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Validated capillary gas chromatographic–mass spectrometric assay to determine 2-methylcitric acid I and II levels in human serum by using a pulsed splitless injection procedure

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Abstract

Background: Despite some clinical applications of 2-methylcitric acid (2-MCA) determination in urine and amniotic fluid, a diagnostic use of 2-MCA levels in serum is not common practice. This could be related to the complexity of the assay, or possibly to unawareness of other feasible clinical applications. *Methods:* The levels of the diastereomers 2-MCA I and II in human serum were determined by GC–MS based on a method using a pulsed splitless injection technique. A stable isotope dilution principle was modified considering the diastereomer ratio and impurities of the internal standard. Precision parameters as well as recovery rates of the assay were determined. Reference intervals for 2-MCA_{total}, 2-MCA I and II levels were obtained in 52 healthy volunteers (31 female, 21 male, mean age 41.7±14.4 years). *Results:* 2-MCA was readily detected in each sample of serum, as well as in urine, cerebrospinal fluid and amniotic fluid. The limit of detection was 10 nmol/l for 2-MCA_{total}. The internal standard showed a diastereomer ratio of 2-MCA II-d_3 to 2-MCA I-d_3 of 0.83±0.05, its chemical purity had to be corrected to 90.5±0.5%. In concentrations of 446, 750 and 1256 nmol/l 2-MCA_{total}, recovery rates of 98.5, 93.7 and 88% with a mean intra-assay RSD of 1.5% were determined. The day-to-day precision was 10% RSD (SD 40 nmol/l) for 2-MCA_{total} obtained with a pooled serum sample at a concentration of 401 nmol/l 2-MCA_{total} over a period of 5 months (n=17). The normal range for 2-MCA_{total} in human serum was calculated as 81–266 nmol/l confirming previous findings. *Conclusions:* The GC–MS assay using a pulsed splitless injection procedure ensures a good response to differing concentrations of 2-MCA in various specimens. Considering exact determination of the diastereomer ratio as well as the purity of the internal standard, the assay offers good precision and recovery for 2-MCA I and II levels in serum.

Keywords: 2-Methylcitric acid

1. Introduction

2-Methylcitric acid (2-MCA) is formed by propionyl-CoA and oxaloacetic acid in a reaction catalyzed by citrate synthase (Fig. 1). The maximal reaction velocity is 10^{-4} times that of its major reaction to form citric acid [1]. The prenatal diagnosis of propionic acidemia and the presence of any type of methylmalonic aciduria can be established by quantifying 2-MCA in the amniotic fluid obtained in the 15–16th week of pregnancy [2,3]. Levels of

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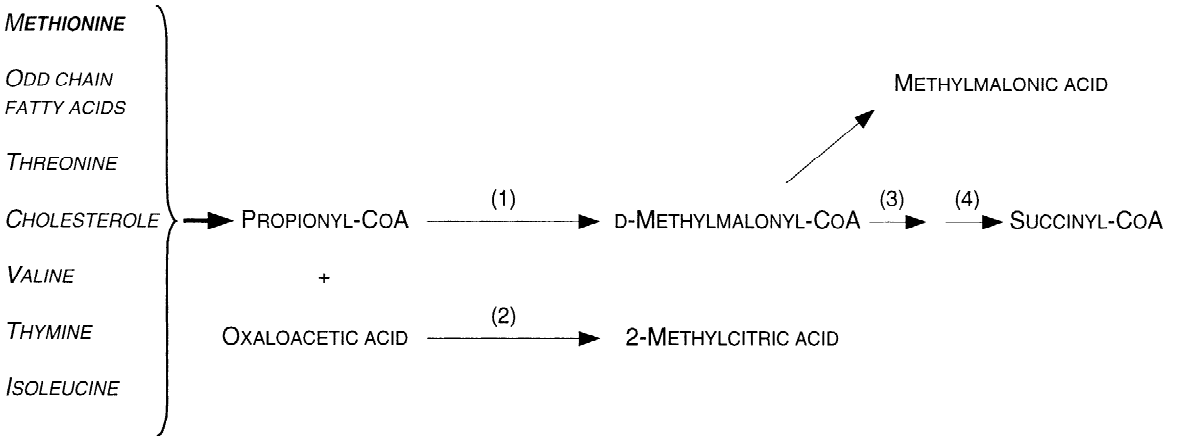

