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# Rapid Screening of High-Risk Patients for Disorders of Purine and Pyrimidine Metabolism Using HPLC-Electrospray Tandem Mass Spectrometry of Liquid Urine or Urine-soaked Filter Paper Strips

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***Background:*** **A rapid and specific screening method for patients at risk of inherited disorders of purine and pyrimidine metabolism is desirable because symptoms are varied and nonspecific. The aim of this study was to develop a rapid and specific method for screening with use of liquid urine samples or urine-soaked filter paper strips.**

***Methods:*** **Reverse-phase HPLC was combined with electrospray ionization (ESI), tandem mass spectrometry (MS/MS), and detection performed by multiple reaction monitoring. Transitions and instrument settings were established for 17 purines or pyrimidines. Stable-isotope-labeled reference compounds were used as internal standards when available.**

***Results:*** **Total analysis time of this method was 15 min, approximately one-third that of conventional HPLC with ultraviolet detection. Recoveries were 96–107% in urine with added analyte, with two exceptions (hypoxanthine, 64%; xanthine, 79%), and 89–110% in urine-soaked filter paper strips, with three exceptions (hypoxanthine, 65%; xanthine, 77%; 5-hydroxymethyluracil, 80%). The expected abnormalities were easily found in samples from patients with purine nucleoside phosphorylase deficiency, ornithine transcarbamylase deficiency, molybdenum cofactor deficiency, adenylosuccinase deficiency, or dihydropyrimidine dehydrogenase deficiency.**

***Conclusions:*** **HPLC-ESI MS/MS of urine allows rapid screening for disorders of purine and pyrimidine metabolism. The filter paper strips offer the advantage of easy collection, transport, and storage of the urine samples.**



Inherited disorders of purine and pyrimidine metabolism have a wide variety of clinical presentations which include, among others, anemia, immunodeficiency, kidney stones, convulsions, mental retardation, autism, and growth retardation *(1)*. Because of this broad clinical spectrum, a simple, rapid, and specific screening method is desired for the diagnosis of these disorders in patients at risk to enable appropriate treatment, genetic counseling, and prenatal diagnosis.

Several methods that use HPLC analysis of urine to screen for disorders of purine and pyrimidine metabolism have been reported *(2–7)*, but to date, these methods have been time-consuming and lack a specific system of detection. Therefore, we developed an analytical method for screening of disorders of purine and pyrimidine metabolism that uses reverse-phase HPLC coupled to electrospray ionization (ESI)[3] tandem mass spectrometry (MS/MS), which can be used for urine as well as for urine-soaked filter paper strips.

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[3] Nonstandard abbreviations: ESI, electrospray ionization; MS/MS, tandem mass spectrometry; 5-OH-Me-ura, 5-hydroxymethyluracil; IS, internal standard; and UV, ultraviolet.

## Materials and Methods

### CHEMICALS

Thymine, hypoxanthine, xanthine, uridine, thymidine, adenine, inosine, adenosine, and guanosine were purchased from Calbiochem. Orotic acid, pseudouridine, 5-hydroxymethyluracil (5-OH-Me-ura), deoxyinosine, deoxyguanosine, and ammonium formate were purchased from Sigma. Deoxyadenosine was purchased from P-L Biochemicals. Uracil was purchased from Fluka. Analytical/reagent-grade methanol and formic acid were purchased from Merck. Deionized water was passed through a MilliQ Labo system (Millipore). Succinyladenosine was prepared essentially according to the method of Jaeken and Van den Berghe *(8)*. In short, 0.5 mL of a solution of 785.8 μmol/L adenylosuccinate and 5 U of *Crotalus adamanteus* venom 5′-nucleotidase, both obtained from Sigma, were mixed in 40 mmol/L Tris-HCl buffer, pH 8.0, containing 4 mmol/L $Mg^{2+}$ and incubated at 37 °C for 2 h.

### INTERNAL STANDARDS

Stable-isotope-labeled reference compounds were used as internal standards (ISs); unfortunately, however, stable-isotope-labeled reference compounds were not available for all compounds of interest. Therefore, each IS was selected based on the similarity of molecular structure, retention time, and fragment pattern with the corresponding compound of interest. An IS mixture of the compounds of interest (580 μmol/L 1,3-$^{15}N_2$-uracil; 567 μmol/L a,a,a,6-$^{2}H_4$-thymine; 482 μmol/L 1,3-$^{15}N_2$-orotic acid; 604 μmol/L ribose-1-$^{13}$C-uridine; 640 μmol/L methyl-$^{13}$C-thymidine; 575 μmol/L 8-$^{13}$C-adenine; and 513 μmol/L ribose-1-$^{13}$C-adenosine) was prepared in 0.01 mol/L $NH_4OH$ adjusted to pH 7.15 with 10 mol/L formic acid. All stable isotopes were purchased from Cambridge Isotope Laboratories, Andover, Massachusetts, USA.

