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# Automated screening system for purine and pyrimidine metabolism disorders using high-performance liquid chromatography

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**Abstract**

An automated screening system for purine and pyrimidine metabolism disorders using high-performance liquid chromatography (HPLC) with column switching is described. The system consists of a reversed-phase column, a cation-exchange column, a column switch, four sets of ultraviolet absorbance detectors, a microcomputer and other conventional equipment. As this system permits the simultaneous determination of urinary orotic acid, uracil, dihydrouracil, pseudouridine, xanthine, 2,8-dihydroxyadenine and succinyladenosine, it offers a useful method for the detection of orotic aciduria, dihydropyrimidine dehydrogenase deficiency, diphydropyrimidinuria, xanthinuria, adenine phosphoribosyltransferase deficiency and adenylosuccinase deficiency.

## 1. Introduction

With the advances in analytical methods in the past 10 years, numerous cases of congenital metabolic disorders such as amino acid and organic acid metabolism disorders have been reported. Compared with these disorders, the number of reported purine and pyrimidine metabolism disorders has been small owing to the complicated analytical procedures required for purines and pyrimidines, making them difficult to screen. If a simple and accurate method for screening purines and pyrimidines could be established, more cases of purine and pyrimidine metabolism disorders would probably be diagnosed.

High-performance liquid chromatographic (HPLC) methods have been widely used in fields such as chemistry, pharmacology and medicine, and we believe that they can also be employed for screening purine and pyrimidine metabolism disorders. This paper describes a method for screening purine and pyrimidine metabolism disorders.

## 2. Experimental

### 2.1. Chemicals and solutions

Xanthine, 2,8-dihydroxyadenine (2,8-DHA),

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orotic acid, pseudouridine, dihydrouracil and uracil were purchased from Sigma (St. Louis, MO. USA). Succinyladenosine was prepared using the method of Jaeken and Berghe [1] using adenylosuccinate and 5′-nucelotidase obtained from Sigma. Analytical-reagent grade sulphuric acid and acetonitrile were purchased from Wako (Tokyo, Japan). Deionized water was passed through a Milli-Q Labo system (Nihon Millipore Kogyo, Yonezawa, Japan).

### 2.2. Urine samples

Urine samples were collected from healthy volunteers and patients with congenital purine and pyrimidine metabolism disorders and were frozen and stored at −70°C. Sample preparation prior to analysis consisted solely of filtration with a Centricut filter (Kurabou, Osaka, Japan). Each analysis was conducted using a 10-$\mu$l sample of urine. The urinary creatinine level was measured with an autoanalyser.

### 2.3. HPLC

The HPLC system is shown in Fig. 1. Two sets of Eyela PLC-5 liquid chromatographs (Tokyo Rikakikai, Tokyo, Japan) were used. Each set was equipped with one pump and two detectors, with a system total of two pumps and four detectors. An SC-15 computerized system controller sent signals to a gradient system (both from Tokyo Rikakikai), a KSP-600 autosampler (Kyowa Seimitu, Tokyo, Japan), a column switch (Tokyo Rikakikai) and the detectors. An on-line ERC-3611 degasser (Erma, Tokyo, Japan) was used for eluent delivery. C-R4A Chromatopac integrators (Shimadzu, Kyoto, Japan) were used for data analysis. An Intelligent UV–Vis detector (Nipponbunko, Tokyo, Japan) was used for peak identification purposes.

