Application of Simple Chromatographic Methods for the Diagnosis of Defects in Pyrimidine Degradation


Recent findings suggest that inborn errors of pyrimidine catabolism are less rare than generally assumed. We propose a complete set of diagnostic methods for these disorders, suitable for the clinical chemistry laboratory, and present relevant reference data. Applications of thin-layer chromatography, high-performance liquid chromatography, and conventional cation-exchange amino acid analysis lead to detection of various defects in pyrimidine degradation, including the recently described deficiencies of dihydropyrimidine dehydrogenase and dihydropyrimidinase. We also illustrate the potential of the methods to analyze for the catabolites expected to be increased in the urine of patients with ureidopropionase deficiency. Possible pitfalls in the diagnosis and ways to prevent misdiagnosis are demonstrated. The methods offer possibilities for clinical chemistry laboratories to extend their diagnostic capacity to the new area of pyrimidine degradation defects.

Indexing Terms: dihydropyrimidines - N-carbamylamino acids - amino acids - heritable disorders - metabolism - urine

In humans, pyrimidines are degraded in four steps (Figure 1), catalyzed by dihydropyrimidine dehydrogenase (DHPD, EC 1.3.1.2), dihydropyrimidinase (DHP, EC 3.5.2.2), β-ureidopropionase (UP, EC 3.5.1.6), and three aminotransferases: R(-)-3-amino-2-methylpropionate-γ-pyruvate aminotransferase (D-3-aminoisobutyrate-γ-pyruvate aminotransferase; AIBPAT, EC 2.6.1.40), β-alanine-γ-pyruvate aminotransferase (BAPAT, EC 2.6.1.18), and 4-amino-γ-butyrate aminotransferase (ABAT, EC 2.6.1.19).1

Inherited deficiencies of DHPD (1) and AIBPAT (2, 3) are well known. A case of dihydropyrimidinuria attributed to a defect in the activity of DHP was recently described (4). Deficiency of ABAT is hypothesized in patients with primary hyper-β-alaninemia (5), but BAPAT and UP deficiencies have not yet been described in humans. UP deficiency has been detected in the C57B1/6 mouse (6).

Recent findings such as the detection of 11 patients with DHPD deficiency in The Netherlands alone, of which details for 8 have been published (1, 7–10), and of the first patient with presumed DHP deficiency (4), suggest that inborn errors of pyrimidine degradation are less rare than has been generally assumed. Therefore, we believe that the clinical chemistry laboratory involved in the diagnosis of inherited metabolic diseases should have available efficient screening methods for these disorders.

Here we present simple methods for detecting the known defects as well as some predicted defects. Some of these methods have been described elsewhere, but we also present additional methods for detecting the as-yet not established defects, as part of an overview of analyses for this group of disorders. Examples of applications of these methods illustrate their usefulness clinically.

Principles of the Methods

DHPD deficiency is characterized by increased excretion of uracil and thymine and in some cases also 5-hydroxymethyluracil. These compounds can be analyzed by two-dimensional thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) after their isolation from urine by anion-exchange column chromatography.

We expect DHP deficiency to be discovered by increased excretion of the dihydropyrimidines dihydrouracil (DHU) and dihydrothymine (DHT), UP deficiency by increased excretion of the N-carbamylamino acids N-carbamyl-β-alanine (NC-β-ala) and N-carbamyl-β-aminoisobutyric acid (NC-β-AIB). Deficiencies of the transaminases AIBPAT and BAPAT or ABAT are characterized by increased excretion of β-aminoisobutyric acid (β-AIB) and β-alanine (β-ala), respectively.

A deficiency of any or all of these enzymes can be detected by methods based on amino acid analysis because the relevant metabolites either are β-ala and β-AIB or can be converted to these β-amino acids (Figure 2). This principle can be used to develop an overall screening method for defects of the pyrimidine degradation pathways beyond the DHPD enzyme. The principle can also be used for the differential diagnosis of the various enzyme defects on the metabolite level by amino acid analysis.

