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Nafion Membrane Tube-based On-line Concentrator. Application to Urinary Orotic Acid Determined by Suppressed Ion Chromatography

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Abstract

This paper describes the performance of suppressed ion chromatography coupled with a Nafion membrane tube-based on-line concentration technique for the determination of urinary orotic acid, which serves as a biochemical indicator for ornithine transcarbamylase deficiency. After the separation of the analyte by the chromatography, it is concentrated while the eluent flows through the Nafion membrane tube, meanwhile, a nitrogen gas flows outside the tube to remove the solvent. The peak of orotic acid was increased with increasing sample residence time, nitrogen gas flow rate, and concentration temperature. With the concentrator, the detection sensibility was improved, and the lower detection limit was obtained compared to that without the concentrator. The optimized system has successfully been applied to the determination of orotic acid in real urinary samples.

Keywords Orotic acid, urine, Nafion tube, concentrator, ion chromatograph

1. Introduction

Orotic acid is an intermediate metabolite in the *de novo* pyrimidine biosynthetic pathway [1]. The measurement of orotic acid in urine is clinically useful than in plasma because the renal excretion of orotic acid is very efficient [2,3]. Imaeda *et al.* [3] analyzed the urinary orotic acid levels in healthy adults, and reported that the mean values were 0.56 μmol/mmol creatinine for male (n = 98) and 0.66 μmol/mmol creatinine for female (n = 67). Urinary orotic acid serves as a biochemical indicator for urea cycle defects, specifically ornithine transcarbamylase deficiency (OTCD) [4,5]. In the patients with OTCD, carbamoyl phosphate that has accumulated in hepatic mitochondria diffuses to the cytosol and enters the pyrimidine biosynthetic pathway. As a result, a large amount of orotic acid is excreted in urine [6]. To date, several approaches for the determination of urinary orotic acid have been proposed, *e.g.*, gas chromatography [7], gas chromatography coupled mass spectrometry [8], high performance liquid chromatography with ultraviolet absorption, fluorometric, or pulsed amperometric detection [9-13], liquid chromatography coupled tandem mass spectrometry [14,15], and capillary isotachophoresis coupled zone electrophoresis [16]. These approaches have respective advantages and drawbacks with the limits of detection (LOD) ranged from 0.05 to 25 μmol L^{-1} (0.008 – 3.9 mg L^{-1}). Ion chromatography is also useful method for the measurement of trace ions in biological samples. In an analysis of urinary sample by ion chromatography, sample dilution is required to prevent an interference from sample matrix. In general, the urinary sample is diluted with pure water by 10 – 1000 fold, and a relatively small amount of the sample diluted (10 – 50 μL) is injected into the chromatographic system [17-19].

In this paper, an analytical column-friendly determination method for urinary orotic acid by suppressed ion chromatography with conductivity detection is proposed. We applied a Nafion membrane tube-based on-line concentration technique [20, 21] so as to improve the detection sensibility of orotic acid. This concentration technique can function not only in the preinjection mode but in the postcolumn position. We decided to use the concentrator at the latter position for keeping an analytical column from deteriorating. The concentrator was evaluated by changing the operating parameters, and was applied to the chromatographic determination of urinary orotic acid.

2. Experimental

2.1. Reagents and urinary sample

All reagents used in this study were of analytical grade. Orotic acid monohydrate (Kishida Chemical Co., Ltd.), ethanol (Kanto Chemical Co., Inc.), *N,N*-dimethylformamide (Kanto Chemical Co., Inc.), and hydrochloric acid (Kanto

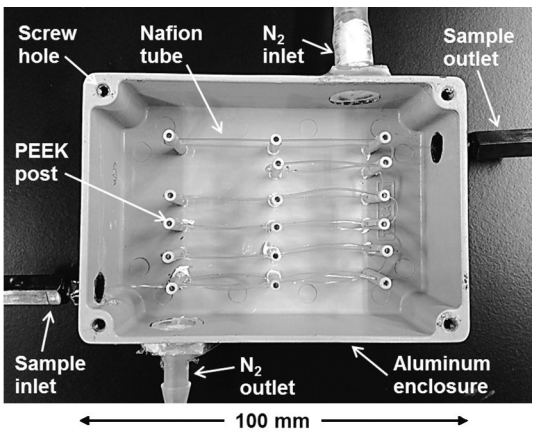

Photo 1 Nafion membrane tube-based on-line concentrator. A top of Aluminum enclosure is not shown.