Fig. 1. Metabolic pathway relations of 2-methylcitric acid with enzymes involved: propionyl-CoA carboxylase (biotin dependent) (1), citrate synthase (2), D,L-methyl-malonyl-CoA racemase (3), L-methylmalonyl-CoA mutase (adenosylcobalamin dependent) (4).

2-MCA in serum and cerebrospinal fluid are elevated in patients with cobalamin deficiencies of different causes [4]. Various forms of methylmalonic aciduria, mostly involving defects of L-methylmalonyl-CoA mutase, go along with increased levels of 2-MCA and methylmalonic acid (MMA) in blood, urine and amniotic fluid [2,5,6]; while defects of the biotin-dependent propionyl-CoA carboxylase, which are a possible result of a holocarboxylase-synthase-deficiency [7], lead to increased levels of 2-MCA and propionic acid [2,3,5,8]. In cases of biotin deficiency, increased amounts of 2-MCA have been found in the urine [9]. Despite these clinical applications, the use of 2-MCA determination in serum is, however, not common practice. This could be related to the difficulty of the assay, as 2-MCA is comprised of a chiral molecule with two asymmetric carbon atoms (Fig. 2). Therefore it exists in the form of two pairs of enantiomers (diastereomers) comprising four enantiomers [10]. Considering the nomenclature of Brandange et al., 2-MCA I consists of the 2*S*,3*R*- and 2*R*,3*S*-enantiomers, while 2-MCA II consists of the 2*S*,3*S*- and 2*R*,3*R*-enantiomers [11]. In vivo, mostly the 2*S*,3*S* and 2*S*,3*R* forms are produced which explains their increase in the case of propionic acidemia [1,3,11]. Today gas chromatography–mass spectrometry (GC–MS) seems to be the method of choice for the determination of 2-MCA and its diastereomers I and II. To our knowledge, the method published by Allen et al., in which 2-MCA is determined together with MMA, is the only technique for the determination of 2-MCA by measuring the concentration of its diastereomers in serum [4]. Unfortunately, no information about the injection procedure is given. Nevertheless, this could be of relevance for the determination of low concentrations of 2-MCA in biological material.

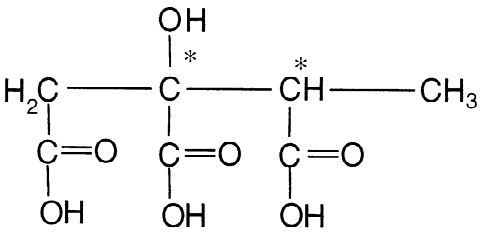

Fig. 2. Structure of 2-methylcitric acid (*chiral carbon atom).

Apart from the findings by Kretschmer and Bachmann regarding the precision of a 2-MCA assay in amniotic fluid [2], nothing is known about the intra-assay and inter-assay precision of a quantitative GC–MS assay of 2-MCA in serum. The precision of the method could be of some relevance for establishing it in clinical use.

In our studies we describe a newly developed quantitative GC–MS assay for 2-MCA and its diastereomers using a pulsed splitless injection technique based on the sample preparation by Allen et al. [4]. In addition, chemical impurities of the internal standard were detected, identified and quantified. To

learn more about the limitations of diastereomer quantification by GC–MS, the assay was tested for its recoveries as well as its intra- and inter-assay characteristics in human serum of various levels of 2-MCA. Reference intervals were determined in a set of 52 age matched healthy volunteers.

