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Review

Diagnostic value of urinary orotic acid levels: applicable separation methods

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Abstract

Urinary orotic acid determination is a useful tool for screening hereditary orotic aciduria and for differentiating the hyperammonemia disorders which cannot be readily diagnosed by amino acid chromatography, thus reducing the need for enzyme determination in tissue biopsies. This review provides an overview of metabolic aberrations that may be related to increased orotic acid levels in urine, and summarises published methods for separation, identification and quantitative determination of orotic acid in urine samples. Applications of high-performance liquid chromatography, gas chromatography, and capillary electrophoresis to the analysis of urinary specimens are described. The advantages and limitations of these separation and identification methodologies as well as other less frequently employed techniques are assessed and discussed.

Keywords: Reviews; Orotic acid

Contents

1. Introduction

Orotic acid (1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidinecarboxylic acid; uracil-6-carboxylic acid) is a minor component of the diet. It is found in whey

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and root vegetables, such as carrots and beets. In contrast to sheep's and goat's milk, cow's milk contains a relatively large amount of orotic acid, while human milk lacks this compound [1–3].

The nutritional importance of orotic acid lies in its role as a growth factor in feed deficiencies and as a protective agent for liver [4]. Orotic acid improves the symptoms due to folate or cobalamin deficiency [5] and increases intracellular levels of nucleotides and nucleic acids. Large doses of magnesium orotate (3 g/day) markedly improve left ventricular function and exercise tolerance in patients with coronary heart disease probably by correcting a relative deficiency of nucleotide precursors or by increasing myocardial energy supply [6]. Orotic acid has been used therapeutically also in the treatment of neonatal jaundice [7], hyperlipoproteinemia [8], degenerative retinal disease [9] and gout [10]. Orotic acid is still referred to as vitamin B13 [11], but it is not really recognised as a vitamin because it is manufactured by the human body and intestinal flora.

Prolonged exposure to elevated orotic acid concentrations may cause noxious effects. Addition of orotic acid to rat diet causes hepatic steatosis [12]. Orotic acid is a promoter of hepatic carcinogenesis in rat treated with 1,2 dimethylhydrazine [13], enhances preneoplastic and neoplastic lesions in hamsters [14] and stimulates proliferation of K 562 leukemic cells in vitro [15]. The estimated LD_{50} of orotic acid in mice is 2000 mg/kg, when administered per os, and 841 mg/kg, when administered via intraperitoneal injection.

1.1. Metabolic pathways and enzymes

Orotic acid is the fourth intermediate in the de novo pyrimidine synthetic pathway which starts with the formation of carbamoyl phosphate from glutamine, carbon dioxide and two molecules of ATP [16]. Carbamoyl phosphate is synthesised by carbamoyl phosphate synthetase II (CPS II), a cytosolic enzyme widely distributed throughout the body which forms a multienzyme complex with the next two enzymes of pyrimidine biosynthesis (aspartate transcarbamylase, which catalyses the irreversible formation of carbamoyl aspartate from carbamoyl phosphate and aspartate, and dihydroorotase, which effects ring closure to form dihydroorotic acid). The enzyme is referred to as CPS II to distinguish it from CPS I, an enzyme present in the mitochondria, which is essential for the urea cycle and confined almost exclusively to the liver, with low activity in kidney, intestinal mucosa and leukocytes (Fig. 1). CPS I, which utilises ammonia instead of glutamine as nitrogen source in the synthesis of carbamoyl phosphate, is regulated by *N*-acetyl-glutamate (NAG), an arginine-sensitive allosteric activator that is the product of glutamate

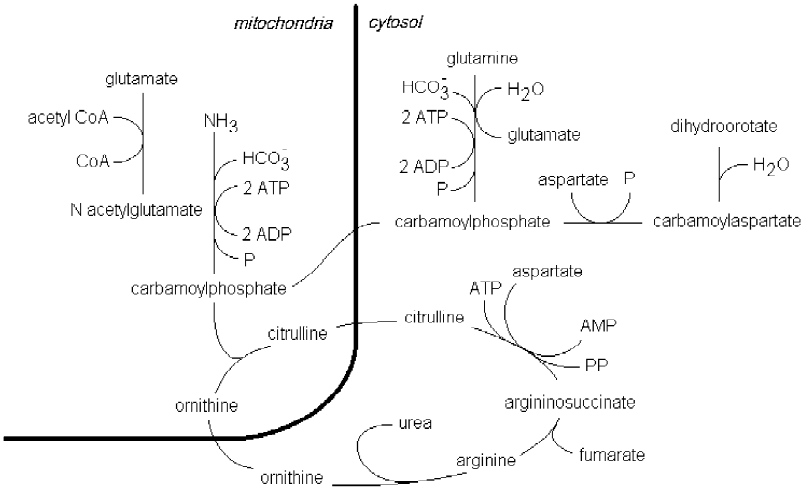

Fig. 1. Metabolic scheme showing the relationship between urea cycle and the first steps of pyrimidine biosynthesis.

and acetyl CoA via NAG synthetase [17,18]. CPS I may be a significant source of liver pyrimidine since, even at optimal conditions, a third of the carbamoyl phosphate synthesised in the mitochondria by this route is exported to the cytosol, with 80% being incorporated into hepatic pyrimidines [19]. The fourth enzyme in the synthetic pathway, dihydroorotic acid dehydrogenase, is located on the outer surface of the inner membrane of the mitochondria and catalyses the oxidation of dihydroorotic acid to orotic acid [20].

