Determination of Urinary Amino Acids by Liquid Chromatography with "Dabsyl Chloride"

Jen-Kun Lin¹ and Chiu-Hwa Wong²

We describe a "high-performance" liquid-chromatographic procedure for measuring amino acids in 0.5 mL of urine. The procedure includes direct derivatization of amino acids in urine samples with "dabsyl chloride" (4-dimethylaminoazobenzene-4'-sulfonyl chloride). An aliquot of this dabsylated amino acid solution is analyzed on a μBondapak C18 column with ethanol/sodium acetate (20 mmol/L, pH 4.0), 4/6 (by vol.), as mobile phase. Dabsylated amino acids are detected by their absorbance at 425 nm and quantitated by measuring peak heights. The procedure allows for the reliable analysis of amino acids in urine at concentrations near 16 mg/L. The sensitivity of this analysis on column approaches 5 ng/sample. Higher urinary tryptophan concentrations were found in the urines of some cancer patients, whereas we saw no significant difference in urinary glucose between cancer patients and control subjects. The present method was shown to be a straightforward procedure for detecting phenylalanine in phenylketonuric urine. Extension of this procedure to screening for other inborn errors of aminoaciduria is recommended.

Additional Keyphrases: heritable disorders · screening · phenylketonuria · cancer

Dabsyl chloride (4-dimethylaminoazobenzene-4'-sulfonyl chloride) is a useful chromophoric labeling reagent for amino acids (1, 2). It reacts readily with all amino acids to form dabsyl amino acids, which can be seen on a thin-layer chromatographic plate and are photostable. The sensitivity of dabsyl chloride is about 60-fold that of 2,4-dinitrofluorobenzene, but about fivefold less than that of dansyl chloride; on the other hand, the stability and visibility of dabsyl amino acids on a thin-layer chromatographic plate are superior to that of either dansyl- or 2,4-dinitrophenyl-labeled amino acids (2).

Recently, we reported the use of dabsyl chloride and thin-layer chromatography on silica gel plates for the evaluation of urinary amino acids in various diseases (2). Our preliminary results indicated that high amounts of tryptophan and glycine occur in the urine of patients with chronic myeloblastic leukemia, acute myeloblastic leukemia, and lymphosarcoma. To assess the biological significance of these increases of these amino acids, we made further quantitative analyses of more urine samples. In doing so, we found that the combination of dabsylation and thin-layer chromatographic procedure was quite satisfactory for qualitative detection, but was quite tedious for quantitative determination of urinary amino acids.

During the past few years, much attention has been focused on new methods for measuring endogenous metabolites in biological materials. The so-called "high-performance" liquid-chromatographic technique has proved particularly useful for separation and measurement of such compounds. In this study, we combined this technique with dabsylation for the qualitative and quantitative assay of urinary amino acids in phenylketonuria and in patients with various cancers. We describe the direct derivatization of urinary amino acids with dabsyl chloride and conditions for their liquid-chromatographic separation.

Materials and Methods

Apparatus

We used a high-performance liquid-chromatographic system (Waters Associates, Milford, MA 01757), consisting of a Model 6000A solvent-delivery system, a Model U6K universal injector, and a Model 450 variable-wavelength detector operating at 425 nm. We used a reversed-phase μBondapak C18 column, 30 cm × 3.9 mm (i.d.). The mobile phase was ethanol in sodium acetate buffer (20 mmol/L, pH 4.0), 4/6 by volume, and the flow rate was 1.0 mL/min at a pressure of 6.9–10.35 MPa (1000–1500 psi).

Chemicals

Dabsyl chloride (m.p. 186–188 ºC) was prepared either by reaction of 4-dimethylaminobenzene with chlorosulfonic acid (2) or by treatment of sodium 4-dimethylaminobenzene-4'-sulfonate with phosphorus pentachloride (1). This reagent is also available from Polysciences Inc., Warrington, PA 18976. An L-amino acid kit (cat. no. LAA-21) was purchased from Sigma Chemical Co., St. Louis, MO 63178. Solvents and other chemicals were of AR grade.

Sampling

Urine samples were obtained from 90 patients with disorders such as acute lymphocytic leukemia, chronic myeloblastic leukemia, acute myeloblastic leukemia, lymphosarcoma, Hodgkin's disease, and polycythemia. Twelve urine samples collected from persons coming to the University Hospital for physical examination served as controls. A sample of a phenylketonuric urine was kindly provided by Professor Yu-Zen Shen, Department of Pediatrics, National Taiwan University Hospital. All urine samples were stored at −30 ºC for two to six weeks before use.

