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Introduction

A growing number of laboratories now rely on mass spectrometry as a routine procedure for the semi-quantitative analysis of a range of amino acids in physiological samples (see AB14). Typically this involves the analysis of dried blood spot samples either in neonatal screening for inborn errors of metabolism or for monitoring individuals with metabolic disorders (eg phenylketonuria) to ensure that their condition is properly controlled. The amino acid profile is acquired in the form of a mass spectrum and this technique is simple and rapid making it ideal as a screening tool. However, one limitation of the method is the inability to differentiate between compounds of identical mass. They appear as a single peak in the mass spectrum. This is of particular importance for leucine, which has three major isomeric forms (leucine, isoleucine and allo-isoleucine) and is also isobaric with hydroxyproline.

Maple Syrup Urine Disease

In neonatal screening, classical Maple Syrup Urine Disease (MSUD) can be detected by an elevation of the concentration of branched-chain amino acids (valine and leucine) in the blood & urine which differentiates the disease from other inborn errors of leucine metabolism that have similar symptoms. The disease has an incidence of approximately 1:200,000 in the UK but can be much more common in other parts of the world. If uncorrected MSUD can produce a range of symptoms including deterioration of the central nervous system, and psychomotor retardation.

In MSUD an increase compared to normal in the total leucine concentration (i.e. leucine + isomers) of at least a factor of three should be apparent (Table 1). However, it is the appearance of allo-isoleucine in the urine & blood that has the greatest diagnostic potential (Table 1).

Currently, an amino acid analyser would be used to measure the concentration of the individual isomers of leucine. These dedicated instruments can take up to 2 hours to complete a single analysis. This note describes a rapid LC-MS-MS method for the measurement of leucine isomers in standard blood spot samples.

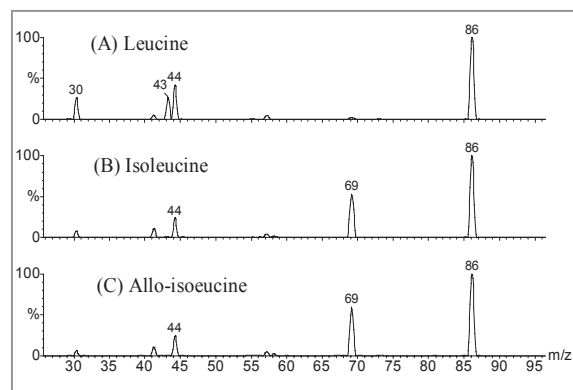


Figure 1: Partial product ion spectra for the butyl esters of leucine (A), isoleucine (B) and allo-isoleucine (C) with conditions optimised for the formation of minor product ions (collision energy 25eV). All three isomers produce a major product at m/z 86 but isoleucine and alloisoleucine also produce a significant product ion at m/z 69 that is almost absent from the leucine spectrum.

	Valine	Isoleucine	Leucine	Alloleucine
Normal	86 – 190	26 – 91	48 – 160	0
MSUD	496 – 1846	199 – 1298	518 – 5091	72 – 310

Table 1: The concentration range ($\mu\text{moles/L}$) of branched chain amino acids in whole blood from normal individuals and from cases of classical MSUD

Methodology

A Quattro LC tandem mass spectrometer with a Z SPRAY ion source was used throughout this study. The instrument was operated in positive ionisation electrospray mode and was interfaced to a Waters 2790 Alliance HT liquid chromatograph. MassLynx NT software was used to control the system and for all aspects of data acquisition and processing.

Hydroxyproline, L-leucine, d_3 -L-leucine, L-isoleucine and L-allo-isoleucine were derivatised using butanolic HCl according to standard procedures and the butyl esters were redissolved at a concentration of $10\mu\text{mol/L}$. Each compound was introduced into the ion source and product ion spectra (Figure 1) generated under varying conditions. For each isomer, the optimum conditions for the formation of the m/z 86 product ion were determined.

Blood spot samples were extracted and derivatised, and a separation was performed using a porous graphitic column (Hypercarb 5 μ 50mm x 4.6mm) eluted at a rate

of 1.5 mL/min with 15% aqueous MeCN containing 0.025% formic acid and 0.05% trifluoro-acetic acid, maintained at 40°C.