2. Experimental

2.1. Chemicals and standards

The internal standard D,L-[methyl-d_3]2-methylcitric acid (2-MCA-d_3) and D,L-2-methylcitric acid were obtained from CDN Isotopes, Quebec, Canada. Acetic acid, methanol, hydrochloric acid and acetonitrile were from Merck, Darmstadt, Germany. The derivatizing agent, *N*-methyl-*N*(*tert*.-butyldimethylsilyl)-trifluoracetamide (MBDSTFA), was obtained from Macherey-Nagel, Düren, Germany.

2.2. Blood samples and healthy subjects

Recovery tests were done using samples of a pooled serum from healthy blood donors.

Reference values were calculated within 52 age matched healthy volunteers (31 female, 21 male, mean age 41.7±14.4 years). All of them had normal renal function as reflected by creatinine levels within the normal range (80–95 μmol/l). None of the controls was acutely ill or revealed clinical signs of a chronic disorder. Volunteers received no vitamin substitution for at least 3 months.

2.3. Sample collection

Blood samples were drawn after an overnight fasting in serum monovettes (Sarstedt, Nümbrecht, Germany) followed by centrifuging at 4 °C (3000 rpm) for 30 min. Serum samples were stored in 2-ml tubes (Sarstedt) at −80 °C.

2.4. Sample preparation

The procedure by Stabler and co-workers [4,12] was modified. Since the assay is based on the stable isotope dilution principle, 50 μl of the internal standard solution containing 363 pmol 2-MCA-$d_{3\,total}$ is pipetted into a 2-ml vial, followed by addition of 400 μl serum and 1 ml water.

Samples were fractionated on a column, containing 100 mg of an anion-exchange resin (AG-MP 1 from Bio-Rad, Munich, Germany). Before use, the resin was purified and activated by washing with 1 *N* HCl and methanol, followed by drying at 60 °C for 30 min. Samples were applied to the columns, which had previously been washed with 1 ml methanol followed by 3 ml water. Each column was washed once with 3 ml water and three times with 3 ml 0.01 *N* acetic acid in methanol. The columns were eluted in a 1.5-ml vial with 1.1 ml of a solution prepared as following: 900 ml of a solution of 250 ml acetic acid (99–100%) and 750 ml methanol were mixed with 100 ml of 1 *N* HCl. Eluates were dried by vacuum centrifugation in an Eppendorf concentrator (series 5301, Eppendorf, Hamburg, Germany) for 90 min at 40 °C. Dried samples were derivatized by incubating for 40 min at 90 °C with 30 μl of a solution containing 10 μl MBDSTFA and 20 μl acetonitrile.

2.5. GC–MS method

GC–MS was performed using a Hewlett-Packard (Waldbronn, Germany, presently called Agilent) 6890 gas chromatograph equipped with a 7683A autosampler and a Hewlett-Packard 5973 mass-selective detector. A Hewlett-Packard HP-5 MS column (30 m×0.25 mm I.D., 0.25 μm film thickness) was used. Injection was done into a focus liner with an internal diameter of 4 mm from SGE, Weiterstadt, Germany, using a pulsed splitless method (see Results and discussion). The sample volume injected was 1 μl. The initial column temperature of 100 °C was kept constant for 1 min, and then increased to 120 °C at a rate of 30 °C/min followed by a rate of 15 °C/min until 300 °C. This temperature was held for 5 min. The injector temperature was 250 °C, source of the mass-selective detector were heated to 230 °C, while the mass detector's quadrupole temperature was 150 °C.

Retention times were determined using the full scan (total ion mode). For quantitative measuring the mass-selective detector was operated in the selected ion monitoring (SIM) mode. Target ions for quantification, were the m/z 605 for endogenous 2-MCA and the m/z 608 for 2-MCA-d_3. The m/z 473 for

2-MCA and the m/z 476 for 2-MCA-d_3 were used as qualifier ions. The amounts of the metabolites were determined by multiplying the integrated ratio of unlabeled to labeled metabolite (target ions were used) with 907.3 nmol/l which was the corresponding concentration of the labeled metabolite in the serum sample of 400 μl.

