

Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 hours

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We compared the screening interpretation of fluorometric analytical results for phenylketonuria (PKU) with tandem mass spectrometry (MS/MS) in filter paper blood spots collected from newborns <24 h of age. In MS/MS, both Phe and Tyr are quantified. Two hundred and eight blood spots collected from infants <24 h of age were retrieved from storage from the California newborn screening program. These samples had been categorized on the basis of fluorometric analysis as initial negative, initial positive for hyperphenylalaninemia with negative determination on recall, or initial positive for hyperphenylalaninemia and confirmed on follow up as PKU or variant hyperphenylalaninemia. The retrieved samples were analyzed in a blinded fashion using MS/MS. Correlation analysis of fluorometry vs MS/MS for Phe concentration was high, with a Pearson correlation coefficient of 0.817. When 180 $\mu\text{mol/L}$ was used as the cutoff Phe concentration for MS/MS and 258 $\mu\text{mol/L}$ was used as the cutoff for fluorometry, all infants with confirmed classical PKU and variant hyperphenylalaninemia were detected. MS/MS analysis reduced the number of false-positive results from 91 to 3. Simultaneous quantification of Phe and Tyr by MS/MS with the use of a cutoff Phe/Tyr molar ratio of 2.5 further reduced the number of false positives to 1. Samples from affected infants showed a discernible trend of increasing Phe concentration and

Phe/Tyr molar ratio with age of collection. These results demonstrate the utility of MS/MS in the routine PKU screening of early-discharge newborns. MS/MS reduces the false-positive rate of fluorometric screening almost 100-fold because of the improved accuracy and precision of Phe measurement and simultaneous confirmation with the Phe/Tyr molar ratio. In addition to the detection of PKU, MS/MS can also detect other aminoacidopathies and disorders of fatty acid and organic acid metabolism with lower false-positive rates than other methods currently used in newborn screening programs.

Routine screening is performed worldwide for phenylketonuria (PKU),⁴ an inborn error of amino acid metabolism. Early diagnosis and treatment of this disorder can prevent mental retardation. If left undiagnosed and untreated for several months, the mental retardation is irreversible. In 1962, when this screening was introduced, blood specimens were collected on filter paper at the time of discharge, generally not earlier than 72 h. More recently, the number of newborns discharged before or at 24 h of age has increased substantially. Because PKU manifests itself as a time-dependent increase in the concentration of Phe in blood, questions concerning the reliability of very early newborn screening for this disease have been raised (1), especially for analytical methods that measure Phe semiquantitatively, such as the Guthrie bacterial inhibition assay. Consequently, concerns have been expressed regarding the efficacy of testing for PKU in infants <24 h postpartum (2).

Few studies have been described that have systemati-

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⁴ Nonstandard abbreviations: PKU, phenylketonuria; MS/MS, tandem mass spectrometry; and Phe/Tyr, phenylalanine-to-tyrosine ratio.

cally examined the quantification of Phe in blood spots collected before 24 h. To reduce or eliminate false-negative results with these early specimens, Cunningham et al. (3) reduced the diagnostic cutoff to 258 $\mu\text{mol/L}$ (4.3 mg/dL) for a fluorometric technique. However, they observed the relatively high rate of 4% for false-positive results. An initial study using HPLC and tandem mass spectrometry (MS/MS) demonstrated the ability to quantify Phe and Tyr simultaneously (4); it has also been suggested that calculation of the ratio of Phe to Tyr (Phe/Tyr) will allow detection of affected newborns under 24 h of age without increasing the rate of false positives (5). This study was designed to test this hypothesis by comparing the results of previous analyses by fluorometry with those by MS/MS, in which Phe and Tyr are quantified, in samples collected from newborns <24 h of age.

