Spurious Elevation of Multiple Urine Amino Acids by Ion-Exchange Chromatography in Patients with Prolidase Deficiency

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Abstract The enzyme prolidase cleaves dipeptides where the C-terminal amino acid corresponds to proline or hydroxyproline. As a consequence, a deficiency of this enzyme leads to accumulation of these dipeptides, which correspondingly are found to be elevated in urine. In fact, the absence of dipeptiduria is sufficient to rule out a diagnosis of prolidase deficiency. However, given the fact that these dipeptides elute at the same position as more common amino acids, the analyzer's software will instead call an elevation of these corresponding amino acids. Thus, an elevation of glycylproline, aspartylproline, glutamylproline, threonylproline and serylproline, valylproline, leucylproline, isoleucylproline, alanylproline, phenylalanylproline, and lysylproline will instead be interpreted as an elevation of leucine, citrulline, methionine, isoleucine, beta-aminoisobutyric acid, gamma-aminobutyric acid, ethanolamine, tyrosine, histidine, and anserine/carnosine, respectively. This particular profile of elevated amino acids, however, can easily be overlooked. We hope that the recognition of this characteristic pattern of falsely elevated urinary amino acids will aid in the recognition of prolidase deficiency.

Introduction

Prolidase deficiency is characterized by skin lesions (typically lower extremity ulcers), recurrent infections (particularly of the skin and respiratory tract), chronic lung disease, dysmorphic facial features, variable intellectual disability, short stature, mild anemia and thrombocytopenia, hypergammaglobulinemia, elevation of liver enzymes, and low complement levels. Only approximately 90 patients have been reported in the medical literature, although many cases probably remain undiagnosed (Ferreira and Wang 2015).

Prolidase cleaves dipeptides where the C-terminal amino acid is proline or hydroxyproline. Such amino acids are known as imidodipeptides, and they can be found in massive amounts in urine of patients with prolidase deficiency. The absence of imidodipeptiduria is sufficient to rule out a diagnosis of prolidase deficiency (Freij and Der Kaloustian 1986). However, the authors are aware of a few instances when the peaks produced by imidodipeptides in urine amino acid analysis were missed. Sometimes, they have been interpreted as non-specific ninhydrin-positive interfering compounds. Other times, the imidodipeptides, due to coelution, are reported as elevations of multiple amino acids in a non-specific pattern. The imidodipeptides have been known to obscure the normal peaks for methionine, isoleucine, leucine, and tyrosine (Buist et al. 1972; Wysocki et al. 1988).

We present the urine amino acid profiles of two patients with prolidase deficiency, and call attention to the fact that this rare condition leads to a characteristic appearance of unusual peaks that elute at the same position as other amino acids, and can thus lead to the erroneous interpretation of elevated leucine, isoleucine, methionine, citrulline, beta-aminoisobutyric acid, gamma-aminobutyric acid, tyrosine, and ethanolamine in urine.

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Methods

The manuscript is a retrospective case series that does not require ethics committee approval at our institution. No identifiable patient information is provided in the manuscript.

A urine sample was obtained from a 24-year-old woman with chronic lower extremity ulcers of unknown etiology since 8 years of age (patient 1). Urine amino acids were quantitated by cation-exchange chromatography with post-column ninhydrin derivatization at a reference laboratory (Mayo Clinic's Biochemical Genetics Laboratory) using a Biochrom amino acid analyzer. Subsequently, we obtained urine samples from a patient with known, molecularly confirmed prolidase deficiency at 5 months and 16 months of age (patient 2), and measured amino acid concentrations by cation-exchange chromatography on a Biochrom 30 analyzer in our laboratory. A running time of 2 h was needed for one complete run of the profile. UV detection was obtained at two wavelengths, 570 nm and 440 nm, according to the type of the amino acids concerned. The free amino groups react with ninhydrin to produce an intense blue or purple color – Ruhemann's purple – which is best detected at 570 nm. The imino group of proline and hydroxyproline, on the other hand, produces a yellow color upon reacting with ninhydrin, that is best read at a wavelength of 440 nm. For patient 1, the internal standards used were glucosaminic acid (GSA) and aminoethylcysteine (AED), while for patient 2 the internal standards were *S*-2 aminoethyl-L-cysteine hydrochloride (ALCH) and D-glucosaminic acid (GLAM). The urine samples were not exposed to acid hydrolysis. Quantification of amino acids was performed using EZChrom Elite software after a calibration curve was obtained.

Results

Multiple amino acids were reported as being elevated in patient 1 (see Fig. 1a and Table 1). The calibration sample is shown in Fig. 1b. This profile was interpreted as an increased excretion of a few unrelated amino acids in a pattern not characteristic for any specific disorder. However, given the clinical suspicion of prolidase deficiency, sequencing for *PEPD* was obtained, and a homozygous canonical splice site mutation was found.

In patient 2, an elevation of similar amino acids was provided on the report (see Supplementary Fig. 1 and Table 1). A control urine amino acid sample processed in the same analyzer is shown in Supplementary Fig. 2.