Dabsylation

Place 0.5 mL of urine, 0.5 mL of 0.5 mol/L sodium bicarbonate, and 2 mL of dabsyl chloride (2 g/L, in acetone) into a 15-mL glass tube; the final pH of this mixture should be 8.5–9.0. Stopper the tube, vortex-mix for 1 min, and allow the tube to stand at ambient temperature (25–26 ºC) for 30 min; then filter it through a 0.45-µm Millipore filter (Millipore Corp., Bedford, MA 01730) and use the filtrate directly for chromatography. If the urine sample is contaminated with amino compounds such as ingested drugs, remove the dabsyl amino compounds thus formed in the reaction mixture by extraction with chloroform.

Prepare standard curves by adding 0.3 mL of authentic amino acid mixtures containing each amino acid in amounts of 0.0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg to 0.2 mL of pooled

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normal urine and treating these samples as described above. Determine the concentrations of amino acids from a graph relating peak heights to the concentrations of known standards.

Results

Separation of Dabsyl Amino Acids

Because high concentrations of urinary tryptophan and branched amino acids were detected in some cancer patients in a previous study (2), we concentrated here on the estimation of these amino acids. Accordingly, we developed a liquid-chromatographic procedure for quantitative determination of these amino acids in urine. An amino acid mixture containing tryptophan, valine, leucine, isoleucine, phenylalanine, and glycine was dabsylated and analyzed as described. Figure 1, a representative chromatogram, shows the sharp, distinct peaks produced by the hydrolyzed product, 4-dimethylaminoazobenzene-4'-sulfonic acid, and by labeled glycine, methionine, valine, tryptophan, and phenylalanine; retention times were 3.6, 6.5, 11.7, 15.5, 17.5, and 21.3 min, respectively. Leucine and isoleucine somewhat overlap in this system.

The highly polar amino acids such as glutamic acid, aspartic acid, threonine, and serine will come out together right after the void volume; the basic amino acids such as lysine and arginine will stay on the column and can be eluted with increased concentrations of ethanol.

Analytical Variables

Linearity and sensitivity. Linear calibration curves passing through the origin were obtained by supplementing blank normal urine with known amounts of amino acids, ranging from 1.6 to 33 mg/L (Figure 2), corresponding to the ranges for urine samples obtained from cancer patients. The minimum detectable concentration of glycine in our samples of urine was about 9.6 mg/L. This produced a 3% deflection of the recorder pen when the recorder was set at 0.1 A full-scale. The volume of sample injected was 5 μL. The sensitivity of this analysis approaches 5 ng/sample if the recorder is set at 0.01 A full-scale.

Analytical recovery. The percentage of analytical recovery of glycine added to six aged human samples (0.1 mg added to 0.2 mL) was 60–70%; however, the mean analytical recovery of glycine added to eight fresh samples (0.105 mg added to 0.2 mL) was 99%, the range 96 to 103%. The ammonia and other amino compounds produced in the aged urine samples could affect the recovery of the amino acid by competing for the available dabsyl chloride. When the ammonia was removed by lyophilizing the specimen, the recovery of amino acid added to urine improved remarkably (80–90%). The mean analytical recovery of tryptophan added to six fresh human samples (87 μg to 0.2 mL) was 96%, the range 94 to 98%.

Precision. We assessed within-run precision of the assay by processing aliquots of dabsylated glycine solution through the procedure during a single day. The coefficients of variation (CVs) were 5.9% at 33 mg/L (n = 10), 6.0% at 11 mg/L (n = 10), and 2.4% at 2.75 mg/L (n = 8). Day-to-day precision was checked during three weeks. The CVs were 5.2% at 33 mg/L (n = 14), 6.4% at 11 mg/L (n = 16), and 7.6% at 2.75 mg/L (n = 13).

Analysis of Urine Sample

Urine samples from 90 cancer patients and 12 ostensibly normal subjects were dabsylated and analyzed by the described procedure. Figure 3B, the chromatogram of a urine sample from a normal subject, demonstrates peaks for glycine and ammonia. The chromatogram in Figure 3A demonstrates the presence of glycine, ammonia, and tryptophan in a urine sample of cancer patient. The presence of glycine and tryptophan in the urine sample has been confirmed by two-dimensional thin-layer chromatography of their dabsylated derivatives on silica-gel plates (9).

All normal and patients' urine samples contained detectable glycine (Table 1), with no significant difference between samples from normal subjects and cancer patients. The range for each group was quite large.

Chromatograms of most urine samples from malignant-disease patients showed a peak behaving like tryptophan (Table 1). Of 90 urine samples from patients with various kinds of malignant diseases, 76 (84%) contained a detectable amount of apparent tryptophan; in contrast, only four of the 12 control urine samples from normal subjects showed this peak.

