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Free amino acid quantification by LC–MS/MS using derivatization generated isotope-labelled standards☆

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ABSTRACT

The further development of derivatizing reagents for plasma amino acid quantification by tandem mass spectrometry is described. The succinimide ester of 4-methylpiperazineacetic acid (MPAS), the iTRAQ reagent, was systematically modified to improve tandem mass spectrometer (MS/MS) product ion intensity. 4-Methylpiperazinebutyryl succinimide (MPBS) and dimethylaminobutyryl succinimide (DMABS) afforded one to two orders of magnitude greater MS/MS product ion signal intensity than the MPAS derivative for simple amino acids. CD_3 analogues of the modified derivatizing reagents were evaluated for preparation of amino acid isotope-labelled quantifying standards. Acceptable accuracy and precision was obtained with d_3-DMABS as the amino acid standards derivatizing reagent. The product ion spectra of the DMABS amino acid derivatives are diagnostic for structural isomers including valine/norvaline, alanine/sarcosine and leucine/isoleucine. Improved analytical sensitivity and specificity afforded by these derivatives may help to establish liquid chromatography tandem mass spectrometry (LC–MS/MS) with derivatization generated isotope-labelled standards a viable alternative to amino acids analysers.

1. Introduction

Amino acids are quantified in an estimated 70% of Clinical Chemistry laboratories by dedicated amino acid analysers. These instruments incorporate ion exchange chromatography, post-column derivatization with ninhydrin and ultra violet (UV) detection at two wavelengths [1]. Chromatographic separation of isomeric amino acids takes up to 150 min which severely limits sample throughput. Replacement of amino acid analysers by a method employing mass spectrometry is challenging because of the large number (>50), structural similarity and functional group diversity of amino acids. The logistics and expense of the necessary isotope-labelled internal standards for isotope dilution quantification is considerable.

Proteomics researchers developed a solution to the standards problem. Derivatization of the terminal amino group of a protein with isotope-labelled tagging reagents is used to generate isotope-labelled standards. Succinimide esters of 4-methylpiperazineacetic acid [2], trimethylaminobutyric acid [3] and a variety of tertiary amine acids [4] are employed. Subsequently a commercial kit, which included two isobaric succinimide esters of 4-methylpiperazineacetic acid (MPAS) and called iTRAQ (isotope coded tag for reactive amine quantitation) reagents [5], was released for quantification of plasma free amino acids. One isobaric reagent derivatizes the plasma amino acids and the second isobaric reagent derivatizes an amino acid standards mixture. The two solutions are combined and analysed by LC–MS/MS, with scheduled multiple reaction monitoring (MRM) experiments, during a 20 min chromatography run. The kit has recently been replaced by another based on a pair of the 8-plex aTRAQ™ reagents [6].

Criticisms of the iTRAQ and aTRAQ™ kits are the high cost of the patented isobaric tags, which contain multiple isotope labels, and the relatively poor MS/MS sensitivity of the derivatives. Measurement precision of some complex amino acids with iTRAQ reagents is poor. The LC column is subject to premature failure because of incomplete removal of protein from the large volume of plasma required. It was reasoned that modifying these derivatives to improve their MS/MS product ion intensity would

Abbreviations: ALA, alanine; ARG, arginine; DMABS, dimethylaminobutyryl succinimide; EI-MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FIA, flow injection analysis; GLU, glutamic acid; GLY, glycine; HPLC, high performance liquid chromatography; iTRAQ, isotope coded tag for reactive amine quantitation; LC–MS/MS, liquid chromatography tandem mass spectrometry; LEU, leucine; MET, methionine; MPAS, 4-methylpiperazineacetyl succinimide; MPBS, 4-methylpiperazinebutyryl succinimide; MS/MS, tandem mass spectrometry; MSUD, maple syrup urine disease; ORN, ornithine; PHE, phenylalanine; SD, standard deviation; TMABS, trimethylaminobutyryl succinimide; TYR, tyrosine; UV, ultra violet; VAL, valine.

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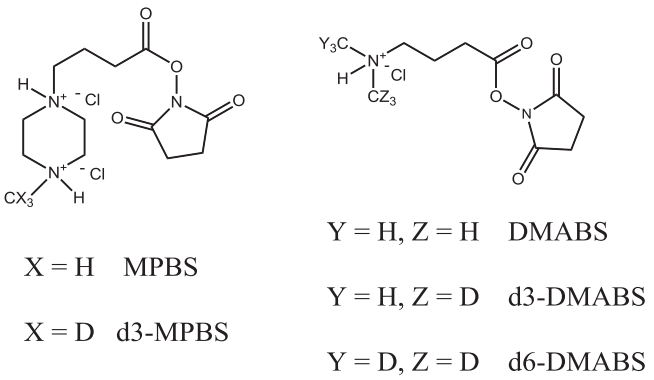

Fig. 1. Structures of the hydrochloride salts of 4-methylpiperazinebutyryl succinimide (MPBS) and dimethylaminobutyryl succinimide (DMABS) and deuterium labelled analogues.

improve accuracy and precision of amino acid measurement. It would also reduce sample volume and increase LC column lifetime. A strategy of making the derivative more hydrophobic, which had proved successful for Girard derivatives of ketones [7], was employed. This led to the synthesis and evaluation of *N*-methylpiperazinebutyryl succinimide (MPBS) and dimethylaminobutyryl succinimide (DMABS) derivatizing reagents (see Fig. 1). Isotope-labelled analogues, d_3-MPBS, d_3-DMABS and d_6-DMABS were synthesised for the preparation of the respective quantifying standards. Performance data for the quantification of amino acids in a test mixture were obtained.