Materials and Methods

The California newborn screening program retrieved 208 filter paper blood specimens from storage (original collection dates ranged from 1992 to 1994). Samples were stored during this period at -20°C . The age (in hours) of the newborn at collection time was recorded; all samples were collected <24 h after birth. The specimens were originally analyzed for hyperphenylalaninemia by fluorometry. Specimens with a Phe concentration ≥ 258 $\mu\text{mol/L}$ (4.3 mg/dL) were classified as positive. An additional specimen was collected from each infant with a positive result. If the subsequent specimen was negative, the result was classified as a false positive. Metabolic specialists made the final diagnoses of cases of classical PKU or variant PKU. The group classified as variant PKU had a mean Phe concentration of 430 $\mu\text{mol/L}$ (7.1 mg/dL) in the second specimen. The group classified as classical PKU had a mean Phe in the second specimen of 1188 $\mu\text{mol/L}$ (19.6 mg/dL). These specimens were assigned study numbers and sent blinded to the Mass Spectrometry Facility at Duke University Medical Center for analysis by MS/MS. The specimens are categorized in Table 1.

Quantitative amino acid profiles were obtained by isotope dilution liquid secondary ion MS/MS, using

methods described previously (4). A cutoff of 180 $\mu\text{mol/L}$ (3.0 mg/dL) was used to identify increased Phe, and a Phe/Tyr molar ratio of 2.5 was used to provide additional evidence of a metabolic defect in the conversion of Phe to Tyr, indicative of a primary hyperphenylalaninemia. These cutoffs were based on values used in a previous study (4) for negative samples collected >24 h post delivery. Results were sent to the California Genetic Disease Branch where the study was unblinded.

Results

Amino acid profiles from filter paper blood spots collected from newborns with an initial negative result (top), a false-positive result (middle), and a positive result from a confirmed case of PKU (bottom) obtained after the MS/MS analysis are illustrated in Fig. 1. Phe and Tyr are distinct quantifiable peaks separated from other amino acids. In addition to Phe and Tyr, a variety of other important amino acids are simultaneously analyzed using MS/MS as illustrated in Fig. 1.

The results of Phe quantification ($\mu\text{mol/L}$) by MS/MS are summarized in Table 2. Fig. 2 is a scatter plot of the data set. It should be noted that all but three specimens originally classified as false positives were correctly categorized by MS/MS as negative. Comparison of results obtained by fluorometry with those obtained by MS/MS reveals a high correlation, with a Pearson correlation coefficient of 0.817. In Fig. 2, the cutoff value for MS/MS is 180 $\mu\text{mol/L}$ (3.0 mg/dL), in comparison with the fluorometric cutoff value of 258 $\mu\text{mol/L}$ (4.3 mg/dL). The results of the Tyr quantification ($\mu\text{mol/L}$) by MS/MS are also summarized in Table 2. A scatter plot of each data point for Tyr (data not shown) showed no partitioning of data points by sample groups, i.e., negative, positive for PKU, or positive for hyperphenylalaninemia, in contrast to that observed for Phe, although the Tyr value for infants with PKU was distinctly lower than those for the other groups. The values for the Phe/Tyr ratio are summarized in Table 2, and a scatter plot of each data point is shown in Fig. 3, using a Phe/Tyr molar ratio of ≥ 2.5 to confirm the diagnosis of hyperphenylalaninemia. The Phe/Tyr ratio eliminated the false-positive designation from two of the three specimens that had remained in the false-positive category when only the Phe value obtained by MS/MS was used. Thus, with values for both Phe and Phe/Tyr, only one specimen that was originally false positive by fluorometric analysis remained false positive by MS/MS, whereas the other 90 specimens originally classified as false positives were negative. The relationships between Phe concentration ($\mu\text{mol/L}$) and Phe/Tyr ratio determined by MS/MS and time of collection (hours) among the groups and in one infant with PKU from whom serial samples were collected during the first 24 h of life are presented in Figs. 4 and 5, respectively. In this infant, both the Phe value and the Phe/Tyr ratio rose consistently during the first 24 h of life. In other infants with PKU or variant hyperphenylalaninemia, the Phe

Table 1. Specimens included in this study.