The metabolic fate of intracellular orotic acid is the conversion to uridine 5′-monophosphate (UMP). The reaction is catalysed by UMP synthase, a bifunctional cytosolic protein containing the activities of orotate phosphoribosyltransferase (OPRT) and of orotate 5′-monophosphate decarboxylase (ODC) [21]. OPRT utilises 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) to add the ribose-5′-monophosphate moiety to orotic acid and produce orotidine 5′-monophosphate (OMP). ODC releases CO_2 from OMP to produce UMP that is converted to all the other required pyrimidine nucleotides. The degradation proceeds largely through the formation of uracil and tymine that are oxidised in the presence of dihydropyrimidine dehydrogenase and then hydrolysed to carbamyl-β-amino acids [22].

The activity of CPS II is the rate-limiting step in the de novo synthesis of UMP except when ATP levels are increased at a time when uridine nucleotides and PP-ribose-P levels are low. Under these conditions, orotic acid accumulates and OPRT is the rate-limiting reaction [23]. Vast increases in the rate of de novo pyrimidine synthesis may be due to increased availability of carbamoyl phosphate produced by CPS I in various urea cycle deficiencies.

Orotic acid is not transported into nucleated cells, but there is a high capacity, nonsaturable transport system for orotic acid into erythrocytes, the rate-limiting step being the conversion of orotic acid to UMP. If nucleated cells (fibroblasts, lymphoblasts) are incubated with erythrocytes, the erythrocytes take up orotic acid and excrete uridine into the medium, where it is utilised by the nucleated cells [24].

Orotic acid concentration in normal plasma is less than 0.5 μmol/l [25]; values higher than 60 μmol/l have been reported for untreated patients with inherited defects in pyrimidine metabolism and urea cycle [26,27]. Very high renal clearance values of orotic acid (up to eight times the glomerular filtration rate) have been observed in patients with ornithine carbamoyl transferase deficiency [27], UMP synthase deficiency [28] or argininosuccinate synthetase deficiency [26]. Evidence for an active mechanism underlying renal secretion of orotic acid is also provided by the large increase in plasma concentration in chronic renal failure, even following hemodialysis [29].

1.2. Metabolic disorders

Because renal excretion of orotic acid is very efficient and urinary values integrate changes over time, orotic acid measurements are more relevant in urine than in plasma. The distribution of the urinary orotic acid values for healthy subjects shows an asymmetrical pattern with a mean value of 1.13 μmol/mmol creatinine and a mode of 0.62 μmol/mmol creatinine [30]. A study on single urine samples from 168 healthy adults in Nagoya General Hospital (Japan) gave a reference range of 0.26–3.20 μmol/mmol creatinine by using HPLC method [31]. Values less than 10 μmol/mmol creatinine have been reported in other studies in healthy adults [25]. Females have higher levels of orotic acid (0.36–3.20 μmol/mmol creatinine) than males do (0.26–1.91 μmol/mmol creatinine). Levels are higher in infants of 1–12 months (0.76–4.10 μmol/mmol creatinine) than in newborns, older children or adults (Table 1) [30,32].

Orotic acid excretion is about half normal during starvation because of a lowered rate of production and utilisation. The starvation adaptation of orotic

Table 1
Reference values of orotic acid in urine

Age	*n*	Orotic acid (μmol/mmol creatinine)	
		Mean	Range
6 days	25	1.77	1.13–2.46
6 months	33	2.61	0.76–4.10
1–5 years	53	1.46	0.68–2.24
6–10 years	47	1.32	0.47–2.19
11–15 years	30	0.66	0.32–0.96
16–40 years	69	0.58	0.41–0.75
≥41 years	97	0.76	0.95–1.05

Modified from Asai et al. [30].

acid excretion occurs more rapidly than does the decrease in urinary nitrogen loss. The response to refeeding of acutely malnourished normal male is an increase in orotic acid excretion with a decrease in whole body protein catabolism [33].

Slight increases in urinary orotic acid have been reported in pregnancy [34] and premature birth [35]. Slight to moderate increases have been observed in patients with heart failure, hypertension, diabetes, malignancy, benign tumors, cerebral infection, and trauma [31,36]. Adult alcoholics show elevated urinary orotic acid-to-creatinine ratios early after drinking episodes, suggesting that liver damage may cause increased orotic acid excretion [37]. An unusually high urinary excretion of orotic acid was found in a patient with hepatocellular carcinoma without cirrhosis, as a result of partial tumor occlusion of the hepatic veins, tumor breakdown and portosystemic shunting [38].

