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Post-column detection by on-line photolysis–electrochemistry in liquid chromatography: application to the determination of phenylalanine in human urine*

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Abstract: On-line, post-column photochemical derivatization for liquid chromatography–electrochemical detection (LC–hν–EC) has been employed for the analysis of phenylalanine in urine. The method is highly sensitive and selective. The quantitative results show good precision and reproducibility. It can be used easily and has the clinical use of diagnosing phenylketonuria disease.

Keywords: *Phenylalanine determination; phenylketonuria; HPLC; electrochemical detection; photolysis of phenylalanine.*

Introduction

Although it is always present in human plasma and urine in a small amount, the concentration of phenylalanine (Phe) in phenylketonuria (PKU) patients is significantly higher than that in a normal person. The analysis of Phe using liquid chromatography (HPLC) for the diagnosis of PKU has been reported previously [1, 3–8]. Of course, the dedicated modern amino acid analyser based on an ion-exchange separation model could be used for this purpose, but the instrument is expensive and the analysis is time-consuming. The application of rapid, highly efficient reversed-phase liquid chromatography (RPLC) in clinical practice has the advantages of being simple, inexpensive and rapid. However, detection methods used in these determinations can be improved. UV detection for the determination of Phe is insensitive, because the extinction coefficient of Phe at 254 nm is only 200 [1]. The use of 210 nm can improve the sensitivity, but is limited due to the strong absorbances of interferences (selectivity of the detection is also reduced), especially in the analysis of plasma or urine samples. Post or pre-column derivatization using the OPA/thiol reagent for FL detection, combined with RPLC separation, provided very sensitive determinations of Phe, but the reproducibility in the quantitation is a problem because of FL quenching of the derivatives [2]. This problem could be even more serious if complex biological fluids were analysed. A stable, simple detection method with high sensitivity and selectivity would be valuable for the determination of Phe in clinical diagnosis.

On-line, post-column photolysis of chromatographic eluents for improved electrochemical detection (hν–EC) has been used for the detection of many classes of compounds and proved to be an easy and efficient means for LC detection [9–14]. The use of RPLC–hν–EC for the analysis of aromatic amino acids and aromatic amino acid containing peptides has been carried out with high sensitivity (same or better than FL detection) and improved selectivity provided by the lamp-on/off EC response differences [14]. It is clear that this work can be immediately employed for the detection of Phe in urine samples for PKU diagnoses. In this paper, the determination of Phe in urine by a RPLC–hν–EC method will be presented with typical chromatograms and quantitative results.

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Experimental

Apparatus

The instrumentation was composed of three parts: a conventional LC system, a photolysis unit, and a conventional amperometric–electrochemical (EC) detector as described in previous papers [9]. The LC system consisted of a Waters solvent delivery pump (model 590, Waters Corp., Milford, MA, USA), a Rheodyne model 7010 injection valve with a 20-μl sample loop (Rheodyne Corp., Cotati, CA, USA) and a LiChrospher C_{18} reversed-phase column (4 × 125 mm) (E. Merck GmbH, Darmstadt, FRG). The post-column, on-line photolysis unit was made of a low pressure mercury lamp as the UV light source (model 816 UV batch irradiator, Photronix Corp., Medway, MA, USA), a knitted open tubular (KOT) reactor knitted with 0.5-mm i.d. × 1.6-mm o.d. Teflon tubing (Alltech Assoc., Deerfield, IL, USA) wrapped around the UV light source, and a power supply accompanying the Photronix batch irradiator. Cyclic voltammetry was carried out by using a CV-1B cyclic voltammograph (Bioanalytical Systems Inc.) with a glassy carbon working electrode, a Ag/AgCl reference electrode and a Pt auxiliary electrode. The EC detector was a model LC-4B dual electronic controller with a dual glassy carbon working electrode, a stainless steel auxiliary electrode, and a Ag/AgCl reference electrode, all obtained from Bioanalytical Systems, Inc. (West Lafayette, IN, USA). A Brown, Boveri and Co. (BBC) model SE 120 dual pen chart recorder (Brown, Boveri and Company, Goerz/Metrawatt Division, Vienna, Austria) and a Hitachi model D-2000 chromatographic integrator (EM Science, Inc., Cherry Hill, NJ, USA) were used for the recording of the chromatograms.

