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Short communication

# Simultaneous diagnostic method for phenylketonuria and galactosemia from dried blood spots using high-performance liquid chromatography-pulsed amperometric detection

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ABSTRACT

We developed a simultaneous diagnostic method for phenylketonuria (PKU) and galactosemia through simultaneous determination of phenylalanine (Phe) and galactose (Gal) by high-performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD). The intra- and inter-day precisions were <5.8%, with satisfactory mean recoveries (98.2–105%). For all PKU-positive samples, Phe levels were above the cut-off value (>30.0 mg/L), but Gal levels were nearly zero. For 77% of galactosemia-positive samples, Phe levels were above the cut-off value, but Gal levels were above the cut-off value (>80.0 mg/L) for all samples. Our HPLC-PAD method can reduce the false-positive rate of misdiagnosis for PKU and galactosemia.



## 1. Introduction

Inherited metabolic disorders (IMDs) lead to the accumulation of toxic metabolites inducing severe irreversible disorders. Especially, phenylketonuria (PKU) and galactosemia are representative IMDs found in newborn [1]. PKU is an autosomal recessive genetic disorder characterized by an enzyme deficiency of phenylalanine hydroxylase. PKU has three types (classical PKU, mild PKU, and non-PKU hyperphenylalaninemia), with a frequency of 1:4500–9000 in Caucasian populations. Untreated PKU with high phenylalanine (Phe) level in blood leads to permanent damage in central nervous system [2]. Galactosemia, a carbohydrate metabolic disorder, also has three types (galactose 1-phosphate uridyltransferase [GALT], galactokinase, and uridine diphosphate galactose 4-epimerase deficiencies), with a frequency of 1:23,000–44,000 in Caucasian populations. Infants with GALT deficiency result in the accumulation of galactose (Gal) in blood with associated liver disease, cataracts, kidney problems, and brain damage [3].

In a previously published paper [4,5], some newborns were misdiagnosed as PKU-positive because of their high levels of Phe in blood; their condition became progressively worse under treatment for PKU. These infants were subsequently diagnosed as galactosemia-positive through a second screening test. Therefore, simultaneous diagnosis for PKU and galactosemia would be ideal for reducing the false-positive result because galactosemia patient samples sometimes contain elevated levels of Phe.

To date, several methods have been reported for separately diagnosing PKU [6–10] or galactosemia [11–14], and a fluorometric method has been used for simultaneous diagnosis of PKU and galactosemia [15]. Here, we introduce a HPLC-PAD-based new method for simultaneous diagnosis of PKU and galactosemia. This method simultaneously measures the Phe and Gal levels in blood spots in order to reduce the false-positive result of diagnosis for the two disorders. Furthermore, we compared the false-positive results by our method to the data obtained by enzymatic colorimetric method

*Abbreviations:* IMD, inherited metabolic disease; PKU, phenylketonuria; GALT, galactose 1-phosphate uridyltransferase; PAD, pulsed amperometric detection; ECM, enzymatic colorimetric method.

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(ECM) [7,11] in order to compare its efficiency, practicality and importance.

## 2. Materials and methods

### 2.1. Reagents

Gal, glucose (Glc), mannose (Man), Phe, and fucose (Fuc) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Acros (Geel, Belgium). Sodium carbonate ($Na_2CO_3$) was purchased from Fluka (Steinheim, Germany). Fructose (Fru) was purchased from TCI (Tokyo, Japan). Sodium acetate (NaOAc) and 50% sodium hydroxide (NaOH) solution were purchased from Fisher Scientific (Fairlawn, NJ, USA). The other reagents and solvents used were of guaranteed or analytical grade. Water used to prepare the solutions was purified by using an Automatic Aquarius AW-1001 (Top Trading, Seoul, South Korea) and solvents were filtered through a Millipore membrane filter (type HA, pore size 0.45 μm, Billerica, MA, USA). Before injection, all samples were passed through a disposable syringe filter (PTFE membrane filters, pore size 0.20 μm, Advantec MFS, Tokyo, Japan).

