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Gas chromatographic–mass spectrometric method for quantitation of phenylalanine and tyrosine in serum

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Abstract

A validated gas chromatographic–mass spectrometric method for quantitation of phenylalanine and tyrosine in serum is described. Quantitation of phenylalanine and tyrosine with a non-labelled non-endogenous internal standard, L-2-chlorophenylalanine, compared favourably with isotope dilution mass spectrometric quantitation. The 95% reference ranges for phenylalanine, tyrosine and the phenylalanine–tyrosine molar ratio in neonate cord blood serum were determined by isotope dilution mass spectrometry and were found to be 77.1–144.7, 33.3–109.3 μmol/l and 1.1–3.0, respectively.

Keywords: Phenylalanine; Tyrosine; Phenylketonuria

1. Introduction

Hyperphenylalaninemia, a disorder resulting from impaired conversion of phenylalanine (Phe) to tyrosine (Tyr), is usually due to deficiencies in the hepatic enzyme, phenylalanine hydroxylase. Phenylketonuria (PKU) manifests in an increased phenylalanine concentration in blood, urine and cerebrospinal fluid. It is essential to diagnose this inborn error of metabolism as early as possible, since untreated PKU can cause mental retardation. Actual injury to brain tissue begins within the second week of life [1]. Screening newborns for PKU is performed by measurement of blood/serum phenylalanine concentrations. The methods used include Guthrie bacterial inhibition assay (BIA) [2], fluorimetry [3,4], enzymatic analysis [5–7], HPLC [8–11], ion-exchange chromatography [12], gas chromatography [13] and tandem mass spectrometry (MS MS) [14–16].

Chromatographic and mass spectrometric techniques facilitate the use of internal standards, contributing to improvement of analytical precision [17], which will result in better diagnostic sensitivity and specificity. The high analytical selectivity of gas chromatography with mass spectrometric detection has established this technique as the confirmation method of choice after a sample was found positive with a screening method [1].

Chromatographic and mass spectrometric techniques allow for simultaneous detection of multiple amino acids. Tandem mass spectrometric analysis with fast atom bombardment ionization [17], has been shown to reduce false positive results by the use of the Phe/Tyr molar ratio but few clinical chemistry laboratories employ this technique. Gas

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chromatography–mass spectrometry (GC–MS) on the other hand is a much more commonly used technique in both private- and governmental laboratories and may also be used to determine the Phe/Tyr ratio. The advantages of GC–MS analysis are its high specificity and accuracy of quantitation through use of the isotope-dilution technique as well as its speed and degree of automation. Analytical accuracy is of paramount importance for the confirmation of PKU and for metabolic follow up of people with PKU on diet.

A validated method is reported for confirmation of PKU by GC–MS. A relative simple sample preparation procedure, comprising an extraction- and derivatization step, was used after which a time-optimised chromatographic analysis was employed. The concept of replacing stable isotope labelled internal standards, by non-labelled amino acid internal standards for GC–MS analysis is addressed. This is done in the context of reducing the instrumental analysis time, which facilitates a higher sample through-put.

2. Experimental

2.1. Chemicals

2.1.1. Amino acid and internal standards

L-phenylalanine (Phe), L-tyrosine (Tyr), L-cycloleucine (cLeu), and L-2-chlorophenylalanine (Phe-Cl) were purchased from Sigma–Aldrich, (Steinheim, Germany); L-phenyl-d^5-alanine (Ph-d5) was purchased from CDN isotopes (Pointe-Claire, Quebec, Canada).

2.1.2. Solvents

Pyridine (derivatization grade) and chloroform were purchased from Pierce (Rockford, IL, USA) and Fluka (Buchs, Switzerland), respectively.

2.1.3. Derivatization reagent

Ethylchloroformate (ECF) was purchased from Merck (Darmstadt, Germany) and was distilled prior to use.

2.2. Sample preparation

2.2.1. Standard solutions

The amino acids were dissolved in hydrochloric acid (0.05 mol/l) to obtain a stock solution with concentration of 2.5 mmol/l. The stock solution was stored at 4°C. Working standard solutions were prepared as required by dilution of the stock solution with hydrochloric acid (0.01 mol/l).