### LIQUID URINE SAMPLES

Urine samples, which were sent to our laboratory for conventional screening of purine and pyrimidine metabolism disorders, were used for the analysis. Fresh urine samples were kept at 4 °C for analysis within 1 week, and others were stored at −20 °C until analysis. The pH of liquid urine samples was adjusted to ~7.5 with 1.0 mol/L $NH_4OH$ before the analysis. In exceptional cases when the pH was higher, 1.0 mmol/L formic acid was used. To avoid contamination of the source and cone of the machine, which may reduce sensitivity, urine samples with creatinine concentrations of 5–10 mmol/L were diluted twofold and urine samples with creatinine concentrations >10 mmol/L were diluted threefold with 0.1 mol/L ammonium formate buffer, pH 7.5. After the addition of 20 μL of IS mixture to 180 μL of the urine or diluted urine, the samples were filtered using centrifuge filters (0.45 μm; Microspin centrifuge filters; Alltech), and 50 μL of filtrate was injected into the HPLC-MS/MS system.

### URINE-SOAKED FILTER PAPER STRIPS

Filter paper strips (12 × 40 mm; type 2992; Schleicher & Schuell) were completely dipped into urine, and then excess urine was removed by wiping it off along the wall of the test tube. The strips were dried completely at room temperature, and 20 μL of IS mixture pipetted onto the center of each strip. After the strips were completely dry, they were cut into small pieces in a 2-mL Eppendorf tube. The compounds of interest were extracted twice with 750 μL of 750 mL/L methanol in water by sonication for 10 min. Both extracts were put together and dried at 40 °C under $N_2$ flow. Dried samples were dissolved in 200 μL of 0.1 mol/L $NH_4OH$ adjusted to pH ~7.5 with 1 mol/L formic acid. After centrifugation at 12 000*g* for 5 min to remove filter paper fibers, 90 μL of the supernatant was

**Table 1. Transitions, cone voltages, and collision energies of each compound in negative ESI mode.**

| Compound | $M_r$ | Parent ion, *m/z* | Daughter ion, *m/z* | Cone voltage, V | Collision energy, eV |
|---|---|---|---|---|---|
| Uracil | 112.1 | 111 | 42 | 20 | 10 |
| Thymine | 126.1 | 125 | 42 | 20 | 10 |
| Adenine | 135.1 | 134 | 107 | 25 | 10 |
| Hypoxanthine | 136.1 | 135 | 92 | 25 | 10 |
| 5-OH-Me-ura | 142.1 | 141 | 42 | 25 | 12 |
| Xanthine | 152.1 | 151 | 108 | 25 | 10 |
| Orotic acid | 156.1 | 155 | 111 | 20 | 10 |
| Thymidine | 242.2 | 287 | 241 | 8 | 10 |
| Uridine | 244.2 | 289 | 243 | 8 | 10 |
| Pseudouridine | 244.2 | 289 | 243 | 8 | 10 |
| Deoxyadenosine | 251.2 | 296 | 45 | 8 | 10 |
| Deoxyinosine | 252.2 | 297 | 251 | 8 | 10 |
| Deoxyguanosine | 267.2 | 312 | 266 | 8 | 10 |
| Adenosine | 267.2 | 312 | 266 | 8 | 10 |
| Inosine | 268.2 | 313 | 267 | 8 | 10 |
| Guanosine | 283.2 | 328 | 282 | 8 | 10 |
| Succinyladenosine | 383.3 | 382 | 134 | 8 | 25 |