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Fig. 1. Pyrimidine degradation pathways

Enzymes: 1, dihydropyrimidine dehydrogenase; 2, dihydroxypirimidinase; 3, ureidopropionate; 4a, R(−)-β-AIB pyruvate aminotransferase; 4b, β-alanine and 4-amino butyrate aminotransferase.

Fig. 2. Illustration of the principle of the methods of screening for pyrimidine degradation defects, showing the relevant β-amino acids and β-amino acid-containing compounds found in urine:

Carnosine, asarline, and Valine are endogenous dipeptides of dietary origin.

acid analysis after chromatographic prefractionation of the urine.

Materials and Methods

Chemicals

Uracil, DHU, thymine, 5-hydroxymethyluracil, DHT, NC-β-ala, N-carbamyl norvaline, asarline, and carnosine were purchased from Sigma Chemical Co., St. Louis, MO. Sulfosalicylic acid and mercuric acetate were from Merck, Darmstadt, Germany; diophenylcarbamate from Baker, Deventer, The Netherlands; norvaline from Serva, Heidelberg, Germany; β-alanine from BDH, Poole, UK; and β-AIB from Fluka, Buchs, Switzerland.

The ion-exchange resins Dowex 50W ×8 (50–100 mesh) and Dowex 1 ×8 (50–100 mesh) were purchased from Fluka and Sigma, respectively. These and all other chemicals were of the highest quality commercially available.

TLC plates, DC-alulofilen, 20 × 20 cm (cellulose layers 0.1 mm), were obtained from Merck.

Apparatus

To isolate the pyrimidines from urine by anion-exchange column chromatography, we monitored the effluent at 254 nm with a Uvicord II detector and collected it with an Ultrorac fraction collector (both from Pharmacia, Bromma, Sweden).

The HPLC system consisted of a gradient solvent delivery system, Series 410 (Perkin-Elmer, Norwalk, CT); a WISP 7108 automatic sampler with cooling accessory (Waters Associates, Inc., Milford, MA); an LC-235 diode-array detector; and a Nelson computing integrator (both from Perkin-Elmer). The 250 × 4.6 mm (i.d.) analytical column, Supelcoil LC-18S, particle size 3 μm, was protected by a 20 × 4.6 mm guard column, Supelguard LC-18S, particle size 5 μm (both from Supelco Inc., Bellefonte, PA).

Amino acid analyses were performed by automated high-performance cation-exchange column chromatography with the Chromakon-500 system (Tegimenta AG, Rotkreuz, Switzerland), combined with a Nelson computing integrator.

Analytical Methods

Pyrimidine bases. The pyrimidine bases uracil and thymine can be determined by two-dimensional TLC or HPLC. 5-Hydroxymethyluracil, a metabolite of thymine, can also be analyzed with these techniques. The TLC method, originally developed to screen for defects of purine and pyrimidine metabolism in general, has been described in detail earlier (17).

Essentially, the pyrimidines are isolated from urine and fractionated on anion-exchange columns into three concentrated salt-free solutions, which are subjected to TLC. The fraction containing the basic and neutral compounds (fraction III) is analyzed on 10 × 10 cm cellulose layers by ascending chromatography with isopropanol/ammonia, 50 mL/L (8/2, vol), and butanol/acetate acid/water (8/2/2, by vol), for the respective two directions of development. The chromatogram is examined under ultraviolet light (254 nm) and sequentially sprayed with a solution of 0.25 g of mercuric acetate in 100 mL of ethanol (960 mL/L) containing 5 drops of acetic acid, and a solution of 0.05 g of diphenylcarbzone in 100 mL of ethanol (960 mL/L). After the TLC plates are heated (120 °C), the compounds become visible.

Alternatively, fraction III can be analyzed by HPLC. The compounds are separated on a reversed-phase column (Supelco LC-18S) by using a gradient system of potassium dihydrogen phosphate (10 mmol/L) and methanol. Details of this method have also been published (12).