### 2.4. Columns and solutions

The first column was a reversed-phase ODS column (Develosil ODS-5, 150 × 6 mm I.D., particle size 5 $\mu$m; Nomura Chemical, Seto, Japan). This column was eluted with 5 m*M* $H_2SO_4$ for 10 min. Next, a 2% acetonitrile–5 m*M* $H_2SO_4$ solution was used for 15 min, the column was then washed with acetonitrile–water (50:50) for 3 min. Finally, it was equilibrated with 5 m*M* $H_2SO_4$ for 10 min. The second column was a cation-exchange column (MCI

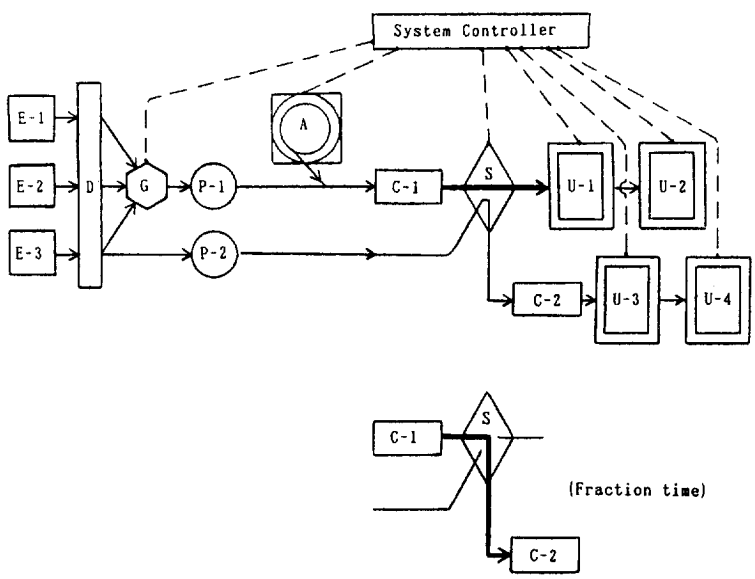


Fig. 1. Automated HPLC screening system. E = Eluent; D = degasser; G = gradient system; P = pump; A = autosampler; C = column; S = column switch; U = ultraviolet absorbance detector. Arrows indicate flows of eluents. Dashed lines indicate signals from a computerized system controller. The two columns are connected from 4.5 to 5.7 min.

GEL CK08EH, $H^+$ form, 300 × 8 mm I.D., particle size 9 μm; Mitsubishi Kasei, Tokyo, Japan). The second column was eluted with 5 m*M* $H_2SO_4$. In both instances the column temperature was 30°C and the flow-rate was 0.8 ml/min.

### 2.5. HPLC procedure

A sample was applied to the first column with the autosampler. The eluate from the first column was continuously monitored at both 270 and 300 nm. Although some compounds could be separated using only the first column, others were not separated from each other. In order to separate such compounds, fractions of the compounds were delivered to the second column. The eluate from the second column was continuously monitored at both 210 and 280 nm. Because the column switch and all detectors were controlled by the system controller (Fig. 1), all procedures were performed automatically and more than 50 samples could be analyzed continuously. The total analysis time for each sample was only about 40 min. For target peak identification, the UV absorbance spectra were recorded manually using the Intelligent UV–Vis detector, which was connected temporarily for this purpose.

## 3. Results

### 3.1. Standards

A preliminary study was conducted using only the first column. Whereas 2,8-DHA, xanthine and succinyladenosine could be separated from each other, the retention times of orotic acid, pseudouridine, dihydrouracil and uracil were almost identical. Hence it was impossible to separate the latter four compounds on the first column. Therefore, the automated column switching system applied a fraction (4.5–5.7 min) containing these four compounds to the second column, where they were well separated.

The column effluents were monitored at two wavelengths each, with one wavelength assigned to each compound. A wavelength of 270 nm was used for xanthine and succinyladenosine, 300 nm for 2,8-DHA, and 280 nm for orotic acid, pseudouridine and uracil, because these wavelengths are close to the unique maximum absorbance of each compound. A wavelength of 210 nm was used for dihydrouracil, which had no unique absorbance.

The retention times of the peaks are given in Fig. 2.

Calibration graphs for the standards were obtained by processing aliquots of an mixed aqueous standard solution at different concentrations (1, 10, 50, 100, 500 and 1000 nmol/ml). The relationships between concentration and peak height were linear in the concentration range 1–1000 nmol/ml. The detection limits were 5 pmol per 10 μl each time the dilution levels of the 1 nmol/ml standard solutions were doubled.