91 initial positive specimens with negative determinations on recall (false positives): 45 specimens collected <12 h post delivery; and 46 specimens collected between 12 and 24 h post delivery
93 initial negative specimens (collected <24 h post delivery)
12 specimens from infants with confirmed classical PKU (collected <24 h post delivery)
7 specimens from infants with confirmed variant hyperphenylalaninemia (collected <24 post delivery)
5 serial specimens from one infant with PKU (collected as cord blood, and 6, 12, 18, and 24 h post delivery)
Total of 208 specimens

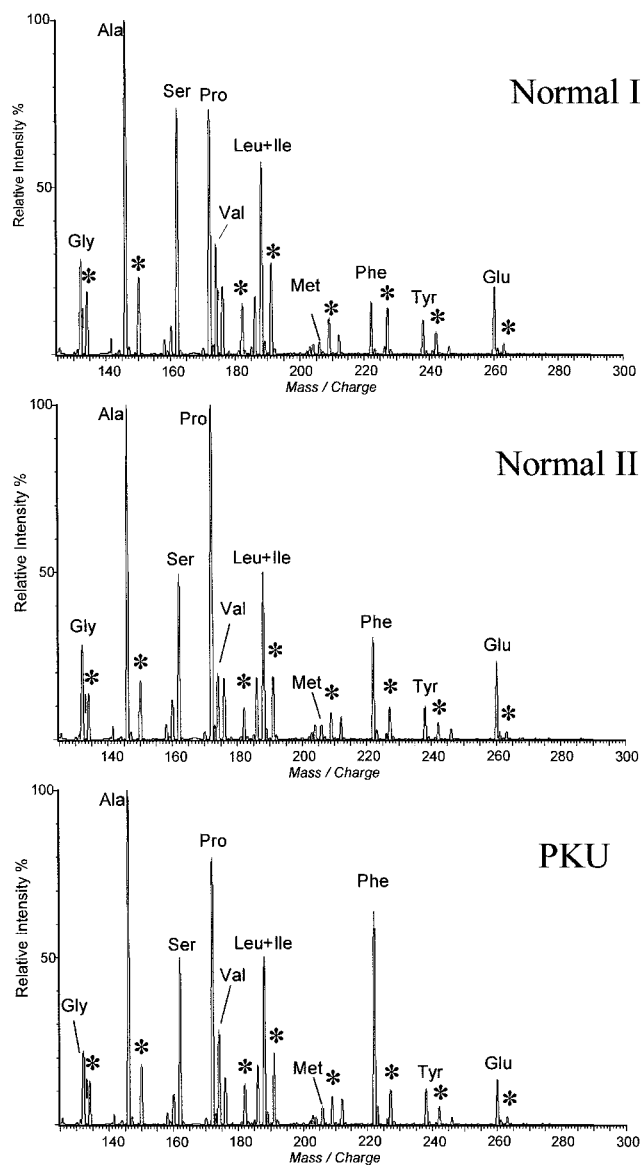


Fig. 1. MS/MS amino acid profiles from neonatal specimens that tested as initial negative (*Normal I*, top panel), false positive (*Normal II*, middle panel), and confirmed PKU-positive (*bottom panel*) in initial fluorometry tests.

The * represent deuterated amino acid internal standards of known concentration. Quantification is achieved by comparing the peak height of the amino acid of interest to its respective internal standard. Each spectrum is normalized to the largest ion signal present. For each of these profiles, the horizontal axis represents the m/z ratio of a protonated ion; the vertical axis represents the intensity of these ion signals normalized to the highest signal produced. The charge on these protonated ions in this ionization mode (liquid secondary ion) is 1; consequently, the horizontal axis represents the mass of a protonated amino acid butyl ester. The mass of the molecular ion of Phe as a protonated butyl ester is observed at m/z 222, whereas that for Tyr is observed at m/z 238. Concentration calculations involve interpolation from a calibration curve; i.e., the ion ratios of the analyte of interest to its respective internal standard is interpolated from a calibration curve of these ion ratios vs known amounts of analyte.

value also tended to be higher at increasing hours of age within the first 24 h (Fig. 4). This was not true for the Phe/Tyr ratio; however, the value at every hour of age sampled was above the cutoff value (Fig. 5).

Table 2. MS/MS results of testing specimens for Phe and Tyr, and the calculated Phe/Tyr ratio.