Dihydroxypyrimidines and NC-β-amino acids: screening. For hydrolysis of the urine, add 2.5 mL of 6 mol/L HCl and 0.05 mL of internal standard solution I (norvaline, 2.5 mmol/L, in doubly distilled water) to 0.5 mL of urine. Hydrolyze at 150 °C for 18 h in a closed tube. After cooling the sample, dry it under reduced pressure and redissolve the residue in 0.5 mL of a 15 g/L aqueous solution of sulfosalicylic acid for deproteinization. Cen-
trifuge at 11,000 × g and use the supernate for amino acid analysis.

Also prepare unhydrolyzed urine, deproteinizing 1 mL of urine with 100 μL of 150 g/L aqueous sulfosalicylic acid, and centrifuging (11,000 × g). Then, to 150 μL of the supernate (from urine or hydrolysate), add 15 μL of internal standard solution II (5-2-aminoethylcysteine, 2.5 mmol/L, in doubly distilled water) and 165 μL of the first elution buffer (lithium citrate buffer, pH 2.3).

We then use the standard procedure for amino acid analysis in physiological fluids stated by the manufacturer; the method is based on cation-exchange chromatography with lithium citrate buffers and postcolumn ninhydrin detection and is mainly according to Moore et al. (13).

Because either amino acid, β-Ala or β-alanine, may occur in unhydrolyzed urine and because the β-alanine-containing dipeptides carnosine, anserine, and balenine may also be present, one must correct for their contributions, if any. To calculate the sum of DHU and NC-β-alanine, correct the concentration of β-alanine measured after hydrolysis by subtracting the values for β-alanine and β-alanine-containing dipeptides (carnosine, anserine, and balenine) determined in the unhydrolyzed urine.

Dihydropyrimidines and NC-β-amino acids: differential analysis. The dihydropyrimidines, NC-amino acids, and other amino acids can also be determined separately by using an amino acid analyzer. However, the compounds first have to be isolated from urine in three different fractions. Subsequently, the first two fractions are hydrolyzed to obtain the corresponding amino acids. Finally, the amino acids in the three fractions are quantified. This method has been described in detail elsewhere (14). Essentially, the isolation and prefractionation is performed by cation- and anion-exchange chromatography with a dual-column system. The dihydropyrimidines, being relatively uncharged, are eluted with 40 mL of doubly distilled water. The amino acids are retained on the cation-exchange column and are subsequently eluted with ammonia. The anion-exchange column retaining the NC-amino acids is eluted with formic acid. The effluents containing the dihydropyrimidines or NC-amino acids are enriched with internal standard (N-carbamoylnorvaline), dried under reduced pressure, and hydrolyzed to obtain the corresponding amino acids.

β-Amino acids. We performed two-dimensional TLC of amino acids according to Wadman et al. (15). To quantify the β-amino acids, we used the above-described amino acid analyzer method.

Validation and Application of the Methods
Pyrimidine Bases

The method for isolation and TLC of pyrimidine bases was originally developed to screen for disorders of pyrimidine as well as purine metabolism (16); therefore, either group of metabolites can be detected on the chromatograms. Technical details concerning stability of the compounds, analytical recovery, and reproducibility have been described (16). An updated map (two-dimensional chromatogram) showing the positions of the pyrimidines and purines as well as normal urinary metabolite patterns has been presented (17).

For HPLC analyses for pyrimidine bases, we prepared calibration curves for each compound and found linear relationships between absorbance peak areas and concentrations up to 2 mmol/L. Analytical recoveries were determined by adding the synthetic compounds to a preanalyzed urine specimen. The recoveries for uracil, thymine, and 5-hydroxymethyluracil were 103%, 100%, and 99%, respectively (concentration range 0–2 mmol/L, n = 5). The interassay reproducibilities (CV) for uracil and thymine were <5.2%, for 5-hydroxymethyluracil 9.5%.

Reference values. In normal children, the ranges for the excretion of uracil and thymine are respectively 7–33 and 0–3 mmol/mol creatinine (n = 93). For children <1 year, the respective ranges are 3–18 and 0–4 mmol/mol creatinine (n = 60). 5-Hydroxymethyluracil is usually undetectable.

