (obtained from Sigma–Aldrich) were prepared and analysed, but without the use of solid phase extraction. No interference was observed for tryptophan, homoarginine, acetyl glutamine, Nα-acetyl lysine or Nε-acetyl lysine but tyrosine did produce a fragment, m/z: 182 > 77, isobaric with that used to determine arginine-d_7. To determine the extent that this might interfere in the quantitation of arginine, tyrosine standards were added to pooled plasma (9 parts plasma to 1 part standard) to increase the endogenous tyrosine concentration by 200, 400, 600, 800 and 1000 μM; this range of tyrosine concentrations greatly exceeds that found in healthy subjects (32–88 μM, [7]) and pathological conditions excluding tyrosinaemia. These preparations were analysed using the method previously described [2]. In addition an aqueous arginine standard, 100 μM, was also spiked with tyrosine and these preparations analysed both including and omitting the solid phase extraction procedure. The results are shown in Fig. 1. If extraction is not used then tyrosine, through isobaric interference, increases the signal produced by arginine-d_7, leading to a decrease in measured arginine concentrations. The solid phase extraction procedure completely removes this interference.

To further investigate the specificity of the extraction step two standards, each consisting of a mixture of seven different amino acids, were prepared and analysed separately. Standard A consisted of proline, leucine, lysine, histidine, arginine, tyrosine and monomethylarginine. Standard B consisted of valine, ornithine, methionine, phenylalanine, citrulline, homoarginine and ADMA. The concentration of each amino acid was 500 μM. Standards were diluted with 0.1 M HCl (1 part standard: 5 parts HCl), and 1 ml of the diluted standard applied to the SPE columns, which were conditioned, washed and eluted as previously described. Each standard was collected as it passed through the SPE column in addition to collecting the fraction eluted by the application of ammonia/methanol. The approximate percentage of each amino acid retained by the column, and then recovered from it after washing and eluting are shown in Table 1.

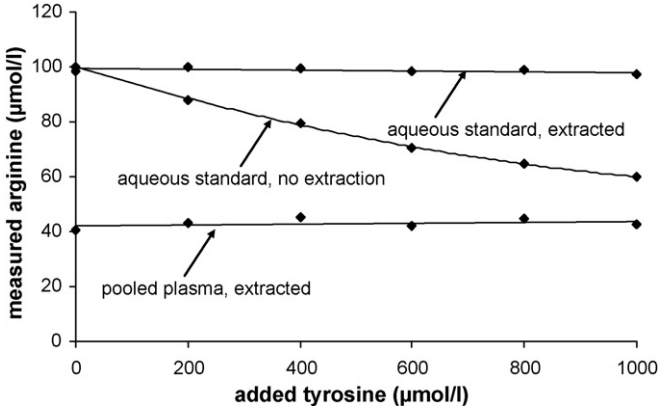

Fig. 1. Effect of tyrosine on measured arginine.

Table 1
Retention and elution of amino acids by strong cation solid phase extraction columns.

	% retained by SPE column	% obtained after washing and eluting column
ADMA	100	100
Arginine	75	75
Citrulline	20	0
Histidine	50	50
Homoarginine	100	90
Leucine	10	0
Lysine	40	30
Methionine	60	0
MMA	90	90
Ornithine	60	50
Phenylalanine	30	0
Proline	5	0
Tyrosine	10	0
Valine	20	0

This experiment was repeated using weak cation exchange columns (also obtained from Sigma). With these columns no extraction of any of the amino acids examined was observed.

Martens-Lobenhoffer et al. [1] also commented that the ion exchange extraction procedure that we have employed in our method is likely to be extremely non-selective. The results of our experiments demonstrate that this is not so and that the converse is in fact true. Of the test substances examined only those that are very basic in nature extract and elute from the SPE columns using the protocol we have described [2].

3.4. Ion suppression

Experiments to investigate the extent of ion suppression were carried out using the solid phase extraction procedure to prepare (a) internal standards and (b) plasma (without the addition of internal standard). The internal standard extract was reconstituted in carrier solution (0.1% formic acid) and this was then used to reconstitute the plasma extract. The internal standard extract and plasma extract were analysed 10 times each and the signals for the internal standards obtained from the two different extracts compared. In the presence of extracted plasma, the signal from each of the internal standards was suppressed by approximately 40%.

3.5. Method refinement

In our method the use of flow injection analysis, rather than in-line chromatography, leads to a broad peak and we have found that for low analyte concentrations there is some difficulty in integrating peaks in a consistent manner, compromising precision. In order to improve the discrimination of our peaks from the baseline we carried out preliminary experiments using a CN guard column cartridge (3 mm × 4 mm, Phenomenex), with the primary aim of limiting peak dispersion. The peaks obtained from injecting a 2 μM ADMA standard (a) using flow injection, flow rate = 50 μl/min, and (b) with use of an in-line CN filter, flow rate = 50 μl/min are shown in Fig. 2.