### 2.2. HPLC

The HPLC equipment consisted of a model Nanospace SI-2/3201 dual pump, and a 3203 autosampler with a 2.5 μL fixed loop (Shiseido, Tokyo, Japan). The PAD system (ICS-3000 series, Dionex, Sunnyvale, CA, USA) was equipped with an Au-Flow cell containing a gold working electrode, a stainless steel auxiliary electrode, and an Ag/AgCl reference electrode. The potential waveform was as follows: E1 = −0.20 V (from 0.00 to 0.04 s); E2 = 0.00 V (from 0.05 to 0.21 s); E3 = +0.22 V (from 0.22 to 0.46 s); E4 = 0.00 V (from 0.47 to 0.56 s); E5 = −2.00 V (from 0.57 to 0.58 s); and E6 = +0.60 V (0.59 s). Data collection was carried out by using the Chromeleon program (Dionex). Chromatographic separation was performed by using an anion-exchange column (Carbopac PA1, 250 mm × 2.0 mm I.D.) connected to a guard column (Carbopac PA1 Guard, 50 mm × 2.0 mm I.D.; Dionex). The elution profiles with gradients of 10 mM NaOH + 3 mM NaOAc (eluent A) and 300 mM NaOH + 25 mM $Na_2CO_3$ (eluent B) were as follows: isocratic elution with eluent A for 3 min, linear gradient elution with eluents A:B in ratios of 100:0 to 0:100 for 1 min, isocratic elution with eluent B for 4 min, linear gradient elution with eluents A:B in ratios of 0:100 to 100:0 for 0.1 min, then equilibration with eluent A for 15 min. The flow rate was 0.25 mL/min and the separation temperature was 30 °C. The injection volume was 2.5 μL. The mobile phase was made on a daily basis, sonicated for 20 min before use, and purged with helium throughout the experiment.

### 2.3. Blood spot specimens

The certified standard blood spots (four different concentrations; S1–4) were purchased from a commercial kit (Quantase™ Neonatal Phe/Gal Screening Assays, Bio-Rad, Hercules, CA, USA). The standard concentrations (mg/L) of Phe and Gal, respectively, were as follows: S1 was 12.3 and 46.7, S2 was 40.2 and 105.9, S3 was 68.4 and 204.8, and S4 was 144.3 and 534.6.

Normal blood samples collected on filter paper within 3 days of birth from 300 Korean newborn babies were kindly provided by the Seoul Medical Science Institute (Seoul, South Korea), a national repository laboratory of samples obtained from general hospitals. The blood samples showing 8 PKU-positive or 13 galactosemia-positive by ECM were also collected from newborns (from the Soonchunhyang University Hospital, Seoul, South Korea for PKU, and from Seoul Medical Science Institute for galactosemia). The blood spots were stored at −20 °C before analysis.

### 2.4. Sample pretreatment

Dried blood spots on filter paper were prepared by punching out a 3.2 mm-diameter circle, corresponded to 3 μL of whole blood. A 3.2 mm-diameter filter paper disc and the internal standard (5 μL of 200 μM Fuc) were placed in each well of a microplate with conical bottoms and Phe and Gal were extracted with 85 μL of 1% (w/v) TCA by shaker for 30 min. For deproteinization, 10 μL of 10% (w/v) TCA was added to the samples, and then the samples were centrifuged for 10 min at 12,400 × *g*. The 2.5 μL of supernatants were subsequently injected into the HPLC system.

### 2.5. Recovery, precision, and accuracy

To determine the precision and accuracy of the method, recovery tests were performed with the blood spot standards containing Phe and Gal ($n = 5$). To determine the inter-day variation, the levels of Phe and Gal in the blood spot standards were measured on five consecutive days.

### 2.6. Method comparison

We compared our HPLC-PAD method to ECM. The test samples were created by the following procedure: standard stock solutions were prepared by putting different concentrations of Phe and Gal into blood from a healthy normal male adult, and the final concentrations of Phe and Gal in the blood were 10.0, 25.0, 50.0, 100.0, 200.0 and 500.0 mg/L. Standard blood spots were produced by pipetting 50 μL of the blood containing each compound concentration onto filter paper (type 903; Schleicher & Schuell). The standard blood spots were dried overnight at room temperature and stored in a zippered plastic bag at 4 °C. The means derived from five independent determinations of each concentration were used to compare the two methods.

