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Short communication

Simple high-performance liquid chromatographic method for the detection of phenylpropionylglycine in urine as a diagnostic tool in inherited medium-chain acyl-coenzyme A dehydrogenase deficiency

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Abstract

Deficiency of medium-chain acyl-CoA dehydrogenase is a frequent and treatable metabolic defect, which can be diagnosed by detection of phenylpropionylglycine in urine after an oral load of phenylpropionic acid. We studied the determination of phenylpropionylglycine in urine by isocratic ion-exclusion chromatography on a cation-exchange column using water–sulphuric acid (pH values between 2 and 4) as mobile phase. Phenylpropionylglycine, phenylpropionic acid and hippuric acid exhibited high retention factors with only a slight decline at increasing solvent pH. This resulted in a good separation from interfering substances after direct injection of urine. We hypothesize that π–π interactions between the aromatic carbonic acids and the ion-exchange resin are responsible for the strong retention on the stationary phase. We conclude that, even in asymptomatic patients, determination of phenylpropionylglycine in urine after a phenylpropionic acid load by ion-exclusion chromatography is a rapid and reliable diagnostic tool for the detection of medium-chain acyl-CoA dehydrogenase deficiency.

Keywords: Phenylpropionylglycine; Acyl-coenzyme A dehydrogenase

1. Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a rather frequent inborn error of mitochondrial β-oxidation (1:15000 live births), which may cause life threatening metabolic crises with severe hypoglycemia and a general derangement of intermediary metabolism with subsequent carnitin depletion. Metabolic crises are usually induced by a catabolic state with mobilisation of free fatty acids e.g., during intercurrent infection. They are associated with a high mortality rate [1,2] in infancies sometimes presenting as sudden infant death [5]. These episodes can easily be prevented by careful avoidance of periods of prolonged fasting [3] and supplementation of carnitin. Under anabolic conditions the patients are usually completely asymptomatic [2]. A reliable confirmation of MCAD deficiency, therefore, is of great importance in the care of patients after an acute life threatening hypoglycemic crisis. Moreover, an early diagnosis is also important in still asymptomatic siblings who may also carry the metabolic defect.

Biochemical diagnosis of this disorder is based on

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the detection of pathognomonic organic acid metabolites, such as octanoylcarnitine or suberylglycine [4,5]. These metabolites can easily be detected by GC–MS in the urine of patients in an acute metabolic crisis but may be absent in remission [2]. Fasting tests in asymptomatic patients in order to provoke catabolism and secretion of pathognomonic metabolites carry a high risk [1]. Therefore, simple and harmless screening tests are needed for the diagnosis of patients in remission. A common mutation (985 A→G transition) has been identified in the gene coding for MCAD which might account for up to 80% of the patients [6,7]. On this basis attempts for mass screening had been undertaken. However, this approach will miss a considerable number of patients.

The oral administration of phenylpropionic acid has recently been proven to be a reliable screening tool for the detection of MCAD deficiency at the metabolite level [8]. In healthy individuals phenylpropionic acid is oxidized by the β-oxidation pathway to benzoic acid, which in turn is conjugated with glycine. The resulting hippuric acid is then excreted in the urine (Fig. 1). The initial step of the β-oxidation of this compound, the 2,3-dehydrogenation is nearly exclusively catalyzed by the mitochondrial MCAD [9]. In patients with this enzyme deficiency phenylpropionic acid is directly conjugated to glycine by N-acyl-glycine-transferase and excreted as phenylpropionylglycine. The detection of this metabolite in urine after a phenylpropionic acid-load has been shown to be diagnostic for MCAD deficiency [8,9].

Phenylpropionylglycine in urine may be detected

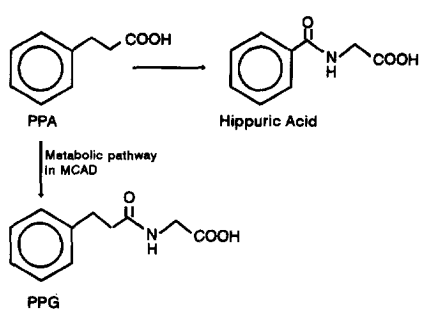

Fig. 1. Metabolic pathway of phenylpropionic acid in normal subjects and in MCAD-deficient patients (PPA: phenylpropionic acid, PPG: phenylpropionylglycine).

by GC–MS or – more readily available and less time-consuming – by HPLC. Rumsby et al. [10] proposed a HPLC-technique for the separation of organic acids in biological fluids, which is based on ion exclusion chromatography. This method was also used for the detection of phenylpropionylglycine in urine [8].