2.6. Pre-experiments

The diastereomer ratio as well as the purity of the internal standard 2-MCA-d_3 had to be determined for exact quantification of the diastereomers. Moreover, as described previously, some serum samples contain an unknown endogenous peak, eluting at the same time as the m/z 608 peak for 2-MCA I-d_3 [4]. Therefore it was necessary to calculate the real area under the curve of that peak from the m/z 608 peak of 2-MCA II-d_3 by dividing its integrated area by the ratio 2-MCA II-d_3/2-MCA I-d_3 indicating a constant diastereomer ratio of the internal standard used.

For determining the diastereomer ratio of 2-MCA_{total} -d_3, 7.4 nmol/l was directly derivatized in 200 μl acetonitrile and 100 μl MBDSTFA to a final concentration of 24.7 mmol/l. This solution was measured 20 times. For the determination of the diastereomer ratio of pure 2-MCA used for the recovery experiments, additional testing in the same manner was done.

2.7. Statistics

Results are given as mean with standard deviation (SD). The precision of the assay is given as the relative standard deviation (RSD).

The Lilliefors test showed no compatibility of the data with normal distributions, except for age in the healthy controls, therefore the Spearman rank correlation test was used for estimating relationships between continuous variables and the *U*-test of Mann and Whitney was used for comparing differences between two independent groups. A P value <0.05 was considered to indicate statistical significance. Normal ranges are given as the mean$\pm$2SD after log normalization to correct for the skewness of the data as well as median with minimum, maximum and central 95% percentile interval.

3. Results and discussion

3.1. Pulsed splitless injection procedure

The HP 6890 gas chromatograph series, equipped with a split/splitless inlet offers the possibility of a pulsed pressure splitless injection as invented by Hewlett-Packard.

The advantage of a splitless injection is to ensure a superior focusing of the sample on the column by circumvent an initial dilution of the sample through purge flow. After injection, the purge flow is opened to rinse the rest of the solvent vapor from the liner to prevent a tailing of the solvent.

An additional convenient possibility to improve the focusing of the sample on the column is reached by an increase of pressure (pressure pulse). By using it, the pressure in the heated inlet is increased to its maximum level until the sample is evaporated leading to a faster focusing on the colder column to prevent any thermal decomposition in the inlet.

The pulsed splitless injection procedure was found to provide best response rates by using the following parameters: a pulsed pressure of 207 kPa for a pulse time of 1.5 min with a purge flow of 25 ml/min during a purge time of 1.5 min resulting in a total flow of 30.6 ml/min. After 2 min, the gas saver mode was used with a total flow of 18 ml/min containing a constant column flow of 0.7 ml/min which was held constant at an average velocity of 31 cm/s and a pressure of 39.7 kPa. These data are of importance as with regard to the literature dealing with GC–MS methods for determining 2-MCA [2,4], no data concerning a pulsed splitless method are published yet, causing long-term empiric search for optimal pressure and flow values. In our experience, the pulsed splitless injection procedure ensures a save determination of 2-MCA in serum as well as in urine, amniotic fluid and cerebrospinal fluid. By its use, the risk of 2-MCA levels not detectable by the assay, as described by Coude et al. in some amniotic fluids of at-risk pregnancies for organic acidurias, could be prevented [5].

3.2. Elution times and mass spectra

When pure 2-MCA was derivatized directly, diastereomers of 2-MCA were eluted after 15.20 and

15.35 min (Fig. 3). According to Allen et al., the first peak, eluted at 15.20 min, was due to 2-MCA II, while the second peak at 15.35 min was attributed to 2-MCA I [4]. The mass spectrum of 2-MCA, obtained under the peak of 2-MCA II, is shown also in Fig. 3. Similar to the literature, major ions of 2-MCA were present at m/z 371, 445, 473 and 605 [2,4]. The labeled counterpart 2-MCA-d_3 shows that major ions were shifted upward to 374, 448, 476 and 608 by three mass units indicating all contained the 2-methyl group which is labeled with deuterium (Fig. 3).

