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Simultaneous measurement of phenylalanine and tyrosine in phenylketonuric plasma and dried blood by high-performance liquid chromatography

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Abstract

Phenylketonuria (PKU) is a disorder characterized by an interruption in the conversion of phenylalanine to tyrosine, a reaction catalyzed by phenylalanine hydroxylase (PAH). Animal models of PKU used in this study were induced by daily subcutaneous injections of pups with α-methylphenylalanine plus phenylalanine in utero and postnatally from day 4 to day 14. Dry blood and plasma were utilized to measure phenylalanine concentration in PKU rats. The results indicated that the concentration of phenylalanine is higher and more stable in plasma than dry blood. Precolumn derivatization of dried blood and plasma free amino acids were conducted with phenylisothiocyanate (PITC). The phenylthiocarbamyl (PTC) derivatives were separated on a reversed-phase C-18 column (15 cm×4.6 mm). A gradient high-performance liquid chromatography method with two eluents, 0.1 *M* sodium acetate buffer and 100% acetonitrile was developed to facilitate the separation of nine amino acids within 11 min. Tyrosine and phenylalanine eluted the column at 5.4 and 9.4 min, respectively. This method provides a quick and reliable technique for neonatal screening.

Keywords: Phenylalanine; Tyrosine

1. Introduction

Phenylketonuria (PKU) is an inborn error of metabolism characterized by the inability of the body to utilize phenylalanine (Phe). The inability to hydrolyze Phe to tyrosine (Tyr) may adversely affect the synthesis of tyrosine dependent neurotransmitter substances [1]. Left untreated, severe mental retardation results [2]. PKU is clinically and experimentally characterized by elevated levels of Phe in the blood. A normal blood Phe level is approximately 1 mg/dl. Levels observed in classic PKU may range from 6 to 80 mg/dl in humans [3].

The diagnosis and treatment of PKU require the measurement of blood Phe levels using high-performance liquid chromatography (HPLC). Many HPLC methods have been utilized for this measurement; however, some of these methods have dis-

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advantages, including sample instability, complex derivatizing agents and difficulties encountered in sample preparation [4]. In contrast, the HPLC method used in this study is simple, sensitive, rapid, accurate and reliable. The objective of this study is to determine the blood Phe level in PKU rats using HPLC.

2. Experimental

2.1. Chemicals

5-Sulfosalicyclic acid (SSA), pyridine, sodium acetate, L-tyrosine, L-phenylalanine, L-norvaline (internal standard, I.S.), L-isoleucine, L-leucine, L-valine, L-proline and L-glycine were obtained from Sigma (St. Louis, MO, USA). Phenylisothiocyanate (PITC) was obtained from Pierce (Rockford, IL, USA). Triethylamine and HPLC-grade acetonitrile were purchased from Fisher (Atlanta, GA, USA), a reversed-phase C-18 column from Supelco (Bellefonte, PA, USA), HPLC system 1100 series was obtained from Hewlett-Packard (Wilmington, DE, USA), PKU test cards (S&S 903) were purchased from Schleicher and Schuell (Keene, NH, USA). Liquid rat diet was purchased from Bio-Serv (Frenchtown, NJ, USA).

2.2. Reagents

The mobile phase for HPLC was prepared from 1 *M* sodium acetate, adjusted to pH 6.5 with acetic acid and diluted to 0.1 *M* sodium acetate. While the coupling solution consisted of acetonitrile–pyridine–triethylamine–water (10:5:2:3, v/v) [5], to which 5 μl of 5 m*M* norvaline solution were added. A fresh vial of PITC reagent was used each day when samples were analyzed.

2.3. Standards and control solutions

Stock solutions of Phe, Tyr and I.S. norvaline, 5 m*M* and 10 m*M* (for Phe and Tyr), respectively, were prepared with deionized water. These stock solutions were good for 6 months when stored at 4 °C. The working solution was prepared from the mixture of stock standards in the ratio of 20 ml of Phe:8 ml of Tyr and brought to a final volume of 100 ml with deionized water, to give working Phe and Tyr standards of 1000 μ*M* and 400 μ*M*, respectively.

2.4. Animals and sample treatment

Four-timed pregnant Sprague–Dawley female rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). Dams were pair-fed daily. Experimental dams were fed with a high liquid diet supplemented with 3% Phe and 0.2% α-methylphenylalanine until the pups were delivered, while control dams were fed with high liquid diet only.