Chemicals and reagents

Reagent grade sodium hydrogen phosphates (monobasic and dibasic) and certified 50% (w/w) sodium hydroxide solution were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). HPLC-grade methanol used in the mobile phase and sample preparation was obtained from EM Science, Inc. (Cherry Hill, NJ, USA), as the Omnisolv™ grade. L-Phenylalanine was obtained from Sigma Chemical Co. (St. Louis, MO, USA) as Sigma grade and used as a standard. Deionized water was prepared in our laboratory using a Barnstead water purification system (Sybron Corp., Boston, MA, USA).

Chromatographic and electrochemical detection conditions

The mobile phase used for isocratic elution was a 0.5:99.5 (v/v) MeOH/phosphate buffer solution. The phosphate buffer was prepared by dissolving 3.0 g Na_2HPO_4 and 3.0 g NaH_2PO_4 in 1 l water (pH 6.8). At times, MeOH–phosphate buffer (1:99, v/v) was also used as a mobile phase. All LC solvents were filtered before use through a Millipore HAWP 0.45-μm filter (Millipore Corp., Bedford, MA, USA) and degassed under vacuum with stirring. The flow rate was 1.0 ml min^{-1}.

The applied potentials for the determinations were +0.72 V and +0.80 V (dual glassy carbon working electrodes). The potentials for the hydrodynamic voltammetry of Phe were varied from +0.30 to +1.20 V. The cyclic voltammograms were obtained in the first run of the scan. The scan was first from 0.00 V to positive +1.35 V, reversed to −0.30 V, and then back to 0.00 V.

Sample preparation

The urine samples were freshly collected and immediately injected after treatment. To 15 ml urine, 3 ml NaOH solution 50% (w/w) was added and the mixture was centrifuged at 7000*g* for 5 min. Then, to the 10 ml supernatant, 10 ml of MeOH was added. The solution was filtered through a 0.22-μm membrane filter. The total dilution of the urine sample was 1:2.4. The final clear solution was analysed by direct injection into the column. The standard solution of Phe was prepared by dissolving Phe in the mobile phase or in a mixture of dilute aqueous sodium hydroxide and methanol.

Results and Discussion

Hydrodynamic and cyclic voltammetry of Phe

The applied potential for hν–EC determination was chosen by performing hydrodynamic voltammetry for the standard Phe solution using post-column photolysis–EC detection at different applied potentials ranging from +0.30 to +1.20 V (with RP column online). Hydrodynamic voltammograms were obtained by plotting the EC current against the applied potential. The hydrodynamic voltam-

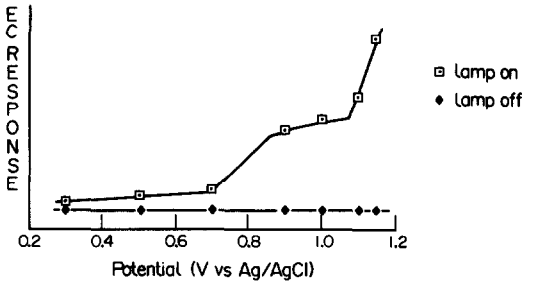

Figure 1
Hydrodynamic voltammograms of phenylalanine. Chromatographic conditions: column, LiChrospher C_{18} (5μm, 125 × 4 mm, i.d.); mobile phase, methanol–phosphate buffer (3 g Na_2HPO_4 + 3 g NaH_2PO_4 in 1 l of water) (1:99, v/v); flow rate, 2.5 ml min^{-1}; injection volume, 20 μl; post-column reactor (KOT), 2.5 ml. Electrochemical detection conditions: glassy carbon working electrode versus Ag/AgCl.