Fig. 2. Standard curves for glycine (Gly), tryptophan (Trp), and phenylalanine (Phe): chromatographic peak-heights, in absorbance units, vs amino acid concentrations (ng/sample)

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During the course of this study, urine samples were collected without conscious order or bias from cancer patients admitted to the University Hospital. Some of them were newly diagnosed and were in a progressive stage; others were being treated with antineoplastic drugs and were in a remissive stage. When we compared our results for these two groups, 89% (63 of 71 cases) of the patients with a progressive stage of disease excreted apparent tryptophan, and 68% (13 of 19 cases) of the patients in remission did so. The mean concentration of tryptophan in normal urine is 12.4 mg/L, far less than that excreted by progressive-cancer patients (Table 1). Patients in remission from chronic myeloblastic leukemia or lymphosarcoma excreted very little apparent tryptophan in their urine. We cannot say whether the excretion of this apparent tryptophan in the urine is unique to cancer patients; some cancer patients did not show it at all.

### Table 1. Urinary Glycine and Tryptophan in Cancer Patients and Controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycine</th>
<th>Tryptophan</th>
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<tbody>
<tr>
<td></td>
<td>No. samples analyzed</td>
<td>No. samples positive</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Acute lymphocytic leukemia</td>
<td></td>
<td></td>
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<tr>
<td>Progressive</td>
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<td>8</td>
</tr>
<tr>
<td>Remissive</td>
<td>7</td>
<td>7</td>
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<tr>
<td>Acute myeloblastic leukemia</td>
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<tr>
<td>Remissive</td>
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<td>3</td>
</tr>
<tr>
<td>Chronic myeloblastic leukemia</td>
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<td>Lymphosarcoma</td>
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<td>Remissive</td>
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</table>
Detection of Urinary Phenylalanine in Phenylketonuria

A urine sample from a phenylketonuric patient was dabsylated and analyzed as described. Figure 4A illustrates the presence of phenylalanine (0.6 g/L) in the patient’s urine, as was further confirmed by co-chromatography of the dabsylated urine sample with dabsyl-phenylalanine (Figure 4B). Under the same experimental conditions, no phenylalanine was detected in normal urine (Figure 3B). Evidently, our procedure is a rapid and suitable one for detecting phenylketonuria.

Discussion

In the present method we made use of the newly developed reagent, dabsyl chloride, to label amino acids. The dabsylated amino acids have maximum absorbance near 425 nm. Thus amino acids in urine samples are easily measured in the visible region, and interferences caused by ultraviolet-absorbing compounds in urine are avoided.

The appreciable amount of ammonia in urine interferes with the determination of methionine and valine. Ammonia reacts with dabsyl chloride to form dabsyaldehyde, which has the same retention time (11.7 min) as that of dabsyl methionine (cf. Figures 1 and 3A). Because the ammonia may consume so much dabsyl chloride that it seriously interferes with the dabsylation of amino acids in the urine samples, it is advisable to remove ammonia by lyophilizing urine samples at pH 8.9 before dabsylation, particularly if the urine specimen has been stored long.

Mean values for urinary glycine and tryptophan of normal subjects are reportedly (3) about 100 and 13 mg/L of urine, respectively—very close to our data (60 and 12.4 mg/L). This seems to be the first time that high urinary tryptophan has been noted in most cancer patients. As has been commonly observed, many cancer patients lose a significant fraction of their stable proteins; the mechanism of the loss is unknown (4). The growth of cancer leads to profound alterations of organs and functions, a consequence of multiple interactions between destruction, attempted repair, homeostasis, and production of biochemical toxins by the tumor. The overall result is the widely recognized, although poorly understood, syndrome known as cachexia (5). It is common to think of tumors as one-way nitrogen traps and to infer that nutritional consequences of tumor growth on the host are dominated by this irreversible step (4). If this is the case, the tumor seems to have a peculiar capability to capture protein molecules from the host tissues. Most amino acids liberated from the captured protein may be utilized by the tumor cells, and a few amino acids such as tryptophan may be excreted in the urine, as demonstrated in the present study; however, the reason that only tryptophan is increased is unknown.

With the present method we can identify phenylketonuria in 52 min. In this study, of 102 samples analyzed, none showed urinary phenylalanine, but analysis of phenylketonuric urine showed urinary phenylalanine unequivocally (Figure 4A). Other inborn-errors of metabolism characterized by aminoaciduria (6) such as maple-syrup urine disease (7), Hartnup disease (8), homocystinuria (9), and hyperglycinemia (10) should be detectable by the present method. Still other amino-acid metabolic defects such as hyperprolinemia (11), hydroxyprolinemia (12), histidinemia (13), hyperargininemia (14), and hyperlysinemia (15) may be detected if a suitable mobile phase is selected. A paper-chromatographic screening test for the detection of disorders of amino acid metabolism was reported by Efron et al. (16). Their procedure involves four color reagents and overnight chromatographic development and is quite tedious. Our method appears to be more sensitive, faster, and simpler for this kind of screening.

Other mobile phases than the one used in this study also gave good results: methanol/water (4/6 by vol) or methanol/sodium acetate buffer (4/6). Furthermore, the methanol systems could operate at 1 mL/min with a lower column pressure (less than 6.9 MPa).

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References

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