Results

The three leucine isomers all fragment to produce a common major product ion (m/z 86). However, if the collision energy is increased, some differences in the product ion spectra can be seen (Figure 1). Both isoleucine and allo-isoleucine produce a fragment at m/z 69 which is almost absent from the leucine spectrum. Although the intensity of the smaller fragment is much less than that of the major product ($\sim 10\%$) this differential fragmentation is useful in the analysis of the isomers.

The regular crystal structure of the porous graphitic packing material provides a stereo-selective medium to separate isomeric and closely related compounds. It is therefore possible to separate hydroxyproline, leucine and isoleucine using a simple, rapid isocratic LC system. Figure 2 shows the separation of a mixture of standards of hydroxyproline, leucine, isoleucine and allo-isoleucine. The two traces represent the reaction monitoring channels for the differential fragmentation described above.

Figure 3 shows the traces acquired from a normal patient (3A) and a patient with MSUD (3B). An internal standard (d_3 -leucine) was monitored for quantification purposes. The middle and lower traces show the presence of allo-isoleucine in the affected sample, where none is observed in the normal.

Summary

A procedure has been described for the differentiation and determination of leucine isomers using the same samples that had previously been used for amino acid and acylcarnitine screening.

References

N Blau, M Duran and M E Blaskovics, in *Physician's Guide to the Laboratory Diagnosis of Metabolic Disease*, Chapman Hall, 1996.

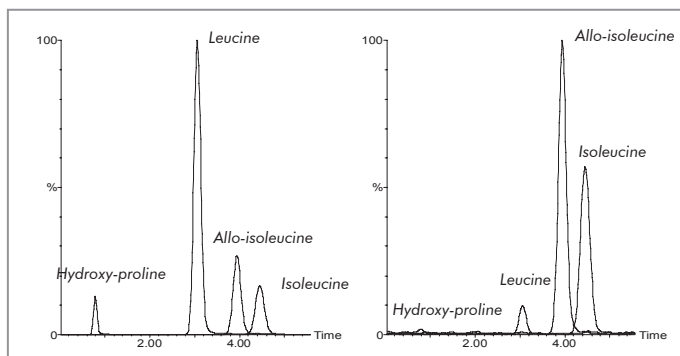


Figure 2: The separation of four isobaric species using a Hypercarb HPLC column as described in the text. Four overlaid chromatograms for hydroxyproline, leucine, isoleucine and allo-isoleucine present in equimolar amounts and acquired using the MRM transition (A) $188 > 86$ and (B) $188 > 69$. Note how the use of the less abundant fragment ($188 > 69$) emphasises the presence of allo-isoleucine but leucine can still be detected.

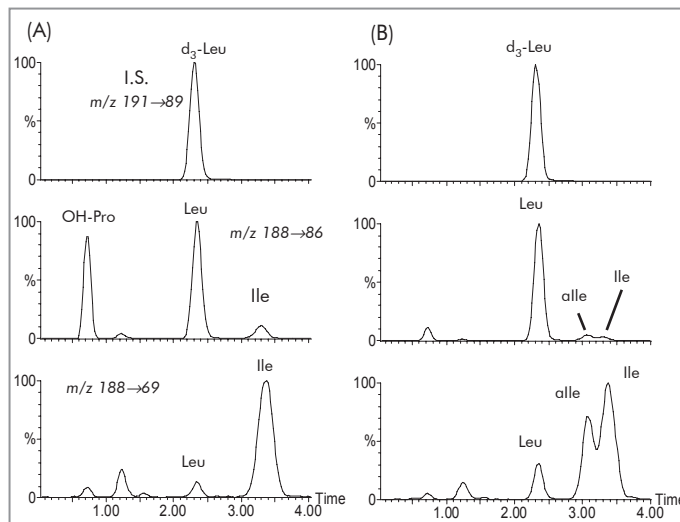


Figure 3: Comparison of (A) normal patient and (B) MSUD patient. Upper trace (m/z 191 \rightarrow 89) is internal standard for quantification. Middle and lower traces are m/z 188 \rightarrow 86 and 188 \rightarrow 69, respectively, to emphasise the different isomers.

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