DHDPD deficiency. Patients affected with this defect present with thymine–uraciluria, because uracil and thymine cannot be converted into DHU and DHT, respectively. In some cases 5-hydroxymethyluracil, a thymine metabolite, is also excreted. Figure 3 shows a characteristic two-dimensional TLC pattern from an affected subject and from a normal subject. The HPLC profile prepared from the same fraction of the affected patient’s urine is presented in Figure 4. This patient excreted uracil and thymine in respective concentrations of 208 and 37 mmol/mol creatinine, as well as a small amount of 5-hydroxymethyluracil. These values were five- and ninefold the normal mean exclutions of uracil and thymine (and 5-hydroxymethyluracil is undetectable in normal urine).

DHP deficiency. The patient with presumed DHP deficiency excreted moderately increased amounts of uracil (49 mmol/mol creatinine) and thymine (12 mmol/mol creatinine) but a normal (undetectable) amount of 5-hydroxymethyluracil.

Dihydropyrimidines and NC-β-Amino Acids: Screening

Recoveries were determined by the standard addition method, with use of samples enriched with synthetic DHDP-defl. Thy normal pattern

Fig. 3. Characteristic two-dimensional TLC of fraction III (see text) of the urine from a DHDP-deficient patient, showing excessive thymine–uraciluria and a small amount of 5-hydroxymethyluracil; a normal pattern is shown for comparison.
compounds in concentrations covering the normal range and the pathological values expected in patients. The overall recoveries established for DHU, DHT, and NC-β-alanine ranged from 91% to 95% (n = 9). The interassay reproducibility was <9.4% (n = 22). For each compound the relationship between absorbance peak area and concentration was linear.

Reference values. We applied the screening method to urine specimens from 125 patients thought to have an inborn error of metabolism and from healthy volunteers. The results are summarized in Table 1. The sum of DHU and NC-β-alanine varies somewhat but on average exceeds the sum of DHT and NC-β-AIB.

DHPD deficiency. As expected, the excretion of the dihydropyrimidines + carbamylamino acids in this case is in the control range (Table 2). The excretion values in the mother and half-brother of the patient are comparable with those in the healthy volunteers.

DHP deficiency. In the patient with presumed DHP deficiency, we found a strongly increased excretion of

<table>
<thead>
<tr>
<th>Table 1. Reference Values for Excretion of DHU plus NC-β-alanine and DHT plus NC-β-AIB in 125 Children Thought to Have an Inborn Error of Metabolism and in 6 Healthy Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual range (and mean ± 2 SD)</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>DHU + NC-β-alanine</td>
</tr>
<tr>
<td>μmol/L</td>
</tr>
<tr>
<td>Children</td>
</tr>
<tr>
<td>(134 ± 155)</td>
</tr>
<tr>
<td>Adults</td>
</tr>
<tr>
<td>(173 ± 84)</td>
</tr>
</tbody>
</table>

the sum of DHU and NC-β-alanine as well as the sum of DHT and NC-β-AIB. His brother excreted normal amounts (Table 2).

UP deficiency. No patient with this defect has yet been detected. We postulate that such a patient will exhibit values similar to those in DHP-deficient patients.

Dihydropyrimidines and NC-β-Amino Acids: Differential Analysis

Recoveries were established by using the standard addition method. For concentrations of DHU, DHT, and NC-β-alanine of 250–1000 μmol/L, the respective analytical recoveries were 91–94%, 99–103%, and 92–100% (n = 10). The interassay reproducibilities varied between 8% and 11% (n = 10). For each compound the relationship between absorbance peak area and concentration was linear.

Reference values. We analyzed urine samples from six control children who were hospitalized for various reasons. The results are presented in Table 3.

Enzyme deficiencies. As Table 3 shows, the DHPD-deficient patient, her mother, and half-brother cannot clearly be discriminated from the control subjects. The patient with presumed DHP deficiency excreted strongly increased concentrations of the dihydropyrimidines but decreased concentrations of the NC-β-amino acids.