2. Materials and methods

2.1. Standards and chemicals

All chemicals, except those mentioned below, were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). All solvents were HPLC grade.

2.1.1. 4-Methylpiperazineacetyl succinimide dihydrochloride (MPAS)

MPAS was synthesised by the method described by Dey et al. [2]. ESI-MS: *m*/*z* 256 $[MH]^+$.

2.1.2. 4-Methylpiperazinebutyryl succinimide dihydrochloride (MPBS)

MPBS was synthesised by a modification to the method described by Dey et al. [2] for MPAS. Ethyl 2-bromobutyrate was substituted for ethyl bromoacetate in the alkylation of 4-methylpiperazine. It was obtained as white crystals, m.p. 192–194 °C. ESI-MS: *m*/*z* 284 $[MH]^+$.

2.1.3. Dimethylaminobutyryl succinimide hydrochloride (DMABS)

A mixture of dimethylaminobutyric acid hydrochloride (168 mg, 1 mmol), *N*-hydroxysuccinimide trifluoroacetate ester (267 mg, 1.2 mmol) and acetonitrile (10 mL) were heated at 65 °C for 16 h, with occasional vortex mixing. The solution was decanted from remaining solid material and evaporated in a stream of nitrogen. Tetrahydrofuran (5 mL) was added and a precipitation of solid material initiated by scratching with a nickel spatula. The solid was washed with tetrahydrofuran (3 × 5 mL) and dried in a stream of nitrogen to afford DMABS (203 mg, 76%) as white crystals, m.p. 168–170 °C. ESI-MS: *m*/*z* 229 $[MH]^+$.

2.1.4. Dimethyl-1,1,1-d_3-aminobutyryl succinimide hydrochloride (d_3-DMABS)

A mixture of methylaminobutyric acid hydrochloride (151 mg, 1 mmole), d_2-formic acid (1 mL), d_2-paraformaldehyde (100 mg) and 35% deuterium chloride in deuterium oxide (100 μL) were heated at 100 °C, under nitrogen, for 16 h. Additional d_2-paraformaldehyde (50 mg) was added and the mixture heated at 100 °C for a further 4 h. The solvent was removed by evaporation in a stream of nitrogen and the residue was re-evaporated with dichloromethane (3 × 3 mL). Acetonitrile (1 mL) was added and the resultant solid which separated was collected and dried under nitrogen. This material was treated with *N*-hydroxysuccinimide trifluoroacetate as described in Section 2.1.3 to afford d_3-DMABS (95 mg, 35%) as white crystals m.p. 167–169 °C. ESI-MS: *m*/*z* 232 $[MH]^+$.

2.1.5. Dimethyl-d_6-aminobutyryl succinimide hydrochloride (d_6-DMABS)

The reactions described in Section 2.1.4 were performed with 4-aminobutyric acid hydrochloride as starting material and with twice the amount of d_2-paraformaldehyde. d_6-DMABS was

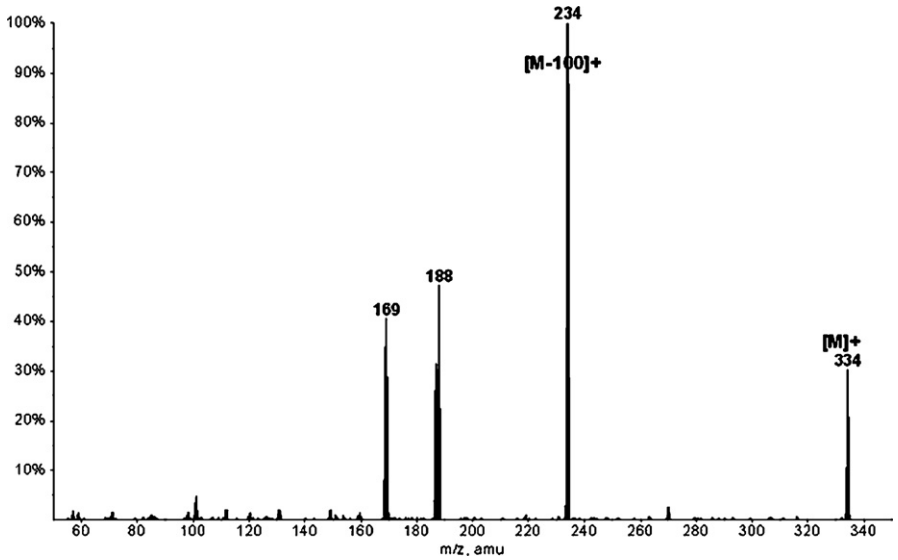

Fig. 2. MS/MS product ion spectrum of the MPBS derivative of phenylalanine. Relative ion intensity is displayed on the *y*-axis.

