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Methylcitric Acid Determination in Amniotic Fluid by Electron-Impact Mass Fragmentography

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Summary: Methylcitric acid, a metabolite of abnormal propionyl-CoA metabolism, is elevated in amniotic fluid when the fetus is affected with propionic acidaemia or methylmalonic aciduria. We developed a method for quantifying methylcitric acid in amniotic fluid by solid-phase extraction, derivatisation to the *t*-butyl-dimethylsilylester, and gas chromatography-mass spectrometry with electron-impact ionisation, using the deuterated analogue of methylcitric acid as an internal standard. The main advantages are a good sensitivity, simple sample preparation, and feasibility on instruments equipped with mass specific detectors.

Introduction

The prenatal diagnosis of propionic acidaemia (propionyl CoA carboxylase[1]) deficiency), and of the methylmalonic acidurias (methylmalonylCoA mutase[1]) deficiency and cobalamine disorders) can be rapidly established by quantifying methylcitrate in the amniotic fluid obtained at the 15–16th week of pregnancy (1, 2) or perhaps even earlier (*L. Sweetman*, personal communication). The method of choice for the quantification of methylcitrate (2-hydroxy-1,2,3-butanetricarboxylic acid), normally present at concentrations below 1 µmol/l, is nowadays gas chromatography mass spectrometry (GC-MS) with stable isotope dilution.

A frequently used derivatisation procedure for gas chromatography of organic acids, and particularly methylcitrate, is silylation (3). Upon electron impact ionisation (EI) with 70 eV, high mass fragments of the trimethylsilyl derivatives are of low abundance and the specifity of lower mass fragments is reduced. In order to circumvent the resulting low sensitivity, chemical ionisation methods have been applied (1, 3). Another possibility would be EI with lower ionisation energy, which however needs more expensive mass spectrometers.

Since GC-MS instruments limited to fixed electron impact ionisation are more readily available, we developed a method suitable for GC-MS with EI, using *t*-butyl-dimethylsilyl derivatives, which offer the advantage of a high yield of heavy and specific (M-57) fragments.

Materials and Methods

Standards, chemicals

Chloroform and *t*-amylalcohol (both p. a.), HCl, ammonia and Extrelute-3 columns were obtained from Merck (Zürich, Switzerland) and used without further purification.

Tricarballylic acid was from Fluka (Buchs, Switzerland), N-methyl-N-(*t*-butyldimethylsilyl)trifluoroacetamide from Pierce (Kontron, Zürich, Switzerland).

The deuterated analogue of methylcitric acid was synthesised according to *Beach* et al. (4). Diethyl 1,3-acetone-dicarboxylate was reacted with d_3-methyliodide (Merck, isotopic purity 99.88% or better) to produce diethyl-2-methyl-3-oxoglutarate. After a cyanohydrin reaction the product was hydrolysed with concentrated HCl to methylcitric acid. Non-deuterated methylcitric acid was obtained by the same procedure, using unlabeled methyliodide (Merck). The described acids were obtained

[1]) Enzymes: Methylmalonyl-CoA decarboxylase (Propionyl-CoA carboxylase), EC 4.1.1.41; Methylmalonyl-CoA mutase, EC 5.4.99.2.

as a mixture of stereoisomers; however, no attempt was made to separate them since the same two isomers appear in about equal amounts in the amniotic fluid, and are readily resolved in the GC-MS-system.

Samples

Amniotic fluid samples were obtained from normal pregnancies, from patients at risk for fetal neural tube defects and from patients at risk for propionic acidaemia or methylmalonic aciduria, mainly at the 16th—19th week of gestation. The specimens were centrifuged and the supernatant stored at −20 °C until processed.

Standard procedure

To 1 ml sample were added: 20 nmol of deuterated methylcitric acid in 100 µl water, 100 nmol of tricarballylic acid in 100 µl water (as an internal standard for control of effective silylation), and 1.8 mmol HCl (300 µl of 6 mol/l HCl).

As a standard, 1 ml of water containing 20 nmol of methylcitric acid was treated as a sample.