1.2.1. Orotic acid overproduction

Abnormally high urinary levels of orotic acid have been found in conditions that evoke hyperammonemia and accumulation of intramitochondrial carbamoyl phosphate, which may diffuse into the cytosol and stimulate de novo pyrimidine synthesis.

With the exception of arginine and ornithine, amino acids as well as ammonia salts induce orotic aciduria in rat [39,40]. Arginine-deficient diet increases liver carbamoyl phosphate and urinary orotic acid level in many species [41,42], while arginine infusion reduces orotic acid excretion in sheep and rat when a high nitrogen diet is given [40,42]. However, it has been reported that an arginine-deficient diet does not cause hyperammonemia or orotic aciduria in adult humans since the de novo arginine synthesis is sufficient for the maintenance of normal cellular metabolism [43].

In most cases, orotic aciduria in humans arises from inherited defects of enzymes involved in the urea cycle after the synthesis of intramitochondrial carbamoyl phosphate. An estimate of the overall incidence of these inborn errors gives a value of 1 per 9400 [44]. Ornithine transcarbamylase deficiency constitutes 70% of the cases, argininosuccinate synthetase deficiency 16%, arginonosuccinate lyase deficiency 12%, and arginase deficiency 2% (Table 2). Clinical presentation of the patients is very similar and related to hyperammonemia, which is common to all these diseases. The variability of the symptoms presumably depends on genomic factors and on the metabolic consequences of the various enzyme deficiencies. For instance, variability in expression of ornithine transcarbamylase deficiency in heterozygous females is related to the proportion of hepatocytes in which the normal or mutant allele is on the active X chromosome. Arginonosuccinate lyase deficiency of a degree similar to ornithine transcarbamylase deficiency may not be as severe a disease because newly synthesised argininosuccinate may serve as a waste nitrogen product. Acute change in consciousness and hyperammonemia in ornithine transcarbamylase deficiency with late onset can simulate Reye's syndrome [45]. However, in Reye's syndrome without inherited transcarbamylase deficiency normal excretion of pyrimidines has been reported [46].

The HHH syndrome (hyperornithinemia, hyperammonemia and homocitrullinuria) is a rare autosomal recessive disease that is related to orotic aciduria in humans [47]. The basic defect is in the transport of ornithine across the inner mitochondrial

Table 2
Urea cycle disorders associated with orotic aciduria

Enzyme deficiency	Relative frequency (%)	Incidence
Ornithine transcarbamylase (OCT deficiency)	70	1:14 000
Argininosuccinate synthetase (citrullinemia)	16	1:57 000
Argininosuccinate lyase (argininosuccinic aciduria)	12	1:70 000
Arginase (argininemia)	2	1:363 000
Total	100	1:9400

Modified from Brusilow and Maestri [44].

membrane into the matrix, which decreases citrulline synthesis and impairs ammonia detoxification. The de novo pyrimidine pathway is stimulated by the same mechanism as ornithine transcarbamylase deficiency. Increased plasma ornithine concentration differentiates the HHH syndrome from urea cycle disorders, while postprandial hyperammonemia and homocitrullinuria distinguish it from ornithine-δ-aminotransferase deficiency (gyrate atrophy).

About 100 patients with lysinuric protein intolerance have been reported and almost half of them are from Finland, where the prevalence of this autosomal recessive disease is 1 per 60 000 [48]. In this inborn error, urinary excretion and clearance of all cationic amino acids, especially of lysine, are increased, and these amino acids are poorly absorbed from the intestine. Because of the ornithine depletion, the patients have periods of hyperammonemia after protein loading that can be abolished by giving citrulline, a neutral amino acid that is well absorbed from the intestine. The enzymes of the urea cycle have normal activities and the orotic aciduria during hyperammonemia supports the view that carbamoyl phosphate is formed in sufficient quantity [49].

A moderate orotic aciduria with homocystinuria, formiminoglutamic aciduria and megaloblastosis has been found in only one girl with severe episodes of ataxia, due to the impairment of the metabolic path responsible for 1-carbon salvage of histidine degradation leading to 5,10-methenyl-tetrahydrofolate [50]. The reactions are catalysed by a bifunctional enzyme containing the activities of glutamate formiminotransferase and of formimino-tetrahydrofolate cyclodeaminase in a single polypeptide chain [51].

It has been reported that some girls with Rett's syndrome and some of their mothers have increased levels of urinary orotic acid following alanine load [52]. The pattern of urinary excretion of orotate was similar to that found in female carriers of ornithine transcarbamylase deficiency. Although Rett initially described hyperammonemia in some of his patients [53], later study revealed no hyperammonemia in the vast majority of cases [54,55].