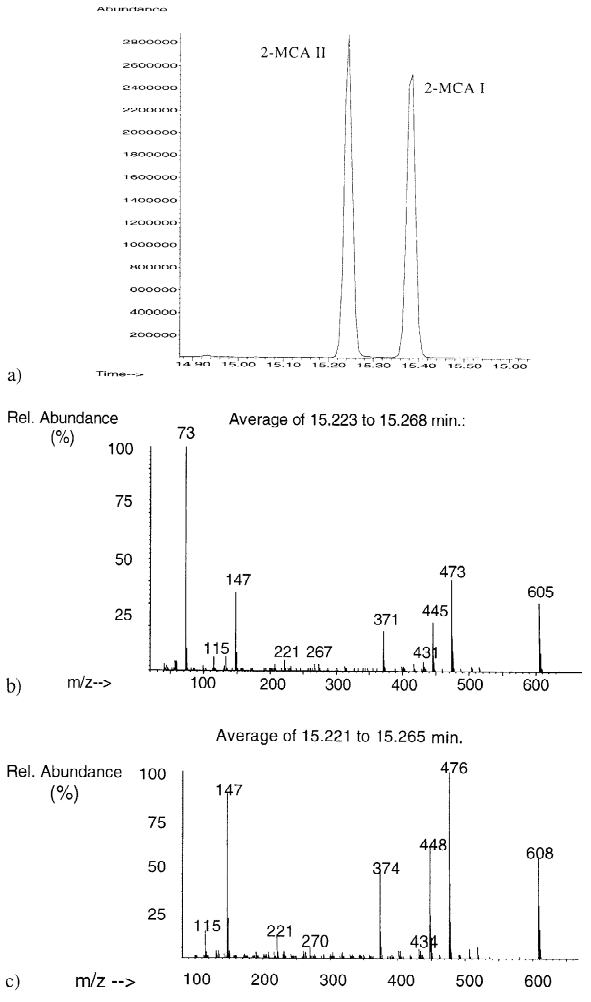

Fig. 3. Total ion chromatograms of the *tert.*-butyldimethylsilyl derivatization product of 2-methylcitric acid (2-MCA) I and II (a) and mass spectra of derivatized 2-methylcitric acid II (b) and 2-methylcitric acid-d_3 II (c). For further information concerning the differences in spectra see the Results and discussion section.

3.3. Diastereomer ratio and purity of 2-MCA-d_3

For the determination of diastereomers of 2-MCA, the ratio of diastereomers in the internal standard had to be determined: The proportion of diastereomers was 54.8±1.6% 2-MCA I-d_3 and 45.2±1.6% 2-MCA II-d_3 comprising a ratio of 2-MCA I-d_3/2-MCA II-d_3 of 1.21±0.05. During these experiments, constant peaks eluted earlier at 15.1 min and later at 15.8/15.9 min in relation to 2-MCA II-d_3 and 2-MCA I-d_3 were detected (Fig. 4). Since no information indicating the chemical purity of 2-MCA-d_3 was given by CDN Isotopes when the deuterated standard was bought in 1998, these peaks had to be classified as chemical impurities of the internal standard. Through spectra analysis, the peaks at 15.1 and 15.8/15.9 min were classified as the MBD-STFA-derivatization products of citric acid and of the two diastereomers of 2,2′-dimethylcitric acid-d_6 (Fig. 4). Impurities were quantified to 9.50±0.52% by calculating the impurities' share of the total area under the curve obtained with all the peaks of the internal standard as were 2-MCA II-d_3, 2-MCA I-d_3 and the peaks of the impurities. The chemical purity of the standard had to be corrected to 90.50±0.52%. Since no additional standards were used for quantifying these substances, the correction we made was just an estimation due to possibly differing response factors. However, the constant detection of the impurities as well as their estimated amount, left no choice in using a standard concentration corrected for chemical impurities. The estimation needed to be checked by the recovery tests.

Since the proportion of deuterated 2-MCA was 99.9% according to product information of CDN Isotopes, no further correction of the purity was done. The internal standard concentration given in the methods section represents the amount after correction for impurities.

