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Note

Quantitative gas chromatographic determination of urinary hydantoin-5-propionic acid in patients with disorders of folate/vitamin B_{12} metabolism

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Hydantoin-5-propionic acid, [1-(2,4-3H,5H)-imidazoledione]propionic acid (HPA), is a metabolite of histidine; it is formed by the oxidation of imidazolonepropionic acid catalysed by aldehyde oxidase [1]. The normal urinary excretion of HPA is rather low; it does not exceed 20 μmol/g of creatinine [2]. Increased levels of urinary HPA have been associated with a deficiency of the enzyme formiminotransferase [2, 3]. In this condition urinary formiminoglutamic acid is generally increased. A semiquantitative analysis of urinary HPA can be achieved with two-dimensional thin-layer chromatography and staining with the mercuric acetate—diphenylcarbazone reagent [4, 5]. A quantitative analysis of HPA was described by Niederwieser et al. [2], who used selected-ion monitoring. This rather complicated method involves isolation of HPA followed by gas chromatography (GC). Our routine GC procedure [6] had a poor reproducibility for HPA, which appeared to be caused by the fact that the extraction yield was strongly influenced by the urea content of the urine. This finding is the basis of the present communication. We describe a quantitative GC method for the analysis of HPA using an ethyl acetate extraction after addition of an excess of urea.

EXPERIMENTAL

Analytical procedure

Hydantoin-5-propionic acid was synthesized from D,L-glutamic acid and potassium cyanate [7]. The final crystalline product contained less than 1%

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of the starting material glutamic acid as measured with an amino acid analyser. The mass spectrum of synthetic HPA (TMS derivative) showed the expected molecular ion at *m/z* 388 and the base peak at *m/z* 257, reflecting the loss of a (CH_2—COOTMS) moiety [3]. All other chemicals and reagents were of analytical grade. The final extraction and derivatization procedure was as follows: to 5 ml of urine were added 5 ml of a saturated sodium chloride solution and 100 mg of urea. The mixture was acidified to pH 1—2 and extracted twice with 20 ml of ethyl acetate. Evaporation of the solvent, trimethylsilylation, and GC were performed as described in detail elsewhere [6]. We used a Varian 3700 instrument equipped with a flame ionization detector and a capillary CPSil 19 CB column (26 m × 0.22 mm I.D., film thickness 0.21 μm). 3-Phenylbutyric acid, eluting at 22 min, was used as an internal standard; HPA eluted at ca. 34 min.

Subjects

Four patients with various metabolic lesions were investigated: (1) a mentally retarded boy with massive formiminoglutamic aciduria [3]; (2) a three-month-old girl with neurological abnormalities who appeared to have combined methylmalonic aciduria and homocystinuria (Cbl C disease); (3) a twelve-year-old mentally retarded girl with megaloblastic anemia, homocystinuria and formiminoglutamic aciduria after loading with L-histidine [8]; (4) an eighteen-month-old girl with familial selective malabsorption of vitamin B_{12} (Imerslund—Grasbeck syndrome). A healthy 33-year-old male who was loaded with 100 mg/kg L-histidine, served as a control.

RESULTS AND DISCUSSION

During the routine GC screening of urinary organic acids, patients 1, 2 and 3 appeared to excrete considerable amounts of HPA (Fig. 1). Attempts to

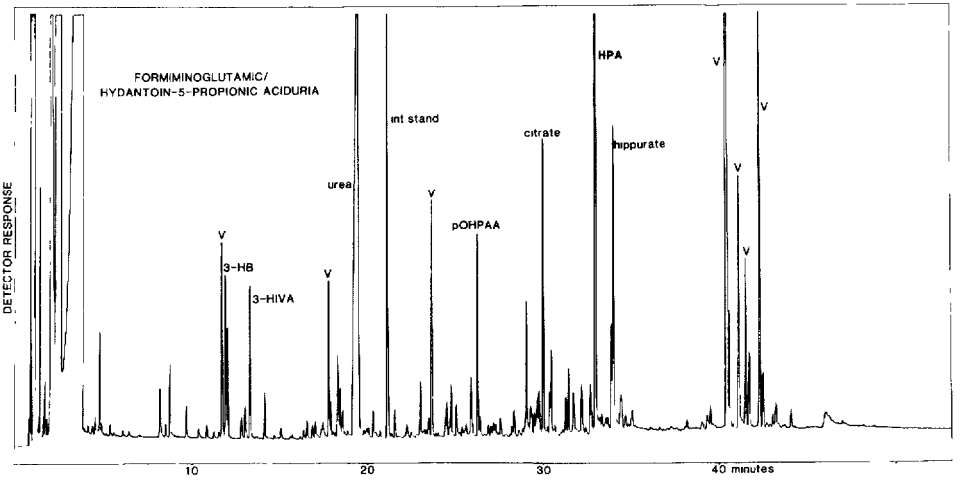

Fig. 1. Urinary organic acids in a patient with formiminotransferase deficiency. In addition to the huge amount of hydantoin-5-propionic acid there were large amounts of valproate metabolites (V); 3-HB = 3-hydroxybutyric acid; 3-HIVA = 3-hydroxyisovaleric acid; pOHPAA = *p*-hydroxyphenylacetic acid; int. stand. = internal standard, 3-phenylbutyric acid.