After a 4-day acclimation period, PKU was induced in a group of ten pups by daily subcutaneous injection of 0.1 ml of 2.4 μmol/g body mass α-methylphenylalanine and 1.2 μmol/g body mass Phe. The dosage level was administered postnatally from day 4 to day 14. Ten control pups were injected with normal saline solution (0.9% NaCl) only. The pups were sacrificed by decapitation on day 14, 2 h after the last injections. Whole blood was collected from each pup in a heparinized test tube and centrifuged at 1000 *g* for 20 min at 4 °C. After centrifugation, the plasma was removed and stored at −20 °C until HPLC analysis. Dried blood spots from both PKU-induced and control rats were prepared by saturating PKU test cards (no. 903, Schleicher and Schuell) with whole blood. The cards were air dried and stored at −20 °C until HPLC analysis.

2.5. Blood or sample preparation

The sample was deproteinized by adding 0.1 ml of 30% sulfosalicyclic acid to 1 ml of plasma [6]. The mixture was centrifuged at 2100 *g* for 20 min at 4 °C. The supernatant, containing free amino acids, was removed and derivatized. Each dried blood spot was removed using a paper punch, placed in a glass tube containing 100 μl of coupling reagent, and vortexed at 133 *g* for 60 min. The extract was removed for derivatization.

2.6. Derivatization

Stable products with minimal interference were produced by derivatization procedure. A 10 μl

volume of plasma free amino acids were dried in an extraction vacuum. Dried free amino acids were resuspended in 100 μl of coupling reagent, vortexed and redried. Residual amino acids were resuspended in 100 μl of coupling reagent, and vortexed, prior to addition of 5 μl PITC. The samples were kept at room temperature for 5 min, then dried. PTC–amino acids were reconstituted in 250 μl of 0.1 *M* sodium acetate buffer. The reconstituted PTC–amino acids were diluted with 1.25 ml of methylene dichloride and centrifuged. The clear layer was removed and analyzed by reversed-phase HPLC with UV detection at 254 nm. The process was repeated using dried blood extracts.

2.7. Quantitation

The 1100 HPLC system used for the measurement of dried blood spots and plasma Phe and Tyr of PKU rats consisted of an autosampler, quaternary pump, and LC UV detector with an emission wavelength of 254 nm. A 25-μl volume of each sample was injected into the HPLC system.

The C-18 column was equilibrated with 100% acetonitrile (solvent A) and 0.1 *M* sodium acetate buffer (solvent B). After 25 μl were injected, the column was equilibrated for 1 min. The elution was followed by a 6-min linear gradient of acetonitrile and sodium acetate (15:85, v/v), followed by 3 min at 28:72 (v/v) and 2 min for 60:40 (v/v) of the above solvents to remove late-eluting amino acids and re-equilibrate the column. The column was monitored at 254 nm with a flow-rate of 1.3 ml/min.

2.8. Calculation

The concentrations of amino acids (Phe and Tyr) were calculated using a peak area ratio of control/peak area ratio of standard. This value obtained is multiplied by the concentration of the amino acid standards (1000 μ*M* for Phe or 400 μ*M* for Tyr) yielded the concentration of specific amino acids in the plasma or in dried blood spots.

3. Results

The chromatogram of the working standards (L-Phe and L-Tyr) and the I.S. (L-norvaline) demonstrated well-resolved peaks with no interference (Fig. 1). The conditions of separation were established after confirming the retention times of Phe, Tyr and L-norvaline. The I.S. norvaline is essential to compare the peak area or the peak height of Phe and Tyr.

The linear range of the calibration curve was determined by using standard aqueous solutions of Phe and Tyr (working standard) at a ratio of 20:8, respectively, and the final volume was increased to 100 ml with deionized water. The lower limit of detection for the curve was 5000 μ*M*. In addition,