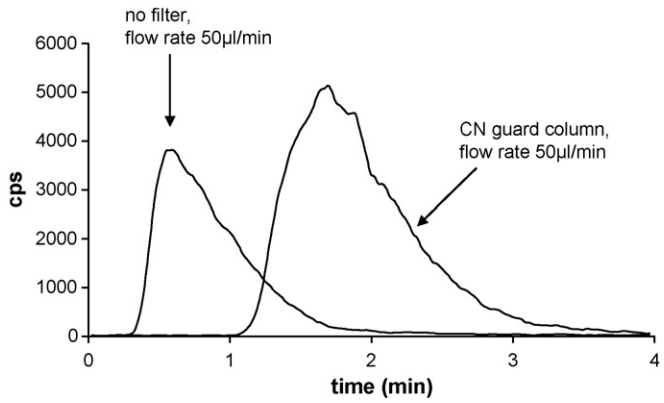

Fig. 2. Effect of on-line CN guard column on signal.

The guard column bed provides a small degree of retention of ADMA (and also SDMA, MMA and arginine—not shown), improving peak shape, but does nothing in terms of separating the individual analytes. Signal suppression was reduced to 10% illustrating that the short column was effective in separating the analytes from ion-suppressing components. It is reasonable to assume that optimising the chromatography conditions would lead to a cleaner signal and better precision.

3.6. Comparability issues

In their comments on comparability issues Martens-Lobenhoffer et al. [1] note that in particular our reported plasma concentrations for arginine in nine apparently healthy young males were higher than values reported by other workers. We have no simple explanation for this. However, two members of the study group said they were taking body building supplements but did not specify the nature or the quantity of the supplements. The samples were tested blind and we cannot identify which samples belonged to which students. Another possible explanation relates to the presence of arginase in red blood cells. Our samples were centrifuged immediately after collection therefore limiting the opportunity for red cell arginase to convert arginine to ornithine thus reducing the concentration of arginine in the plasma. We have previously observed decreases in plasma arginine related to delayed red cell separation (unpublished work).

The legend to Fig. 5 in our paper [2] states that it shows "analysis of a typical plasma sample" in fact this is an example of a diluted sample with approximate ADMA and SDMA concentrations of 0.125 μM. We thank Martens-Lobenhoffer et al. [1] for bringing this error to our attention.

3.7. Specific fragments

We suggested a probable fragmentation pattern and structure for the ADMA daughter ion with $m/z = 46$ [2]. Following experimental studies Martens-Lobenhoffer et al. [1] have proposed a more convincing fragmentation pattern to account for this ion that we readily accept. However, the analytical usefulness of this ion is not in dispute and the fragmentation mechanism has no relevance as to the discussion of the necessity of chromatography.

4. Conclusion

Martens-Lobenhoffer et al. [1] raised the question "Is LC superfluous?" Based on our experience [2] and experiments reported here, we conclude that with an appropriate solid phase extraction procedure in conjunction with the additional selectivity conferred by the tandem mass spectrometer that LC it is not essential to MS/MS use. In particular we maintain that the formation of the unique daughter ions m/z: 203 > 46 for ADMA and m/z: 203 > 172 for SDMA provide a useful and reliable means for determining these two substances without the necessity of either derivatising them or separating them chromatographically.

In the clinical environment analytical procedures involving protein precipitation, derivatisation, heating, drying, etc. are likely to be rejected in favour of less complicated, and sometimes less precise, methods.

References

[1] J. Martens-Lobenhoffer, E. Schwedhelm, D. Tsikas, J. Chromatogr. B 877 (2009) 3261.
[2] G. Weaving, B.F. Rocks, M.P. Bailey, M.A. Titheradge, J. Chromatogr. B 874 (2008) 27.
[3] E. Schwedhelm, R. Maas, J. Tan-Andresen, F. Schulze, U. Riederer, R.H. Böger, J. Chromatogr. B 851 (2007) 211.
[4] C. Fleck, F. Schweitzer, E. Karge, M. Busch, G. Stein, Clin. Chim. Acta 336 (2003) 1.
[5] C. Fleck, A. Janz, F. Schweitzer, E. Karge, M. Schwertfeger, G. Stein, Kidney Int. 59 (Suppl. 78) (2001) 14.
[6] C.R. Mallet, Z. Lu, J.R. Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.
[7] S. Letellier, J.P. Garnier, J. Spy, B. Bousquet, J. Chromatogr. B 696 (1997) 9.

Gary Weaving[a,*], Bernard F. Rocks[a], Michael P. Bailey[a], Michael A. Titheradge[b]
[a] *Royal Sussex County Hospital, Clinical Biochemistry and Immunology Department, Eastern Road, Brighton, East Sussex BN2 5BE, United Kingdom*
[b] *School of Life Sciences, Sussex University, Falmer, Brighton, East Sussex BN1 9RH, United Kingdom*

[*] Corresponding author.
E-mail address: gary.weaving@bsuh.nhs.uk
(G. Weaving)

17 June 2009
Available online 10 July 2009