These data provide detailed information on how to establish a quantitative GC–MS assay for 2-MCA including its diastereomers which considers also impurities of the internal standard as a basis for good precision and recovery.

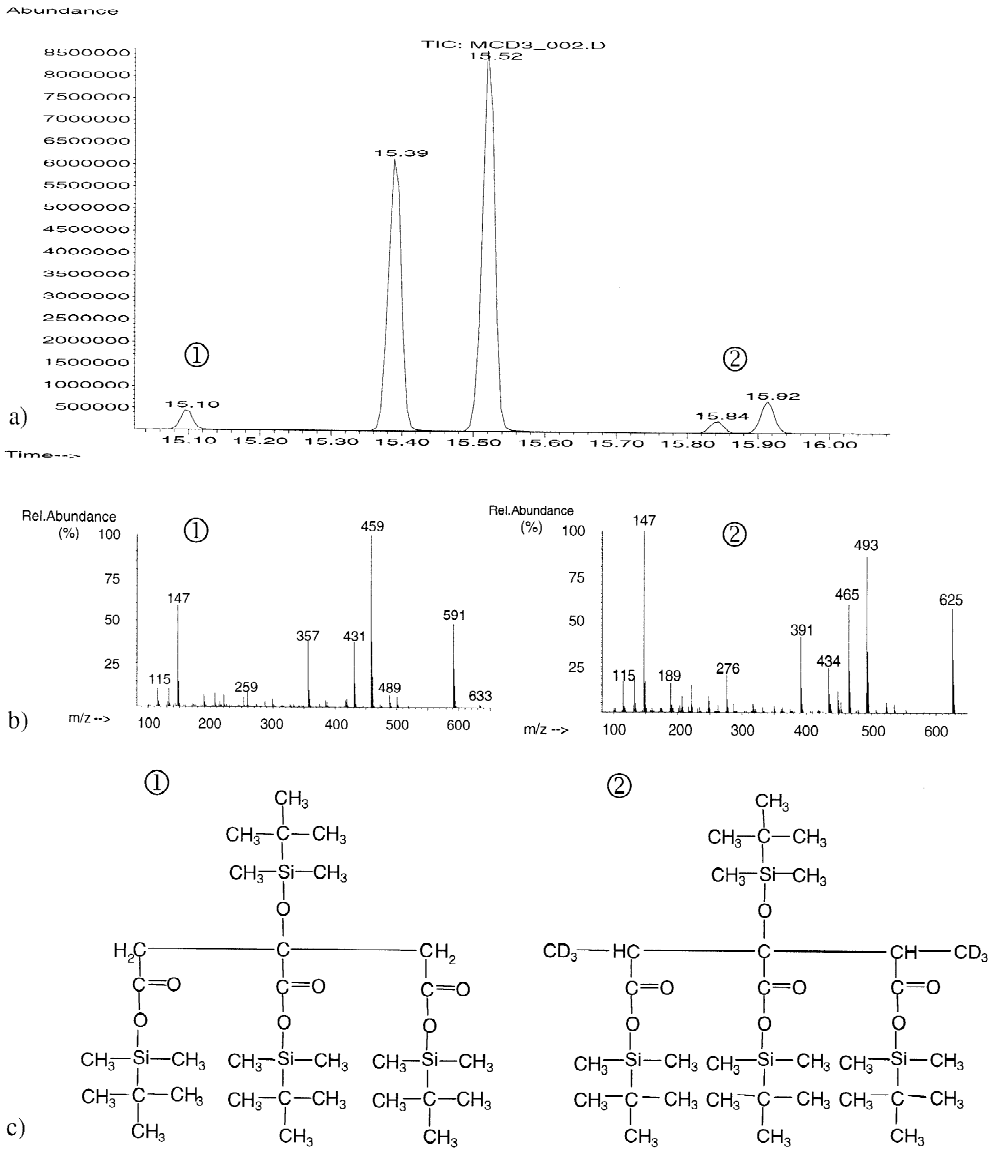

Fig. 4. Total ion chromatogram (a), mass spectra (b) and formula (c) of the *tert*.-butyldimethylsilyl derivatives of the impurities citric acid 1 and 2,2′-dimethylcitric acid-d_6 2 of the internal standard.