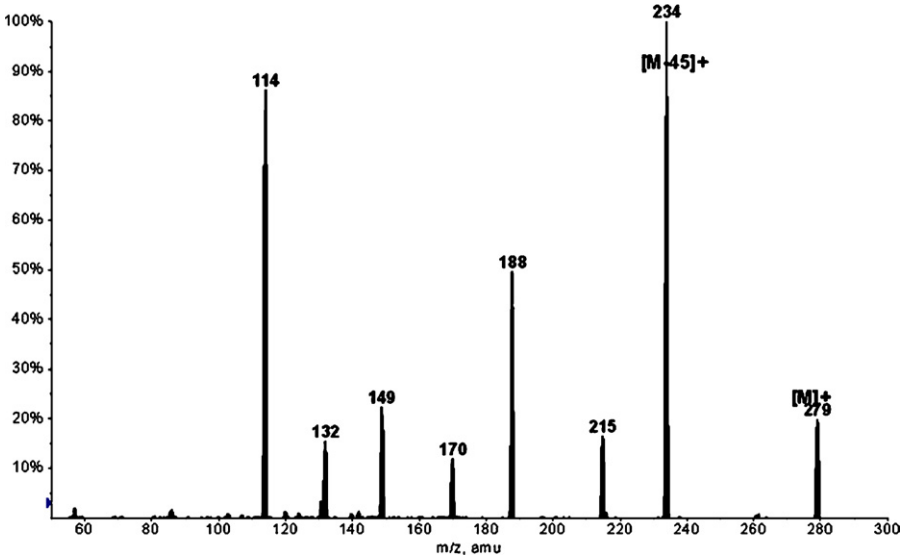

Fig. 3. MS/MS product ion spectrum of the DMABS derivative of phenylalanine. Relative ion intensity is displayed on the *y*-axis.

obtained in 59% yield as white crystals, m.p. 168–170 °C. ESI-MS: *m*/*z* 235 $[MH]^+$.

2.1.6. 4-(Methyl-d_3)-piperazinebutyryl succinimide dihydrochloride (d_3-MPBS)

d_3-MPBS was prepared by the reductive alkylation reaction described in Section 2.1.4 on piperazinebutyric acid dihydrochloride followed by succinimide ester formation described in Section 2.1.3. d_3-MPBS was obtained as white crystals. ESI-MS: *m*/*z* 287 $[MH]^+$.

2.1.7. Trimethylaminobutyryl succinimide hydrochloride (TMABS)

TMABS was prepared by the method of Morano et al. [8]. ESI-MS: *m*/*z* 243 $[M]^+$.

2.2. Sample preparation

To dried protein precipitated plasma (2–5 μL plasma), dried methanol extracted 3 mm bloodspot or amino acid standards in acetonitrile (50 μL) is added MPAS, MPBS or DMABS (50 μL, 2.0 mM in acetonitrile containing 1% trifluoroacetic acid). The trifluoroacetic acid aids dissolution of MPAS and MPBS but is not used for DMABS except for comparison experiments. The mixture is heated at 65 °C for 30 min, quenched with hydroxylamine (2 μL, 12% in water) and then diluted with water/acetonitrile/formic acid (500 μL, 50:50:0.1 (v/v/v)) for flow injection analysis or with 1% formic acid in water for LC–MS/MS analysis. An isotope labelled standard mixture is prepared by reacting measured amounts of amino acids with d_3-MPBS, d_3-DMABS or d_6-DMABS under the same conditions, quenching and diluting in an equal volume of solvent. The two solutions are mixed and the resultant mixture analysed by either flow injection ESI-MS/MS or by LC–MS/MS.

2.3. ESI-MS/MS analysis

ESI-MS/MS analysis was performed on an AB SCIEX (Concord, Ontario, Canada) API4000 instrument equipped with a TurboV ion source operated in positive ion mode. Ion source temperature was 450 °C, collision gas was nitrogen at a pressure of approximately

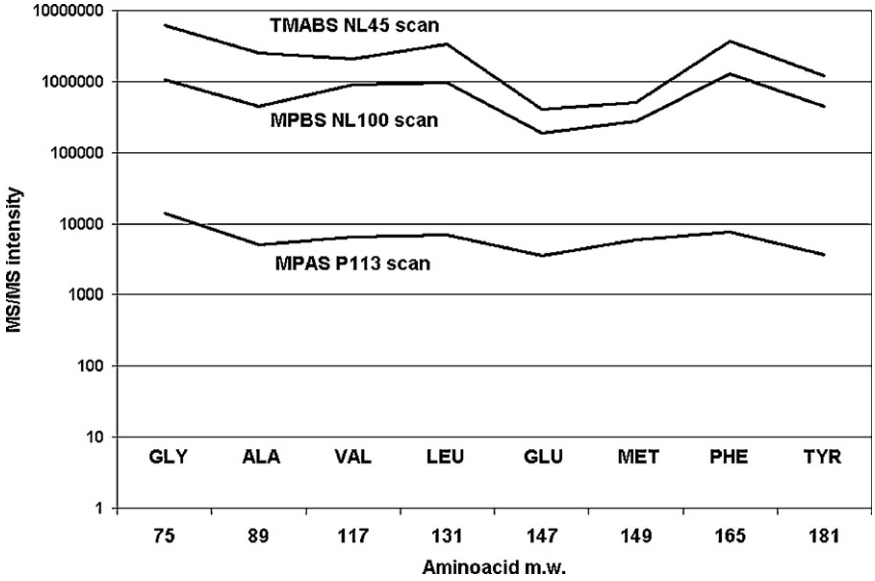

Fig. 4. Plot of the comparative ion intensities for the MPAS, MPBS and DMABS derivatives of eight amino acids. They were measured by MS/MS precursor 113 ion, neutral loss 100 Da and neutral loss 45 Da scans respectively.