**Table 2. Recoveries and reproducibility for liquid urine supplemented with the various compounds of interest.**

| Compound | IS[a] | Added concentration, μmol/L | Recovery | | |
|---|---|---|---|---|---|
| | | | Mean, μmol/L | % | CV, % |
| Uracil | 1,3-$^{15}N_2$-Uracil | 82.9 | 80.6 | 97 | 6.1 |
| Thymine | $^{2}H_4$-Thymine | 82.9 | 82.8 | 100 | 5.3 |
| Adenine | 8-$^{13}$C-Adenine | 82.9 | 83.8 | 98 | 7.6 |
| Hypoxanthine | 8-$^{13}$C-Adenine | 82.0 | 71.3 | 64 | 2.7 |
| 5-OH-Me-ura | 1,3-$^{15}N_2$-Uracil | 82.9 | 80.6 | 97 | 8.8 |
| Xanthine | 8-$^{13}$C-Adenine | 88.3 | 79.6 | 79 | 7.9 |
| Orotic acid | 1,3-$^{15}N_2$-Orotic acid | 87.4 | 88.1 | 100 | 2.9 |
| Thymidine | Me-$^{13}$C-thymidine[b] | 92.8 | 92.9 | 100 | 2.7 |
| Uridine | Rib-1-$^{13}$C-uridine | 82.0 | 79.8 | 96 | 6.6 |
| Pseudouridine | Rib-1-$^{13}$C-uridine | 92.8 | 182.0 | 104 | 6.5 |
| Deoxyadenosine | Rib-1-$^{13}$C-adenosine | 64.9 | 69.2 | 107 | 4.2 |
| Deoxyinosine | 8-$^{13}$C-Adenine | 60.4 | 58.3 | 97 | 7.0 |
| Deoxyguanosine | Rib-1-$^{13}$C-adenosine | 85.6 | 82.9 | 97 | 4.4 |
| Adenosine | Rib-1-$^{13}$C-adenosine | 108.1 | 108.7 | 100 | 3.9 |
| Inosine | 8-$^{13}$C-Adenine | 72.1 | 76.2 | 103 | 5.2 |
| Guanosine | 8-$^{13}$C-Adenine | 89.2 | 95.3 | 107 | 5.5 |

[a] IS, added for calculation of concentrations. Data derived from 10 injections.
[b] Me, methyl; Rib, ribose.

diluted twofold to measure creatinine by the conventional alkaline creatinine picrate method *(9)*. Based on the creatinine concentrations, the residual supernatants were diluted in the same way as the liquid urine samples, and 50 μL of each supernatant was injected into the HPLC-MS/MS system.

### HPLC-MS/MS

The HP 1100 series HPLC system consisted of a binary gradient pump, a vacuum degasser, and a column temperature controller (Hewlett Packard), and was connected to a Gilson 231 XL sampling injector (Gilson).

The Supelcosil LC-18S analytical column [250 × 2.1 mm (i.d.); particle size, 5 μm; Supelco], was protected by a 20 × 2.1 mm (i.d.) guard column of the same material (Supelguard cartridge; Supelco). The column temperature was maintained at 20 °C. The mobile phases were as follows: eluent A, consisting of 0.05 mol/L ammonium formate (pH 5.0); and eluent B, consisting of a 1:1 (by volume) mixture of eluent A and methanol. The eluents

**Table 3. Recoveries and reproducibilities for urine-soaked filter paper strips.**

| Compound | IS[a] | Urine-I[b] (n = 10) | | | Urine-II (n = 9) | | |
|---|---|---|---|---|---|---|---|
| | | Mean, μmol/L | Recovery, % | CV, % | Mean, μmol/L | Recovery, % | CV, % |
| Uracil | 1,3-$^{15}N_2$-Uracil | 12.7 | 104 | 3.5 | 91.0 | 99 | 3.7 |
| Thymine | $^{2}H_4$-Thymine | 12.1 | 102 | 6.0 | 83.5 | 91 | 5.9 |
| Adenine | 8-$^{13}$C-Adenine | 13.4 | 101 | 11 | 94.5 | 101 | 2.8 |
| Hypoxanthine | 8-$^{13}$C-Adenine | 18.9 | 89 | 9.4 | 76.1 | 65 | 7.7 |
| 5-OH-Me-ura | 1,3-$^{15}N_2$-Uracil | 9.9 | 91 | 6.2 | 73.5 | 80 | 12 |
| Xanthine | 8-$^{13}$C-Adenine | 9.3 | 95 | 9.5 | 83.6 | 77 | 6.8 |
| Orotate | 1.3-$^{15}N_2$-Orotate | 16.3 | 99 | 1.5 | 90.2 | 93 | 4.4 |
| Thymidine | Me-$^{13}$C-thd[d] | 11.8 | 110 | 4.3 | 94.0 | 91 | 2.7 |
| Uridine | Rib-1-$^{13}$C-uridine | 9.8 | 108 | 5.4 | 84.9 | 91 | 6.9 |
| Pseudouridine | Rib-1-$^{13}$C-uridine | 6.9 | 106 | 4.0 | 181.6 | 101 | 9.0 |
| Deoxyadenosine | Rib-1-$^{13}$C-ado | 8.7 | 110 | 6.1 | 71.2 | 99 | 3.8 |
| Deoxyinosine | 8-$^{13}$C-Adenine | 8.2 | 92 | 12 | 64.8 | 97 | 7.5 |
| Deoxyguanosine | Rib-1-$^{13}$C-ado | 7.9 | 99 | 5.8 | 94.9 | 98 | 3.5 |
| Deoxyadenosine | Rib-1-$^{13}$C-ado | 8.7 | 105 | 9.8 | 111.6 | 93 | 3.0 |
| Inosine | 8-$^{13}$C-Adenine | 8.2 | 89 | 5.9 | 79.5 | 96 | 5.2 |
| Guanosine | 8-$^{13}$C-Adenine | 7.9 | 95 | 7.0 | 94.0 | 95 | 6.3 |