### 3.2. Urine samples

The chromatogram of urine from a healthy infant is shown in Fig. 3. Peaks are observed for xanthine at 270 nm, dihydrouracil at 210 nm, and orotic acid, pseudouridine and uracil at 280 nm. With the Intelligent UV–Vis detector, it was

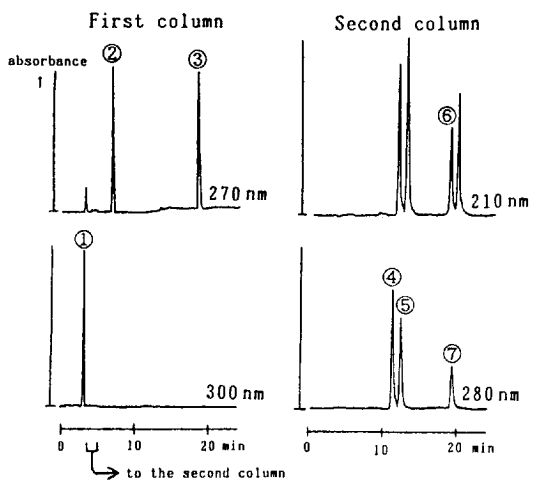


Fig. 2. Chromatogram of the standard mixture (concentration 100 nmol/ml). Upper left, first column chromatogram, monitoring at 270 nm; lower left, first column chromatogram at 300 nm; upper right, second column chromatogram at 210 nm; lower right, second column chromatogram at 280 nm. Peaks: 1 = 2,8-DHA; 2 = xanthine; 3 = succinyladenosine; 4 = orotic acid; 5 = pseudouridine; 6 = dihydrouracil; 7 = uracil.

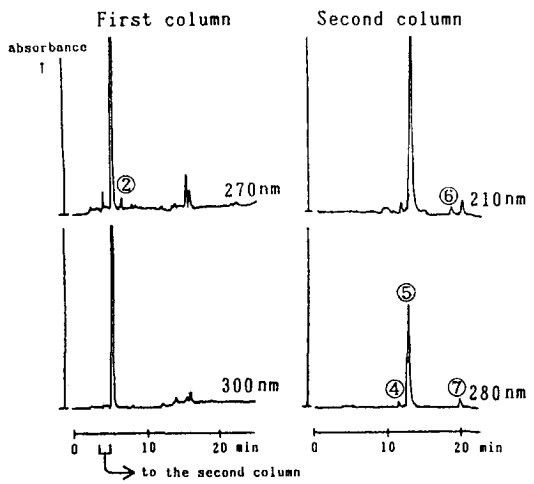


Fig. 3. Chromatogram of urine from a healthy infant. Peak numbers as in Fig. 2.

confirmed that the UV absorbance spectra of xanthine, orotic acid, pseudouridine and uracil corresponded to those of the standards (data not shown). We could not examine the absorbance spectrum of dihydrouracil because dihydrouracil has no unique absorbance. The preliminary data for the reference values of healthy infants ($n$ = 35, 2–6 years) are as follows: the concentration ($\mu$mol/g creatinine) of urinary xanthine was $247 \pm 103$ (mean ± S.D.), that of orotic acid $18.2 \pm 13.0$, that of pseudouriudine $724 \pm 251$ and that of uracil $191 \pm 89.0$. The concentration of dihydrouracil was calculated to be $108 \pm 55.8$, assuming that the dihydrouracil peak in the chromatogram was pure.