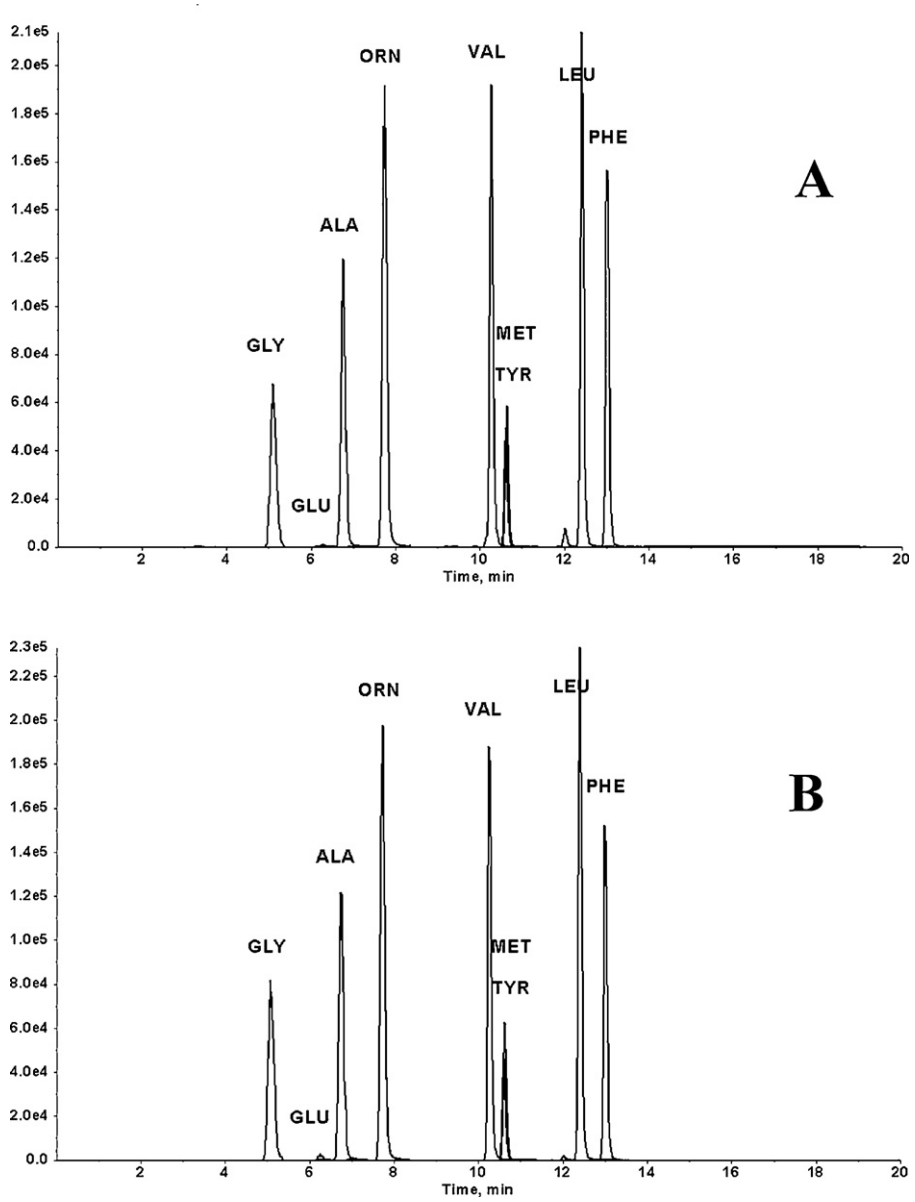

Fig. 5. Composite MRM ion chromatograms for (A) amino acids derivatized with DMABS and (B) amino acids derivatized with d_3-DMABS obtained after LC–MS/MS analysis of equal amounts of amino acids derivatized with DMABS and d_3-DMABS and mixed. Ion intensity is displayed on the *y*-axis.

$2.5\,e^{-5}$ torr and collision energy was 25 eV. Samples (20 μL) were introduced from 96 well plates with a Gilson 215 autosampler (Middleton, IL, USA) into the solvent flow supplied by an Agilent 1100 HPLC system (Waldbronn, Germany). Data analysis was with Analyst 1.5.1 software.