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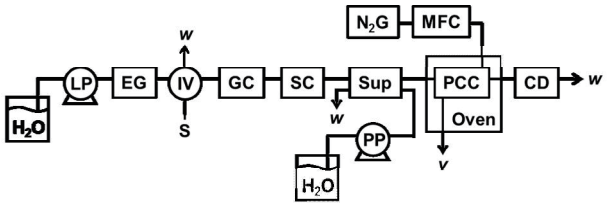

Fig. 1 Schematic of urinary orotic acid determination system by Nafion tube-based post-column concentrator coupled suppressed ion chromatography. LP, liquid pump; EG, eluent generator; IV, injection valve; GC, guard column; SC, separation column, Sup, suppressor; PCC, post-column concentrator; CD, conductivity detector; N_2G, N_2 generator; MFC, mass flow controller; PP, peristaltic pump; s, sample; *w*, waste; *v*, vent.

Chemical Co., Inc.) were purchased as indicated and were used without further purification. Sartorius arium 611DI grade deionized water (>18 MΩ cm) was used throughout. Urine samples were collected in paper cups from a healthy volunteer (twenty-something male). The samples were immediately filtered through disposable disk filters with 0.20 μm pore size (Sartorius Stedim Biotech GmbH). The filtrates were diluted 100-fold by the deionized water, and were analyzed on the same day as the collection of samples.

2.2. Nafion membrane tube-based on-line concentrator

Photo 1 shows the inner portion of the Nafion membrane tube-based concentrator used for the on-line concentration of samples. Before assembling the device, a Nafion membrane tube (0.33 mm i.d., 0.51 mm o.d., 80 cm length, TT-020, Perma-Pure LLC) was boiled in a mixture of ethanol and *N,N*-dimethylformamide (50% v/v) for 5 min, and manually extended to be 100 cm in length. This treatment makes the tube thickness thin and increases a water evaporation rate of the device. In order to convert the membrane to a H^+ form, the Nafion tube was boiled in 1 mol L^{-1} hydrochloric acid for 5 min, and washed thoroughly with water. Seventeen PEEK posts (0.75 mm i.d., 1.6 mm o.d., 2.5 cm length, Dionex Corp.) with two or three apertures (1.0 mm dia.) were affixed in a Diecast Aluminum enclosure (10 cm × 7 cm × 3 cm, TD7-10-3, Takachi Electronics Enclosure Co., Ltd.). There were four holes for nitrogen (N_2) gas inlet/outlet and sample inlet/outlet on the sides of enclosure. The Nafion tube was strung through the apertures so as to prevent the tube from touching the enclosure wall. Both ends of the Nafion tube were inserted in a FEP tubing sleeve (0.61 mm i.d., 1.58 mm o.d., 2.54 cm length, F-245X, M&S Instruments Inc.) for sample inlet/outlet connections. The sample flows through the Nafion tube, meanwhile, the N_2 gas flows outside the tube to eliminate the water vapor that emanates from the tube. As a result, involatile compounds are on-line concentrated more and more as time proceeds.

2.3. Chromatographic system coupled with on-line concentrator

An ICS-2000 ion chromatography system (Dionex Corp.) configured with a liquid pump, electrodialytic KOH generator, 6-port injection valve, IonPac AG20 2-mm guard column, IonPac AS20 2-mm separation column, ASRS300 2-mm electrodialytic suppressor, and conductivity detector (cell volume: 1 μL) were used for the determination of orotic acid. A five-microliter of sample was injected into a 35 mmol L^{-1} KOH eluent flowing at a range of 0.17 to 0.35 mL min^{-1}. The guard/separation columns and conductivity cell were maintained at 30°C. The suppressor was operated with an external water mode at a water flow rate of 1 mL min^{-1}. The system operation was controlled by a software (Chromeleon Ver. 6.80). The Nafion tube-based concentrator was placed between the suppressor and conductivity detector as shown in Fig. 1, and was housed inside an oven (CTO-6A column oven, Shimadzu Corp.) for controlling the concentration temperature. A N_2 gas supplied by a N_2 generator (AT-5NP-CB, Air-Tech Corp.) was controlled with a mass flow controller (model 8500, Kofloc Co. Ltd.). A copper tube (4 mm i.d., 6 mm o.d., 2 m length) was also placed in the oven allowed for preheating the N_2 stream.