The pretreated samples and the standard probe were adjusted with 1300 µl of water to a total volume of 2800 µl and absorbed on an Extrelut-3 column for 10 minutes. The elution was performed with 15 ml of a mixture of chloroform and *t*-amylalcohol (1 + 1, by vol.), into conical 20-ml flasks containing 2.4 mmol of ammonium hydroxide solution (800 µl, 3.0 mol/l), and mixed briefly on a vortex mixer.

The whole eluates were evaporated to dryness under a stream of nitrogen in a waterbath at 50 °C. Then 150 µl of N-methyl-N-(*t*-butyldimethylsilyl)trifluoroacetamide were added to the dry residues, and the flasks were carefully stoppered and maintained at 70 °C for 1 h. After cooling, 4 µl of the solution were injected into the GC-MS system.

Gas chromatography-mass spectrometry

A Hewlett-Packard HP5990 GC-MS system with floppy disc software was used. For gas chromatography, a 30 m capillary column of Durabond DB-5 (J + W Scientific, Rancho Cordova, CA, USA), 0.32 mm ID, coated with 0.25 µm SE54, chemically bonded, was installed in the GC-MS system. The injector temperature was 250 °C. The carrier gas was helium at 3 ml/min. The temperature was programmed for 3 minutes at 200 °C, followed by heating at 8 °C/minute to 270 °C. Under these conditions, the retention times for the methylcitric acid isomers were 7.4 and 7.6 minutes, respectively. Effluent was transferred to the MS by a jet separator, and ionisation was by electron-impact (70 eV).

The effectiveness of silylation was controlled by monitoring the peak area of the tricarballylic derivative which in our experience is sensitive to interferences. The mass fragmentography (dwell times 50 ms) was performed so as to record the abundances of ions (M-57) of the methylcitrate and d_3-methylcitrate derivatives, m/z 605 and m/z 608, respectively. The integration was done using the standard software of the system. All the calculations were made by adding the areas of the two peaks of the methylcitrate stereoisomer derivatives. Owing to the good linearity of the isotopic dilution standard curve of the ratio of m/z 605 to m/z 608, further calculations of the samples were made using only one standard ratio.

Results

Mass spectra of deuterated and nondeuterated methylcitrate are depicted in figure 1. The intra-series precision of the method amounted to 1.9% CV (n = 9) measured at a concentration of 2.53 µmol/l, the day to day precision was 3.6% CV (n = 12), with a mean of 2.50 µmol/l. The recoveries were tested by adding increasing amounts of methylcitrate to 1 ml amniotic fluid (tab. 1). While recoveries of 105% were found at the lowest concentration, they systematically decreased to 88% at 30 nmol added.

The limit of detection was lower than 50 nmol/l.

The time required for performing the analysis was about 6 h for 8 samples.

Tab. 1. Recovery of nonlabelled methylcitrate added to 1 ml of amniotic fluid:

Standard added (nmol)	Observed concentration (µmol/l)	Recovery (%)
0	0.43	—
1.11	1.59	105.0
1.67	2.21	107.0
3.33	3.84	102.3
8.88	8.86	95.0
16.6	16.2	94.6
33.3	29.6	87.5