1.2.2. UMP synthase deficiency

Massive orotic aciduria, crystalluria and obstruction of the urinary tract may be found in the inherited deficiency of UMP synthase, the dimeric protein which catalyses the last two steps in pyrimidine synthesis [25]. The defect is very rare and transmitted as an autosomal recessive trait. In most of the cases the inborn error affects both the phosphoribosylation of orotic acid and the decarboxylation of OMP (hereditary orotic aciduria type I). In only one patient, orotate phosphoribosyltransferase activity was normal or relatively high, while OMP decarboxylation was barely detectable (hereditary orotic aciduria type II) [56]. The finding of normal phosphoribosyltransferase activity was corroborated by high urinary excretion of orotidine. It has been suggested that, if the dimeric structure of UMP synthase is blocked or otherwise inhibited, the protein may lose only the OMP decarboxylase activity, while deficiency of the overall enzyme activities should result from an anomalous configuration of the monomeric subunit [57].

An increase in urinary excretion of orotic acid and orotidine, as reported for the hereditary orotic aciduria type II, may be obtained upon administration of allopurinol or oxipurinol [58,59]. Orotidinuria results from inhibition of OMP decarboxylase by the nucleotide derivatives of the drug. The orotic aciduria may be due to an inhibition of orotate phosphoribosyltransferase by the increased concentration of OMP or of the metabolites of allopurinol and oxipurinol. In addition, orotate phosphoribosyltransferase activity may be reduced by a transient decrease in the intracellular content of PP-ribose-P, which occurs 3–5 h after allopurinol administration.

The administration of 6-azauridine in patients with nonterminal malignant disease is also accompanied by a striking increase in excretion of orotic acid and orotidine, following the enzymatic conversion of the drug to 6-azauridine-5′-phosphate, a specific competitive inhibitor of OMP decarboxylase [60]. Patients receiving both 6-azauridine and allopurinol excreted less orotic acid and orotidine than when 6-azauridine was given alone. Experiments with spleen slices from anaemic mice show that brief exposure to 6-azauridine stimulates carbamoyl phosphate production and de novo pyrimidine biosynthesis by increasing the intracellular level of PP-ribose-P, a potent positive effector for carbamoyl phosphate synthetase II [61]. Moreover, experiments with fibroblast from a patient with hereditary orotic aciduria type I show that 6-aza-UMP stabilises the

Table 3
Protonation constants of orotic acid at 25 °C

log K ($\mu=0.1$)	log K ($\mu=0$)	ΔH ($\mu=0$) (kcal/mol)	ΔS ($\mu=0$) (cal/(mol×degree))
1.96	1.8	−0.5	6
9.34	9.6	−8.7	14
>13			

Constants have been extrapolated to zero from measurements at low-ionic-strength (μ) by using Davies equation. Modified from Martell and Smith [64] and Sober [65].

structurally altered UMP synthase against heat denaturation and proteolytic degradation, leading to an increase in UMP synthase protein in the cells [62].

There is a report of one mentally-retarded infant with markedly decreased PP-ribose-P synthetase activity in erythrocytes who had persistently low levels of uric acid in body fluid and increased levels of urinary orotic acid because of the impairment of orotate phosphoribosyltransferase activity [63].

2. Analytical methods in clinical chemistry

Orotic acid is soluble in water up to 1.7 g/l and negatively charged even under strongly acidic conditions because of mesomeric delocalization of the negative charge of the carboxyl group by the pyrimidine ring due to the presence of two hydroxyl groups in the structure. Protonation constants and corresponding enthalpy and entropy values are reported in Table 3 [64,65].

Measurements of urinary orotic acid have been carried out using an ion trap mass spectrometer in negative mode. In a selected part of the full mass spectrum, a dominant peak with m/z 155 clearly identifies the metabolite, while fragmentation in the ion trap allows to distinguish among compounds with identical masses without chromatographic separation [66,67]. Although direct analysis of untreated urine is possible in some cases, suppression of ionization due to matrix is observed and off-line clean-up is necessary for routine analyses.

The absorption spectrum of orotic acid in the ultraviolet region is characterised by a broad absorption band around 280 nm (Table 4) and this makes ultraviolet spectroscopy the method of choice for measuring orotic acid concentration, if the metabolite is isolated by procedures that minimise spectral interference. The ^{1}H NMR spectrum of orotic acid is characterised by a singlet resonance at 6.22 ppm, a region where only few metabolites present in body fluids show resonance. Therefore, ^{1}H NMR is a valuable screening tool for future studies of inborn errors that are accompanied by increased levels of orotic acid [68].