3. Results and Discussion

3.1. Operating parameters of on-line concentrator

The Nafion tube-based on-line concentrator was evaluated by changing the eluent flow rate to the concentrator, N_2 gas flow rate, and concentration temperature. Figure 2 shows the orotic acid peak with and without the concentrator. The representative peaks are shown here, though the experiment was repeated 3 times at each experimental condition. While the peak height with the concentrator is decreased with the eluent flow rate resulting from the reduction of sample residence time in the concentrator (Fig. 2b), the peak height with the concentrator is linearly increased with the N_2 gas flow

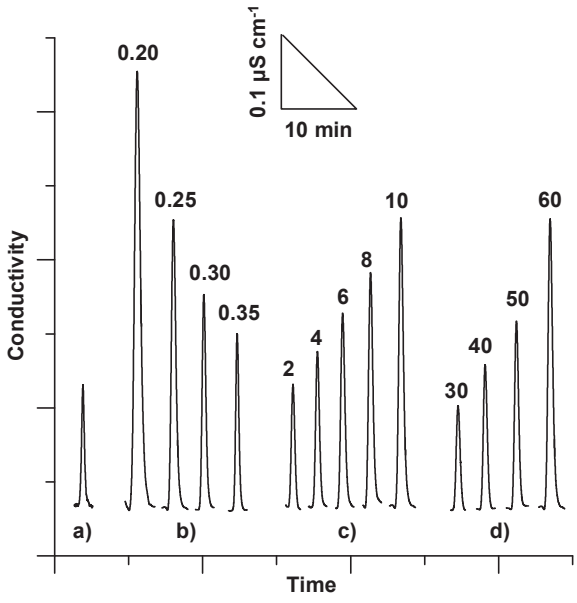

Fig. 2 Comparison of 1 mg L^{-1} orotic acid peak (a) without and (b – d) with Nafion tube-based concentrator. Unless otherwise noted, the chromatographic system was operated at 60°C concentrator temperature with 0.25 mL min^{-1} eluent flow rate and 10 L min^{-1} N_2 flow rate. (b) Effect of influent flow rate, 0.20 – 0.35 mL min^{-1} as indicated. (c) Effect of N_2 flow rate, 2 – 10 L min^{-1} as indicated. (d) Effect of concentrator temperature, 30 – 60°C as indicated.

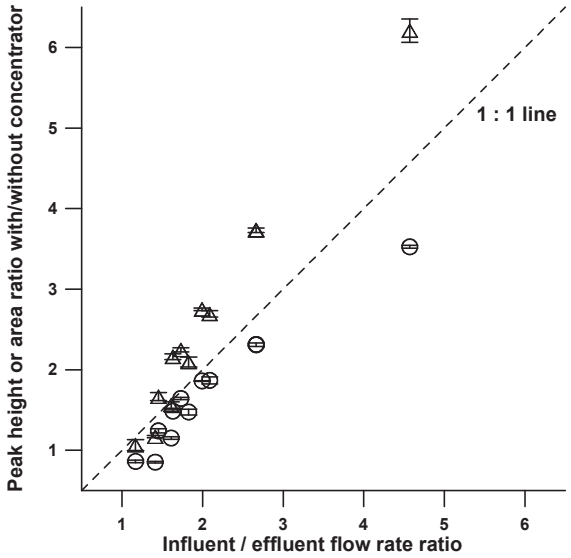

Fig. 3 Relationship between the peak height and area ratios of orotic acid vs the eluent influent/effluent flow rate ratio. Open circle, peak height ratio; open triangle, peak area ratio. The error bars shown are one standard deviation, n =3.

rate (Fig. 2c, r^2 = 0.993). The concentrator was evacuated by a vacuum pump, and was evaluated without N_2 gas flow. In this case, saturated water droplets were observed on the tube connected to the N_2 gas outlet, and the amount of water droplets was increased with the concentration temperature. Therefore, the dry gas is clearly-needed for continuous running of the concentrator. With increasing the concentration temperature, the peak height is increased as shown in Fig. 2d. One may notice that the peak height with the concentrator at 30°C in Fig. 2d is lower than the peak height without the concentrator (Fig. 2a). This is because of the desperation of sample during the concentration process. In fact, the peak area with the concentrator at 30°C is increased by a factor of 1.17 ± 0.01 (n = 3) compared to that without the concentrator.