## 3. Results and discussion

### 3.1. Optimum analytical conditions for Phe and Gal

Sugars and amino acids are separated through anion-exchange column under high pH condition. The simultaneous determination of sugars and amino acids using HPLC-PAD was carried out by Carbopac PA1 (250 mm × 2.0 mm I.D.), an anion-exchange column suitable for sugars. The chromatogram showed a stable baseline with rapid elution of Phe and high resolution between Gal and Glc peaks (Fig. 1A). Simultaneous determination required a gradient elution system, because Phe and Gal show quite different retention behaviors. This gradient elution system employed eluent A (10 mM NaOH + 3 mM NaOAc) and eluent B (300 mM NaOH + 25 mM $Na_2CO_3$). In this eluent condition, Phe, Gal and internal standard (Fuc) were detected within 8 min (Fig. 1A). The running time for each sample was less than 24 min, including the equilibration time.

We tested whether residential sugars (Glc, Man, Fru) or 40 free amino acids interfered Phe and Gal detection. The peaks of Phe, Gal and internal standard did not overlap with any of the similar sugars (Fig. 1B) or the free amino acids in the blood (Fig. 1C). Especially, the separation between Gal and Glc is very important factor in reducing a rate of false-positive errors, because Glc is usually contained in the blood at a level 20 times higher than Gal, and has the similar structure with Gal, epimer. Our method produced a clear baseline separation of Gal and Glc with high resolution ($Rs = 1.85$) (Fig. 1B).

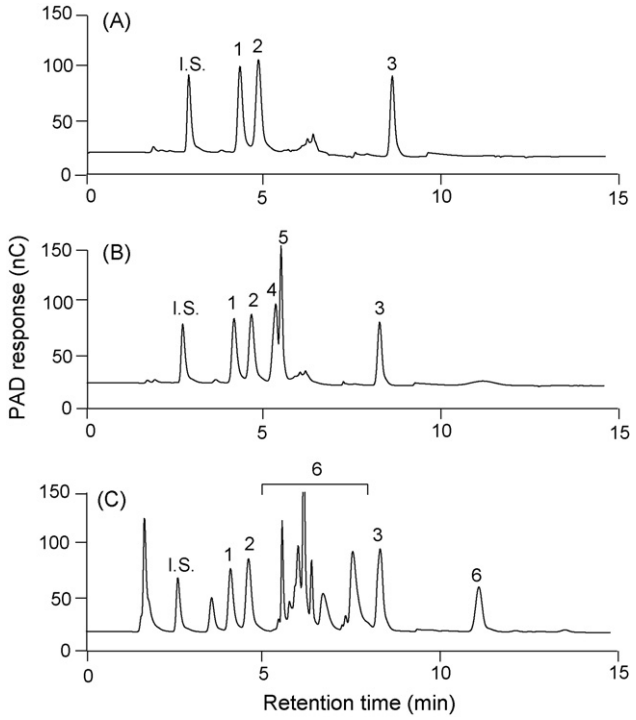


**Fig. 1.** Separation chromatograms of Phe, Gal and internal standard (A), separation with other sugars (B) and amino acids (C). Peaks: (1) Gal; (2) Glc; (3) Phe; (4) Man; (5) Fru; (6) free amino acids mixture; (I.S.) Fuc.

There are three different types of waveforms for PAD; triple-potential, quadruple-potential, and six-potential. The triple- and quadruple-potential waveforms have been used for carbohydrates [16]. The quadruple-potential waveform has better reproducibility than the triple-potential waveform because products from the oxidation of carbohydrate can be cleaned off the electrode at a high negative potential [16]. The recently developed six-potential waveform has been used for amino acids or amino sugars [17]. We tested the three types of waveforms to find the most suitable mode for Phe and Gal.