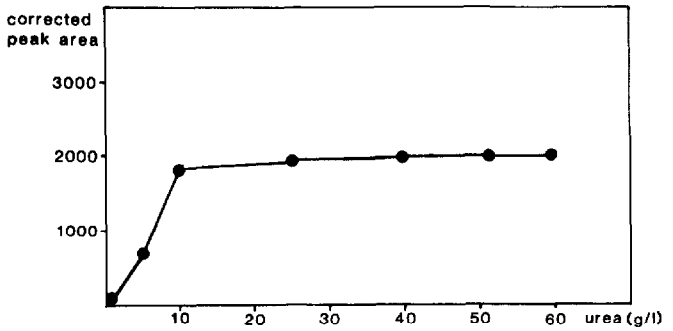

Fig. 2. Relationship between the urea concentration and the recovery of hydantoin-5-propionic acid during ethyl acetate extraction from aqueous solution. The peak area was corrected for the recovery of the internal standard 3-phenylbutyric acid expressed in integration units.

quantitate the amount of HPA were unsuccessful because of the large variation in the recovery during the ethyl acetate extraction. We had the impression that extraction was more efficient from "concentrated" urine samples than from "dilute" urine samples. The solute present in the highest concentration in urine is urea, which is extracted with ethyl acetate in considerable amounts. We estimated the solubility of urea in ethyl acetate to be 1.4 g/l. It was decided to investigate whether urea influenced the ethyl acetate extraction of HPA from aqueous solutions. The effect of increasing urea concentration is shown in Fig. 2. The analytical method was linear up to a HPA concentration of 15 mmol/l. The detection limit was estimated to be 25 μmol/l. Urine samples which appear to contain more HPA than 15 mmol/l have to be re-analysed after appropriate dilution. We tested the reproducibility of the method by analysing a urine sample from a control subject to which synthetic HPA had been added (final concentration 2.4 mmol/l). The within-assay precision was estimated from five analyses; the relative standard deviation was 3.9%. Roughly the same value was observed when aliquots were analysed on five consecutive days: the relative standard deviation was 2.9%. The recovery of HPA taken through the whole procedure was calculated to be 37% when compared with a sample of the authentic substance which was derivatized and analysed as such. Urine samples for the analysis of organic acids are routinely stored at -20°C. Storage for up to two weeks at this temperature did not lead to a loss of HPA. On the other hand, samples that had been stored at room temperature for two weeks lost 11% of their HPA content.

Addition of urea to urine samples that already contain a large amount of this solute ("concentrated urines") is not an absolute necessity, but its routine addition for the quantitation of HPA minimizes mistakes. It is not advisable to add urea to all urine samples for organic acid screening because a large urea peak in the gas chromatogram may obscure a number of important compounds. The addition of urea does not have a positive effect on the extraction recovery of other hydrophilic compounds, such as orotic acid and uracil: probably the latter compounds are better isolated by an ion-exchange procedure [5]. Apparently, urea-containing ethyl acetate is a better solvent for HPA than ethyl acetate itself. The HPA may interact with a urea molecule via hydrogen bridges

and thus becomes less polar and more readily soluble in ethyl acetate. Interaction between the two types of molecules may well be understood because of the structural relationship between urea and part of the hydantoin ring.

The position of trimethylsilylated HPA in the capillary gas chromatogram (Fig. 1) is between those of 4-hydroxyphenyllactic acid and hippuric acid. No interfering organic acids are presently known to us. Patient 1 was treated with the anti-epileptic drug sodium valproate; accordingly he excreted large amounts of valproate metabolites which are denoted by V in the chromatogram (Fig. 1). Characteristic urinary excretion values are given in Table I. Because 24-h urine collections were not always possible, the values are given in mmol per mol of creatinine. The concentrations in the patient with formiminotransferase deficiency are in good agreement with previously obtained values using selected-ion monitoring [3].

TABLE I

URINARY EXCRETION OF HYDANTOIN-5-PROPIONIC ACID (HPA) AND FORMIMINOGLUTAMIC ACID (FIGLU) IN VARIOUS PATIENTS

Patient	Age (years)	Excretion (mmol/mol of creatinine)	
		HPA	FIGLU
1. Formiminotransferase deficiency	5.5		
Basal excretion		418	158
L-Histidine loading (100 mg/kg)		712	689
2. Methylmalonic aciduria—homocystinuria	0.3	350	1348
3. Folate metabolic defect	12		
Basal excretion		42.9	13.4
L-Histidine loading (300 mg/kg)		580	252
4. Vitamin B_{12} deficiency	1.5	21 0	N.D.*
5. Control: L-histidine loading (100 mg/kg)	33	14.5	N.D.

*N.D. = not detectable.

There was a very sharp increase in HPA excretion in patient 3 with the presumed defective folate/B_{12} metabolism after histidine loading. The basal level in this patient was higher than that in the methylmalonic aciduria—homocystinuria patient 2. The patient with vitamin B_{12} deficiency had a considerably lower HPA excretion than the above-mentioned patients, the value being similar to that observed in the control subject after a histidine load.

An abnormal HPA excretion is often accompanied by an increase of the urinary formiminoglutamic acid (FIGLU) excretion. As such both compounds can be used as an index of a disturbed folate/vitamin B_{12} metabolism. The concentration of urinary HPA and FIGLU were of the same order of magnitude in the patients studied, although there was no linear relationship. The urine samples of subjects 4 and 5 were stored at $-20°C$ for several years. We presume that all FIGLU in these samples has decomposed during this period. The fact that FIGLU decomposes rapidly, especially at alkaline pH, favours the

analysis of HPA. Moreover, the latter substance can be found in the gas chromatogram of urinary organic acids, which is part of the standard screening programme for the investigation of inherited metabolic disorders nowadays.

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