Orotic acid can be converted to barbituric acid by bromination and reduction in the presence of ascorbic acid. Barbituric acid is coupled with *p*-dimethylaminobenzaldehyde to form an orange product, 5-(*p*-dimethylaminobenzylidene) barbituric acid, which absorbs light at 480 nm [69,70]. Both orotic acid and orotidine are measured by this method unless the metabolites are separated chromatographically before analysis. This simple colorimetric assay may be subject to interference from a variety of compounds [71,72]. At the concentrations found in urine, only histidine has significant absorbance around 480 nm. Since histidine does not produce colour without bromination, non-brominated controls fail to correct for histidine interference. Interfering compounds are unlikely to be a problem when orotic acid is measured for the diagnosis or for monitoring of therapy in hereditary orotic aciduria because the concentrations involved are high. Chromatographic clean-up of urine before analysis is needed for

Table 4
Spectral properties of orotic acid

pH	λ_{max} (nm)	ε_{max} ($\times10^{-3}$)	λ_{min} (nm)	Spectral ratios						
				230	240	250	260	270	280	290
1	280	7.5	241	0.61	0.41	0.54	1.00	1.54	1.82	1.56
7	279	7.7	241	0.68	0.43	0.57	1.00	1.49	1.71	1.36
12	286	6.0	244	1.36	0.80	0.80	1.00	1.38	1.71	1.72

The ratio columns give absorption ratio at the wavelength given to that at 260 nm. Modified from Sober [65].

determination of urea cycle disorders or carrier status in hereditary orotic aciduria.

The effect of positive interference compounds other than orotic acid in the colorimetric method can be eliminated with a blank, which contains orotate phosphoribosyltransferase and orotate 5′-monophosphate decarboxylase and converts orotic acid to UMP [73]. In protein-free samples, the enzymatic conversion of orotic acid to UMP can be monitored directly at 295 nm [74] and this makes unnecessary the colorimetric determination of the metabolite. The reaction is started by adding P-ribose-PP, unless P-ribose-PP is present in the sample. If the sample contains P-ribose-PP, the assay must be started with enzyme addition and a suitable correction must be performed for the absorbance change due to the enzyme. High concentrations of inorganic salts (e.g. 0.2 mol/l NaCl) and allopurinol ribonucleotide (0.7 μmol/l) strongly inhibit the reaction.

Another enzymatic assay of orotic acid utilises the reaction catalysed by dihydroorotic acid dehydrogenase [75]. However, this method, which involves the addition of the flavoenzyme together with reducing agents to the assay system, is only possible under special conditions, e.g. measurements at 282 nm, use of pure enzyme, inclusion of standards.

Table 5 summarises the assay procedures that are employed to quantify orotic acid in urine extracts which have been purified by chromatographic or electrophoretic methods.

2.1. Chromatographic methods

Partial purification of the urinary samples on a Dowex 1X2 column has been employed for rapid screening of patients suspected of having very high level of orotic acid as a result of an inborn error of metabolism [76]. Under these conditions, orotic acid is monitored in the effluent at 280 nm without significant interference from other UV-absorbing trace metabolites. When the orotic acid level is relatively low, amberlite CG 120 [71] or dry silica gel [77] have been employed to remove some of the urinary metabolites that interfere with the colorimetric assay of orotic acid.

High-performance liquid chromatography (HPLC) of the urine sample on a μ-Bondapak C_{18} column with 4 mmol/l Na-phosphate, pH 6.0, allows higher separation efficiency [78], but the elution profile is inadequate because the orotic acid peak is very near to the uric acid peak and the front of analysis. An improved method requires the purification of the urine specimen on a strong cation exchanger (amberlite CG 120), followed by the isocratic elution of the prepurified sample from a reversed-phase HPLC column (Superspher RP 18) with a buffer containing 2% (v/v) acetonitrile and 5 mmol/l tetrabutylammonium phosphate, pH 7.4 [79]. Another procedure of analysis consists of the purification of the urinary sample with Dowex 1X8 [80], followed by two HPLC steps on a μ-Bondapak NH2 and a μ-Bondapak C_{18} column [81].

An automatic screening system consisting of two HPLC columns and a column switch has been described [30–32,82,83]. Each column is connected to one pump and two UV absorbance detectors with a system total of two pumps and four detectors. The equipment is governed by a computerized controller that allows consecutive analysis of more than 100 urine samples. The samples are cleaned up through a Centricut filter before the analysis. The first chromatographic run is carried out on a reversed-phase column (Develosil ODS-5), which is eluted consecutively with 5 mmol/l H_2SO_4 for 10 min and with 5 mmol/l H_2SO_4 containing 2% (v/v) acetonitrile for

Table 5
Detection methods employed for orotic acid analysis

	Separation methods	References
Colorimetric assay	Low-pressure liquid chromatography	[71,77]
UV absorbance detector	Low-pressure liquid chromatography	[76]
	High-performance liquid chromatography	[78,79,81–84]
	Capillary electrophoresis	[86–88,90–94]
	Capillary isotachophoresis and zone electrophoresis	[89,96]
Mass spectrometry	Gas chromatography	[85]

15 min and then washed with acetonitrile–water (50:50) for 3 min. The 4.5–5.7 min fraction coming from the first column is automatically delivered to a cation-exchange column (MCI Gel CK08EH) and eluted with 5 mmol/l H_2SO_4, which allows separation of orotic acid from pseudouridine, uracil and dihydrouracil. The total time of analysis for each sample is about 40 min.