### 3.2. Linearity

In order to evaluate our HPLC method, we tested the level of Phe and Gal contained in four certified blood spot standards (S1–4). Calibration curves were constructed using the concentration range of 12.3–144.3 mg/L for Phe and 46.7–534.6 mg/L for Gal. Linearity was defined by the following equations: $y = 0.0575x - 0.0130$ and $r^2 = 0.9996$ for Phe, $y = 0.0438x - 0.0083$ and $r^2 = 0.9999$ for Gal. For both analytes, $y$ represented the peak area ratio (analytes/internal standard), and $x$ represented the concentration of the analyte. One disc (3 μL) from each blood spot was diluted approximately 33 times before the HPLC analysis, and the injected amount of each sample was 0.92–10.82 ng for Phe and 3.50–40.10 ng for Gal. The limits of detection (LOD) and limits of quantification (LOQ), from the blood spots were 180 (72 μg/L) and 600 pg (240 μg/L) for Phe and 90 (36 μg/L) and 270 pg (108 μg/L) for Gal, with signal-to-noise ratios of 3 and 10, respectively.

### 3.3. Recovery, precision, and accuracy

The recovery of Phe and Gal from blood spot standards (12.3, 40.2, 68.4 and 144.3 mg/L for Phe, and 46.7, 105.9, 204.8 and 534.6 mg/L for Gal) for an intra- and inter-day assay is summarized

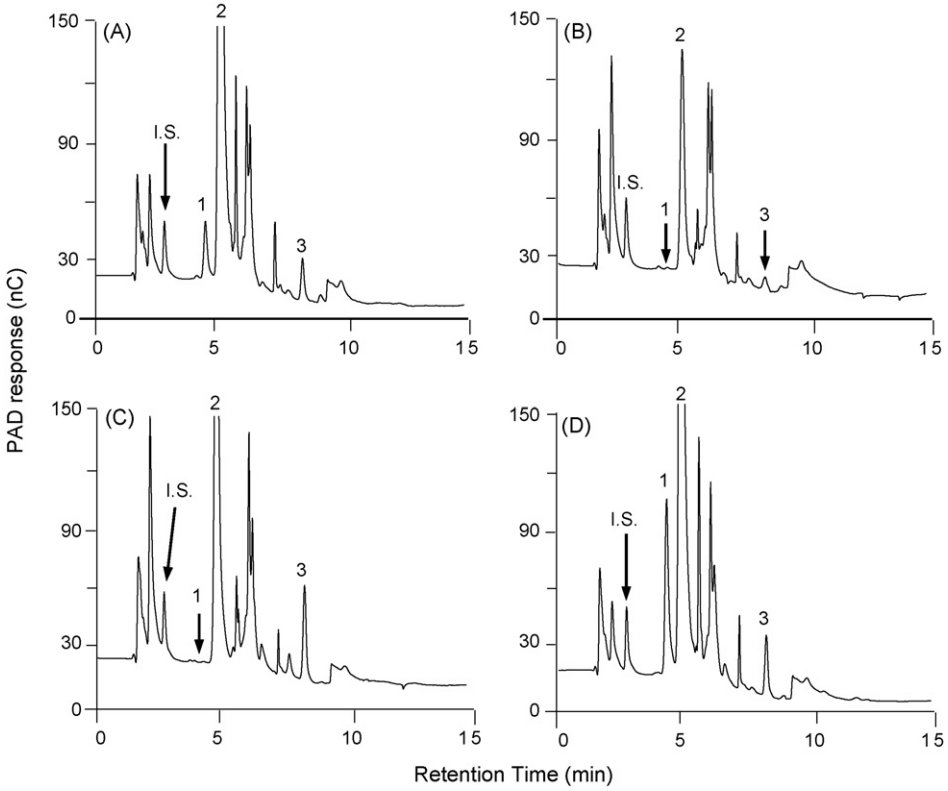


**Fig. 2.** Typical chromatograms of blood spot specimens. (A) Standard blood spot containing Gal (105.6 mg/L) and Phe (40.2 mg/L), (B) normal blood spot (4.1 mg/L Gal, and 13.0 mg/L Phe), and patients blood spots of (C) PKU (Gal; not detected, and 164.5 mg/L Phe,) and (D) galactosemia (319.7 mg/L Gal, and 116.5 mg/L Phe). The concentrations were obtained by HPLC-PAD. Peaks: (1) Gal; (2) Glc; (3) Phe; (I.S.) Fuc.