Usually, there was no succinyladenosine peak at 270 nm, although a small peak was occasionally observed with a retention time corresponding to that of the succinyladenosine standard. However, the UV absorbance spectrum of the peak did not correspond to that of the standard. Consequently, the peak seemed to be an unknown compound. However, as its peak height was smaller than that of the 20 $\mu$mol/g creatinine value of succinyladenosine, the unknown compound did not interfere with the screening process. Similarly to succinyladenosine, a small peak was sometimes observed at 300 nm. Although the retention time of this peak corresponded to 2,8-DHA, the UV absorbance of the peak differed from that of 2,8-DHA. Its peak height was extremely small, and it did not interfere with the determination of 2,8-DHA.

The chromatogram for a sample from a patient [2] with adenylosuccinase deficiency (McKusick 10305) is shown in Fig. 4, and indicates a high peak of succinyladenosine (10 980 $\mu$mol/g creatinine). The UV absorbance spectrum of succinyladenosine in the urine corresponded to that of the standard.

The chromatogram for a sample from a patient [3] with hereditary orotic aciduria (McKusick 258900) is shown in Fig. 5, and indicates an extremely high peak of orotic acid (78 892 $\mu$mol/g creatinine). The UV absorbance spectrum of orotic acid in the urine corresponded to that of the standard.

The chromatogram for a sample from a patient [4] with dihydropyrimidinuria (McKusick 222748) is shown in Fig. 6, and indicates a high peak corresponding to dihydrouracil. Because dihydrouracil had no unique absorbance, we used gas chromatography–mass spectrometry and confirmed that the abnormal peak was dihydrouracil [4]. The urinary concentration of dihydrouracil was 5541 $\mu$mol/g creatinine.

The chromatogram for a sample from a patient

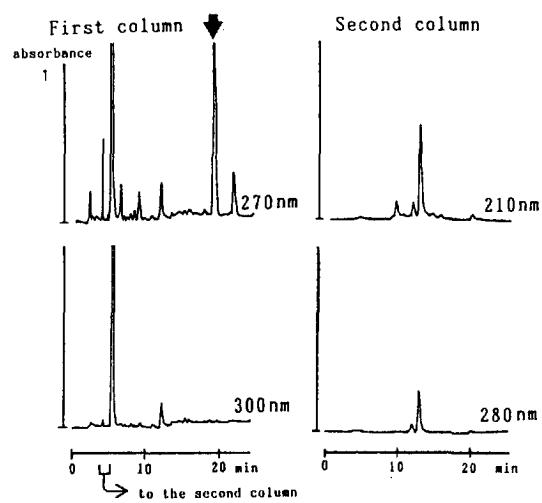


Fig. 4. Chromatogram of urine from a patient with adenylosuccinase deficiency. Arrow indicates an abnormal peak of succinyladenosine.

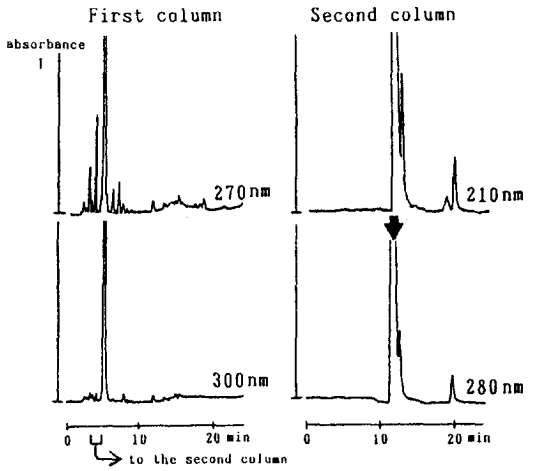


Fig. 5. Chromatogram of urine from a patient with hereditary orotic aciduria. Arrow indicates an abnormal peak of orotic acid.