3.2. Concentration factor of orotic acid with on-line concentrator

The concentration factors based on peak height/area of orotic acid (CF_H, CF_A) were, respectively calculated as

$$CF_H = H_w / H_{wo} \quad (1)$$

$$CF_A = A_w / A_{wo} \quad (2)$$

where H_w and H_{wo} are the peak heights of orotic acid with/without concentrator, A_w and A_{wo} are the peak areas of orotic acid with/without concentrator. Figure 3 shows the concentration factors of orotic acid as a function of the ratio of eluent influent rate to effluent flow rate. The CF_H is lower than the gravimetrically measured influent/effluent flow rate ratio because of the axial dispersion of the sample. On the other hand, most of the CF_A is higher than the flow rate ratio. This is because volatile substances such as carbonic acid presented in the eluent were lost during the concentration process, and as a result, the increase of background conductivity was suppressed [21]. In other words, the concentrator functions as a suppressor being used in ion chromatography. No evaporation loss of orotic acid occurred during the sample concentration process because the orotic acid is a relatively strong acid (pK_a = 1.8 ± 0.2) [22].

3.3. Application to urinary sample

In order to obtain high detection sensibility, the Nafion tube-based concentrator operated at 60°C concentrator temperature with 0.17 mL min^{-1} eluent flow rate and 10 L min^{-1} N_2 flow rate. With this operating parameters, the calibration curves were constructed from seven standard solutions containing 0 – 10 mg L^{-1} orotic acid. The peak height and area of the conductivity signal (H in μS cm^{-1}, A in μS cm^{-1}·min) can be expressed as follows.

$$H = 0.771C - 0.023, \quad r^2 = 0.999 \quad (3)$$

$$A = 0.809C - 0.070, \quad r^2 = 0.999 \quad (4)$$

where C is the concentration of orotic acid (mg L^{-1}). The LOD (3σ/S, σ and S are the residual standard deviation and slope of the regression line) was calculated to be 0.188 mg L^{-1} (corresponding to 0.94 ng orotic acid) for peak height and 0.247 mg L^{-1} (1.24 ng orotic acid) for peak area, respectively. With the concentrator, the LODs were respectively improved by a factor of 2.19 for peak height and 1.78 for peak area while the slopes of calibration curves were respectively increased by a factor of 3.37 for peak height and 6.42 for peak area compared to those without the concentrator.

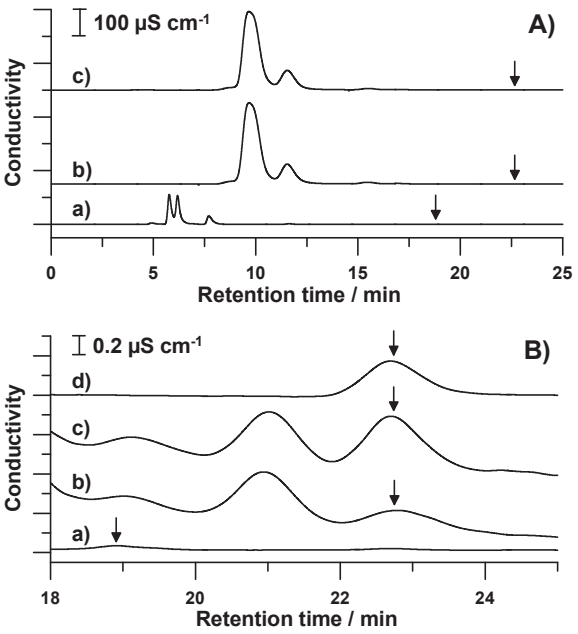

Fig. 4 Chromatogram of postcolumn concentration with Nafion tube-based concentrator operated at 60°C concentrator temperature with 0.17 mL min^{-1} eluent flow rate and 10 L min^{-1} N_2 flow rate. The graph B) shows the response around the peak of orotic acid. (a) 100-fold diluted urine sample without concentrator, (b) 100-fold diluted urine sample with concentrator, (c) 0.5 mg L^{-1} orotic acid spiked 100-fold diluted urine sample with concentrator, and (d) 0.5 mg L^{-1} orotic acid standard solution with concentrator. The arrows point the orotic acid peaks.