2.4. HPLC separation

Samples derivatized with DMABS were separated on an AB SCIEX AAA C18 column (150 × 4.6 mm, 5 μm particle size). The flow rate was 650 μL/min and column temperature 25 °C. The solvents

Table 1
Accuracy and precision (±SD) of amino acid measurement, for a test mixture ($n = 6$). Identical amounts of an amino acid mixture derivatized with DMABS and a deuterium labelled DMABS were combined and analysed by MS/MS MRM experiments. Results from the use of d_3-DMABS and d_6-DMABS were compared. Mixtures employing d_3-DMABS were analysed by both flow injection analysis (FIA) and LC–MS/MS.

	d_3-DMABS FIA		d_3-DMABS LC–MS/MS		d_6-DMABS FIA	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Glycine	93	1.7	90	3.8	104	8.7
Alanine	97	2.9	89	4.7	100	5.1
Ornithine	107	5.2	108	6.5	117	17.2
Valine	99	2.4	96	5.7	111	3.7
Leucine	94	2.6	98	4.3	103	3.9
Methionine	84	1.7	101	6.2	121	5.4
Phenylalanine	98	3.0	112	5.2	96	4.2
Tyrosine	109	11.7	101	5.8	102	8.6

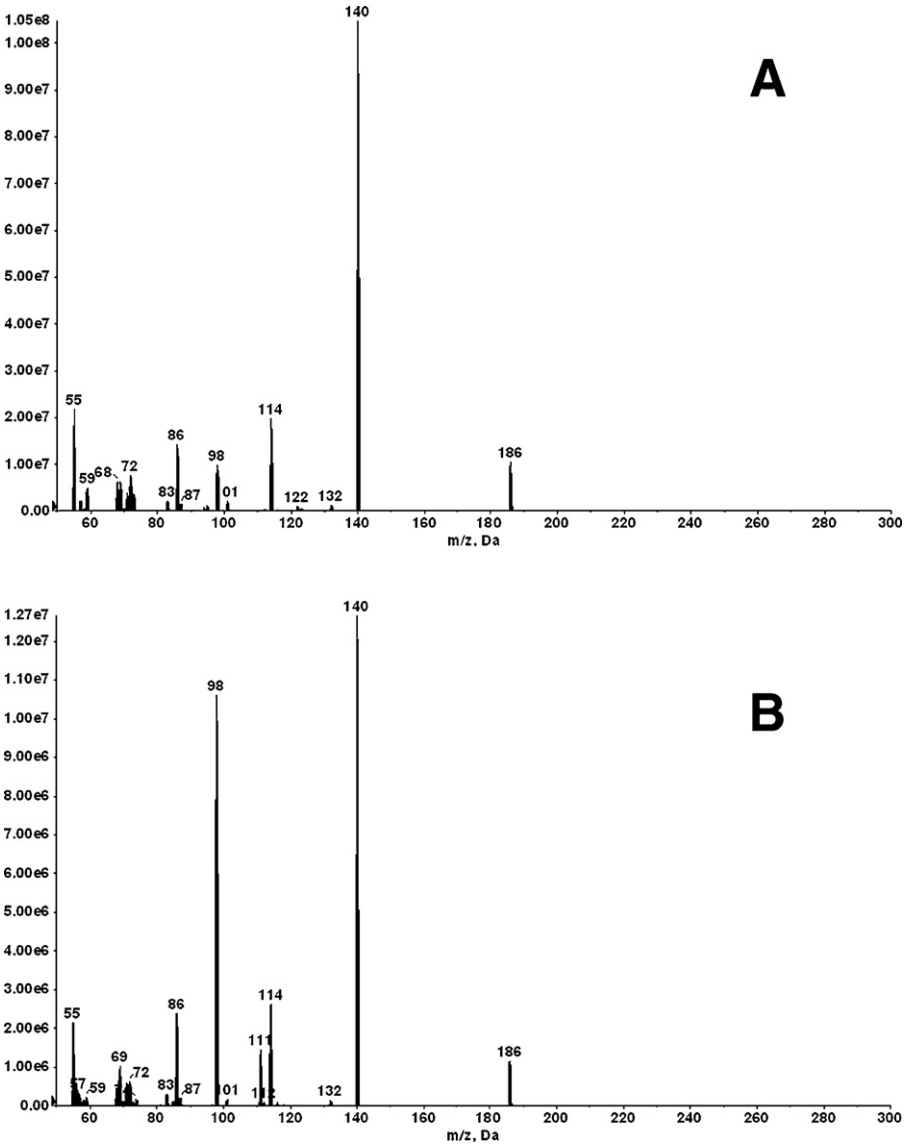

Fig. 6. MS/MS product ion spectra of the DMABS derivatives of A) valine and B) norvaline. Ion intensity is displayed on the *y*-axis.

were (A) water containing formic acid (0.1%) and heptafluorobutyric acid (0.01%) and (B) acetonitrile containing formic acid (0.1%) and heptafluorobutyric acid (0.01%) with a gradient over 20 min as follows: 0–0.1 min (5% B), 9–10 min (25% B), 11–15 min (100% B), 16–20 min (5% B). The same column and conditions were used to separate underivatized leucine isomers [9].