[a] IS added for calculation of concentrations.
[b] Supplemented with low concentrations of the relevant compounds.
[c] Supplemented with high concentrations of the relevant compounds. Urine-II is the same urine as described in Table 2.
[d] Me, methyl; thd, thymidine; Rib, ribose; ado, adenosine.

**Table 4. Mean recoveries and CV for duplicate samples of liquid urine (n = 10) or urine-soaked filter paper strips (n = 9).**

| Compound | Sample type | IS | Added concentration μmol/L | Recovery | |
|---|---|---|---|---|---|
| | | | | Mean, % | CV, % |
| Uracil | Liquid | 1,3-$^{15}N_2$-Uracil | 78.2 | 99 | 4.1 |
| | Filter paper | 1,3-$^{15}N_2$-Uracil | 92.0 | 100 | 4.8 |
| Thymine | Liquid | $^{2}H_4$-Thymine | 78.2 | 100 | 8.2 |
| | Filter paper | $^{2}H_4$-Thymine | 92.0 | 100 | 2.3 |
| Adenine | Liquid | 8-$^{13}$C-Adenine | 78.2 | 102 | 3.2 |
| | Filter paper | 8-$^{13}$C-Adenine | 92.0 | 97 | 6.9 |
| Hypoxanthine | Liquid | 8-$^{13}$C-Adenine | 77.4 | 105 | 13 |
| | Filter paper | 8-$^{13}$C-Adenine | 91.0 | 103 | 12 |
| 5-OH-Me-ura | Liquid | 1,3-$^{15}N_2$-Uracil | 78.2 | 88 | 7.8 |
| | Filter paper | 1,3-$^{15}N_2$-Uracil | 92.0 | 95 | 3.7 |
| Xanthine | Liquid | 8-$^{13}$C-Adenine | 83.3 | 98 | 13 |
| | Filter paper | 8-$^{13}$C-Adenine | 98.0 | 87 | 8.7 |
| Orotic acid | Liquid | 1,3-$^{15}N_2$-Orotic acid | 82.5 | 93 | 2.9 |
| | Filter paper | 1,3-$^{15}N_2$-Orotic acid | 97.0 | 98 | 6.2 |
| Thymidine | Liquid | Me-$^{13}$C-thymidine[a] | 87.6 | 92 | 1.6 |
| | Filter paper | Me-$^{13}$C-thymidine | 103.0 | 90 | 1.6 |
| Uridine | Liquid | Rib-1-$^{13}$C-uridine | 77.4 | 98 | 4.8 |
| | Filter paper | Rib-1-$^{13}$C-uridine | 91.0 | 96 | 4.3 |
| Pseudouridine | Liquid | Rib-1-$^{13}$C-uridine | 87.6 | 92 | 8.6 |
| | Filter paper | Rib-1-$^{13}$C-uridine | 103.0 | 91 | 11 |
| Deoxyadenosine | Liquid | Rib-1-$^{13}$C-ado | 61.2 | 101 | 9.2 |
| | Filter paper | Rib-1-$^{13}$C-ado | 72.0 | 100 | 7.0 |
| Deoxyinosine | Liquid | 8-$^{13}$C-Adenine | 57.0 | 98 | 5.1 |
| | Filter paper | 8-$^{13}$C-Adenine | 67.0 | 90 | 6.0 |
| Deoxyguanosine | Liquid | Rib-1-$^{13}$C-adenosine | 80.8 | 102 | 5.6 |
| | Filter paper | Rib-1-$^{13}$C-adenosine | 95.0 | 101 | 12 |
| Adenosine | Liquid | Rib-1-$^{13}$C-adenosine | 102.0 | 92 | 1.8 |
| | Filter paper | Rib-1-$^{13}$C-adenosine | 120.0 | 95 | 2.7 |
| Inosine | Liquid | 8-$^{13}$C-Adenine | 83.3 | 99 | 8.1 |
| | Filter paper | 8-$^{13}$C-Adenine | 80.0 | 93 | 7.8 |
| Guanosine | Liquid | 8-$^{13}$C-Adenine | 84.2 | 102 | 13 |
| | Filter paper | 8-$^{13}$C-Adenine | 99.0 | 97 | 6.6 |
| Succinyladenosine | Filter paper | 8-$^{13}$C-Adenine | 78.5 | 99 | 13 |