Sample classification	n	Median	Phe, $\mu\text{mol/L}$	
			Minimum	Maximum
Negative	93	68	45	124
FP ^a <12 h	45	112	56	177
FP >12 h	46	107	29	290
PKU	12	274	201	391
Variant hyperphenylalaninemia	7	305	197	354
			Tyr, $\mu\text{mol/L}$	
Negative	93	97	36	208
FP <12 h	45	100	52	273
FP >12 h	46	118	67	310
PKU	12	62	39	133
Variant hyperphenylalaninemia	7	82	48	119
			Phe/Tyr, molar ratio	
Negative	93	0.73	0.29	2.4
FP <12 h	45	1.1	0.31	2.1
FP >12 h	46	0.91	0.3	3.0
PKU	12	4.1	2.8	7.2
Variant hyperphenylalaninemia	7	3.7	2.7	5.0

^a FP, false positive.

Discussion

In this study, Phe measurement by MS/MS, with a cutoff of 180 $\mu\text{mol/L}$ (3 mg/dL), or by fluorometry, with a cutoff of 258 $\mu\text{mol/L}$ (4.3 mg/dL), detected all variant and classical cases of PKU. One variant and one classical case of PKU would have been missed if the MS/MS cutoff was moved to 249 $\mu\text{mol/L}$ (4.1 mg/dL). At the cutoff of 180 $\mu\text{mol/L}$, however, MS/MS analysis sharply reduced the number of false-positive results originally classified by fluorometry. When the Phe value alone was used, none of the 45 specimens collected before 12 h and only 3 of the 46 specimens collected 12–24 h post delivery originally classified as false positive by fluorometry were positive by MS/MS.

The addition of the Phe/Tyr molar ratio determined by MS/MS with a cutoff of 2.5 allowed the accurate detection of PKU and variant hyperphenylalaninemia in all infants originally determined to be positive, with only one false-positive result. Thus, although both fluorometry and MS/MS detected classical PKU or hyperphenylalaninemia in specimens collected <24 h post delivery, including one collected as early as 4 h post delivery, MS/MS greatly reduced the number of false-positive identifications.

The differences in quantitative cutoff values between MS/MS and fluorometry may be attributed to predictable fluorescent interference, which raises the concentration of Phe. However, we have found that MS/MS results are slightly lower when compared with other methods as well, specifically HPLC and the bacterial inhibition assay. Additional work is in progress to compare MS/MS with

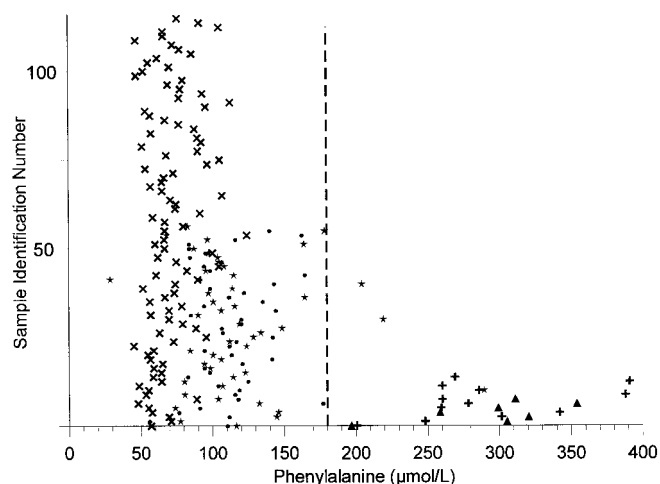


Fig. 2. Scatter plot of quantitative Phe results for the data set.

The horizontal axis represents the concentration of Phe ($\mu\text{mol/L}$), whereas each point on the vertical axis is the sample identification number. (X), specimens that tested initial negative; (●), false-positive specimens collected <12 h post delivery; (*), false-positive specimens collected >12 h post delivery; (+), classical PKU; and (▲), hyperphenylalaninemia (variant). The cutoff using MS/MS analysis is marked by a dotted vertical line.

these methods. However, previous work has shown a good correlation for HPLC and MS/MS for the measurement of Phe in plasma samples (4). Additional comparative studies are in progress.

The measurements of Phe concentration in unaffected newborns by MS/MS and fluorometry are tightly clustered around the median of $68 \mu\text{mol/L}$ (Fig. 2). The phenylalanine hydroxylase deficiency that produces classical PKU or variant hyperphenylalaninemia dictates a high Phe/Tyr ratio in association with the increased Phe.