Addition standards with concentrations as indicated in Table 1 were used to alter the concentrations of pooled neonate serum for use as calibration standards. The addition standards contained internal standards L-cycloleucine, L-2-chlorophenylalanine and L-phenyl-d^5-alanine at fixed concentrations corresponding to that of the third addition standard. (Table 1).

An internal standard mixture, containing L-cycloleucine, L-2-chlorophenylalanine and L-phenyl-d^5-alanine at fixed concentrations corresponding to that of the third addition standard was used to spike the neonate serum samples.

2.3. Serum

Serum (0.5 ml), internal standard solution (500 μl), concentrated hydrochloric acid (75 μl), and chloroform (75 μl) were mixed in a vial with a Teflon-lined cap. The mixture was incubated at 75°C for 5 min and centrifuged for 10 min (4000 revolutions per min at 4°C). An aliquot of the supernatant (200 μl) was extracted with hexane (100 μl). The mixture was vortexed for 20 s after which the hexane layer was discarded. The aqueous phase, containing

Table 1
Concentrations of addition standards

Addition standard no.	cLeu (μmol/l)	Phe-Cl (μmol/l)	Phe-d5 (μmol/l)	Phe (μmol/l)	Tyr (μmol/l)	Concentration (mg/dl)
1	132	85	100	18	17	0.3
2	132	85	100	48	44	0.8
3	132	85	100	103	94	1.7
4	132	85	100	158	144	2.6
5	132	85	100	182	166	3

the amino acids, was subjected to the derivatization procedure.

2.4. N,(O)-ethoxycarbonyl amino acid derivatization procedure

The procedure reported by Husek and co-workers [18,19] was used: 200 μl of a mixture of 2.5 m*M* hydrochloric acid–ethanol–pyridine (60:32:8, v/v), ECF (100 μl) and cleaned-up sample (60 μl) was mixed in a 2-ml vial with a Teflon-lined cap. The solution was shaken gently for 3–5 s and left at room temperature for 10 min. CO_2 evolved during this period. A mixture of ECF in chloroform (100 μl, 1%) was added to the derivatized sample and vortexed for 20 s. An aliquot (100 μl) was dried under a stream of nitrogen at room temperature and dissolved in chloroform (100 μl).

2.5. Equipment:

A HP6890 GC system fitted with a HP7683 Auto injector and HP5973 Mass Selective Detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis. Data collection and integration was performed with HP Chem Station software.

A Zebron 1701 (Torrance, CA, USA), capillary column was used (length: 15 m; I.D.: 320 μm; film thickness: 0.25 μm).

2.6. Gas chromatographic and mass spectrometric detection (GC–MS) procedure

Two μl derivatized sample was injected in the pulsed splitless mode with a pulse time of 1.5 min. The inlet temperature was set at 250°C and helium carrier flow-rate at 2 ml/min. The oven temperature was ramped from 100 to 265°C at a ramp rate of 20°C/min with an initial isotherm of 1 min. The oven temperature was ramped immediately from 265 to 300°C at a ramp rate of 100°C/min with a final isotherm of 2 min. The total chromatographic time was 11.6 min.

The MSD transfer line temperature was set at 280°C and that of the quadrupole, 106°C. The source temperature was 230°C. A solvent delay time of 4 min was used to allow solvent elution before the source was switched on. All mass spectra were recorded at 70 eV. Ions with the following mass: charge ratios (m/z) were used in the selected ion-monitoring mode for quantitation: L-cycloleucine: 156; L-phenylalanine: 176; L-phenylalanine-d^5: 181; L-tyrosine: 192; L-2-chlorophenylalanine: 210. The dwell time on each ion was 0.100 s.

2.7. Calculations

2.7.1. Calibration curves

Five point calibration curves were set up in pooled neonate serum by standard addition. Known amounts of standards (Phe and Tyr) were added to pooled cord blood serum to obtain five different concentration levels. The endogenous concentrations of Phe and Tyr in the pooled serum were ascertained by extrapolation of the calibration curves. The x-intercepts represented the original endogenous amount. The sum of the original- and added amount was used to calculate the concentration of Phe and Tyr in the spiked serum standards. The concentrations and corresponding abundance ratios, Phe and Tyr relative to the internal standards, were used to set up a calibration curve. The concentrations of Phe and Tyr in the 120 cord blood serum samples of the population study were calculated by reference to the appropriate standard curves.