**Table 1**
Intra-day and inter-day variations of Phe and Gal in standard blood spots.

| Compounds | Added amounts (mg/L) | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|---|
| | | Mean ± SD | RSD (%) | Mean recovery (%) | Mean ± SD | RSD (%) | Mean recovery (%) |
| Phe | 12.3 | 12.1 ± 0.7 | 5.8 | 98.7 | 12.3 ± 0.4 | 3.3 | 99.9 |
| | 40.2 | 41.3 ± 0.5 | 1.3 | 102.0 | 40.5 ± 0.6 | 1.6 | 101.0 |
| | 68.4 | 67.1 ± 1.1 | 1.7 | 98.2 | 68.5 ± 0.5 | 0.7 | 100.0 |
| | 144.3 | 144.6 ± 0.5 | 0.3 | 100.0 | 144.4 ± 0.3 | 0.2 | 100.0 |
| Gal | 46.7 | 48.1 ± 1.7 | 3.6 | 103.0 | 49.1 ± 1.7 | 3.4 | 105.0 |
| | 105.9 | 106.2 ± 1.7 | 1.6 | 100.0 | 105.4 ± 1.4 | 1.3 | 99.6 |
| | 204.8 | 202.5 ± 2.6 | 1.3 | 98.9 | 201.9 ± 3.1 | 1.5 | 98.6 |
| | 534.6 | 535.5 ± 0.7 | 0.1 | 100.0 | 535.6 ± 0.9 | 0.2 | 100.0 |

**Table 2**
Method comparison between HPLC-PAD and ECM.

| Phe and Gal conc. (mg/L) | HPLC-PAD[a] | | | | ECM[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Phe | | Gal | | Phe | | Gal | |
| | Found amount | Recovery (%) | Found amount | Recovery (%) | Found amount | Recovery (%) | Found amount | Recovery (%) |
| 10.0 | 9.8 | 97.5 | 10.5 | 106.0 | 23.0 | 230.0 | 17.0 | 170.0 |
| 25.0 | 25.2 | 101.0 | 24.6 | 98.3 | 36.0 | 144.0 | 43.0 | 172.0 |
| 50.0 | 46.9 | 93.8 | 47.3 | 94.6 | 35.0 | 70.0 | 43.0 | 86.0 |
| 100.0 | 101.4 | 101.0 | 99.0 | 99.0 | 110.0 | 110.0 | 94.0 | 94.0 |
| 200.0 | 203.0 | 102.0 | 204.9 | 103.0 | 181.0 | 90.5 | 146.0 | 73.0 |
| 500.0 | 498.9 | 99.8 | 498.3 | 99.7 | 383.0 | 76.6 | 299.0 | 59.8 |
| $r^2$ | | 0.9999 | | 0.9998 | | 0.9899 | | 0.9893 |
| Mean ± SD | | 99.1 ± 3.0 | | 99.9 ± 3.7 | | 120.0 ± 60.1 | | 109.0 ± 49.3 |

[a] High-performance liquid chromatography with pulsed amperometric detection.
[b] Enzymatic colorimetric method.

in Table 1. The ranges of intra-day precisions from the samples S1–4 were 98.2–102.0% (CV < 5.8%) for Phe and 98.9–103.0% (CV < 3.6%) for Gal, respectively. For the inter-day analysis, the mean recovery ranges for Phe and Gal were 99.9–101.0% (CV < 3.3%) and 98.6–105.0% (CV < 3.4%), respectively.