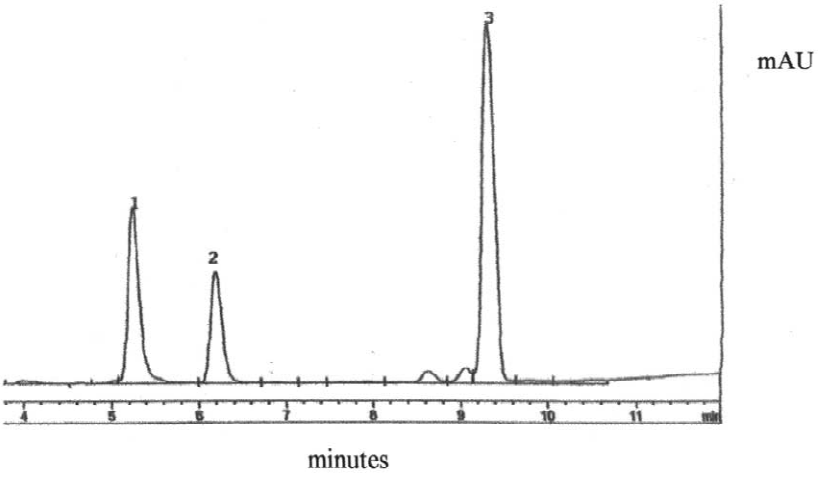

Fig. 1. Chromatographic separation of norvaline (I.S.), tyrosine and phenylalanine (standards) by HPLC. 1=Tyr, 2=Norval, 3=Phe.

the standard curve was linear to at least 10 000 μ*M* for Phe and Tyr in dry blood and plasma.

The chromatographic method used resulted in the detection of the three peaks Tyr, Phe and the I.S. norvaline. Tyr eluted the column at 5.4 min, the I.S. L-norvaline at 6.2 min and phenylalanine at 9.4 min. The chromatographic separation was complete within 10 min.

The analysis of the amino acids eluted from plasma of a control rat by HPLC is shown (Fig. 2A). This chromatogram shows that the peak height of Tyr is greater than that of Phe, suggesting that Phe hydroxylase is enzymatically active.

Fig. 2B is a chromatogram of the derivatized amino acids from the plasma of a PKU induced pup. In contrast to Fig. 2A, Fig. 2B demonstrates a greater peak height for Phe versus Tyr. The alteration in peak height indicates that Phe hydroxylase was deficient and PKU was induced in the pup.

Furthermore, dry blood Phe levels were analyzed to determine blood stability. A chromatogram of dry blood from untreated pups (Fig. 2C) parallel those observed in (Fig. 2A). The peak for Tyr is greater in height than the peak for Phe. These findings indicate that the normal biochemical process of Phe conversion is occurring. In contrast, the opposite effect is demonstrated in experimental samples (Fig. 2D). In this chromatogram, the peak height of Phe is greater than the peak height of Tyr. Therefore, Fig. 2B and 2D demonstrates that the methodology used in these studies permit induction of PKU in experimental animals.

Fig. 2B and 2D represents a comparison between the experimental results of Phe eluted from dry blood versus levels present with the plasma isolated from the blood specimen of the same PKU induced pup. In both figures, PKU was induced and illustrated a substantial peak for Phe when compared with the peaks for Tyr. The peaks of interest, as well as other PTC–amino acids, were slightly lower in height in dry blood than in plasma, suggesting that Phe in plasma is more stable than Phe in dry blood. Overall, the levels of Phe determined in dry blood and plasma are comparable.

Because the number of amino acids present in plasma and in dried in blood, gradient mobile phase system was used to measure their levels in elutes of dried blood spots. This system facilitates the analysis of Phe and Tyr, diagnosis of PKU and other inherited disorders [7,8].

However, the changeover of solvent that took place in the gradient mobile phase system resulted in baseline instability and some peak resolution in gradient HPLC chromatographies [9]. The observation in this investigation is comparable to those reported by others.

Table 1 compares plasma concentrations of Phe and Tyr among control and experimental groups of pups. The control or untreated pups expressed low levels of Phe in plasma and the concentrations were <96 μ*M*. As expected in the normal (control) samples the concentration of Tyr is greater than Phe, indicating that the enzyme is active (Table 1). In contrast, the experimental group in Table 1 illustrates the opposite effect. The significantly high concentrations of Phe and the relatively low concentrations of Tyr indicate that the normal conversion process has been interrupted. Subsequent exposure of the experimental group to an inhibition of Phe hydroxylase activity altered the biochemical pathway for the conversion of Phe to Tyr. These results are evidence that PKU was induced in the experimental pups.