2.8. Evaluation of the internal standard characteristics and performance

The characteristics and performance of the internal standards were evaluated by comparison of the linear regression parameters of the respective calibration curves. Ten samples of the same neonate pooled serum sample were analysed to evaluate the precision that resulted from the use of each internal standard.

2.9. Stability of derivatives

The stability of the ethoxycarbonyl derivatives were studied over a period of 10 h by injecting the same sample ten times, once every hour. The response of Phe and Tyr was measured relative to Phe-Cl internal standard and the coefficient of variation calculated.

2.10. Population study

One hundred and twenty randomly selected cord blood serum samples were analysed. The samples were obtained originated from a neonatal hypothyroidism routine screening program. The reference range concentrations of Phe, Tyr and their ratio in cord blood were calculated by applying non-parametric statistical data analysis [20].

3. Results and discussion

A chromatogram recorded by single ion monitoring mass spectrometric detection is shown in Fig. 1.

In addition to decreased detection limits, the enhanced selectivity in the selected ion detection mode has the potential of decreasing the chromatographic separation time. Chromatographic resolution can theoretically be traded for separation speed to a point where overlap with other chromatographic peaks contribute to the analytical signal, due to the presence of ions with the same mass:charge ratio (m/z). Fig. 1 indicates that the chromatographic resolution is more than required. Trading resolution for a faster separation will result in a shorter analysis time. A capillary column with a small diameter (~100 μm) will also contribute to reduced analysis time. A separation time of 4 min has been reported [18]. Faster elution will also yield better detection limits since the mass spectrometer is a mass flow sensitive detector. With GC–MS being highly automated, shortened separation times will increase sample through-put.

The mass spectra of the N,(O)-ethoxycarbonyl derivatives of L-phenylalanine, L-2-chlorophenyl-