### 3.4. Analysis of normal, PKU-positive, and galactosemia-positive blood samples

We analyzed newborn blood spots from 300 normal, 8 PKU-positive, and 13 galactosemia-positive. The Phe and Gal cut-off values for PKU and galactosemia were 30.0 and 80.0 mg/L, respectively [8,13]. Fig. 2 shows typical chromatograms for simultaneous analysis of Phe and Gal in blood spot. The analytical time required per sample was 8 min. The detection of changes on the peak intensities of Phe and Gal in standard blood spot (A), normal (B), PKU-positive (C), and galactosemia-positive (D) samples clearly indicates the validity for the clinical diagnosis of PKU and galactosemia. As shown in Fig. 3, Phe and Gal levels in the blood from normal newborns were below 30.0 and 80.0 mg/L for Phe and Gal, respectively. For blood samples from the PKU-positive newborns, Phe levels were above the cut-off value, and Gal levels were nearly zero in all tested 8 samples. These results suggested that the measurement of blood levels of Gal and Phe are critical factors in determining PKU. For blood samples from the galactosemia-positive newborns, the Phe levels were above the cut-off value (>30.0 mg/L) for 10/13 samples, and Gal levels were above the cut-off value (>80.0 mg/L) for all 13 samples. Because galactosemia-positive patients show elevated Phe levels by liver damage, these patients are sometimes misdiagnosed as PKU-positive based on the Phe levels measured. Both Phe and Gal levels in a newborn are very important factors for diagnosing PKU or galactosemia.

To our best knowledge, there is no report for performing simultaneous tests for the two metabolic disorders, PKU and galactosemia. Most newborn screening laboratories relied on the independent data from each PKU and galactosemia diagnostic method. Therefore, our method, monitoring Phe and Gal simultaneously will be practically useful for identifying PKU from galactosemia by a single run.

### 3.5. Comparative evaluation

We analyzed six different concentrations of blood spot standards and compared the results to data obtained by ECM (Table 2). ECM showed reasonable linearity ($r^2$ = 0.9899 and 0.9893 for Phe and Gal, respectively) and poor accuracy and precision for quantifi-

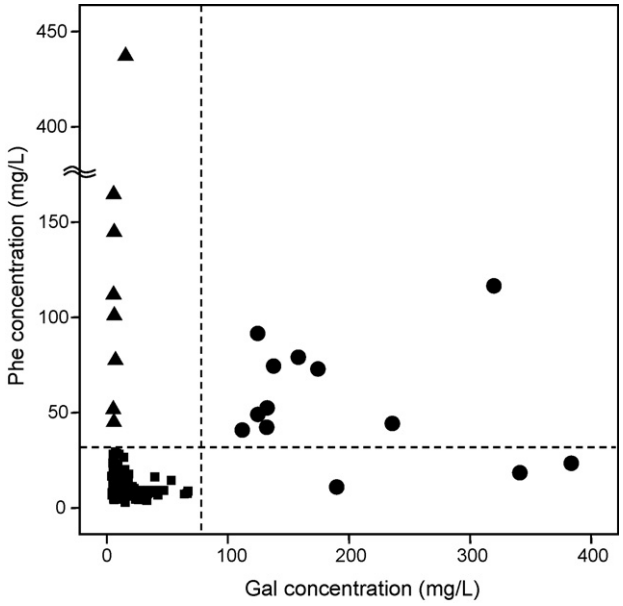


**Fig. 3.** Distribution maps of normal and patient samples in PKU and galactosemia. Dashed line means cut-off value of Galactosemia (80.0 mg/L Gal) and PKU (30.0 mg/L Phe). Symbols indicates normal (■), PKU (▲) and galactosemia (●).

cation of Phe and Gal in low concentrations (10.0–25.0 mg/L). The recovery ranges in ECM method were 70.0–230.0% and 59.8–172.0% for Phe and Gal, respectively, with mean standard deviations of 60.1% and 49.3%. Our HPLC-PAD method showed excellent linearity ($r^2$ = 0.9999 and 0.9998 for Phe and Gal, respectively) with high accuracy and precision for quantification of Phe and Gal in blood spots in low concentrations. The recovery ranges were 93.8–102.0% and 94.6–106.0% for Phe and Gal, respectively, with mean standard deviations of 3.0% and 3.7%. Comparison of the two methods enabled us to reconfirm our HPLC-PAD method more accurate than ECM.

## 4. Conclusions

We developed a simultaneous diagnostic method for PKU and galactosemia through simultaneous determination of Phe and Gal in blood by HPLC-PAD. The anion-exchange column suitable for sugar (Carbopac PA1) gave an optimal condition on simultaneous analysis of Gal, Glc and Phe. The total running time for each sample was less than 24 min, including the equilibration time. This method can make minimal the false-positive rate of misdiagnosis for PKU and galactosemia, with high selectivity, sensitivity, and repeatability.

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