3. Results and discussion

3.1. Modification of iTRAQ reagent

Increasing the hydrophobic nature of the derivatizing reagent, by systematically extending the linking chain, was a successful strategy for improved MS/MS product ion intensity with Girard derivatives of ketosteroids [7]. In like manner, a series of succinimide esters of 4-methylpiperazinealkyl acids, were synthesised by modification of the methods of Dey et al. [2]. Phenylalanine, as a test amino acid, was derivatized with each succinimide ester and the optimized MS/MS product ion spectrum examined. Highest signal intensity of the major product ion was obtained with the succinimide ester of 4-methylpiperazinebutyric acid (MPBS, see Fig. 1), with a linking chain of 3 methylene units. Fig. 2 shows the MS/MS product ion spectrum of phenylalanine MPBS derivative with a high mass product ion from neutral loss of 100 Da (methylpiperazine).

It was reasoned that further signal intensity improvement was possible by reverse engineering the neutral loss of a smaller molecule from the derivative in the collision cell of the MS/MS. Accordingly the succinimide ester of dimethylaminobutyric acid (DMABS, see Fig. 1) was prepared. This derivative has previously been identified as a possible tagging reagent for proteins [4] but testing with free amino acids has not been reported. Fig. 3 shows the MS/MS product ion spectrum of phenylalanine DMABS derivative showing the major product ion from neutral loss of 45 Da (dimethylamine).

Quaternary ammonium derivatives usually afford higher MS/MS ion intensities than tertiary amine derivatives. The MS/MS analytical sensitivity of very long chain fatty acids is improved tenfold by converting dimethylaminoethyl esters to trimethylaminoethyl esters [10]. From this reasoning the trimethylaminobutyryl succinimide (TMABS) derivative should be superior to the DMABS derivative. Indeed it has been used as an alternative to iTRAQ

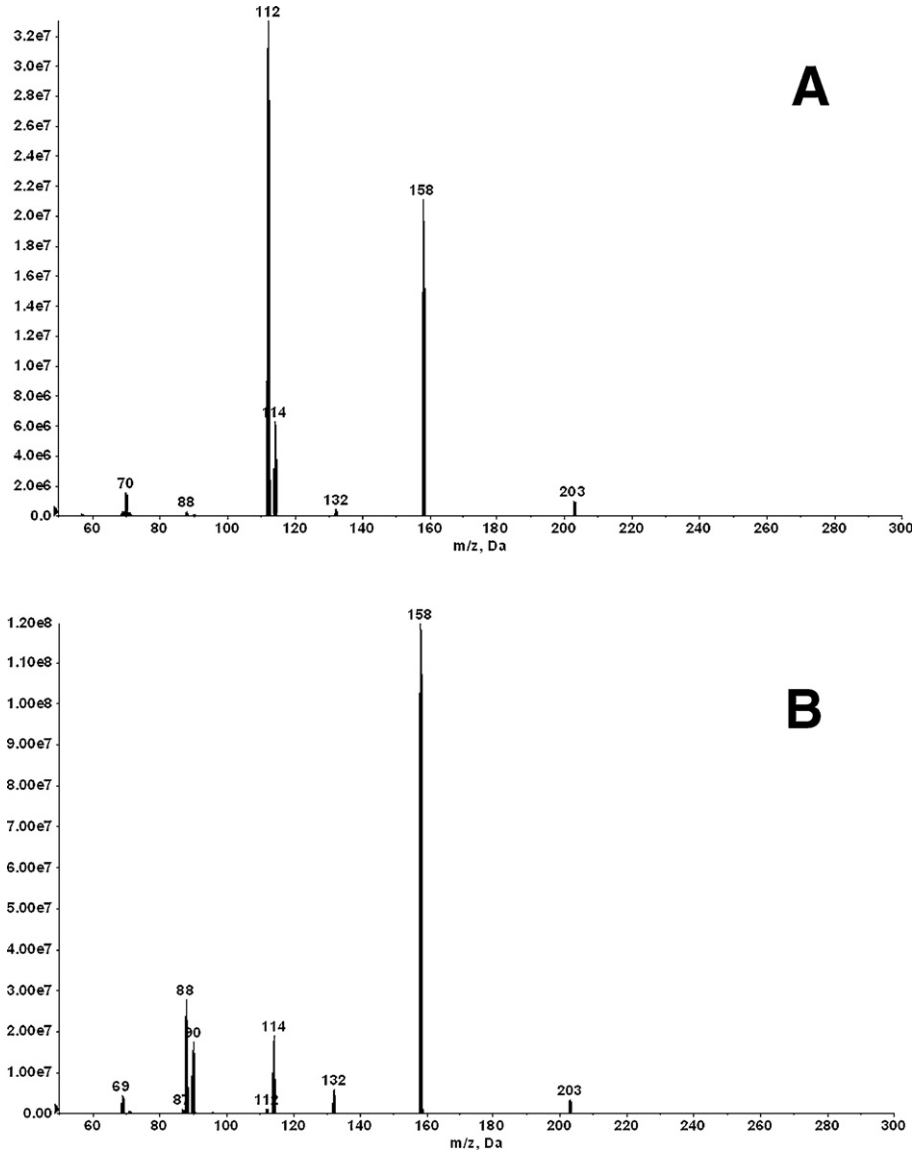

Fig. 7. MS/MS product ion spectra of the DMABS derivatives of A) alanine and B) sarcosine. Ion intensity is displayed on the *y*-axis.

in proteomics research [8]. Accordingly the TMABS derivative of phenylalanine was prepared. The optimized product ion spectrum showed the major product ion with very low mass (*m*/*z* 57) which is unsuitable for quantification of free amino acids.