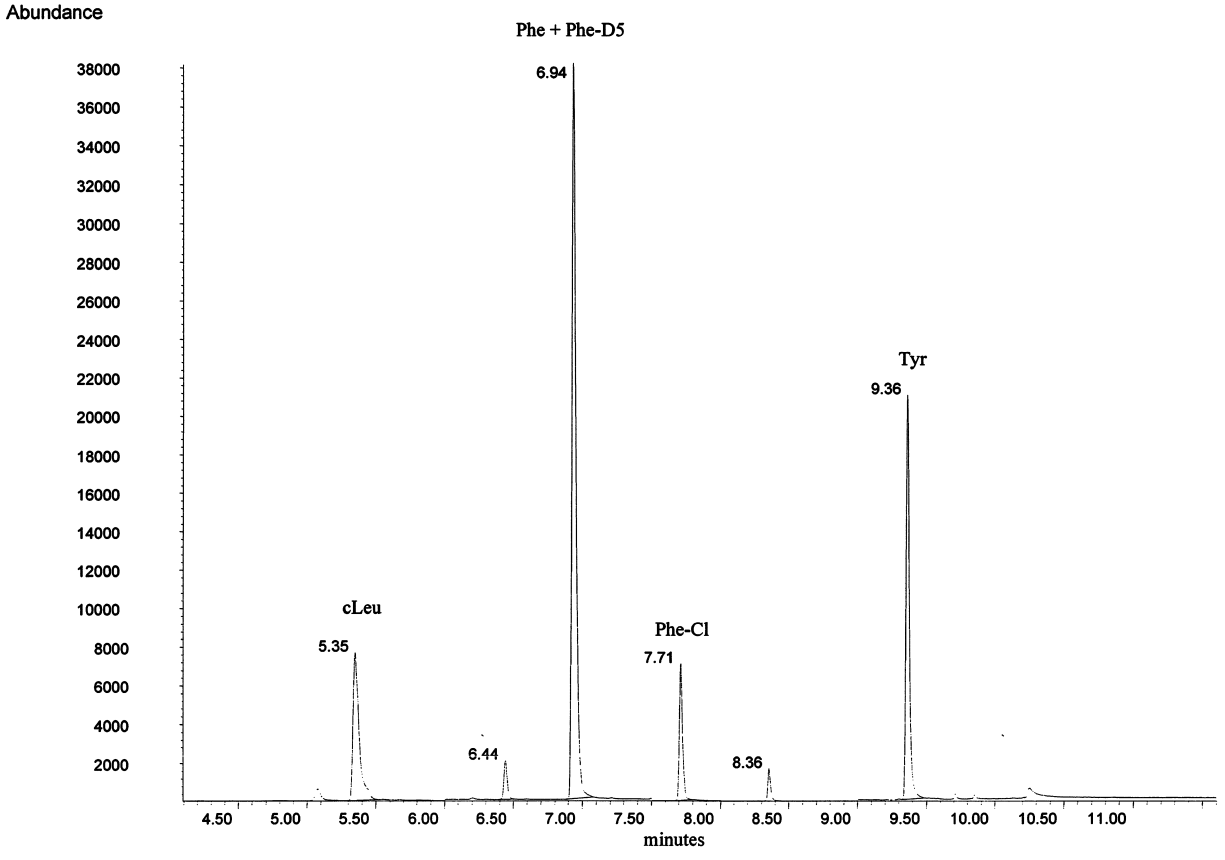

Fig. 1. Chromatogram of serum extract of ethoxycarbonyl amino acid derivatives.