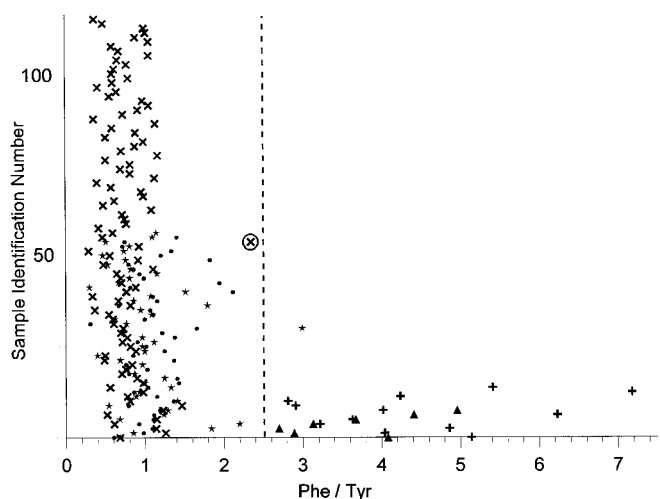


Fig. 3. Scatter plot of Phe/Tyr molar ratio for the data set.

The horizontal axis represents the Phe/Tyr ratio; each point on the vertical axis is the sample identification number. (X), initial negative samples; (●), samples collected <12 h post delivery that gave false-positive results; (*), samples collected >12 h post delivery that gave false-positive results; (+), classical PKU; and (▲), hyperphenylalaninemia (variant). The cutoff using MS/MS analysis is marked using a dashed vertical line. ⊗, sample from a newborn on intravenous nutrition.

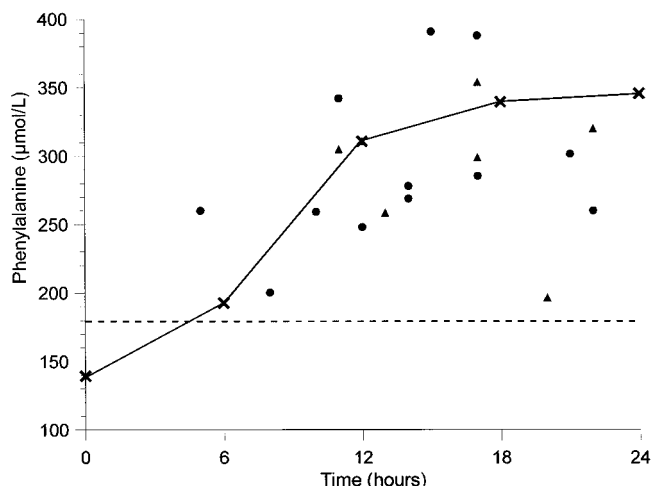


Fig. 4. Scatter plot of specimen collection time vs Phe concentration of specimens collected for newborn screening from infants with PKU (●), infants with hyperphenylalaninemia (▲), and a selected infant with PKU whose blood samples were collected every 6 h for 24 h (X).

The horizontal axis represents collection time in hours; the vertical axis represents Phe concentration. Data points for the single infant with PKU are connected using a single solid line. The MS/MS analysis cutoff value is a dotted horizontal line.

As shown in Fig. 3, the initial negative (normal) samples are tightly clustered around a median molar Phe/Tyr ratio of 0.73. There is one negative sample (⊗) that is clearly an outlier and near the cutoff value of 2.5. This sample had increased Met as well as increased Phe and decreased Tyr. Review of the case identified this specimen as being from a newborn receiving intravenous feeding. The abnormal Leu/Phe and Met/Phe ratios indicate that the sample did not have either classical PKU or variant