To evaluate the modified amino acid derivatives, an amino acid test mixture was derivatized under identical conditions with the MPAS, MPBS and DMABS reagents. The ion intensities for MS/MS precursor 113 ion, neutral loss 100 Da and neutral loss 45 Da experiments respectively were plotted on a logarithmic scale in Fig. 4. The MPBS and TMABS derivatives afforded intensities one to two orders of magnitude greater than the MPAS derivative. LC–MS/MS of the MPBS and DMABS (see Fig. 5A) test mixtures showed comparable separation of most amino acids to that observed with the MPAS derivative. Experimentation showed that the minimum derivatization reaction time for all derivatives is 30 min which is not reduced with higher temperature. Derivatization of amino acids containing two amine groups, such as ornithine and lysine, gives predominantly a single derivative with MPBS and DMABS. In contrast, MPAS gives a higher proportion of double derivatives.

3.2. Preparation and testing of isotope-labelled derivatizing reagents

A study with deuterium labelled TMABS derivatives of proteins [8] indicated that the isotope-labelled derivatizing reagent for the standards does not need to be isobaric to the analyte derivatizing reagent for accurate quantification. More easily prepared CD_3 analogues of MPBS and DMABS may suffice. Accordingly MPBS labelled with three deuterium atoms on the 4-methyl group was synthesised by a reductive alkylation reaction described in Materials and Methods. Two isotope labelled versions of DMABS were prepared containing one and two CD_3 groups. Deuterium enrichment of these compounds was 97–98%. These compounds, as well as the unlabelled versions, show no observable deterioration when stored in a desiccator at room temperature for one year.

To test the CD_3 analogue hypothesis equal amounts of amino acid test mixture were reacted with labelled and unlabelled DMABS then mixed and analysed by ESI-MS/MS, using MRM experiments. The $[MH]^+/[MH\text{-}45]^+$, $[MH]^+/[MH\text{-}48]^+$ and $[MH]^+/[MH\text{-}51]^+$ ion pairs were used for DMABS, d_3-DMABS and d_6-DMABS respec-

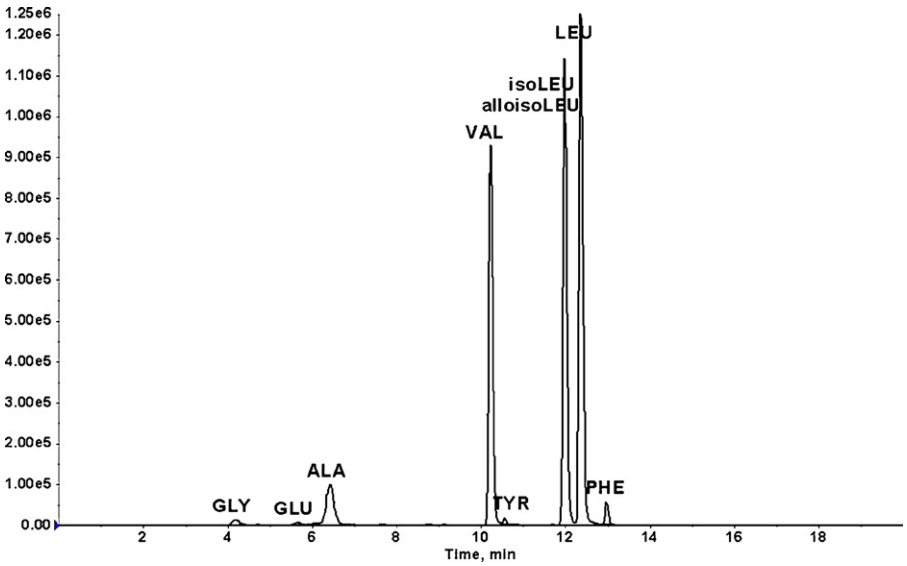

Fig. 8. LC–MS/MS ion chromatogram of a methanol extracted and DMABS derivatized 3 mm blood spot from an MSUD patient. Ion intensity is displayed on the *y*-axis.

tively. Accuracy/precision data for replicate analysis ($n = 6$) are shown in Table 1 for both isotope-labelled reagents. Results from flow injection MS/MS and LC–MS/MS analysis for d_3-DMABS are also included. With flow injection MS/MS analysis, d_3-DMABS afforded accuracy and precision data comparable with those usually observed when individual standards, isotope labelled within the amino acid, are used for quantification. A typical LC–MS/MS analysis, with separate composite traces for unlabelled and labelled derivatives, of a 1:1 mixture of amino acid DMABS and d_3-DMABS derivatives is shown in Fig. 5. Use of d_6-DMABS with flow injection analysis led to poorer precision and poor accuracy with valine attributable to a deuterium isotope effect (see Table 1). The poor accuracy for methionine by flow injection analysis is only observed for analysis of the amino acid test mixture but not when methionine alone is derivatized and analysed nor when the amino acids are separated during LC–MS/MS analysis.

