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Note

Sensitive and specific gas chromatographic method for the determination of methylmalonic acid in the plasma and urine of ruminants

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Methylmalonic acid (MMA) is an intermediate in the metabolic conversion of propionic acid to succinate, an essential step in the carbohydrate metabolism of ruminants. Reduced tissue levels of the vitamin B_{12}-dependent enzyme methylmalonyl CoA mutase (EC 5.4.99.2) result in elevated levels of MMA in plasma or urine. There is consequently considerable interest in the measurement of both plasma and urine MMA as a diagnostic tool for cobalt deficiency in ruminants and a need for a practical, specific and reproducible method.

Published methods [1, 2] for determining MMA in ruminant urine have proved unsatisfactory. These are susceptible to interference by other compounds [3] and lack sensitivity. Other methods have been published for determining MMA in tissues and body fluids using various analytical techniques. Colorimetry subsequent to an ion-exchange chromatographic clean-up step has been developed [4], refined [5] and semi-automated [6, 7]. A paper chromatographic method [8] and a sensitive thin-layer chromatographic (TLC) technique [9] with in situ visualization of MMA have been developed for screening human urine and plasma samples. A variety of gas chromatographic (GC) methods using either the methyl [10, 11], butyl [1, 12], cyclohexyl [13] or silyl [14] ester derivatives have been used. For screening of human amniotic fluid, an ultra-sensitive specific gas chromatographic—mass

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spectrometric (GC—MS) method using stable isotope dilution has been described [15].

The method we have developed is convenient, sensitive, specific and reproducible. It uses an organic extraction step followed by gas—liquid chromatography to allow accurate quantitation of MMA in sheep urine or plasma. Ethylmalonic acid is used as an internal standard. The specificity of the assay has been checked using GC—MS together with ion ratio methods.

EXPERIMENTAL

Apparatus

We used a Model 5880 gas chromatograph with a level 4 integrator—thermal printer, flame-ionization detector and 7671A autosampler, all from Hewlett-Packard (Altringham, U.K.). An HP5995A bench-top gas chromatograph—quadrupole mass spectrometer was used with a Part No. 18964A open-split GC—MS interface. The system was operated via an HP9825B computer using the floppy disk-based computer software as supplied. Print-out was via an HP9876A matrix printer.

Chromatographic column

An Ultra 1 (option 012) methyl silicone bonded phase column (25 m × 0.3 mm I.D.) with a film thickness of 0.15 μm was obtained from Hewlett-Packard.

Ancillary equipment

Standard 3-ml Quickfit tubes with ground-glass stoppers were used. Special 1-ml glass tubes with ground-glass stoppers were prepared by Ulster Scientific (Carrickfergus, U.K.). A standard vortex mixer and a needle manifold for 36 tubes to allow evaporation under nitrogen were also used. Autosampler vials (Type 03VC) with crimp-on caps were obtained from Chromacol (London, U.K.).

Chemicals

Acetone, hexane, ethyl acetate, sulphuric acid, hydrochloric acid, butan-1-ol, acetyl chloride and sodium chloride were all AnalaR grade and obtained from BDH (Poole, U.K.). Ethylmalonic acid was obtained from Aldrich (Gillingham, U.K.). Methylmalonic acid was obtained from Sigma (Poole, U.K.).

Blood and urine

Whole blood was collected in heparinized collection tubes from sheep on local farms where a clinical trial into the effects of cobalt deficiency was being carried out. Plasma was separated by low-speed centrifugation within 4 h of collection. Urine (20 ml) was collected into 2 ml of 40% hydrochloric acid from the same animals. All samples were stored at - 20°C prior to analysis.

Reagents

Reagent A consisted of 0.5 *M* sulphuric acid saturated with sodium chloride. Reagent B: ethylmalonic acid (EMA), internal standard (1 mmol/l), was prepared by diluting a 10 mmol/l stock standard (1.32 g/l EMA in acetone)

with acetone. Both standards were stored at 4°C. Reagent C: methylmalonic acid standard (0.5 mmol/l) was prepared by diluting a 10 mmol/l stock standard (1.18 g MMA/l in acetone) with acetone. Both standards were stored at 4°C. Reagent D: butylating reagent was prepared fresh before use by adding 1 part of acetyl chloride slowly with mixing to 10 parts of butan-1-ol.