[a] Me, methyl; Rib, ribose; ado, adenosine.

were filtered through 0.45 μm membrane filters (MF-Millipore, HA type; Millipore). The compounds were eluted from the column with a linear gradient from 100% eluent A to 100% eluent B over 7 min. After 0.4 min of isocratic elution with 100% B, the mobile phase was switched back by a linear gradient to 100% A in 0.1 min. The injection interval was ~15 min. A flow rate of 0.3 mL/min was applied, and the eluate was introduced into the mass spectrometer at a rate of 10–20 μL/min.

A Quattro II tandem mass spectrometer I (Micromass) in the negative ESI mode was used. The collision gas was argon, and the cell pressure was 0.26 Pa. The source temperature was maintained at 80 °C, and the capillary voltage was 3.1 kV. Multiple-reaction monitoring, which detects a particular pattern of fragmentation of each substance, was used for the detection. The transition, cone voltage, and collision energy established for each compound are listed in Table 1.

### VALIDATION

To evaluate the efficiency of the extraction procedure, 200 μL of a calibration mixture containing all of the compounds mentioned in Tables 2, 3, and 4 was pipetted onto filter paper strips. The strips were dried at room temperature and extracted in the same way as described above. Subsequently, the extracts were analyzed by reverse-phase HPLC and ultraviolet (UV) detection as has been described previously *(2)*.

The linearity and detection limit for each compound were obtained from injections of the calibration mixtures with different concentrations. Quantification was based on the peak-area ratios of each compound to the IS that had been selected because of the similarity of the retention time, molecular structure, and/or fragment pattern. These ratios were used to calculate the concentrations by use of a calibration curve. This also compensated for the contribution of the first isotope of the unlabeled endogenous