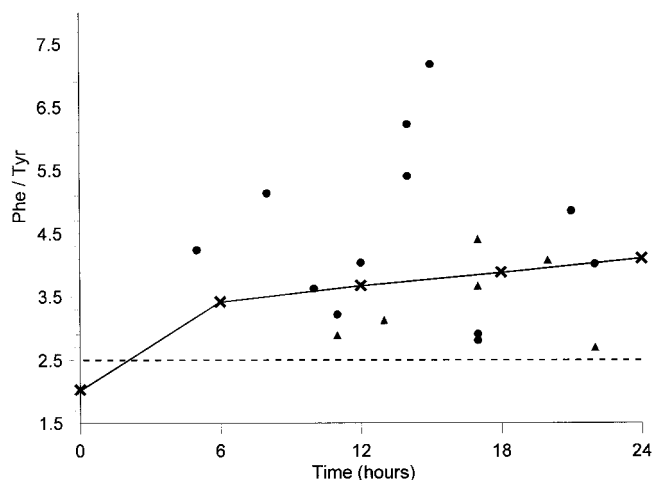


Fig. 5. Scatter plot of collection time vs Phe/Tyr molar ratio for samples collected for newborn screening from infants with PKU (●), infants with hyperphenylalaninemia (▲), and a selected infant with PKU whose blood samples were collected every 6 h for 24 h (X).

The horizontal axis represents collection time in hours; the vertical axis represents the Phe/Tyr ratio. Data points for the single infant with PKU are connected using a solid line. The MS/MS analysis cutoff value is a dotted horizontal line.

hyperphenylalaninemia. The range of the Phe/Tyr ratio for initial false-positive samples is 0.91–1.07, which is only slightly increased compared with initial negative samples. Only one false-positive sample was greater than the cutoff of 2.5. All of the classical PKU or variant hyperphenylalaninemia patient specimens had Phe/Tyr ratios >2.5.

It is noteworthy that 88 of 91 false-positive results had a Phe concentration within the reference interval by MS/MS, suggesting that most false-positive results previously detected by fluorometry were attributable to interference from other amino acids or from other compounds present in the blood, rather than an actual increase of Phe. This result also supports previous work that indicated that MS/MS is a more accurate method for measuring Phe concentration. Moreover, the Phe/Tyr ratio determined by MS/MS in this study is a molar ratio, rather than the signal-intensity ratio used by many other methods. Therefore, data using a Phe/Tyr molar ratio may not be directly comparable to the ratios of signal intensities published in other articles.

In specimens collected at various times during the first 24 h post delivery from infants with PKU or variant hyperphenylalaninemia, as depicted in Figs. 4 and 5, the concentration of Phe increases over time, becoming >180 $\mu\text{mol/L}$ at 6 h. The Tyr concentration does not show a trend toward decreasing concentration with time (data not shown). In the one infant studied serially, the Phe/Tyr ratio became greater than the cutoff value by 6 h and then plateaued. The rate of increase of the Phe/Tyr ratio over time is not as noticeable as the rate of change for Phe.

When MS/MS was used, the newborn detection of infants confirmed to be positive for PKU or variant hyperphenylalaninemia was equally valid using either Phe or the Phe/Tyr ratio. The precision of the method, however, was greater for the Phe/Tyr ratio than for Phe alone (data not shown). These results demonstrate the utility of MS/MS in the detection of PKU in newborns discharged early and the reduction of false-positive results achieved through the higher accuracy of the Phe measurements and the simultaneous determination of the Phe/Tyr ratio.

MS/MS can diagnose PKU with an extremely low false-positive rate, only $\sim 1/100$ that of fluorometry, with excellent accuracy and precision, as described previously (4). With the cutoff values that we have used, it is anticipated that few if any false-negative results will occur. Because the expected false-positive rate is so dramatically lower, the cutoff concentration could be set to <180 $\mu\text{mol/L}$ with only a small increase in the false-positive rate. However, this is a decision that must be made in individual laboratories and through experience. At this time, with the experience of testing >550 000 blood specimens from newborns by MS/MS at Neo Gen Screening, no known false-negative results have occurred.

In addition to detection of PKU, MS/MS can also

detect other aminoacidopathies, including maple syrup disease (6) and homocystinuria (7). Because a single analysis measures not only Phe and the Phe/Tyr ratio but also amino acids other than Phe and Tyr, the method is very cost-effective. In addition to other aminoacidopathies, MS/MS adds newborn screening for the organic acid disorders and disorders of fat metabolism, such as medium chain acyl-CoA dehydrogenase deficiency (8, 9). The technique is cost-effective and meets the current need to expand methods of preventive medicine. Because of its low false-positive rates, this method is an efficient way to screen for large numbers of disorders in individuals while minimizing the cost of follow up because of low false-positive rates. The cost-benefits of MS/MS and its applications in newborn screening seem to be solidly based (10).

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