Method

Either 250 μl of plasma, 25 μl of urine or 50 μl of standard MMA (reagent C) were placed in a 3-ml glass quickfit tube. The urine sample and standard was diluted to 250 μl with water. To each were added 50 μl of EMA standard (reagent B) followed by 250 μl of acetone while vortexing. Reagent A (1.0 ml) was added followed by 0.5 ml of ethyl acetate. This mixture was vigorously shaken for 25 s and then centrifuged at 1200 *g* for 10 min. The ethyl acetate layer was transferred to a 1-ml glass tube. The ethyl acetate extraction was repeated, and the two extracts were combined. The ethyl acetate was removed from the extracts by evaporation under nitrogen at room temperature (20°C) using a multi-needle manifold. When dry, 50 μl of butylating reagent (reagent D) were added to the residue and the samples vortexed. The tubes were stoppered and heated on a water bath at 70°C for 20 min. After the tubes had cooled, 150 μl of hexane were added, followed by 400 μl of distilled water. The tubes were shaken and the layers allowed to separate. The upper hexane layers were then transferred to the autosampler vials which were capped and sealed.

Gas chromatography

The derivatized sample (10 μl) was injected onto the column from the autosampler. The capillary injector was used in the split mode to give an injection ratio of 10:1. The helium carrier gas pressure on the column was 0.85 bar, giving a flow-rate of 1.5 ml/min. The injector port and the flame-ionization detector temperatures were 200 and 280°C, respectively. The oven temperature programme used for chromatography was 120°C with an initial time of 2 min, followed by a temperature ramp of 5°C/min to 150°C. Post-run, the temperature was held at 280°C for 5 min to flush the column.

Calibration was carried out at the beginning of each analytical run by analysing a combined MMA and EMA standard solution that had been taken through the complete analytical procedure. Calibration was rechecked at the end of each sample batch.

Integration of the peak areas and calculation of results was carried out using the GC systems own integrator and report generator. The EMA internal standard was used to compensate for losses and variations accumulated during the analytical procedure. The final results were expressed at μmol/l MMA in plasma or urine.

Analytical recovery was also determined routinely by adding 20 μl of the 0.5 μmol/ml MMA standard (reagent C) to either a plasma or urine sample before the addition of the acetone.

Gas chromatography–mass spectrometry

The same procedures for sample preparation and derivatization were carried

out as already described for gas chromatography. Samples (5 μl) were injected manually. The capillary injection port was operated in the splitless mode at 200°C. Instrument conditions were as follows: oven injection temperature 70°C followed by a ramp rate of 17°C/min to 110°C, then 5°C/min to a final value of 160°C. The transfer line, source and analyser temperatures were 280, 200 and 150°C, respectively.

RESULTS

A typical gas chromatogram from two plasma samples A and B containing 7 and 73 μmol/l MMA, respectively, a urine sample containing 120 μmol/l