β-Amino Acids

The methods for two-dimensional TLC and quantification of β-amino acids have long been in routine use in many laboratories. For detailed technical information and applications, the reader is referred to the literature (17–19).

In normal children, the range for the excretion of β-AIB is 0–79 mmol/mol creatinine (n = 57). β-Ala is not detected by the usual methods.

In cases of DHPD, DHP, and UP deficiency, a low excretion of the β-amino acids is expected. However, some β-AIB originating from valine will be detected. Some examples are given in Table 3. Deficiencies of the transaminases AIBPAT, BAPAT, and ABAT will be discovered by increased amounts of the relevant β-amino acids in the urine.

Pitfalls

Besides the primary enzyme deficiencies, other conditions may cause similar abnormalities in metabolite
patterns. False interpretation of these abnormalities can lead to misdiagnosis.

Thymine–uraciluria, which can indicate either DHPD or DHP deficiency, may also result from an increased tissue degradation if the capacity of DHPD has been exceeded. However, this situation may also be detected by hyper-\(\beta\)-aminoisobutyric aciduria and hyper-\(\beta\)-alaminuria (18, 19) (e.g., see Table 4).

In DHP-deficient patients, bacterial contamination of the urine may enhance the thymine–uraciluria to the amounts found in DHPD deficiency, because thymine and uracil can be partly rehydrolyzed from the corresponding dihydropyrimidines by bacterial metabolic activity. In the example shown in Table 4, an additional contribution to uracil may have originated from pseudouridine. Even more complicating is the production of NC-\(\beta\)-amino acids from the dihydropyrimidines by bacteria, which may suggest UP deficiency (see Table 4).

Discussion

The methods we have presented can easily be applied in the clinical chemistry laboratory equipped with an amino acid analyzer. Relatively simple facilities are required for the two-dimensional TLC methods. Quantitative analyses of the pyrimidine bases with HPLC are useful but not strictly necessary because TLC provides for semiquantitative estimation.

These methods have proven reliable in applications to patients with defects of pyrimidine degradation. The combination of the methods allows the detection of a possible defect of each step in the catabolic pathway.

Methods for assaying pyrimidine bases and amino acids have been in use for many years in laboratories doing metabolic investigations, but methods for assays of the dihydropyrimidines and the NC-\(\beta\)-amino acids are new. These methods provide the opportunity to detect previously undiscovered defects.

Some analytical aspects of the methods for the analysis of the dihydropyrimidines and the NC-\(\beta\)-amino acids should be mentioned. In the screening method, a negative value is sometimes found for the difference in concentration of \(\beta\)-AIB in urine before and after hydrolysis. In our experience this is caused by an unknown compound co-eluting with \(\beta\)-AIB, which disappears after hydrolysis; \(\beta\)-AIB itself is not affected by the hydrolysis. The contribution of the interference can be estimated by visual inspection of the \(\beta\)-AIB spot on the TLC chromatogram. NC-norvaline is added to the urine in the screening method for the dihydropyrimidines and NC-\(\beta\)-amino acids as an internal standard to control for the efficiency of the hydrolysis step. In the differential analysis this internal standard is added to each of the isolated fractions for the same purpose. However, because it appears to be mostly eluted in the water effluent containing the dihydropyrimidines, NC-norvaline is not suitable for sole control of the differential isolation.

Applying the screening method to a population of children thought for various reasons to have an inborn error of metabolism yielded a set of reference values for the sum of the uracil- or thymine-derived dihydropyrimidines and NC-\(\beta\)-amino acids excreted. For practical purposes, the use of the actual ranges is preferred. Nevertheless, the established ranges remain far below the values found in the DHP-deficient patient, thus validating their reliability for screening. By differential analysis of the individual compounds, the block in the degradation pathway can easily be located.