Finally, we applied the proposed method to real urinary sample. Five-microliter of 100-fold diluted urine sample and 0.5 mg L^{-1} orotic acid spiked 100-fold diluted urine sample were injected into the chromatographic system with and without the concentrator. Figure 4A shows the output signals. The large matrix peaks such as chloride, sulfate, and phosphate peaks appeared in the first half of chromatograms. The retention time of orotic acid was 18.9 min for the system without the concentrator, and shifted to 22.7 min with the concentrator. Figure 4B shows the response signals around the peak of orotic acid. Without the concentrator, the orotic peak appeared but it was below the LOD (Fig. 4Ba). On the other hand, as shown in Fig. 4Bb, the orotic acid peak was increased to the detectable level with the concentrator (0.238 ± 0.2 mg L^{-1}, n = 3) though further improvement is clearly needed for quantification. In addition, good recoveries (96.8 ± 2.8%, n = 2) were obtained for the analyte spiked in the sample (Fig. 4Bc). These retention times of the orotic acid peak in urinary sample were consistent with that of the standard sample (Fig. 4Bd).

In summary, we have presented the analytical column-friendly detection method for urinary orotic acid by suppressed ion chromatography. The chromatographic system coupled with the Nafion membrane tube-based on-line concentrator enabled us to detect the orotic acid in 100-fold diluted urine sample.

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References

[1] M. Löffler, L. D. Fairbanks, E. Zameitat, A. M. Marinaki, H. A. Simmonds, *Trends Mol. Med.*, **11**, 430 (2005).
[2] C. Salerno, C. Crifò, *J. Chromatogr. B*, **781**, 57 (2002).
[3] M. Imaeda, K. Kidouchi, S. Sumi, K. Hayashi, S. Ohba, M. Kobayashi, Y. Wada, *Purine and Pyrimidine Metabolism*, **22**, 19 (1998).
[4] F. Scaglia, Q. P. Zheng, W. E. O'Brien, J. Henry, J. Rosenberger, P. Reeds, B. Lee, *Pediatrics*, **109**, 150 (2002).
[5] R. Koch, *Pediatrics*, **68**, 271 (1981).
[6] M. E. Brosnan, J. T. Brosnan, *J. Nutr.*, **137**, 1656S (2007).
[7] H. Kataoka, K. Tamura, M. Maeda, M. Makita, Bunseki Kagaku, 38, 327 (1989).
[8] R. Hušková, P. Barták, L. Čáp, D. Friedecký, T. Adam, *J. Chromatogr. B*, **799**, 303 (2004).
[9] S. Ohba, K. Kidouchi, T. Katho, T. Kibe, M. Kobayashi, T. Wada, *J. Chromatogr.*, **568**, 325 (1991).
[10] A. Fioravanti, M. Flaviani, C. Gambelunghe, A. Micheletti, M. Sposito, S. Rufini, *J. Chromatogr. B*, **703**, 263 (1997).
[11] H. Niu, Y. Chen, J. Xie, X. Chen, J. Bai, J. Wu, D. Liu, H. Ying, *J. Chromatogr. Sci.*, **50**, 709 (2012).
[12] S. Yin, S. Dragusha, V. Ejupi, T. Shibata, T. Kabashima, M. Kai, *J. Fluoresc.*, **25**, 1005 (2015).
[13] T. R. I. Cataldi, D. Nardiello, R. Ciriello, A. Guerrieri, *J. Chromatogr. A*, **1107**, 130 (2006).
[14] G. la Marca, B. Casetta, E. Zammarchi, *Rapid Commun. Mass Spectrom.*, **17**, 788 (2003).
[15] W.-Y. Hsu, C.-M. Chen, F.-J. Tsai, C.-C. Lai, *Clin. Chim. Acta*, **420**, 140 (2013).
[16] A. Procházková, L. Křivánková, P. Boček, *J. Chromatogr. A*, **838**, 213 (1999).
[17] Y. Shimada, *J. Chugoku Junior College*, **32**, 101 (2001).
[18] F. Zhao, Z. Wang, H. Wang, M. Ding, *J. Chromatogr. B*, **879**, 296 (2011).
[19] Dionex Corporation, Application Note 107.
[20] M. Takeuchi, P. K. Dasgupta, J. V. Dyke, K. Srinivasan, *Anal. Chem.*, **79**, 5690 (2007).
[21] M. Takeuchi, K. Yoshioka, Y. Toyama, A. Kgami, H. Tanaka, *Talanta*, **97**, 527 (2012).
[22] E. M Woolley, R. W. Wilton, L. G. Hepler, *Can. J. Chem.*, **48**, 3249 (1970).

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