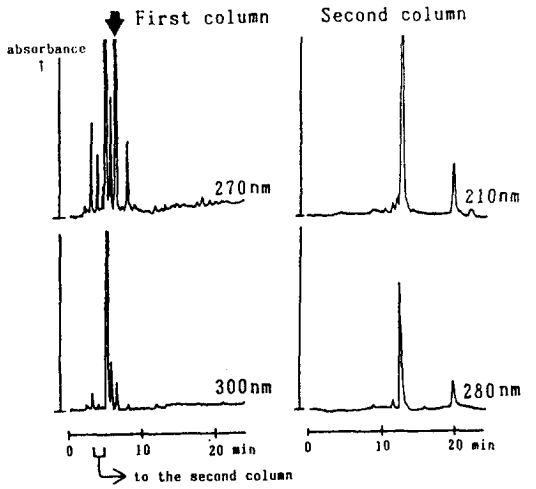


Fig. 7. Chromatogram of urine from a patient with hypoxanthine–guanine phosphoribosyltransferase partial deficiency, treated with allopurinol (10 mg/kg per day). Arrow indicates an abnormal peak of xanthine.

with hypoxanthine–guanine phosphoribosyltransferase partial deficiency (McKusick 308000) is shown in Fig. 7. The enzyme activity of erythrocytes from the patient was 4.22% that of the normal controls. Becuase the patient had hyperuricaemia, he was treated with allopurinol (10 mg/kg per day). A previous report [5] indicated that allopurinol often induced secondary xanthinuria in the disease. In keeping with this, the chromatogram showed an extremely high peak of xanthine (10 788 μmol/g creatinine).

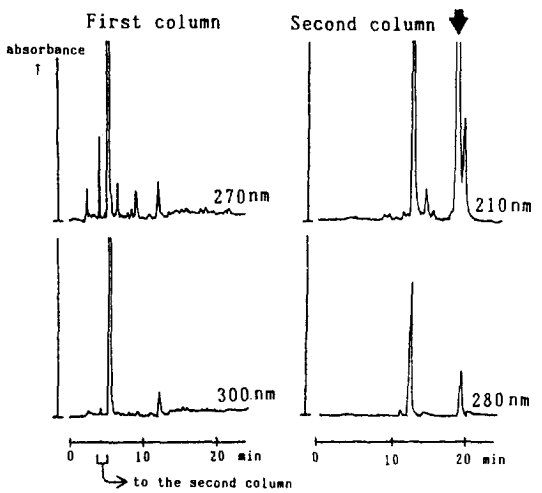


Fig. 6. Chromatogram of urine from a patient with dihydropyrimidinuria. Arrow indicates an abnormal peak of dihydrouracil.

## 4. Discussion

Purine and pyrimidine metabolism disorders have recently been the focus of considerable attention. Adenylosuccinase deficiency is accompanied by severe mental retardation, and often also autistic features. The patients tend to excrete excessive amounts of succinyladenosine and succinylaminoimidazole carboxamide riboside in their urine. De Bree et al. [6] developed a thin-layer chromatographic method for studying adenylosuccinase deficiency in urine from high-risk populations, but the method is not suitable for screening other diseases.

Dihydropyrimidine dehydrogenase deficiency (McKusick 27427) presents a wide heterogeneity in clinical expression, ranging from severe mental retardation to severe 5-fluorouracil toxicity [7], with the patients typically excreting large amounts of uracil and thymine in their urine. Lu et al. [8] reported a screening method which entails measuring dihydropyrimidine dehydrogenase activity. This method is acceptable with

small groups, but difficulties arise when dealing with large populations owing to the complicated procedure for measuring enzyme activity.

Dihydropyrimidinuria is a dihydropyrimidinase defect, which is the next step following dihydropyrimidine dehydrogenase. The patients excrete large amounts of dihydrouracil and dihydrothymine in their urine. The first case was reported in 1991 [9], and details of the disease are still unknown. Although Duran et al. [9] warned of severe 5-fluorouracil toxicity in the patients, a useful screening method for the disease has not been reported.