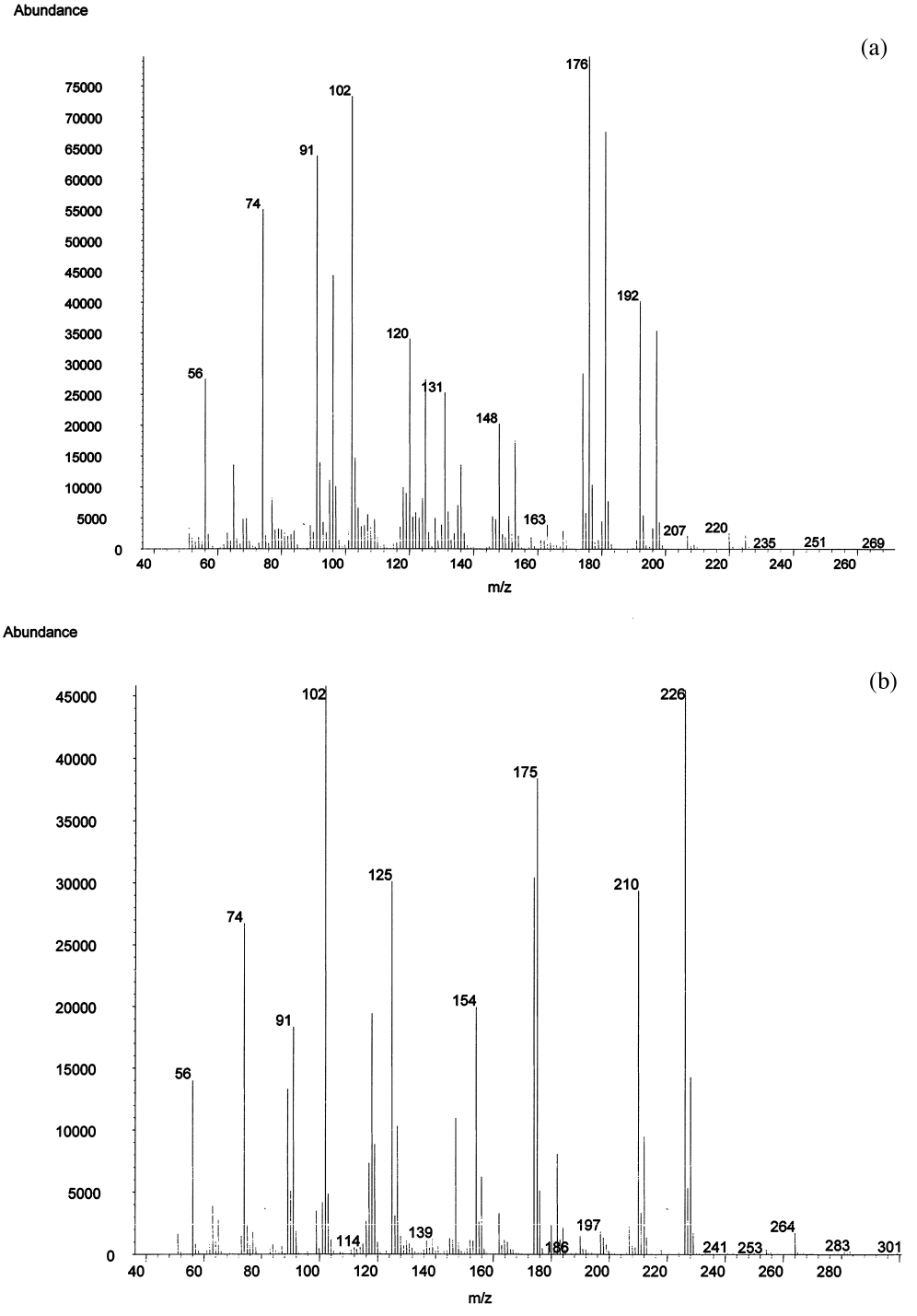

Fig. 2. (a) Mass spectra of the N,(O)-ethoxycarbonyl derivative of L-phenylalanine (M_r: 265). (b) Mass spectra of the N,(O)-ethoxycarbonyl derivative of L-2-chlorophenylalanine (M_r:300). (c) Mass spectra of the N,(O)-ethoxycarbonyl derivative of L-tyrosine (M_r: 353).

alanine and L-tyrosine are shown in Fig. 2. It can be seen that the abundance of the molecular ions are low. The characteristic ions used for single ion monitoring, during quantitation of Phe and Tyr in neonate serum, are shown in Fig. 3 [21,22].

Mass spectrometric detection permits the use of stable isotope labelled internal standards. These compounds resemble the stability and reactivity of the analytes exactly since it has the same chemical structure as the endogenous compounds. The regression analysis parameters of the calibration curves with Phe-Cl and Phe-d5 as internal standards are shown in Table 2. The use of these two internal standards resulted in nearly similar regression parameters. This indicates that the non-labeled Phe-Cl is suitable for use as an internal standard as compared to Phe-d5 which is used in isotope dilution mass spectrometric quantitation. The use of cLeu resulted in a linear correlation coefficient (r^2) of below 0.9 and a coefficient of variation (C.V.) above 30%. Its use for Phe and Tyr quantitation in neonate serum was for this reason not considered since it will result in poor analytical precision and accuracy. The difference in characteristics of cLeu and Phe-Cl can be attributed to the difference in their chemical reactivities. Even though both are cyclic amino acids, the first has an aliphatic structure and the latter an aromatic structure.

Stable isotope labeled Tyr will be the ultimate internal standard for the quantitation of endogenous Tyr. From the r^2 values reported in Table 2, it can be seen that even the Phe-d5 internal standard does not mimic the behaviour of Tyr precisely. Quantitation of Tyr against Phe-d5 internal standard resulted in an expected loss of accuracy and precision as compared to Phe.

The signal-to-noise ratios of the endogenous signals for Phe and Tyr in the samples with the lowest