Discussion

The imidodipeptides that accumulate in patients with prolidase deficiency coelute with other amino acids. The presence of these broad, unusual imidodipeptide peaks is thus reported instead as an elevated concentration of various urine amino acids, as seen in our patients. We are indeed aware of several instances where the laboratory has reported a non-specific elevation of various amino acids in a pattern not characteristic for any known inborn error of metabolism, as occurred in patient 1.

We inferred the relative position of the different imidodipeptide peaks (Table 2) from the previously published literature (Buist et al. 1972; Duran 2008; Goodman et al. 1968; Lou and Hamilton 1979; Nusgens and Lapiere 1973; Powell et al. 1974). Despite coelution of the imidodipeptides with several amino acids, the 570/440 absorbance ratio, a constant for each amino acid, was in each case much lower than expected. This is due to increased heights of the peaks at 440 nm, in turn secondary to the fact that the imidodipeptides contain proline, which reacts with ninhydrin to form a yellow compound with absorption maximum at 440 nm. A simple visual inspection of the 440 nm channel in affected patients (Fig. 1a and Supplementary Fig. 1) as compared to controls (Fig. 1b and Supplementary Fig. 2) can attest to the increased height of these peaks – leading to the aforementioned decreased 570/440 ratio.

This peculiar pattern of urine amino acid elevation is noted on unhydrolyzed samples. When the urine samples are exposed to an equal volume of 6N hydrochloric acid and subsequently hydrolyzed by heating at 100°C for 20–24 h, then the unusual peaks will disappear, giving rise instead to a marked increase in proline, hydroxyproline, and glycine (Ferreira and Wang 2015).

Although not performed, we would expect similar profiles to the patients presented here (Fig. 1a and Supplementary Fig. 1) on any of the dedicated amino acid analyzers such as Beckman and Hitachi instruments given the similarities of the methodologies to the Biochrom.

Fig. 1 (a) Urine amino acid chromatogram from patient 1. (b) Calibration sample. The 570 nm channel appears on top in green, and the 440 nm channel at the bottom of each figure, in blue

Conversely, we would not expect to see the imidodipeptide peaks using LC-MS or LC-MS/MS methods as the multiple reaction monitoring (MRM) pairs for these compounds are not typically incorporated into these methods and had the urine been analyzed this way, it likely would have been interpreted as normal pattern of urine amino acids.

It is valuable to note that the order of the tallest peaks, even in the same patient, varied over time. However, the tallest peaks tend to be those coeluting with leucine, isoleucine, methionine, beta-aminoisobutyric acid, and citrulline, followed by smaller peaks coeluting with gamma-aminobutyric acid, tyrosine, and ethanolamine.

Table 1 Reported concentration of urine amino acids (in nmol/mg creatinine)

Amino acid	Patient 1	Reference range	Patient 2 (5 months)	Patient 2 (16 months)	Reference range
Citrulline	1,084	8–50	5,281	3,411	0–124
Methionine	411	38–210	7,024	4,736	0–644
Isoleucine	1,896	16–180	12,131	5,144	0–259
Leucine	3,516	30–150	10,055	5,998	18–220
Tyrosine	278	90–290	2,477	1,363	0–934
BAIBA	1,482	10–510	6,581	8,855	46–680
GABA	NR	–	2,691	3,273	0–166
Ethanolamine	NR	–	1,789	2,913	0–1,448

Values in boldface are elevated with respect to the reference range

BAIBA beta-aminoisobutyric acid, GABA gamma-aminobutyric acid, NR not reported

Table 2 Urinary dipeptides and their corresponding coeluting peaks

Dipeptide	Neighboring peak
Asx-Pro (Asp-Pro or Asn-Pro)	Citrulline
Glx-Pro (Glu-Pro or Gln-Pro)	Methionine
Glx-Pro, Thr-Pro, and Ser-Pro	Isoleucine
Gly-Pro	Leucine
Ala-Pro	Tyrosine
Val-Pro	β-aminoisobutyric acid (BAIBA)
Leu-Pro	Gamma-aminobutyric acid (GABA)
Ile-Pro	Ethanolamine/ammonia
Phe-Pro	Histidine/1-methylhistidine
Lys-Pro	Anserine/carnosine

This is in keeping with the known excretion pattern seen in patients with prolidase deficiency, where the more abundant imidodipeptides are Gly-Pro, Asx-Pro, and Glx-Pro (Hechtman 2014).

Conclusion

Prolidase deficiency leads to an increased excretion of urinary imidodipeptides that appear as broad, unusual peaks in the chromatogram. These peaks elute at the same position as other amino acids, leading to an incorrectly increased quantitation of urinary leucine, isoleucine, methionine, beta-aminoisobutyric acid, and citrulline – and to a lesser extent also of gamma-aminobutyric acid, tyrosine, and ethanolamine. We hope that our report will

help elicit suspicion of this rare inborn error of metabolism.

A Concise One Sentence Take-Home Message

Prolidase deficiency can lead to a particular pattern of spurious elevation of several urine amino acids, given their coelution with uncleaved imidodipeptides.

Compliance with Ethics Guidelines

Conflict of Interest

- Carlos R. Ferreira and Kristina Cusmano-Ozog declare that they have no conflict of interest.

- No identifying information about patients is included in the article.
- This article does not contain any studies with animal subjects performed by the any of the authors.
- Writing, preparation, and critical review of the manuscript: CRF and KCO.

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