One must be aware of conditions that may complicate the interpretation of results obtained with the various methods presented. In addition to the situations dis-

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**Table 3. Differential Analysis of Dihydropyrimidines and NC-\(\beta\)-Amino Acids in Patients with Pyrimidine Degradation Defects and Their Family Members**

<table>
<thead>
<tr>
<th></th>
<th>DHU</th>
<th>DHT</th>
<th>NC-(\beta)-ala</th>
<th>NC-(\beta)-AIB</th>
<th>(\beta)-ala</th>
<th>(\beta)-AIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPD-deficient patient</td>
<td>26</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>n.d.</td>
<td>46</td>
</tr>
<tr>
<td>Mother</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>n.d.</td>
<td>19</td>
</tr>
<tr>
<td>Sister</td>
<td>42</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>n.d.</td>
<td>84</td>
</tr>
<tr>
<td>Half-brother</td>
<td>42</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>n.d.</td>
<td>18</td>
</tr>
<tr>
<td>DHP-deficient patient</td>
<td>622</td>
<td>406</td>
<td>4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>53</td>
</tr>
<tr>
<td>Brother</td>
<td>21</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>n.d.</td>
<td>46</td>
</tr>
<tr>
<td>Control subjects*</td>
<td>16–110</td>
<td>2–28</td>
<td>6–71</td>
<td>2–6</td>
<td>Trace</td>
<td>0–79</td>
</tr>
</tbody>
</table>

n.d., not detected.

* Range of results for six unaffected children.

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**Table 4. Two Examples of Confounding Conditions in Diagnosis of Pyrimidine Degradation Defects**

<table>
<thead>
<tr>
<th></th>
<th>Patient with Burkitt lymphoma</th>
<th>Patient with &quot;DHP deficiency&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>During treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td></td>
<td>1st day</td>
<td>2nd day</td>
</tr>
<tr>
<td>Uracil</td>
<td>1672*</td>
<td>949</td>
</tr>
<tr>
<td>Thymine</td>
<td>165</td>
<td>73</td>
</tr>
<tr>
<td>Pe-urid</td>
<td>211</td>
<td>51</td>
</tr>
<tr>
<td>DHU</td>
<td>91</td>
<td>50</td>
</tr>
<tr>
<td>DHT</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>NC-(\beta)-ala</td>
<td>47</td>
<td>23</td>
</tr>
<tr>
<td>NC-(\beta)-AIB</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>(\beta)-AIA</td>
<td>89</td>
<td>26</td>
</tr>
<tr>
<td>(\beta)-AIB</td>
<td>1040</td>
<td>826</td>
</tr>
</tbody>
</table>

* Excretion values in mmol/mol creatinine.

n.a., not available; pe-urid, pseudouridine.
cussed in *Pitfalls* (Table 4), dihydrokynuridines can be converted to NC-β-amino acids in dihydrokynuridinuria (20).

Isolated hyper-β-AIB-uria due to AIBPAT is the most common of the β-amino acid transaminase deficiencies. Patients with this defect excrete almost exclusively the R-enantiomer of the amino acid, which originates from thymine. The excretion of the valine-derived S-enantiomer is not altered (18). The high-excretor phenotype, predominantly occurring in Mongoloid populations, is a metabolic polymorphism. Isolated hyper-β-alanineuria has not been described. Patients with BAPAT deficiency also excrete excess R-β-AIB. In contrast, patients with ABAT deficiency excrete excessive S-β-AIB in addition to β-alanine. In these patients, concentrations of γ-aminobutyric acid are also increased in the body fluids (5).

Clinically, defects of pyrimidine degradation can be associated with psychomotor retardation, convulsions, and epileptic conditions—as can be deduced from the data available for the patients described with DHPD deficiency (10) and for the two known patients with presumed DHP deficiency (4; and H. A. Simmonds, personal communication). Although, as in many inborn errors of metabolism, the causal relationship between clinical symptoms and biochemical abnormalities has not yet been established, screening for pyrimidine degradation defects is indicated in patients presenting with any of these symptoms. A special category of patients who should be screened for pyrimidine degradation defects is those cancer patients being treated with pyrimidine analogs such as 5′-fluorouracil and 5′-fluorouridine. For example, defects leading to a reduced catabolism of 5-fluorouracil could cause life-threatening situations in these patients because of greatly enhanced toxicity.

References