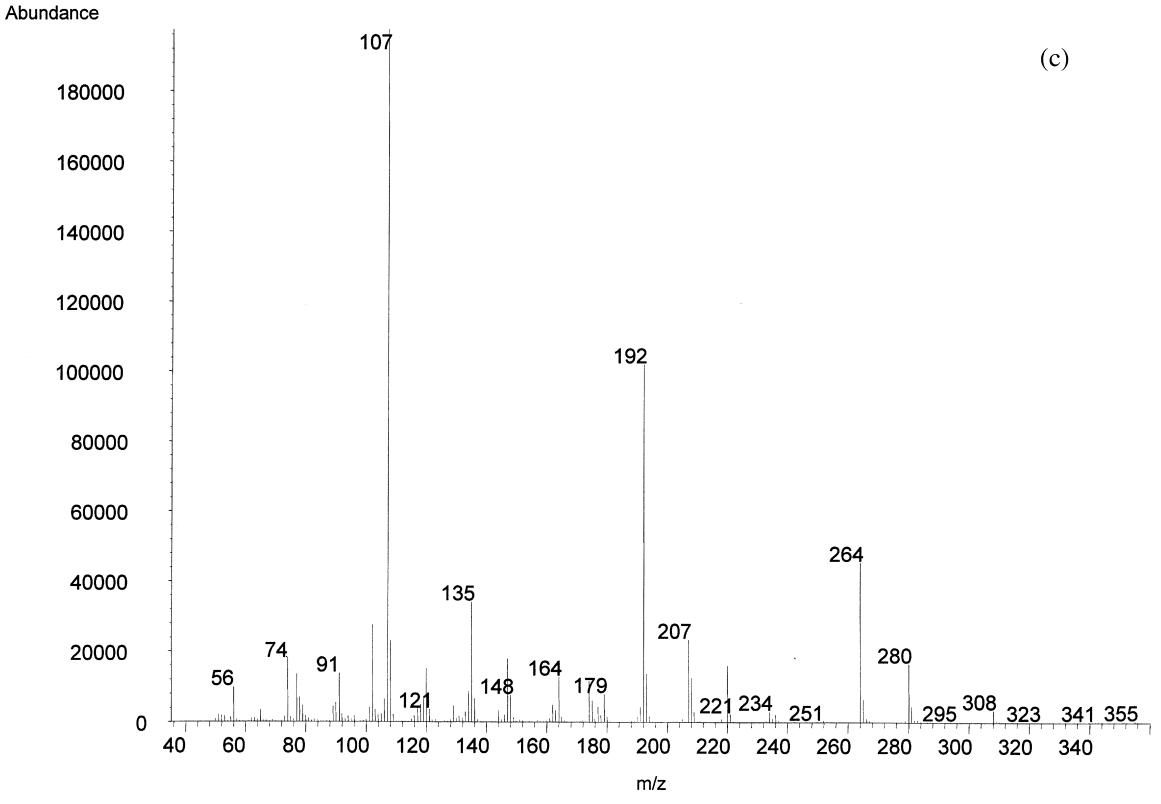

Fig. 2. (*continued*)

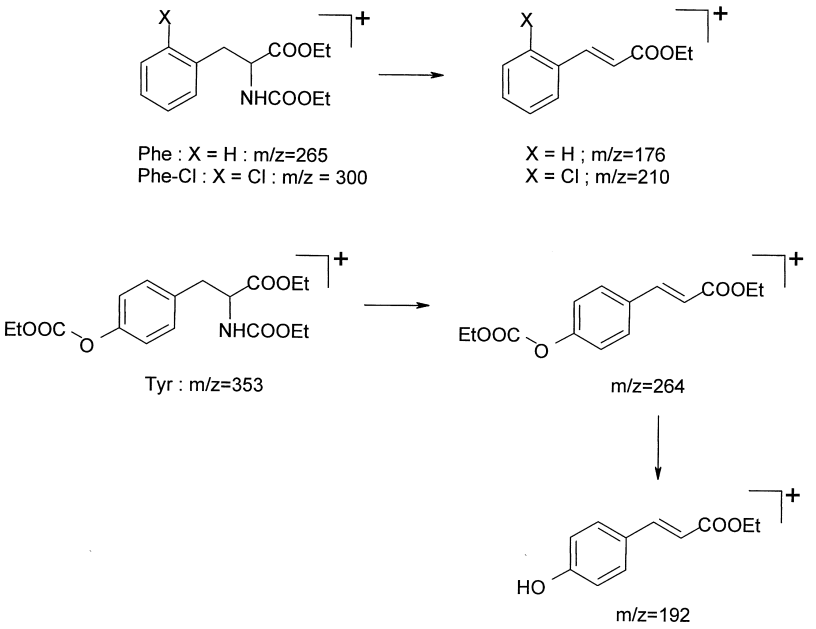

Fig. 3. Characteristic ions chosen for single ion monitoring [21,22].

concentrations were 2333:1 and 750:1, respectively. The estimated minimum quantification limits to give a signal-to-noise ratio of 10:1 correspond to 0.30 and 0.38 μmol/l, respectively. This is well below the physiological reference ranges for Phe and Tyr in cord serum.

An important prerequisite for routine chromatographic batch analysis, is that the amino acid derivatives should be stable. If the amino acid derivatives are unstable, isotope labelled internal standards will compensate for sample decay. On the other hand non-labeled internal standards can be used effectively provided that the derivatives are stable. Fig. 4 shows a plot illustrating the stability of the ethoxycarbonyl derivatives over a period of 10 h. The response of Phe and Tyr was measured relative to that of Phe-Cl. The relative response coefficient of variation for Phe and Tyr was 1.3 and 1.5%, respectively, indicating stability of Phe-Cl with respect to Phe and Tyr.