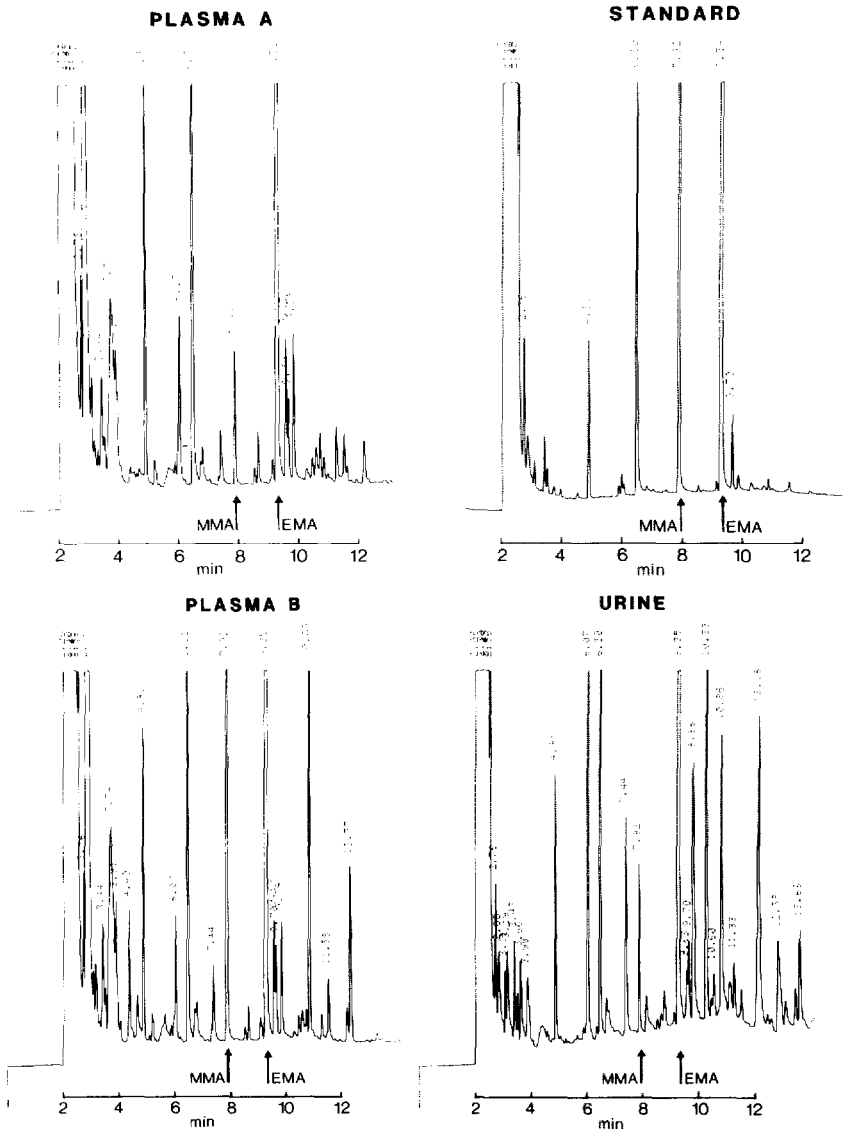

Fig. 1. Gas chromatogram showing the butyl derivatives of MMA and EMA in two plasma samples, a urine sample and a standard.

and a standard representing 100 μmol/l MMA in plasma or 1000 μmol/l MMA in urine is shown in Fig. 1. MMA elutes at 7.9 min and EMA at 9.3 min.

Analytical accuracy and precision were determined by dosing a plasma and urine sample containing low levels of MMA, with MMA so that the levels obtained were within the diagnostically significant range. The results are shown in Table I. Recoveries ranged from 86 to 101% and coefficients of variation ranged from 1.3 to 7.6%.

TABLE I

RECOVERY AND PRECISION OF MMA IN PLASMA AND URINE

$n = 5$.

MMA added (μmol/l)	MMA found (μmol/l)	MMA recovered (μmol/l)	Recovery (%)	S.D.	Coefficient of variation (%)
Plasma					
0	0.77	—	—	0.04	4.7
10	9.38	8.61	86	0.34	3.6
50	51.25	50.48	101	0.67	1.3
Urine					
0	19.2	—	—	1.46	7.6
100	114.5	95.3	95	1.73	1.5
500	506.1	486.9	97	12.8	2.5

Various other organic acids such as benzoic, oxalic, malonic, malic and succinic acids have been reported as causing possible interference in the determination of MMA by GC [3]. These were checked by carrying standard solutions of each through the procedure. The peaks obtained had different retention times to those of both MMA and EMA, showing that there was no interference from these compounds.

To further validate the method and check for interference from other compounds, 30 plasma and urine extracts were analysed by both GC and GC—MS. For GC—MS analyses, selected-ion monitoring (SIM) was used to measure the ions at 74, 119 and 175 a.m.u. for MMA and at 115 a.m.u. for EMA. The electron-impact (EI) mass spectra of the bis-dibutyl esters of MMA and EMA are shown in Fig. 2. Linear regression analyses of the combined data showed good agreement between both methods, where y (MMA in μmol/l by GC–MS) = $2.09 + 0.998x$ (MMA in μmol/l by GC). The correlation coefficient (r) was 0.998.

EMA is used as an internal standard in the assay since it is expected to have a similar chemical behaviour to MMA and enables quantitation to be carried out more accurately. However, the validity of this depends on there being little or no endogenous EMA present in the plasma or urine samples. To test this, 36 plasma and urine samples from grazing sheep, which had various levels of MMA, were carried through the procedure without adding EMA internal standard to the samples. For plasma, the EMA levels found ranged from 0.3 to 3.5 μmol/l, with a mean of 1.66 μmol/l. For urine, the EMA levels ranged from 4.3 to 19.0 μmol/l, with a mean of 10.9 μmol/l. In the assay, levels of EMA added as an internal standard to plasma and urine are equivalent to 200