3.4. Diastereomer ratio and purity of 2-MCA

For recovery testing, the serum had to be spiked up with pure 2-MCA of a known diastereomer ratio and purity. Therefore, 2-MCA was tested in the same manner as 2-MCA-d_3 did, despite its chemical purity of 95% given by CDN Isotopes. The purity of 2-MCA was corrected to 94±0.14%. The difference of only 1% between our results and the amount of impurity given by CDN, supported our estimation regarding the impurities of 2-MCA-d_3.

The proportion of diastereomers was

51.90±0.27% 2-MCA I and 48.10±0.27% 2-MCA II comprising a ratio of 2-MCA I/2-MCA II of 1.08±0.01.

3.5. Recoveries and intra-series precision

Apart from data concerning recovery rates for 2-MCA in human serum [4] and precision parameters of a stable isotope dilution assay of 2-MCA in human amniotic fluid [2], the precision of the assay in human serum remains unclear. We believe that the following data could be of relevance to estimate the usefulness of a quantitative GC–MS assay for 2-MCA II and 2-MCA I in serum as well as in amniotic fluid.

For recovery testing, a pooled serum sample containing 243 nmol/l 2-MCA_{total} with a diastereomer ratio of 1.13 (2-MCA I 129 nmol/l/2-MCA II 114 nmol/l) was spiked with 86, 216 and 431 pmol/l of pure 2-MCA with the purity and diastereomer ratio given above to concentrations of 446, 750 and 1256 nmol/l. Three different sample preparations of each concentration were each measured 10 times; recoveries of 98.5, 93.7 and 88% in concentrations of 446, 750 and 1256 nmol/l 2-MCA_{total} with a mean RSD of 1.5% (determined including the RSD of baseline concentration) were obtained (Table 1). For the corresponding 2-MCA I levels of 234, 392 and 655 nmol/l, recoveries were 98.4, 94 and 88.6% with a mean RSD of 1.6%. The recoveries for the related 2-MCA II levels were 98.6, 93.3 and 87.4%, obtained with a mean RSD of 1.7%. These intra-assay parameters of lower than 2% are comparable with the findings by Kretschmer and Bachmann in amniotic fluid [2]. The mean recovery of 98.5% for 2-MCA I and II levels determined in the lowest spiking level was satisfactory with regard to the estimated impurity of the standard. In comparison to our data, Allen et al. presented nearly constant recovery rates of 105, 102 and 103% for 2-MCA_{total} in concentrations of 125, 250 and 500 nmol/l in serum, whereas the decreasing recovery rates found in our assay are comparable to previous findings in amniotic fluid [2]. The decrease of the recovery rate in higher concentrations could be interpreted in part due to a relative overload of the gas chromatographic column. By determining the levels of 2-MCA in 52 hemodialysis patients [median age 53 (21–64) years] (data not shown), a maximum 2-MCA_{total} level of 1994 nmol/l was detected while keeping an excellent peak configuration, indicating that the decrease of recovery is not a serious disadvantage [13].

3.6. Inter-assay precision (day-to-day)

The day-to-day precision was 10% RSD (SD 40 nmol/l) for 2-MCA_{total} obtained with a pooled serum sample at a concentration of 401 nmol/l 2-MCA_{total} over a period of 5 months ($n=17$). 2-MCA I at a corresponding concentration of 212 nmol/l was determined with an RSD of 7.5%, 2-MCA II with an RSD of 14.3% at 189 nmol/l.