Derivatization of orotic acid to its methyl ester increases the relative retention by a factor of three in reversed-phase chromatography and permits a reliable separation from other urine constituents by means of a single HPLC column [84]. However, the procedure, which enables quantitative analysis of orotic acid in the normal range, is quite complex and increases substantially the total time of analysis. The urinary sample is eluted through a C_{18} cartridge to remove low-polarity compounds that could interfere with the methylation reaction. The eluate is dried at 55 °C under vacuum, dissolved in 0.1 mmol/l sulfuric acid in methanol and incubated for 20 min at 80 °C with continuous agitation. After cooling, the acidic derivatized sample is diluted in 10 vol. of a-mixture of 0.1 mol/l triethanolamine–HCl and acetonitrile (95:5), pH 3.0, and eluted isocratically through the HPLC column (Spherisorb ODS).

Another method that requires orotic acid derivatization is based on isotope dilution of the sample with 1,3-[$^{15}N_2$]orotic acid and analysis by gas chromatography–mass spectrometry (GC–MS) [85]. The urine sample containing a known amount of 1,3-[$^{15}N_2$]orotic acid is saturated with NaCl and acidified to pH$<$1 with HCl. The organic acids are extracted twice with ethyl acetate, dried under a nitrogen stream and derivatized to form trimethylsilyl derivatives with a mixture of *N,O*-bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (10:1). The derivatized urine residue is then injected into a gas chromatograph equipped with a cross-linked 5%-phenylmethylsilicone capillary column (Ultra 2). The oven temperature program is started at 100 °C and increased by 10 °C/min up to 270 °C. Flow-rate of the helium carrier is 35 cm/s. The natural orotic acid derivative is monitored with a quadrupole mass spectrometer at m/z 254 and 357, while the stable isotope internal standard is monitored at m/z 256 and 359.

2.2. Electromigration methods

Capillary electrophoresis may provide an excellent tool for detecting orotic aciduria because of the high separation efficiency and flexibility. Background electrolytes with relatively high pH and ionic strength allow minimal amount of specimen preparation. When the electrophoresis is used in the cationic mode, the urine could be injected directly into the instrument even if the samples have some insoluble material [86,87]. Dilution of urine samples in 10 vol. water as well as alkalinization by NaOH (final concentration 0.1 mol/l; pH 12) is needed to prevent loss of constituents due to precipitation [88,89] and to improve peak-separation quality [88]. To remove waste material from fused silica, the capillary should be washed with 0.1 mol/l NaOH, water, and separation buffer between runs [86–88,90]. When low pH background electrolytes are employed in anionic mode, the capillary is prone to problems such as unstable migration time and poor assay precision [91]. Examination of the capillary under a low power light microscope reveals that the instability is often accompanied by the deposition of a brown amorphous precipitate on the inner surface of the capillary inlet. This material is presumably denatured protein and can be removed by periodically breaking off 2–5 mm of the capillary tip. Centrifugation in a cutoff-type filter works well for many patient specimens, although a significant number clogs the filter before a sufficient volume of urine filtrate is collected.

The background electrolyte composition substantially affects metabolite separation by capillary electrophoresis. When the instrument is used in the cationic mode at relatively high electroosmotic flow, all the compounds that are present in the urine are carried out towards the cathode. Borates and germanates provide good resolution via their well-known complexation with *cis*-diol species [90]. Separation performed in 20–60 mmol/l sodium borate gives better resolution of urinary metabolites [86–88,90]. Separation of urine components improves with increasing pH, but at pH$>$10 the very high electrophoretic mobility of orotic acid prolongs the analysis. Moreover, at pH$>$9.7, run-to-run reproducibility is reduced as a consequence of a lowering of the buffer capacity because the ionization pK of boric

acid is 9.23. The effect of capillary temperature on the separation quality at pH 9.2 has been investigated in the range between 15 and 35 °C [92]. High temperatures cause faster migration times without influencing the migration order. The resolution improves with increasing voltage that causes a drastic reduction of migration time. The increase in voltage is limited by the increasing current levels and the Joule heating effects. The optimal separation is achieved at 20–30 °C with a voltage of 20–30 kV (0.35–0.52 kV/cm) [86–88,90,92].

To improve resolution and reproducibility, the effect of different counterions, surfactants and organic modifiers has been studied [90]. Excellent resolution was achieved using 2-amino-2-methyl-1-propanol instead of Na^+ as counterion in the borate buffer because of the higher buffering capacity and the lower conductivity. Higher reproducibility can also be expected in a system utilising surfactants because it prevents interactions of urine proteins with the capillary wall. Comparison of assays performed with and without sodium dodecyl sulfate (SDS) revealed that zone electrophoresis rather than a micellar separation mechanism prevailed for the separation of urinary purines and pyrimidines. Unfortunately, in the presence of SDS, orotic acid gave rise to a broad peak very late in the electropherogram and this makes SDS–capillary electrophoresis unsuitable for detecting orotic acid in urinary specimens. Acetonitrile, methanol and cyclodestrins worsened peak separation.