DMABS can also function as a dehydrating agent. Complex amino acids such as ornithine which can form stable ring structures by intramolecular cyclization, involving loss of water, show some dehydrated product when the reaction is performed at room temperature. Total dehydration of ornithine occurs when the derivatization is performed at 65 °C, the conditions used in obtaining data for Table 1. The parent ion which is monitored is therefore $[MH\text{-}18]^+$. MRMs of [228.2/183.2] and [231.2/183.2] were employed to measure ornithine (unlabelled and d_3-labelled derivative respectively) in the LC–MS/MS ion chromatogram shown in Fig. 5.

A useful property of MPBS and DMABS derivatives of amino acids is the ability to generate alternative lower mass product ions which can be used for confirmation, better sensitivity, to avoid interfering ions or to distinguish structural isomers. DMABS derivatives all afford a strong product ion at m/z 114 (see Fig. 4) from cleavage of the amide group and retention of charge on the tertiary amine. The corresponding ions for d_3-DMABS and d_6-DMABS derivatives are m/z 117 and m/z 120, respectively. This is a better product ion to use for quantification of arginine and other complex amino acids. Fragmentation of the DMABS derivative is sensitive to small changes in amino acid structure. Valine and norvaline derivatives are distinguished by the intensity of the m/z 98 product ion (see Fig. 6) and alanine and sarcosine by the intensity of the m/z 112 product ion (see Fig. 7). Leucine DMABS derivative affords a product ion with m/z 125, at a collision energy of 35 eV, which is virtually absent in the product ion spectra of isoleucine, allo isoleucine and norleucine. This is a property that could be exploited for rapid analysis of amino acid mixtures without chromatography.

The improved MS/MS signal intensity of the DMABS derivatives (relative to iTRAQ) enables analysis of amino acids in smaller samples of blood such as 3 mm bloodspots punched from Guthrie card filter paper collected for Newborn Screening programs. Fig. 8 shows the LC–MS/MS analysis of a 3 mm bloodspot from an MSUD (maple syrup urine disease) patient collected at 2 days of age. Elevated concentrations of branched chain fatty acids valine, leucine and leucine isomers are seen. HPLC baseline separation of leucine and isoleucine + allo isoleucine is observed. Use of a derivative with high sensitivity under electron impact conditions has recently been described for fast GC/MS analysis of these amino acids in bloodspots [11]. Another positive attribute of the DMABS derivatizing reagent for bloodspot analysis is its lack of reactivity with amide groups. Amide containing amino acids such as glutamine cannot be quantified by Newborn Bloodspot Screening programs that employ butanol/hydrogen chloride for butyl ester formation [12].

Examination of the MPBS and DMABS derivatized amino acid profiles from a wide selection of amino acid disorders is required to confirm MRM ion pairs for less common amino acids and further test the new derivatives. The MS/MS fragmentation properties of the derivatives provide some flexibility in the product ion used to monitor each amino acid. As with the MPAS derivative, separation of isoleucine and allo isoleucine must be performed by concurrent separation of underivatized plasma extract mixed with the derivatized extract [9].

4. Conclusions

Derivatization generated isotope-labelled internal standards should be considered for use in LC–MS/MS methods when isotope-labelled analytes are unavailable, expensive or required in large numbers, as is the case with amino acids. It is essential that derivatization proceed to completion in a reasonable time, analytes and the standards be derivatized under identical conditions, excess derivatizing reagents completely destroyed, and the solutions mixed without loss.

This study shows that derivatizing reagents for MS/MS analysis are often best suited to a limited analyte mass range. A derivative designed for protein analysis may not be the most suitable for small molecule analysis. Strategies are available to optimize the analytical sensitivity and selectivity of the derivative [7]. Modification

of the MPAS structure to afford the MPBS and DMABS derivatives led to a significant improvement in the MS/MS product ion intensity for derivatized simple amino acids. Sample volume size was consequently reduced and blood spot analysis could be performed. The MPBS and DMABS derivatives form diagnostic product ions for structural isomers during MS/MS analysis. Provided only a single CD_3 group is incorporated in the isotope-labelled reagent, to react with amino acid standards, acceptable analytical accuracy and precision is obtained.

Will LC–MS/MS analysis with derivatization generated isotope-labelled internal standards replace amino acid analysers for a full amino acid screen? Cost, speed, complexity, accuracy and precision must be considered. A suitable LC–MS/MS instrument is typically twice the price of an amino acid analyser. The cost per sample for the iTRAQ and aTRAQ™ kits is high but reduced with the DMABS/d_3-DMABS derivatives. Chromatography time drops from 150 to 20 min but the LC–MS/MS method requires much of that time difference for amino acid extraction, derivatization, quenching of reagents and mixing and for data processing. The LC–MS/MS method requires greater care and technical skill. There is a large intensity range of the monitored MS/MS product ion for different amino acids with some complex amino acids measured with poorer accuracy by LC–MS/MS than with the amino acid analyser. Additional complications are side reactions during succinimide ester derivatization and ESI-MS/MS analysis and integration of amino acid isomer separation.

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