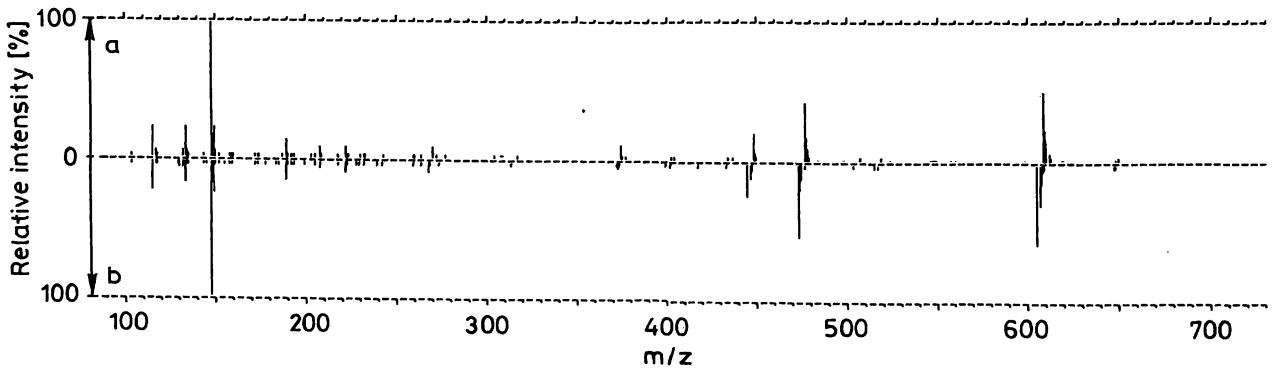

Fig. 1. Mass spectra of the deuterated (a) and the nondeuterated methylcitrate derivatives (b; for conditions see text).

Reference values

Methylcitrate was readily detected in all amniotic fluid samples. The distribution of the data from samples obtained at 14–19 weeks of gestation was tested for normality by the *Kolmogoroff-Smirnov* and the χ^2 tests. No significant deviation from a *Gaussian* distribution was found. The upper limit of the reference range can thus be estimated at 530 nmol/l (tab. 2).

Tab. 2. Reference values for methylcitrate in amniotic fluid:

14th–19th week of gestation: n = 31	Range 110–480 nmol/l Median value 350 nmol/l Mean ± SD: 340 ± 94 nmol/l
31st–36st week of gestation: n = 4	550, 580, 710, 600 nmol/l

In 4 samples taken at the 31st to the 36th weeks of gestation for testing foetal lung maturity, we measured a significant elevation (*Wilcoxon* test, $p < 0.01$) of the methylcitrate concentration compared with the samples obtained at 14–19 weeks of gestation; the median was at 590 nmol/l with a range of 550–710 nmol/l.

The method proved to be useful for the detection of patients affected as shown in table 3. Using the same samples, parallel determinations were done by *L. Sweetman* in San Diego with chemical ionisation GC-MS; the results were practically identical.

Tab. 3. Methylcitrate in amniotic fluid of patients at risk (nmol/l):

Non-affected:	n = 3:	430, 290; 250 (270)[a]
Affected[b]:	n = 2:	11 500; 6800 (6600)[a]

[a] Values obtained in parallel in a reference laboratory
[b] Propionyl-CoA carboxylase deficiencies

Discussion

The use of Extrelute for the sample preparation for GC-MS is well established (5, 6) in our laboratory. To extract organic acids, the sample has to contain an amount of HCl corresponding to the volume of the column rather than to the sample volume; we observed a strong decline in the extraction efficiency when less than 250 µl of HCl (6 mol/l) were added to the sample.

An adequate silylation could not be obtained at room temperature, even after 20 h reaction time. The same reagent with *t*-butyldimethylsilylchloride (10 g/l), or with the addition of acetonitrile, did not lead to better results, but complete derivatisation was achieved by heating at 70 °C for at least 40 minutes.

This method illustrates well the advantages of using GC-MS together with isotopic dilution and a solid-liquid extraction method. The sensitivity and the selectivity of the GC-MS, together with the deuterated internal standard, which also acts as a carrier, allow a fast and simple specimen preparation.

The methylcitrate concentration in amniotic fluid of patients with inherited defects of propionate metabolism is much higher than that of controls. Thus the systematic decrease in recovery of high concentrations is not a serious drawback for diagnosis. The use of a standard curve with several concentrations (including high ones) could improve the accuracy, but is more time consuming.

The antenatal diagnosis of inherited metabolic disorders by the quantification of critical metabolites in amniotic fluid is now well documented (2, 3, 7); if possible, it is performed in parallel with the determination of enzymatic activity in cultured foetal cells obtained from amniotic fluid. The metabolite determination, however, does not have the disavantage of the time delay of tissue culture.

The present method uses simple sample preparation, and does not need especially sophisticated instrumentation. It thus could be used by a large number of clinical laboratories interested in inborn errors. Nevertheless we would recommend – as for other analyses used for prenatal diagnosis – that the analyses or enzyme determination be performed in parallel in an independent laboratory for confirmation, because of the prognostic implications of the results.

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