In this paper we describe the analytical characteristics (i.e., precision, accuracy, detection limit) of a modification of this technique. Furthermore, the impact of solvent pH on retention behavior of aromatic carbonic acids and on the resolution of these compounds from interfering substances is reported.

2. Experimental

2.1. Reagents and chemicals

Phenylpropionylglycine was synthesized from phenylpropionylchloride (purchased from Aldrich, Steinheim, Germany) by Schotten–Baumann reaction according to Dakin [11]. Sulfuric acid (Suprapur) and HPLC-grade water (Lichrosolv) was obtained from Merck (Darmstadt, Germany), phenylpropionic acid from Aldrich and hippuric acid from Sigma (Deisenhofen, Germany).

2.2. Equipment

The HPLC equipment was purchased from Merck–Hitachi (Darmstadt, Germany) and consisted of an isocratic pump (655-A11) connected to an autosampler (655-A40) and a variable-wavelength UV detector (655A). Data plotting and analysis was done with a D-2000 chromatointegrator.

The column was an Aminex HPX-87 H cation-exchange column, 300×7.8 mm I.D., 9 μm particle size with a cation H 30×4.6 mm I.D. cartridge as guard-column (Bio-Rad Laboratories, Munich, Germany).

2.3. Chromatographic conditions

Chromatography was performed under isocratic conditions. To prepare the mobile phase HPLC-grade water was adjusted to the desired pH (2.0, 2.6, 3.0)

with concentrated sulfuric acid. For the more basic mobile phase 1 m*M* disodium phosphate in water was adjusted to pH 4.0 with sulfuric acid. Flow-rate was set to 0.8 ml/min and column temperature to 50°C. Injection volume was 50 μl. Column effluent was monitored at 210 or 260 nm.

Urine samples were diluted with water to a creatinine concentration of 1 mmol/l and injected directly onto the column.

2.4. Calculations

The detection limit was calculated at a signal-to-noise ratio of 3 and the lowest limit of reliable quantification at a signal-to-noise ratio of 10. Specificity of the method was defined as the concentration of analyte at which a positive peak was clearly separable from false positive peaks. This was calculated as the mean concentration of interfering peaks at the respective retention time plus 3 standard deviations. Retention factors were calculated by the formula $k=(t_x-t_0)/t_x$, where t_x is the retention time of the analyte and t_0 the retention time of an unretained peak (lithium sulfate).

3. Results

3.1. Influence of mobile phase pH on retention

Mobile phases with a different pH, ranging from pH 2 to pH 4, were used for chromatography of an aqueous solution of 25 μmol/l each of phenylpropionic acid, phenylpropionylglycine and hippuric acid (Table 1). In general, the aromatic carbonic acids had high retention factors which showed only a slight decrease with increasing solvent pH. In urine, chromatography yielded good separation of phenylpropionylglycine from hippuric acid and other interfering substances, that was not dependent on solvent pH. A representative chromatogramm of a patients urine containing 0.4 mmol/l phenylpropionylglycine is shown in Fig. 2.

3.2. Optimal wavelength for detection

Phenylpropionylglycine exhibits absorption maxima at 210 and 260 nm. Detection at 210 nm resulted in a 70 times more intense signal than detection at 260 nm, as determined for phenylpropionylglycine at a concentration of 50 μmol/l ($n=3$). Since phenylpropionylglycine was readily separated from other compounds, we consecutively used 210 nm as detection wavelength due to its superior sensitivity, although 260 nm may be more specific for aromatic compounds.