mograms recorded under both lamp-off and lamp-on conditions are shown in Fig. 1. Phe had no inherent oxidative EC activity below +1.2 V (Fig. 1). When the lamp was off the maximum tolerated potential of the glassy carbon electrode was +1.30 V. On the other hand, sensitive EC detection could be gained when the lamp was on, if the proper potential was used. From the lamp-on voltammogram (Fig. 1), the optimal applied potential for the Phe standard solution sample should be more than +0.90 V, since the EC response reached a plateau after +0.90 V. The applied potential for urine sample determination, however, should be lower than that for the standard sample due to the matrix effect. So the potentials for urine samples were chosen as +0.80 and +0.72 V (dual glassy carbon electrodes). The cyclic voltammetries of Phe were also studied under both lamp-off and lamp-on conditions, shown in Fig. 2, using a glassy carbon electrode. Phe showed no EC signals without irradiation. However, it did show an EC oxidative peak after photolysis. Also, the CV peak height changed with the irradiation time, but it did not show an increase in peak height when 5 min or longer irradiation times were employed. This agreed with the results of a residence–time study, in which the maximum EC responses were found to correspond to 1.5–2 min photolysis.

Chromatographic separation and hν–EC detection of urine sample

The chromatograms of a urine sample obtained under both lamp-off and lamp-on conditions are shown in Fig. 3. In Fig. 4, the addition of standard Phe solution to the urine sample increased the peak height of Phe in the urine. This was suggestive of the presence of Phe in the normal volunteer. Both Phe peaks in Figs 3 and 4 were compared with the Phe peak resulting from the injection of standard Phe solution, in terms of the retention time for further confirmation of the Phe peak. More than this, another comparison was made for urine samples and standard Phe solutions in terms of the EC response differences under lamp-off and lamp-on conditions. In Fig. 5, the chromatograms for standard Phe solutions

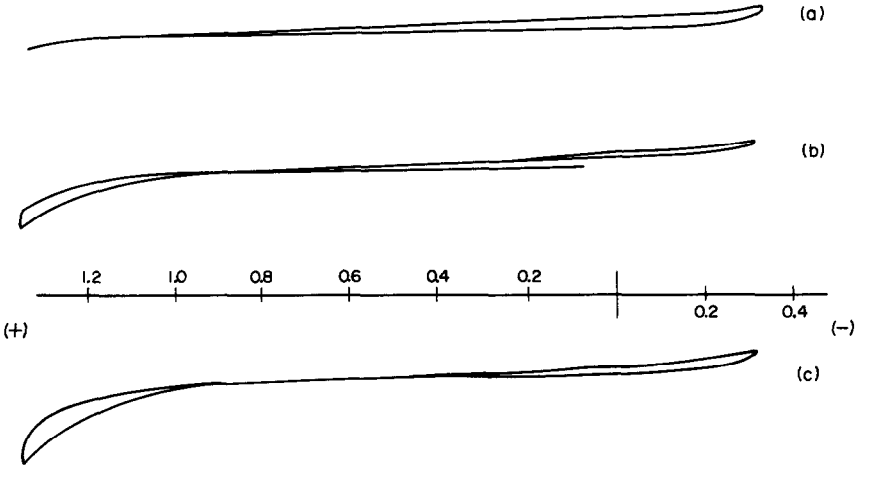

Figure 2
Cyclic voltammograms of phenylalanine. Scan rate, 300 mV s^{-1}; glassy carbon working electrode, Ag/AgCl working electrode, Pt auxiliary electrode. Phenylalanine concentration, 1 mg ml^{-1} in methanol–phosphate buffer (3 g Na_2HPO_4 + 3 g NaH_2PO_4 in 1 l of water) (1:99, v/v). (a) No irradiation; (b) 1 min irradiation; (c) 2 min irradiation.

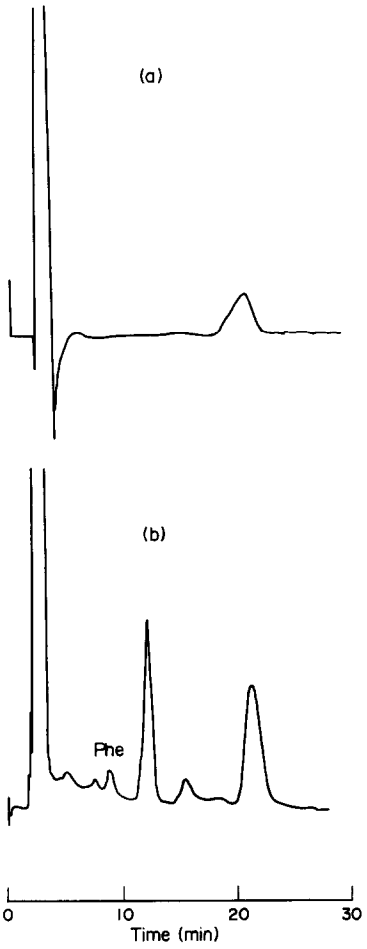

Figure 3
Chromatogram of human urine by RPLC–hν–EC. Conditions as in Table 1. (a) Lamp off, (b) lamp on.