Methods employing alkaline buffers are inherently not free of interference because all compounds present in urine pass the detector, regardless of charge, and some of them may puzzle the analyst. When the instrument is used in anionic mode with low pH background electrolytes, migration is restricted to a limited number of small molecular mass anions having significant ionisation in acidic pH range. Because of the extremely low ionization p*K*, orotic acid migrates towards the positive electrode (anode) under these conditions [93,94]. There is a limited number of buffers useful at very low pH. Phosphate buffer (p*K* 2.1) is low absorbing in ultraviolet region and therefore is suitable for separation with a spectrophotometric detector. Oxalate (p*K* 1.3) and malate (p*K* 1.9) have good buffering capacity, but absorb relatively strongly at short wavelength and so their use results in poor detection limits. Assays at pH $<$ 1.6 are associated with a high generated current and excessive Joule heating. Best results have been obtained in 200 mmol/l Na-phosphate buffer, pH 1.8 at 25 °C, with a voltage of 8 kV (0.3 kV/cm) [94].

Capillary electrophoresis methods based on direct measurements of orotic acid in untreated urine have been validated by means of samples from patients with different types of orotic aciduria. Off-line clean-up and preconcentration techniques enable quantitative analysis of orotic acid also in the normal range, but prolong substantially the total analysis time. Good assay precision was achieved by cleaning up the urine sample with a reversed-phase C_{18} column equilibrated with 0.1 mol/l barbituric acid buffer, pH 4.4, which competitively inhibits orotic acid interactions with the column, thus favouring its passage with the eluate [91]. Electrophoresis was carried out in anionic mode in 0.1 mol/l Na-phosphate buffer, pH 3.0 at 35 °C, by using a polyvinyl alcohol coated capillary, which needs little conditioning with the acidic electrolyte and does not require flow reverse agents, such as cetyltrimethylammonium bromide that is often necessary to optimise anion analysis [95].

On-line combination of isotachophoresis and capillary electrophoresis was used to detect traces of orotic acid in urine samples [89,96]. Isotachophoresis provides an enhanced sample load capacity to the separation system (a 30-μl sample injection volume), concentrates the analyte, and serves as an on-line clean-up technique. On the other hand, capillary electrophoresis performs final separation of the analyte from matrix constituents present in the isotachophoresis pre-treated sample and provides favourable conditions for its detection and identification. The method was optimised by Danková et al. [89]. By using phosphate and citrate as discrete spacers, the entrance of 60–90% of urine matrix constituents from isotachophoresis into the electrophoresis stage was prevented. Capillary electrophoresis was performed in 30 mmol/l aspartate, pH 3.5, and diethylenetriamine was used as pH buffering counterion. Addition of α-cyclodestrin (30 mmol/l) and β-cyclodestrin (10 mmol/l) in the background

electrolyte systems of isotachophoresis and capillary electrophoresis, respectively, provided considerable selectivity in the separation of orotic acid from urine matrix constituents.

2.3. Quantitation and validation

Orotic acid can be determined in liquid urine samples or in dried filter papers [67,85,90,94]. Filter papers offer the advantage of easy collection, transport, and storage of urine specimens. The dried urine spot can be dissolved in deionized water [90] or in a mixture of methanol and water (75:25) [67] with recovery efficiencies of 93–99%. Ethyl acetate extraction, which is employed for orotic acid determination by GC–MS analysis, gives recovery efficiencies of 25–30% for both dried filter papers and liquid urine samples [85].

The detection limit of urinary orotic acid (with a signal-to-noise ratio of 3) for HPLC instruments equipped with ultraviolet detectors is around 0.3–0.5 μmol/l [79,83,84]. The value is approximately 10 times lower than those reported for capillary electrophoresis (2.5–5.2 μmol/l) [88,90,91,93,94]. By using isotachophoresis sample pretreatment, multiwavelength photometric diode array and current correction and smoothing procedures, the sensibility of capillary electrophoresis can markedly improve and the detection limit can be reduced to about 0.2 μmol/l [89]. The assay response is linear up to 500–1000 μmol/l with HPLC and capillary electrophoresis [83,88,90,91,94]. In the latter case, however, peak broadening reduces resolution from nearby peaks at high orotic acid concentrations [91]. Orotic acid determination by GC–MS shows a detection limit of 1.09 μmol/l provided that the stable isotope 1,3-[$^{15}N_2$]orotic acid is added to the urine sample as internal standard [85].