A Levy-Jennings graph for phenylalanine quantitation over a period of 2 weeks illustrating the day–day repeatability is shown in Fig. 5. Single ion monitoring with Phe-d5 and Phe-Cl as internal

Table 2
The regression analysis parameters of the calibration curves with Phe-Cl and Phe-d5 internal standards (n=5)

Internal standard		r^2	Slope (RRF analyte/IS)	y-Intercept	Within batch C.V.: 10 samples (1.7 mg/dl) (%)	Between batch C.V.: 10 samples (1.7 mg/dl) (%)	Recovery at 1.7 mg/dl concentration (%)
Phe-Cl	Phe	0.993	2.216	−0.00005	5.2	7.3	103.1
	Tyr	0.968	2.044	−0.0002	15.3	12.3	88.8
Phe-d5	Phe	0.995	0.764	−0.00001	3.72	6.1	102.2
	Tyr	0.959	0.702	−0.00002	13.5	12.2	87.7

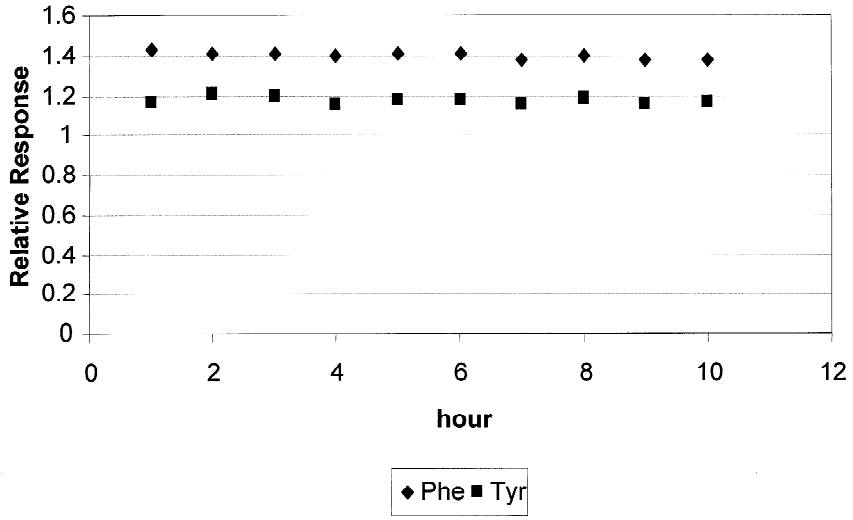

Fig. 4. Graph of the response the ethoxycarbonyl derivatives of Phe and Tyr relative to Phe-Cl. The same sample was injected over a period of 10 h.

standards was performed. The coefficients of variation were found to be 3.7 and 5.1%, respectively

The cord serum 95% reference ranges for Phe, Tyr and Phe/Tyr ratio are listed in Table 3. By definition, the three samples in the upper 2.5% percentile would be positive in a screening programme. However, when applying the additional condition of the Phe/Tyr ratio to be below a certain value [17] they might prove to be false positive.

This method is suitable for confirmation of PKU cases and follow up of diagnosed PKU patients. It may even be possible to use the method for PKU screening in cord blood samples. The validity of performing neonatal Phe and Tyr analysis on cord blood for PKU diagnosis by this method needs further investigation. The results of such a study should be compared with that of a screening program, performed in parallel on day 5 neonates, with a validated alternative analytical technique. Scriver et al. [23] found that while cord blood phenylalanine was slightly higher in infants with PKU than in controls, the overlap was too great to allow for reliable screening. Utilizing the Phe/Tyr ratio, which may determined accurately with this method, as an additional exclusion condition might render PKU screening on cord blood effective [17].

The lower limit of the Phe reference range in Table 3, namely 77.1 μmol/l, corresponds to a signal-to-noise ratio of approximately 2500:1. Minimum detection limits reported in literature with enzymatic methods are in the order of 20–40 μmol/l (signal-to-noise ratio=2) [5,6,9]. This translates into a minimum quantifiable level of 100–200 μmol/l (signal-to-noise ratio=10), which is inadequate for accurate and reliable quantitation of Phe in cord blood.