3.3. Lowest detectable concentration and detection limit

Blank urine samples spiked with decreasing concentrations of phenylpropionylglycine were measured (eluent pH 2.6, detection wavelength 210 nm, $n=5$ each). The lowest detectable concentration of phenylpropionylglycine was 0.25 μmol/l. Thus, the lowest limit of quantification was 0.83 μmol/l corresponding to 0.04 nmol injected. In order to determine specificity, eleven urine samples from normal persons were chromatographed under the same conditions. In three urine samples a small interfering peak at the retention time of phenylpropionylglycine was detected. When calculated as phenylpropionylglycine by external standardization the mean concentration of this false positive peak was 1.8±0.15 μmol/l (mean±standard

Table 1
Retention factors (k) of the different aromatic carbonic acids at different eluent pH values

Eluent	k		
pH	Hippuric acid	Phenylpropionylglycine	Phenylpropionic acid
2.0	5.0	6.9	10.7
2.6	4.8	6.7	10.7
3.0	4.3	5.9	10.5
4.0	3.5	5.1	9.9

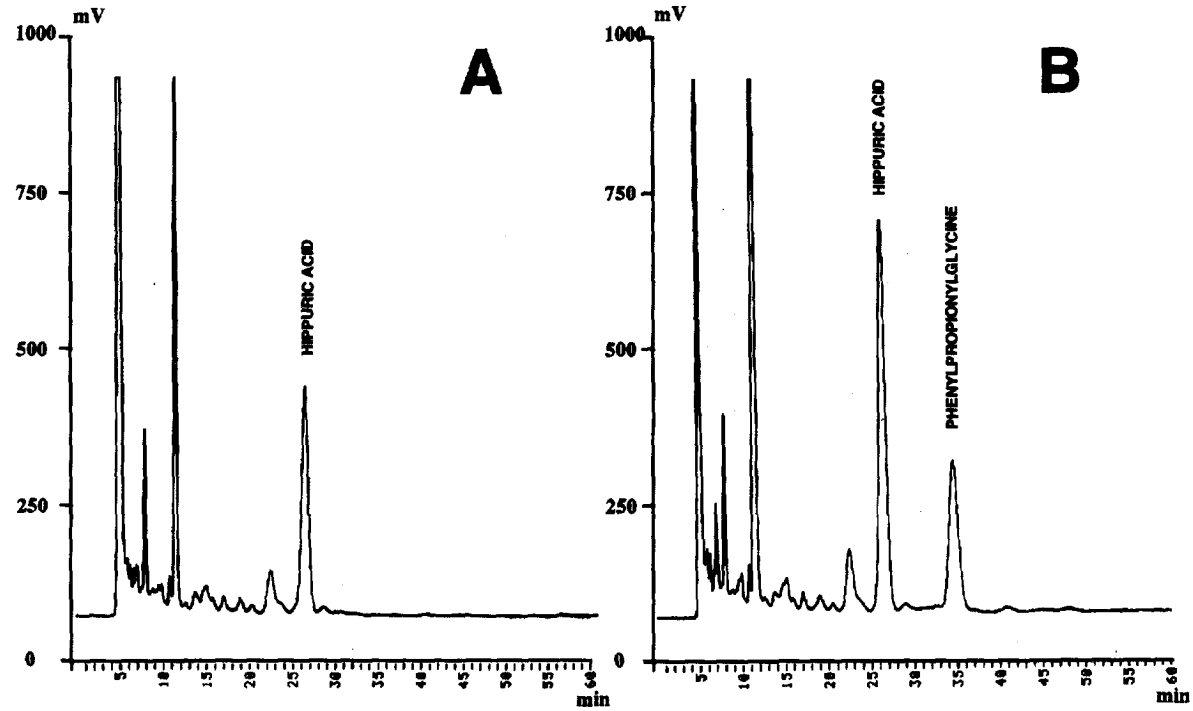

Fig. 2. Phenylpropionic acid-loading test (25 mg/kg body weight) in a MCAD-deficient patient. Urine samples prior to (A) and 0–6 h after (B) phenylpropionic acid administration were chromatographed at pH 4.0 and a detection wavelength of 210 nm. The appearance of phenylpropionylglycine (PPG) after the load is clearly visible. In addition, there is a moderate increase in hippuric acid (hippuric) excretion. The retention times for hippuric acid and phenylpropionylglycine were 26.3 and 34.6 min, respectively.

deviation). Thus, peaks exceeding 2.25 μmol/l were regarded as phenylpropionylglycine.

3.4. Linearity of response

Aqueous standard solutions of phenylpropionylglycine (twelve different concentrations ranging from 5 to 100 μmol/l) were chromatographed as well as urine spiked with phenylpropionylglycine (six different concentrations from 5 to 50 μmol/l). In the aqueous solution linear regression analysis yielded a slope of 5.73±0.09 with a y-intercept of −16.27±5.03 and an x-intercept of 2.84. The respective values in urine were 4.88±0.53, 14.33±16.15 and −2.94. The measurement was linear over the entire concentration range tested. Correlation coefficients (r^2) in water and urine were 0.998 and 0.955, respectively.