Table 6 shows the urinary orotic acid levels that are usually found in patients and heterozygotes with abnormal pyrimidine metabolism. In hereditary orotic aciduria, urinary orotic acid levels are always high and homozygotes excrete up to 600–1500 mg orotic acid per day [30,25]. In ornithine transcarbamylase (OCT) deficiency, three populations can be differentiated [30,46,97]: hemizygous males with a very marked overexcretion of orotic acid; female patients with varying amounts of residual enzyme activity (as expected from the Lyon hypothesis) and urinary orotic acid overexcretion generally less than in hemizygous males; asymptomatic heterozygous women who excrete traces or slightly increased amounts of orotic acid. In citrullinemia (arginonosuccinate synthetase deficiency) the orotic acid excretion closely reflects the clinical expression of the disease [30,46,97]: it is very pronounced in the neonatal form and increased in the classical type, while the patient with benign course shows no abnormal elevation. All the patients with argininosuccinic aciduria (argininosuccinate lyase de-

Table 6
Reported range of urinary orotic acid levels in patients and heterozygotes with abnormal pyrimidine metabolism [30,50,97,88,46]

Inborn error	Number of patients	Orotic acid (μmol/mmol creatinine)
Hereditary orotic aciduria homozygote with treatment	1	181.3–283.4
Hereditary orotic aciduria homozygote without treatment	1	8929
Hereditary orotic aciduria heterozygote	6	3.5–30.5
OCT deficiency hemizygote with hyperammonemia	12	10.9–871.4
OCT deficiency hemizygote without hyperammonemia	1	1.0–1.3
OCT deficiency heterozygote	5	0.2–5.6
Citrullinemia homozygote with hyperammonemia	6	0.6–1049
Citrullinemia homozygote without hyperammonemia	2	1.4–6.7
Argininosuccinic aciduria homozygote with hyperammonemia	7	5.3–38.4
Argininosuccinic aciduria homozygote without hyperammonemia	1	0.6
Argininemia homozygote with hyperammonemia	4	650–831
HHH syndrome without hyperammonemia	1	23–106
Lysinuric protein intolerance	2	5.5–112.1
Formiminotransferase/cyclodeaminase deficiency	1	6.4–21.4

ficiency) have barely increased levels of urinary orotic acid [46,97]. In contrast, patients with argininemia (arginase deficiency), in whom blood ammonia elevation is comparable to that found in the patients with argininosuccinic aciduria, show a very marked increase in orotic acid in their urine [97]. High urinary orotic acid levels may be observed in patients with HHH syndrome [88] and lysinuric protein intolerance [46] during episodes when the clinical conditions are out of dietary balance. A slight increase in orotic acid excretion has been reported in one case of formiminotransferase/cyclodeaminase deficiency [50].

3. Biological relevance of the analytical results

Urinary orotic acid determination is a useful tool for screening hereditary orotic aciduria and for differentiating the hyperammonemia disorders which cannot be readily diagnosed by amino acid chromatography, thus reducing the need for enzyme determination in tissue biopsies.

Hyperammonemia is caused by congenital disorders of the urea cycle and it is frequently found in organic aciduria and several aminoacidopathies. The amino acid analysis in plasma and urine allows the diagnosis of citrullinemia, argininosuccinic aciduria, argininemia, gyrate atrophy, HHH syndrome, lysinuric protein intolerance, and non-ketonic hyperglycinemia [97]. However, the amino acid pattern is non specific in *N*-acetylglutamate synthetase deficiency, carbamoyl phosphate synthetase deficiency, ornithine transcarbamylase deficiency, and organic acidurias. Among this latter group of diseases, the elevation of orotic acid may be useful for identifying ornithine transcarbamylase deficiency. If orotic acid is not elevated, organic acidurias should be searched for by determining short fatty acids and organic acids in plasma and urine [98]. Diagnosis of *N*-acetylglutamate synthetase deficiency and carbamoyl phosphate synthetase deficiency can be made by assaying the enzymes in liver biopsy and in rectal or duodenal tissue, respectively [99–101].

It has been reported [102] that orotic acid excretion associated with ornithine transcarbamylase deficiency increases in the morning, whereas those in healthy subjects remain at baseline. It has been suggested that the increase in orotic acid excretion in the patients is due to the protein intake at breakfast after starvation throughout the whole night. Because orotic acid may go undetected in patients with ornithine transcarbamylase deficiency in the afternoon of a non-hyperammonemic period, urinary orotic acid concentration does not appear to be an ideal diagnostic index for the inherited disease. Urinary uracil has been recently proposed, instead of orotic acid, for a more useful indicator of ornithine transcarbamylase deficiency [30,88,103].

Orotic acid determination in the allopurinol loading test (300-mg oral dose) may be a useful diagnostic tool to establish the carrier status of women at risk for ornithine carbamoyltransferase deficiency [104]. Although the predictive value of the test is good, both orotic acid and orotidine should be measured to reduce the risk of misclassification [105]. Moreover, orotic aciduria is not as sensitive or specific an indicator of heterozygosis as the presence of orotidinuria. Finally, an increased excretion of orotic acid in sick children is relatively frequent and a positive allopurinol load test may not indicate a specific urea cycle defect [106].

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