4. Conclusions

A validated quantitative gas chromatographic mass spectrometric method for assessment of Phe and Tyr concentrations in serum is described. Quantitation of phenylalanine and tyrosine against a carefully selected, non-labelled, non-endogenous internal standard compared favourably to isotope dilution mass spectrometric quantitation. The high selectivity and sensitivity of GC–MS complements this method for PKU confirmation.

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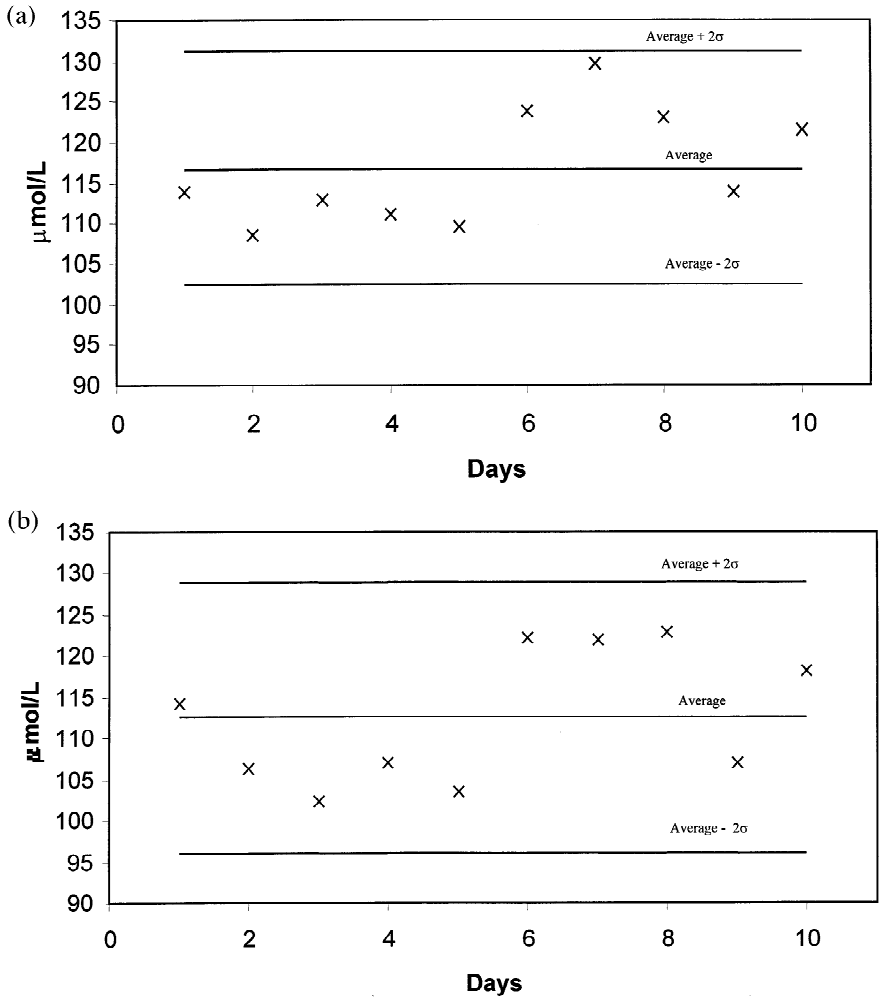

Fig. 5. (a) Levy-Jennings graph of Phe quantitation with Phe-d5 internal standard over a period of 2 weeks illustrating the day–day repeatability. (b) Levy-Jennings graph of Phe quantitation with Phe-Cl internal standard over a period of 2 weeks illustrating the day–day repeatability.

Table 3
Reference range concentration values for Phe and Tyr in cord blood serum (Mean±2 SD)

		Internal standard	
		Phe-d5	Phe-Cl
Phe	Mean	107.5	103.8
(μmol/l)	95% Reference range	77.1–144.7	71.9–142.7
Tyr	Mean	64.2	61.7
(μmol/l)	95% Reference range	33.3–109.3	32.4–105.5
Phe/Tyr	Mean	1.8	1.8
(molar ratio)	95% Reference range	1.1–3.0	1.1–3.0

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