Table 2 is a comparison of Phe and Tyr concentrations in dry blood samples. A higher concentration of Tyr was observed in the blood of the control group when compared with Phe levels. On the contrary, the concentration of Phe in dry blood is significantly higher when compared to the concentration of Tyr in the experimental specimens. It is likely that the concentrations of Phe and Tyr in Tables 1 and 2 fluctuate because of variations in body masses of the pups. The concentrations of Phe recorded in Table 2 are slightly lower than those in Table 1 supporting the conclusion that the stability of Phe in dry blood decreases with time [7]. Three samples from control rats and two from experimental rats were not analyzed because they were contaminated during sample preparation.

3.1. Reproducibility and calibration curve values

The calibration to determine the reproducibility of the method was carried out using standard mixtures of Phe and Tyr at different concentrations (625, 1250, 2500, 5000 and 10 000 μ*M*). The standard mixture (Tyr and Phe) was prepared in the same way

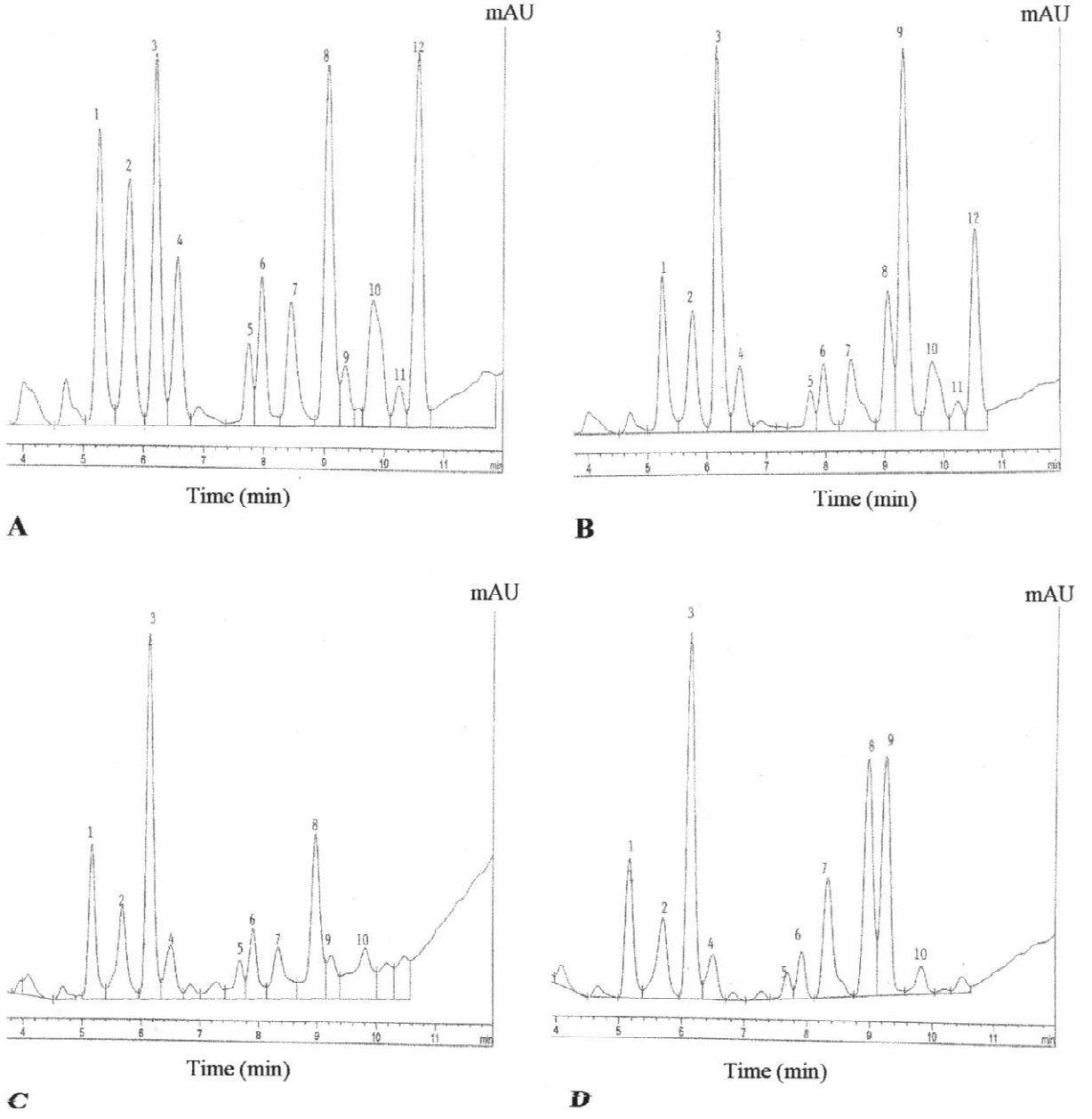

Fig. 2. (A) Chromatogram of plasma free amino acids from control pup. (B) Chromatogram of plasma free amino acids from PKU pup. (C) Chromatogram of dry blood spot from control pup. (D) Chromatogram of dry blood spot from PKU treated rat. 1=Tyr, 2=Val, 3=Norval, 4=Arg, 5=Ile, 6=Leu, 7=Lys, 8=His, 9=Phe, 10=Trp, 11=Cys, 12=Asn.

as those used for samples assayed by HPLC. The values obtained were analyzed using ANOVA. For all the amino acid levels of concentration, the inter-day CV was less than 14.8% except for Tyr at the lower concentration (15.4% for 1250 μ*M*). The inter-day levels of concentrations show that the CV was <13.3% except for tyrosine (16.7% at 1250 μ*M*) (Table 3). The method used in these analyses

Table 1
Comparison of phenylalanine and tyrosine concentrations (μM) from the analyses of plasma samples by HPLC methods from 20 pups

Control	1	2	3	4	5	6	7	8	9	10
Phe	90	75	96	86	37	56	45	32	14	63
Tyr	134	154	239	711	185	238	179	257	145	54
Experimental	1	2	3	4	5	6	7	8	9	10
Phe	404	309	672	317	377	433	407	175	272	394
Tyr	140	146	136	78	167	131	140	97	107	129