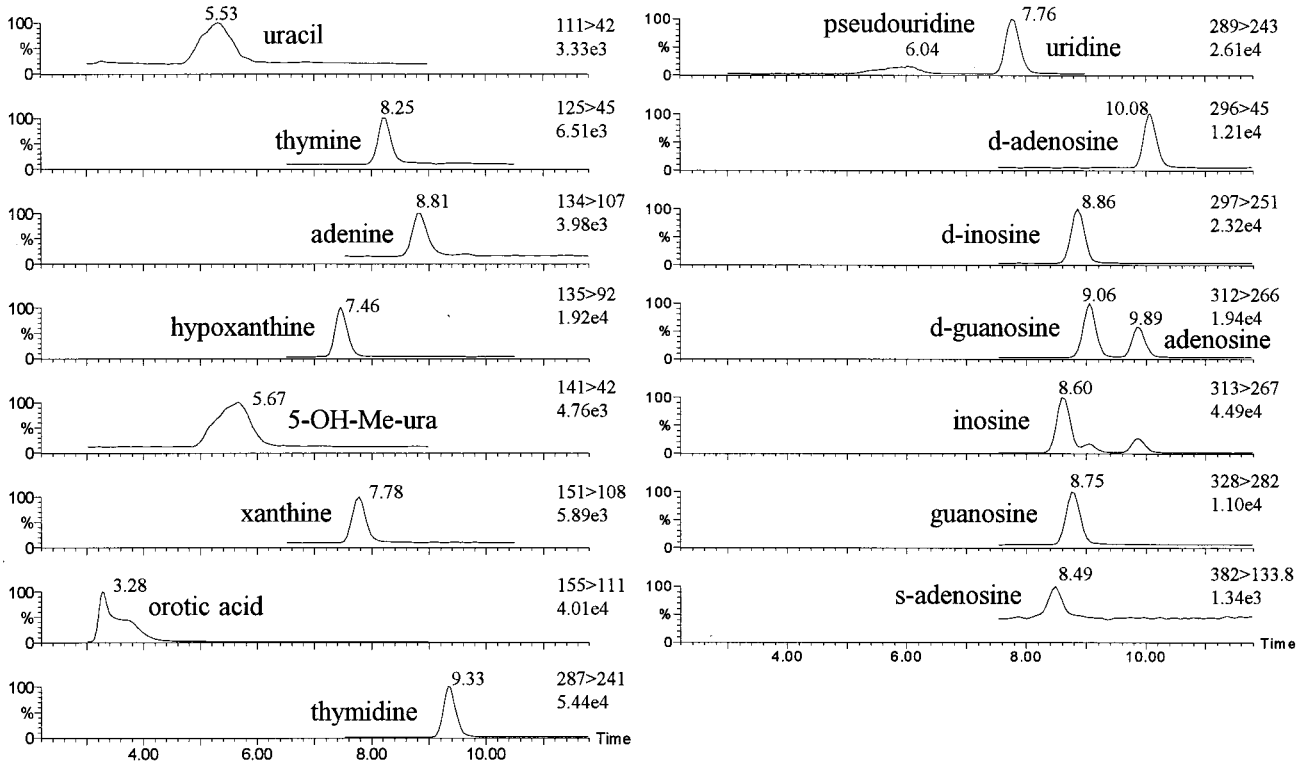


Fig. 1. HPLC-ESI MS/MS chromatograms of each compound from the calibration mixture with retention times and transitions.
The transition and response (peak height) are given at the *right* of each chromatogram. *d-*, deoxy; *s-*, succinyl.

compound to the peak of the labeled IS. The detection limit was defined as a signal-to-noise ratio of 3. Reproducibility and recovery were evaluated by analyzing (10 times) 1 liquid urine from a healthy individual and 3 urine-soaked filter paper samples enriched with synthetic compounds and by analyzing (in duplicate) 9 different liquid and 10 filter paper samples soaked in urine from a healthy individual, with and without the addition of IS compounds. Succinyladenosine was analyzed only in samples of filter paper strips soaked in 10 different urine samples from healthy individuals to evaluate the methanol extraction efficiency, reproducibility, and recovery.

The usefulness of this method was evaluated by analyzing urine samples from patients with established purine nucleoside phosphorylase deficiency, ornithine transcarbamylase deficiency, molybdenum cofactor deficiency, adenylosuccinase deficiency, and dihydropyrimidine dehydrogenase deficiency.

## Results

As shown in Table 1, specific transitions were obtained for a large number of purines and pyrimidines, making their separation by HPLC unnecessary. Therefore, separation by HPLC was used only to separate compounds with the same molecular mass and transition (two pairs, i.e., uridine and pseudouridine, and adenosine and deoxyguanosine) from each other and from interfering substances, including salt. The HPLC profiles are shown in Fig. 1. Carryover was evaluated by analyzing urine samples with very low concentrations of the compounds of interest after urine samples with added compounds. No carryover was detected.

The efficiency of the extraction of the various compounds of the calibration mixture from filter paper strips was 88–103%, with CVs of 0.7–2.2% (n = 10). No differences in the extraction yields of IS compounds were found between urine supplemented with IS mixture and subsequently soaked into filter paper, and dried urine-soaked filter paper strips to which the IS mixture was added. The extraction efficiency of creatinine with this method was 87%, with a CV of 6.3% (n = 10). The linearity of each compound was good in the range 2.5–250 μmol/L ($r^2$ = 0.984–0.999). The detection limit was adequate to detect patients with slightly increased concentrations of these metabolites (2.5 μmol/L xanthine; 1.6 μmol/L succinyladenosine; 1.0 μmol/L uracil, adenine, hypoxanthine, 5-OH-Me-ura, and pseudouridine; 0.5 μmol/L for all other compounds).

The recoveries of the compounds added at concentrations of ~100 μmol/L to liquid urine and soaked filter paper strips, which were prepared from a urine sample with creatinine of 1.6 mmol/L, are listed in Tables 2 and 3, respectively. In addition, low concentrations of compounds (~10 μmol/L) were added to a urine sample with a creatinine concentration of 0.9 mmol/L, and filter paper strips were prepared. The results are listed in Table 3. As