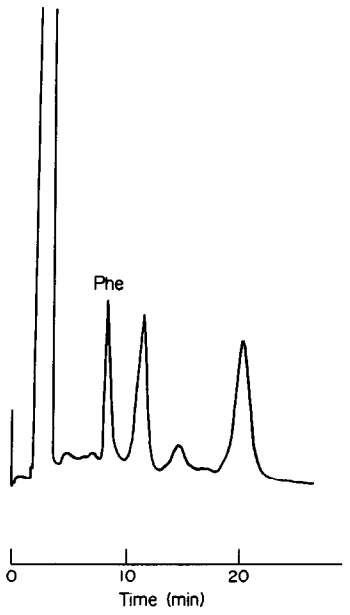

Figure 4
Chromatogram of human urine by RPLC–hν–EC after spiking with Phe. Conditions as in Table 1.

were obtained under the same conditions as in Fig. 3. The Phe peak disappeared when no photolysis was performed (Fig. 5a), although it could be detected under lamp-on conditions (Fig. 5b). This was also true in the case of a urine sample (Fig. 3). This was further evidence that the small peak in Fig. 3b was Phe. Actually, several authors have reported the appearance of Phe peak in the chromatograms for urine and plasma samples under the same elution conditions (MeOH–phosphate buffer) used here [1, 7].

The separation of Phe from other peaks was achieved using isocratic elution. Most of the inorganic and organic compounds in the urine were either precipitated during the sample preparation or strongly retained on the precolumn or analytical column. Other strong ionic species in the sample eluted in the solvent front. The peaks seen on the chromatograms

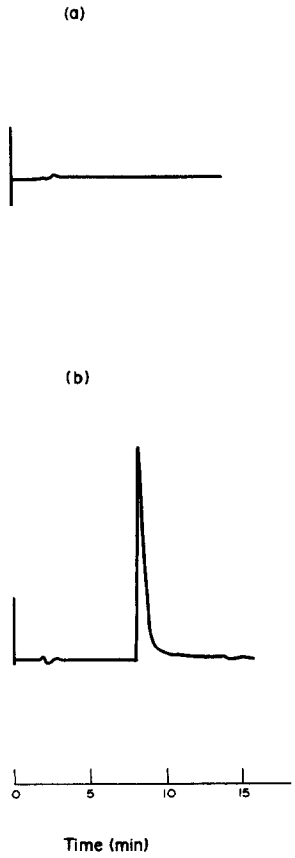

Figure 5
Chromatogram of standard Phe by RPLC–hν–EC. Conditions as in Table 1. (a) Lamp off, (b) lamp on.

under the mobile phase conditions used were only some more polar substances (but not polar enough to be eluted in the solvent front). Phe was one of them and was well retained and resolved from other peaks with reasonable retention time and peak shape. Washing the column with strong solvent after a period of use, or changing the pre-column, therefore is recommended to remove the retained species in urine from the column.