The day-to-day precision of our assay is higher compared to an RSD of 3.9% found in amniotic fluid

Table 1
Intra-assay precision parameters (RSD, SD) and recovery for 2-methylcitric acid total determined in human serum

Spiking level[a] (nmol/l)	Mean[b] (nmol/l)	Recovery (%)	SD (nmol/l)	Mean±2SD (nmol/l)	RSD (%)	2-MCA I/ 2-MCA II[a]	2-MCA I/ 2-MCA II[b]
0[c]	243	–	3.1	237–249	1.3	0	1.13
446[c]	439	98.5	9.8	419–459	2.2	1.11	1.11
750[c]	702	93.7	12.3	677–727	1.8	1.10	1.10
1256[c]	1106	88	7.3	1091–1121	0.7	1.09	1.10

[a] Calculated concentrations and diastereomer ratios according to baseline level after spiking up with defined amounts of pure 2-MCA as described in the Results and discussion section (0=baseline).

[b] Mean concentrations and diastereomer ratios determined.

[c] Means and precision parameters given per line are mean values resulting from three sample preparations, each measured 10 times (30 measurements total) comprising the intra-assay (intra-series) precision.

[2]. This could be related to the different properties of the material as well as the use of various chromatographic columns and the higher number of measurements within a longer observation time used for the determination of day-to-day RSD in this study.

3.7. Limit of detection

By using the parameters mentioned above, a normal serum sample containing only 45 nmol/l 2-MCA II and 39 nmol/l 2-MCA I, showed excellent peak configuration. The three times overnoise detection limit was found to be 10 nmol/l for 2-MCA_{total}.

3.8. Normal subjects

2-MCA was detected in the serum of 52 healthy volunteers as described in the methods. The distribution of age was considered to be normal ($P=0.2$ according to Lilliefors test).

The normal range for 2-MCA_{total} in human serum was calculated as 81–266 nmol/l. It is given together with those for 2-MCA I and 2-MCA II in Table 2. The normal ranges for 2-MCA_{total} as well as 2-MCA I and 2-MCA II confirm previous findings by Allen et al. [4]. The corresponding levels in both studies can be estimated as an additional marker for the precision of the assay. To our knowledge, no further consistent information concerning normal values of 2-MCA in human serum have been published yet.

As expected, 2-MCA_{total} showed a strong positive correlation with 2-MCA I ($r=0.871$, $P<0.001$) and 2-MCA II ($r=0.924$, $P<0.001$). 2-MCA I correlated with 2-MCA II ($r=0.666$, $P<0.001$). Only 2-MCA II showed a weak positive correlation with age ($r=0.289$, $P=0.038$).

Male controls revealed higher 2-MCA I levels compared to females (70±18 nmol/l in male vs. 59±12 nmol/l in female, $P=0.042$). This is in contrast to the findings of Allen et al. who found no significant differences between men and women [4]. The correlation of 2-MCA II but not 2-MCA I levels with age, as well as the sex related difference found only for 2-MCA I levels, remains unclear.

In conclusion, the GC–MS assay using a pulsed splitless injection technique is suitable for the rapid determination of 2-MCA levels in serum as well as in urine, cerebrospinal fluid and amniotic fluid. If the diastereomer ratio and the chemical purity of the internal standard 2-MCA-d_3 have been determined, the method provides good precision and recovery for the determination of 2-MCA I and II levels in serum. The clinical impact of routinely determining 2-MCA I and II levels in serum as well as in urine or amniotic fluid, for the diagnosis of organic acidurias as well as for deficiencies of cobalamin and biotin, needs further investigation.

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Table 2
Reference ranges for 2-MCA_{total}, 2-MCA I and 2-MCA II, calculated from 52 healthy volunteers (31 female, 21 male), mean age 41.7±14.4 years

	Mean (SD)	Median	Minimum	Maximum	95% percentile-interval (central)	Mean±2SD after log-transformation
2-MCA_{total}(nmol/l)	151 (34)	149	84	258	87–243	81–266
2-MCA I (nmol/l)	63 (15)	61	37	107	38–103	38–99
2-MCA II (nmol/l)	87 (22)	84	45	151	47–149	51–140
2-MCA I/2-MCA II	0.75 (0.15)	0.74	0.42	1.30	0.46–1.24	0.49–1.08

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