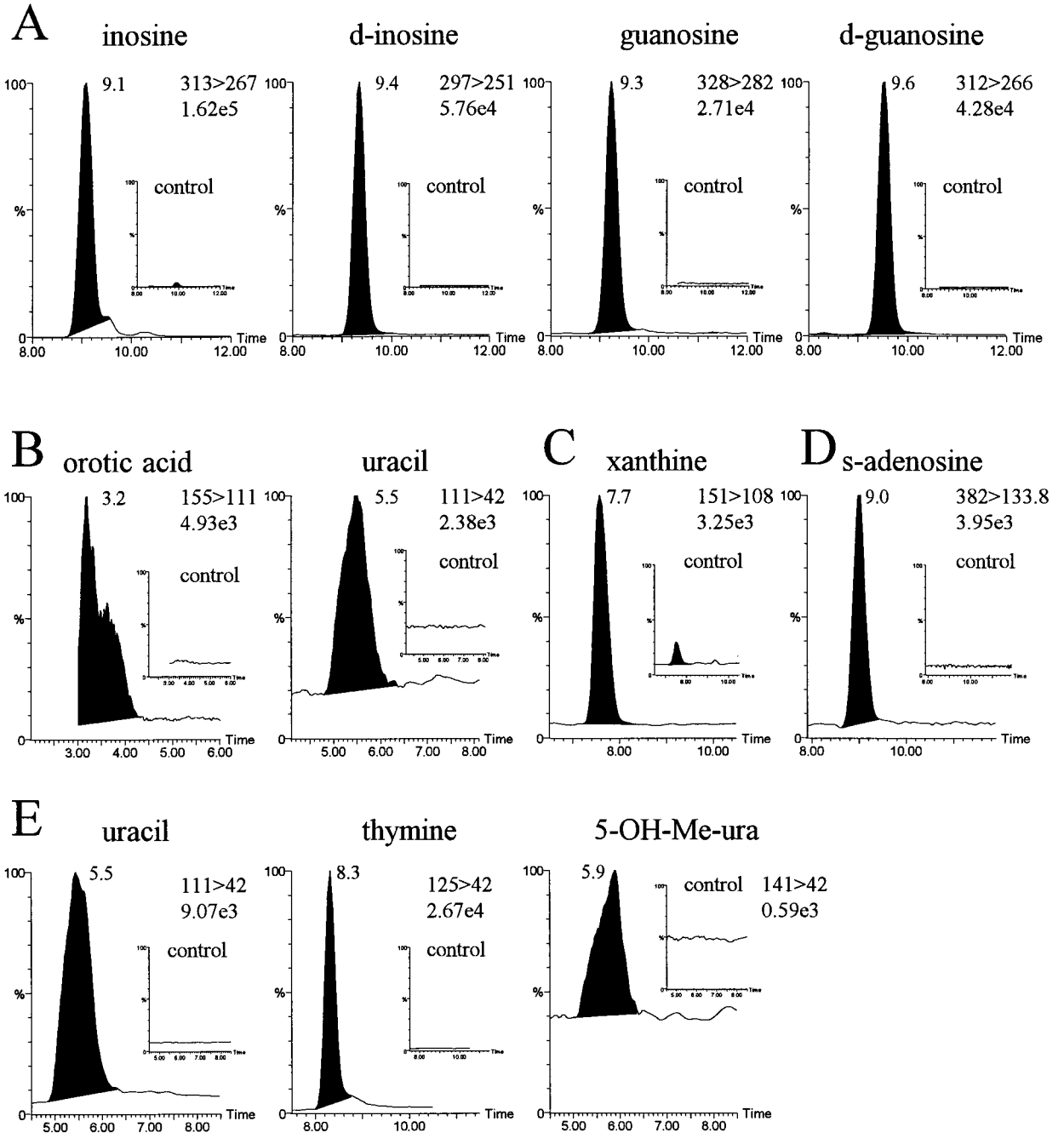


Fig. 2. HPLC-MS/MS profiles of urines from patients diagnosed with purine or pyrimidine metabolic disorders.

(*A*), male infant with purine nucleoside phosphorylase deficiency; (*B*), 22-year-old female with ornithine transcarbamylase deficiency; (*C*), 1-month-old male with molybdenum cofactor deficiency; (*D*), male infant with adenylosuccinase deficiency; (*E*), 7-year-old female with dihydropyrimidine dehydrogenase deficiency. The transition and response (peak height) are given at the *right* of each chromatogram. The peak heights for the patients and corresponding controls (*insets*) are at the same scale. Peak heights of the ISs in patients and controls were comparable and, therefore, are not shown. *d-*, deoxy; *s-*, succinyl.

can be seen, recoveries for liquid urine samples were 96–107% except for hypoxanthine and xanthine (64% and 79%, respectively). Recoveries from urine-soaked filter paper strips were 89–108% for concentrations of ~10 μmol/L and 91–101% for concentrations of ~100 μmol/L, except for hypoxanthine, xanthine, and 5-OH-Me-ura (65%, 77%, and 80%, respectively). The CVs were ≤12% for all compounds in either type of sample. A third urine sample was enriched with ~2 μmol/L of each compound and analyzed 10 times. The original concentrations of all compounds were very low except for uracil and pseudouridine. Recoveries were 70–130% for all compounds except 5-OH-Me-ura and succinyladenosine (64% and 59%, respectively). The CVs were 10–35%.