Analytical figures of merit for Phe using LC–hν–EC

In Table 1, the standard deviation (SD) is listed with dual electrode response ratios for a Phe solution (dissolved in H_2O–NaOH–MeOH). All EC responses were measured as peak heights with three repeat injections (n = 3). That RSDs were within 1.5% indicated the good precision of the method. The linear response range for Phe using LC–hν–EC detection was from 90 ng ml^{-1} to 180 μg ml^{-1}, 3.3 orders of magnitude, with r^2 = 0.9993. The detection limit was 90 ng ml^{-1} or 10.9 pmol injected (S/N = 3), which is comparable to, or better than, FL detection. The dual electrode response ratios (RR) for different sample concentrations were obtained by monitoring the Phe standard solutions at two different potentials (+0.72 and +0.80 V) and taking the ratio of these responses (peak heights). The RR values, taken within the linear response ranges, were essentially constant (the differences were within experimental error). Beside the selectivity provided by the chromatographic separation, the choice of different applied potentials, and lamp-off/lamp-on conditions, the dual electrode response ratio offers another mode for selectivity, as in conventional EC detection. The RR value is unique for each analyte separated by chromatography and can be used for the identification of analytes. For example, the RR value (at potentials of +0.72 and +0.80 V) of Phe peak in a urine sample was found to be the same as RR values listed in Table 1 for standard Phe, that was 1.50 with an RSD <2% (n = 3).

Method validation was confirmed by the analysis of single-blind spiked phenylalanine in the H_2O–NaOH–MeOH (5:1:6, v/v) medium, because NaOH–MeOH were used for the sample treatment. In Table 2, the comparison of found concentrations and actual spiked ones is shown. Three single blind spiked Phe samples were analysed with three repeat injections for each sample, having good reproducibility. The relative errors for the determinations were within 1%, suggesting good accuracy of the method.

Quantitation of Phe in urine

Standard addition was used for the quantitation of Phe in urine to reduce errors caused by the matrix effect. Two different volumes of 1 mg ml^{-1} standard Phe solution (0.1 and 0.2 ml) were used for the addition (5.0 ml urine sample). Three repeat determinations were made for each sample used. The total number of determinations was six. In Table 3, the calculated results for the concentration of Phe in the urine samples (after dilution) are

Table 1
Summary of dual electrode response ratios*

ppm	Peak height 0.72 V (SD)	0.80 V (SD)	Response ratio (SD)
5.0	15.3 ± 0.2	22.8 ± 0.1	1.49 ± 0.02
20.0	67.0 ± 0.8	101.9 ± 0.6	1.52 ± 0.01
70.0	233.7 ± 0.8	356.8 ± 4.8	1.53 ± 0.01

Chromatographic conditions: column, LiChrospher C_{18} (5 μm, 125 × 4 mm, i.d.); mobile phase, methanol–phosphate buffer (3 g Na_2HPO_4 + 3 g NaH_2PO_4 in 1 l water) (0.5:99.5, v/v); flow rate 1.0 ml min^{-1}; injection volume, 20 μl; post-column reactor (KOT) 1.8 ml. Electrochemical detection conditions: glassy carbon working electrode; applied potentials +0.8 and +0.72 V versus Ag/AgCl.

*Phe as analyte, n = 3, and EC responses were measured in peak heights.

Table 2
Spiked Phe determinations using LC–hν–EC

Phe	Found concentration*	Actual concentration†	Relative error (%)‡
No. 1	5.02	5.0	0.4
No. 2	19.9	20.0	0.5
No. 3	70.2	70.0	0.3

Chromatographic and EC conditions as in Table 1.
*Concentration in ppm, RSD <3% (n = 3).
†Spiked in H_2O–NaOH–MeOH, in ppm.
‡(Found concentration − actual concentration)/actual concentration × 100.

Table 3
Summary of determinations of Phe in urine samples

Number	Phe concentration (ppm)*
1	Mean 4.3
2	4.3
3	4.3
4	4.1
5	4.2
6	
	Mean 4.2
	SD 0.1
	RSD 2.3%

Chromatographic and EC detection conditions as in Table 1.

*Due to the dilution during sample preparation, the Phe concentration in the original sample was 2.4 times that listed, i.e. 10.3 ppm.

listed with the mean, standard deviation and RSD values for $n = 6$. Good precision was obtained for the determinations with RSD <3% ($n = 6$). The injection-to-injection reproducibility was within 6% ($n = 3$). Considering the dilution of the urine sample during sample preparation, the Phe concentration in the original sample was 10.32 μg ml^{-1}, which is within the normal range of 5–20 μg ml^{-1} [8]. For PKU patients, the Phe concentration in urine is at least 10-fold higher. At these high concentrations of Phe, the method should give reproducible and accurate quantitative results with the simple sample treatment mentioned above for diagnosis of PKU.

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