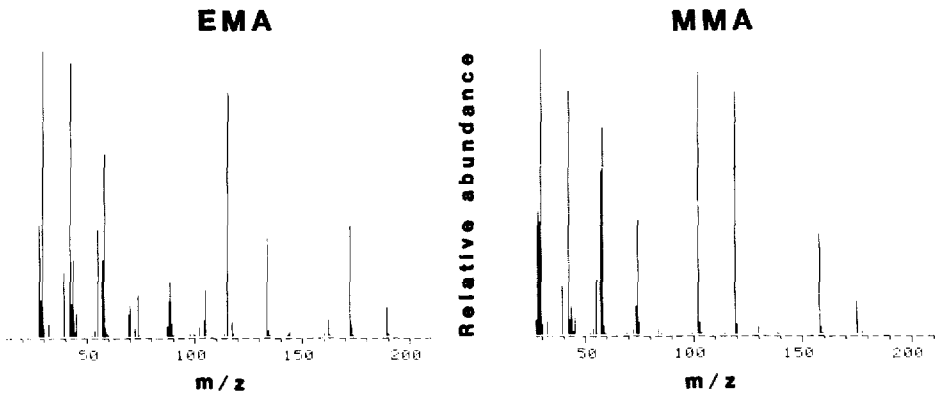

Fig. 2. Electron-impact mass spectra of the bis-dibutyl esters of MMA and EMA.

and 2000 μmol/l, respectively. For plasma, therefore, the errors arising from endogenous EMA present in these samples would lead to the MMA being underestimated by a mean value of 0.8% and a maximum value of 1.7%. In urine, the errors would be 0.5% mean and 0.9% maximum.

DISCUSSION

The method we have developed has established the practicality of using a direct capillary GC method for the determination of methylmalonic acid in either urine or plasma from sheep. It had already been shown that the colorimetric methods were unsatisfactory for the determination of MMA concentrations in human serum and urine. A six-fold reduction in the apparent MMA concentration was observed [10] when the colorimetric method was compared with a GC procedure.

We therefore considered that a procedure based on GC would offer the best possible opportunity for developing a method which had the qualities required for clinical diagnostic work and also had the potential throughput to accommodate both clinical and experimental material. In examining other published methods, we first of all tried the original GC method of Millar and Lorentz [1], which uses a boron trifluoride—butanol mixture for derivatization. This method proved to be unsatisfactory on a number of counts. First of all, the butanol derivative eluted on the shoulder of the broad solvent peak. We were unable to improve this by using a variety of capillary columns to modify the chromatographic resolution of the system. The fact that the MMA was not separated from the solvent peak meant that the assay was insensitive, a problem which had already been noted by others [10, 11].

This problem has been circumvented in the method described here by using a mixture of acetyl chloride and butanol to prepare the bis-butyl derivative. This gives a much cleaner chromatogram than boron trifluoride—butanol derivatives and allows MMA and EMA to be well separated from interfering peaks. This, together with the use of capillary columns rather than packed columns, has considerably improved the sensitivity and specificity of the assay. The specificity has been confirmed by the close correlation obtained in the comparison between GC and GC—MS. In addition, we have shown that oxalate and benzoate do not interfere as they did in another GC assay [3].

The internal standard used in an assay should preferably be a compound similar in all respects to the substance being measured, and one which is not present in the original sample. In our assay, we tried a number of compounds such as succinic and phenylmalonic acid as suitable internal standards but rejected them since they did not behave similarly to MMA. EMA was found to be most suitable, and although small amounts of endogenous EMA are found in plasma and urine, these are extremely small compared to the amounts added.

Losses of MMA can occur during evaporation of the ethyl acetate extracts under nitrogen; originally, this was carried out at 60°C for speed but it was found that up to 30% MMA was lost at this temperature. Although this loss was compensated for by an equal loss of EMA in the assay, it was considered better to carry out this step at room temperature (20°C), where less than 4% of MMA is lost; again, this is compensated for by equal losses of EMA.

Precipitation and removal of plasma proteins prior to extraction was not found to be necessary in our assay. Plasma proteins are precipitated upon addition of acetone and since these were found not to interfere with subsequent extraction steps they were not removed separately.

The detection limits obtained from our assay are 0.5 μmol/l for plasma and 5 μmol/l for urine. This is at least ten times more sensitive than colorimetric or other GC methods previously described. This degree of sensitivity allows accurate measurement of the levels of MMA found in both clinically normal (cobalt sufficient) and abnormal (cobalt deficient) sheep.

The assay has been in use in this laboratory for about two years and has proved itself to be both convenient (about 40 samples per day), accurate and reproducible.

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