Nine or 10 different urine samples with creatinine concentrations of 0.8–17.1 mmol/L were measured in duplicate [creatinine concentrations, mean ± SD, for liquid urine, 5.6 ± 5.5 mmol/L (n = 9); for urine-soaked filter paper strips, 6.2 ± 5.5 mmol/L (n = 10)]. The mean recoveries and CVs of the various purines and pyrimidines from these samples are listed in Table 4. The mean recoveries were 87–105%, and the CVs were 1.6–13% for all compounds in either type of sample.

The chromatograms of urine samples from patients with established diagnoses are shown in Fig. 2. In all cases, the correct diagnosis was easily found.

## Discussion

For the selective screening of patients with suspected purine or pyrimidine metabolic disorders, two-dimensional thin-layer chromatography, reverse-phase HPLC, and gas chromatography–mass spectrometry analyses of urine are mainly used *(4, 5)*. These methods require complicated sample preparation, prefractionation, and adequate separation, and therefore, are time-consuming. Reverse-phase HPLC, which is the most common method for screening, needs relatively long analytical times to achieve sufficient separation of the compounds. Furthermore, many substances that have UV absorbance, such as hippuric acid, aromatic amino acids, methylated purines (e.g., caffeine and theophylline), and some kind of drugs (e.g., 6-mercaptopurine and methotrexate) may interfere in the analysis *(5)*. The connection of reverse-phase HPLC with the MS/MS enables specific analysis of most compounds by multiple-reaction monitoring. In multiple-reaction monitoring, the parent ion is detected by the first MS, and a specific daughter ion is detected by the second MS after the fragmentation in the collision cell.

Detection by MS/MS also shortens the analytical time because a complete separation of all compounds is not necessary. For the compounds mentioned in this report, only two pairs of compounds (uridine and pseudouridine, and adenosine and deoxyguanosine) had to be separated because of the same molecular mass and transition. The analysis time for one sample with our new method was approximately one-third of that of the conventional screening method using HPLC-UV *(2)*. As shown in Fig. 1, some peaks are broad with poor shapes compared with conventional HPLC-UV methods. This may have been caused by the low buffering effect of the eluent. However, high concentrations of salts in the mobile phase quenched the signals of the compounds of interest, reducing the sensitivity. By measuring the total areas of the peaks and using appropriate ISs, we obtained adequate recoveries and reproducibilities as indicated by the CV values. The lower recoveries for hypoxanthine, xanthine, and 5-OH-Me-ura in the urine supplemented with high concentrations of compounds are probably attributable to the lack of corresponding ISs, which can be used to compensate for matrix effects. However, this will not cause a problem for the detection of patients with xanthinuria or dihydropyrimidine dehydrogenase deficiency because of the high concentrations of these compounds in the urine of such patients. For the other compounds, the values are comparable to those obtained by the conventional HPLC method *(2)*.

For the analysis of acylcarnitines and amino acids by MS/MS, a loop-injection method has been reported *(10–12)*. However, it was very difficult to develop such a method for the purine and pyrimidine compounds because other urinary ions, such as salts, which substantially quench the signal of purines and pyrimidines, could not be removed adequately by ordinary methods.

Although urine samples are suitable for screening because abnormal metabolites usually accumulate in this body fluid, it is sometimes difficult to collect this material, especially from neonates and infants. Moreover, transportation of the urine samples to the specialized metabolic laboratories requires special preservation methods and transportation facilities, making it more expensive. As mentioned by some authors *(13–15)*, the use of urine-soaked filter paper strips to collect urine from babies is very easy because the strips can be placed in a baby's diaper. In addition, the strips can be transported easily and stored with lower costs. For that reason, we developed a method that uses filter paper urine samples. In this method, a simple methanol extraction is used, and therefore, it may easily be combined with other methods for newborn and/or infant screening for genetic metabolic diseases and other diseases such as neuroblastoma *(16)*.

The reproducibility and accuracy of the new method is adequate for the screening of disorders of purine and pyrimidine metabolism that are characterized by increased excretion of one or more of the compounds mentioned in Table 1. This was illustrated clearly by the application of the method to urine samples from patients with established diagnoses of purine and pyrimidine metabolism. The use of urine-soaked filter paper strips may offer the possibility of neonatal screening of disorders that cannot be detected in blood. Because no high-throughput method is currently available, the present method, because it can be completely automated, could be used for neonatal screening of disorders of purine and pyrimidine metabolism. This will allow diagnosis in an early stage, which may prevent irreversible damage by enabling adequate treatment in some of these diseases.

In conclusion, HPLC-ESI MS/MS of urine allows rapid screening for disorders of purine and pyrimidine metabolism. Liquid urine as well as urine-soaked filter paper strips can be used for this purpose. The latter offers the advantage of easy collection, storage, and transport of urine samples.

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