Table 2
Comparison of phenylalanine and tyrosine concentrations (μM) in dry blood samples by HPLC methods; five samples were contaminated (three control and two experimental); no distinct peaks for Phe and Tyr

Control[a]	1	2	3	4	5	6	7	8	9	10
Phe	0	119	76	35	58	0	46	*	*	*
Tyr	38	192	164	224	245	342	49	*	*	*
Experimental[b]	1	2	3	4	5	6	7	8	9	10
Phe	204	355	179	525	299	452	244	395	*	*
Tyr	103	151	113	185	125	140	88	275	*	*

*, Contaminated samples not used.
[a] $n=7$.
[b] $n=8$.

showed good linearity over the concentration ranges examined (Table 4).

3.2. Limit of detection

The HPLC 1100 series is very sensitive. The limit of detection per injection of this system is <10 μM for these amino acids. When high blood concentrations are found, simple dilution of the extract can be conducted in order to quantitate the amino acid.

3.3. Recovery

When Phe and Tyr working standard was added to normal blood sample to final concentration of 1000 μM each, dry blood spots were prepared on the filter paper and eluted. The remaining blood sample was

Table 3
Intra- and inter-day reproducibilities

Standard	Concentration (μM)	Intra-day variability ($n=10$)		Inter-day variability ($n=10$)	
		Detected (μM) (mean±SD)	RSD (%)	Detected (μM) (mean±SD)	RSD (%)
Tyrosine	625	45±6	13.3	36±2	5.6
	1250	52±8	15.4	42±7	16.7
	2500	51±4	7.8	46±6	13.0
	5000	51±5	9.8	49±6	12.2
	10 000	50±4	8.0	48±5	10.4
Phenylalanine	625	54±5	9.3	60±8	13.3
	1250	100±8	8.0	102±7	6.9
	2500	209±31	14.8	223±16	7.2
	5000	362±12	3.3	405±50	12.3
	10 000	736±31	4.2	742±80	10.8

Table 4
Calibration curve values for the method

Standard (μM)[a]	Regression equation[b]	Correlation coefficient
Plasma		
Phenylalanine	$y = 2.35907 \cdot 10^{-1}x$	0.9998
Tyrosine	$y = 4.25083 \cdot 10^{-2}x$	0.9997
Norvaline	$y = 1.0x$	0.0000
Dry blood		
Phenylalanine	$y = 3.45838 \cdot 10^{-1}x$	0.9999
Tyrosine	$y = 8.78217 \cdot 10^{-1}x$	0.998
Norvaline	$y = 1.0x$	0.0000

[a] 5000 or 10 000.
[b] x, amount ratio; y, area ratio.

centrifuged and plasma was used for plasma recovery studies. Recovery values of Phe and Tyr for blood spots and plasma were 98% and 95% for Phe and 104% and 93% for Tyr. These results support the findings of others [8,10].