3.5. Accuracy and precision

Urine spiked with 50 μmol/l phenylpropionylglycine was repeatedly chromatographed (six times each and on 5 different days). The concentration was determined by comparing the peak area with an external aqueous standard of 50 μmol/l phenylpropionylglycine. The mean recoveries in intra- and inter-assay experiments were 96.0% and 102.8%, respectively. The intra-assay and inter-assay coefficients of variation were 1.3% and 9.5%, respectively.

3.6. Examination of patient urine

Urine samples were collected prior to and 0–6 h after phenylpropionic acid load (25 mg/kg) in four patients with known MCAD deficiency. In addition, three patients were investigated in order to exclude

this condition. Chromatography was performed at an eluent pH of 2.6 and with detection at 210 nm. Two of the MCAD deficient patients showed urinary excretion of large amounts of phenylpropionylglycine (1.3 and 1.2 mmol/mmol creatinine). In the two other patients, exhibiting a partial defect, only 0.018 and 0.281 mmol phenylpropionylglycine/mmol creatinine were found. In the other urines no measurable quantities of the metabolite were detected. In addition, there was a large increase of the hippuric acid excretion that confirmed a sufficient intestinal resorption of phenylpropionic acid.

4. Discussion

The determination of phenylpropionylglycine in urine after phenylpropionic acid load by ion-exclusion chromatography is a reliable and very simple method for selective screening of MCAD deficiency. The biochemical basis of this loading test is well established. Rinaldo et al. [9] could demonstrate that phenylpropionic acid is nearly exclusively dehydrogenated by MCAD. Patients with MCAD deficiency excrete large amounts of phenylpropionylglycine after an oral load with phenylpropionic acid. In contrast, we were not able to detect this substance in urine of normal controls and of patients with other metabolic disorders. Phenylpropionic acid is a rather hydrophobic compound and might be poorly resorbed from the gastrointestinal tract especially when administered in an aqueous solution. The method described allows the simultaneous measurement of hippuric acid as a simple control of phenylpropionic acid resorption.

We also could demonstrate that the isocratic separation of phenylpropionylglycine from hippuric acid and other concomitants in urine can easily be achieved, even when omitting a prior extraction of the sample. Detection at 210 nm resulted in an increased sensitivity as compared to detection at 260 nm. The reliable quantitation of aromatic carbonic acids in crude urine at a sensitive but rather unspecific wavelength is possible due to the high retention factors of these compounds. The strong retention of the phenylated carbonic acids and the only slight decrease of retention factors with increasing eluent pH is different from the behavior of aliphatic carbonic acids. These compounds show a much steeper decrease of retention factors with increasing solvent pH [12]. This may be explained by their increased solute charge thereby increasing repulsion from the negatively charged ion-exchanger matrix of the stationary phase. Moreover, the high-molecular-mass of the phenylated carbonic acids should reduce retention due to size exclusion effects [13]. Therefore, independent of eluent pH, a relatively strong force, which counteracts electrostatic repulsion and size exclusion, must be postulated. This force may account for the highly reduced retention factors of the aromatic acids, that remained stable over a wide pH range. The drop of the retention factors is more pronounced for aliphatic carbonic acids as compared with aromatic carbonic acids. Therefore, this force should be different from a simple hydrophobic interaction (van der Waals interaction). We suspect that interaction between the benzene nucleus of the aromatic carbonic acids and the π-electronic system of the polystyrene matrix of the ion-exchanger is responsible for this strong retention. These electron donor–acceptor complexes between planar π-systems have been reported to strongly influence chromatographic behavior [14,15].

The chromatographic characteristics enabled us to separate phenyplpropionylglycine from hippuric acid and other concomitants in urine in a very simple manner. Despite its simplicity, this procedure is sensitive and specific and exhibits high precision. Therefore, the quantification of phenylpropionylglycine in urine by HPLC after a phenylpropionic acid loading test is a simple and widely applicable diagnostic tool for the detection of MCAD deficiency, even in asymptomatic patients.

Moreover, the recognition of the unique chromatographic behavior of aromatic carbonic acids on polystyrene based cation exchangers may be of use for the development of other applications.

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