Hereditary orotic aciduria results in excessive excretion of orotic acid. Although the patients display megaloblastic anaemia and mental retardation, early treatment with uridine can prevent these symptoms completely [3]. Moreover, certain urea cycle disorders, such as ornithine transcarbamylase deficiency, can induce secondary orotic aciduria. Screening for orotic aciduria is therefore an important factor in the diagnosis of urea cycle disorders. Although a spectrophotometric screening method for urinary orotic acid has been reported [10], it is not capable of screening for other urinary compounds.

Patients with hereditary xanthinuria (McKusick 278600) or secondary xanthinuria excrete large amounts of xanthine in their urine and often produce xanthine stones in their urinary tracts. Patients with adenine phosphoribosyltransferase deficiency (McKusick 102600) excrete large amounts of 2,8-DHA and also produce 2,8-DHA stones in their urinary tracts. Separate screening methods for each of these diseases have been reported [11,12], but screening for both diseases at the same time has not been possible.

As mentioned above, purine and pyrimidine metabolism disorders show various kinds of clinical symptoms, and early diagnosis may lead to effective treatment. Although certain screening methods for specific diseases have been reported, a systematic method capable of screening for a number of diseases simultaneously has not been available to date. In this paper, we have described a systematic screening method for the detection of orotic aciduria, dihydropyrimidine dehydrogenase deficiency, dihydropyrimidinuria, xanthinuria, adenine phosphoribosyltransferase deficiency and adenylosuccinase deficiency. We introduced two technical features to the HPLC method: an automated column-switching system [13] and a multiple UV absorbance detector system for monitoring the maximum absorbance wavelength of each compound. As a preliminary cut-off line for screening, we adopted the averages plus two standard deviations for orotic acid, uracil, dihydrouracil and xanthine and 20 $\mu$mol/g creatinine for 2,8-DHA and succinyladenosine. Because patients showed excretions of urinary compounds much larger than the cut-off line, it seems to be easy to distinguish patients from normal subjects. However, a final cut-off line will be determined after the accumulation of further data. We believe that this method will contribute significantly to the srceening process for patients with purine and pyrimidine metabolism disorders.

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## References

[1] J. Jaeken and G.V.D. Berghe, Lancet, 2 (1984) 1058.
[2] P.K. de Bree, S.K. Wadman, M. Duran and H.F. de Jonge, Clin. Chim. Acta, 156 (1986) 279.
[3] H. Morishita, M. Kokubo, S. Sumi, M. Suchi and Y. Wada, Nippon Shonika Gakkai Zasshi, 90 (1986) 2775 (in Japanese with English abstract).
[4] S. Ohba, K. Kidouchi, S. Sumi, N. Takeda, K. Kodama, K. Yamanaka, K. Kobayashi and Y. Wada, Adv. Exp. Med. Biol, in press.
[5] T. Mizuno, M. Segawa, T. Kurumada, H. Maruyama and J. Onisawa, Neuropaediatrie, 2 (1970) 38.
[6] P.K. de Bree, S.K. Wadman, M. Duran and F. de Jonge, Chin. Chim. Acta, 156 (1986) 279.
[7] A.P. Lyss, R.C. Lilenbaum, B.E. Harris and R.B. Diasio, Cancer Invest., 11 (1993) 239.
[8] Z. Lu, R. Zhang and R.B. Diasio, Cancer Res., 53 (1993) 5433.

[9] M. Duran, P. Rover, P.K. de Bree, C.H. Schreuder, H. Berkenhorst, L. Dorland and R. Berger, J. Inher. Metab. Dis., 14 (1991) 367.
[10] L.E. Roger and F.S. Porter, Pediatrics. 42 (1968) 423.
[11] T. Nisida, R. Sakurai and T. Kojima, Purine Pyrimidine Metab., 16 (1992) 166.
[12] L.A. Johnson, R.B. Gordon and B.T. Emmerson, Biochem. Genet., 15 (1977) 265.
[13] S. Ohba, K. Kidouchi, T. Katoh, T. Kibe, M. Kobayashi and Y. Wada, J. Chromatogr., 568 (1991) 325.