4. Discussion

Newborns in the USA are screened for PKU [4]. The capillary blood used for the determination is obtained by puncture or pricking the heel or finger, applied to special paper card and air dried [7]. The techniques used in this screening include Guthrie bacterial inhibition assay (BIA) [11], tandem mass spectrometry and fluorometric analysis [12]. These established methods are not very reliable because of the potential to yield false positive results [12,13]. The Guthrie assay is semiquantitative and has a false positive rate of about 5%, coupled with the fact that its accuracy can be affected by many factors. The fluorometric microplate assay has a false rate of about 0.6% while its sensitivity is 30 μM [12]. Although the sensitivity of tandem mass spectrometry is 3 mM for Phe, its repeat and analysis rate is drastically reduced [12]. A false positive result causes unnecessary anxiety and cost to families until a definitive diagnosis is established. Such situations can be avoided by utilizing quantitative and precise tests to confirm the diagnosis and monitor PKU in patients [10]. The laboratory method investigated here has demonstrated that a quantitative analysis which requires a very small sample volume, derivatization and gradient-HPLC will produce reliable, rapid and cost effective results when compared to the three methods mentioned.

Phe stability in dried filter paper blood spots decreased when tested over a 10-day period [7]. In contrast, plasma Phe is stable [10,14]. This quality of plasma phenylalanine has been demonstrated through collection, transportation and storage of this sample before the sample is received in many PKU screening centers and laboratories in the UK and The Netherlands. It has been shown that residual plasma Phe of initial blood and registered plasma Phe collected from PKU infants under 3 weeks old have been utilized for quantitation and diagnoses instead of recalling a patient for another diagnostic visit [10,14]. This suggests that plasma Phe is stable if properly stored.

Phenylisothiocyanate (PITC) reagent was used for the precolumn derivatization of the amino acids. This reaction is performed in aqueous solutions prior to HPLC analysis. With this derivatizing agent, high resolution, increased sensitivity and stability at retention times were better achieved than with *o*-phthalaldehyde [15]. The PITC and methylene dichloride used in this method made it possible for the removal of excess and interfering reagents [8,16].

The present method could serve as a model for reliable or accurate screening of newborns for PKU in reference laboratories since the three currently used techniques have led to false positive results. Based upon our results, the peaks and molar concentrations of Phe in dried blood and plasma of PKU rats are considerably higher than those of Tyr. This suggests that degree of PAH inhibition is increased and hepatic function is impaired. These findings have the potential to lead to revisions of current procedures. Another model or a new approach to screening newborns for PKU appears feasible.

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References

[1] R. Surtees, N. Blau, Eur. J. Pediatr. 159 (2000) S109.
[2] A. Folling, Z. Physiol. Chem. 222 (1934) 167.
[3] A. Diamond, V. Ciaramitaro, E. Donner, S. Djali, M. Robinson, J. Neurosci. 14 (1994) 3072.
[4] National Institutes of Health Consensus Development Panel, Pediatrics 108 (2001) 972.
[5] J. Meulendijk, W. Underber, Chromatogr. Sci. 48 (1990) 261.
[6] T. Graser, H. Godel, Anal. Biochem. 151 (1985) 142.
[7] R. Roesel, P. Blankenship, F. Hommes, Clin. Chim Acta 156 (1986) 91.
[8] J. Rudy, J. Rutledge, S. Lewis, Clin. Chem. 33 (1987) 1152.
[9] A.D. Bruce, B.K. Yamamoto, Life Sci. 43 (1988) 913.
[10] D.A. Ngel, A. Green, Clin. Chem. 34 (1988) 2241.
[11] R. Guthrie, A. Susi, Pediatrics 32 (1963) 338.
[12] A. Clague, A. Thomas, Clin. Chim. Acta 315 (2002) 99.
[13] S.G. Natallja, I.V. Steklova, T. Tunminen, Clin. Chem. 35 (1989) 2112.
[14] P.H. Verkerk, F.J. van Spronsen, M. van Houten, G.P. Smit, R.C. Sengers, Acta Paediat. Suppl. 407 (1994) 70.
[15] M. Hariharan, S. Naga, T. VanNoord, J. Chromatogr. 621 (1993) 15.
[16] A. Donzanti, B. Yamamoto, Life Sci. 43 (1988) 913.