Journal of Chromatography, 566 (1991) 215–222
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5797

Short Communication

Analysis of serum methylmalonic acid for the determination of cobalt deficiency in cattle

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(First received September 26th, 1990; revised manuscript received December 29th, 1990)

ABSTRACT

An improved method has been developed and validated for the determination of methylmalonic acid (MMA) in serum to determine cobalt deficiency in cattle. Serum samples were extracted with ethyl acetate and derivatised to form the propyl esters using 14% boron trifluoride–propanol as derivatising reagent. Derivatised samples were analysed by capillary gas chromatography using split injection, a DB-17 30 m × 0.25 mm I.D. capillary column and flame ionisation detection. The detection limit for the method was 0.5 μmol/l and precision, determined by replicate analyses of spiked serum samples, was less than 2% relative standard deviation. When cobalt deficiency is defined as a MMA serum concentration of more than 2 μmol/l, the method was able to detect clinical deficiency of cobalt in animals with symptoms such as "coasty" coats and low weight gain.

INTRODUCTION

In ruminant animals volatile fatty acids (VFAs) are produced by the digestion of forage and are the most important source of energy. Propionic acid is metabolised via methylmalonic acid (MMA) to succinic acid and then ultimately to glucose. The conversion of MMA to succinic acid is catalysed by the enzyme methylmalonyl-coenzyme-A-isomerase which requires vitamin B_{12} as a cofactor. Vitamin B_{12} is synthesized by microbes in the rumen and cobalt is required for its production. When cobalt supply is limiting, vitamin B_{12} production is reduced [1]. Although cattle can store relatively large amounts of vitamin B_{12} in the liver [2], long-term shortage of cobalt leads to a reduction of the methylmalonyl-coenzyme-A-isomerase activity and therefore, a reduction in the conversion rate of MMA to succinic acid, an increase in serum MMA concentration and an overall reduced conversion rate of propionic acid to glucose.

Cobalt status in sheep is normally assessed by analysis of serum vitamin B_{12} concentrations [3–6] for which kits are commercially available. However, in the bovine rumen, inactive analogues of vitamin B_{12} are also produced [7]. These

0378-4347/91/$03.50

analogues interfere with vitamin B_{12} analysis; and hence serum vitamin B_{12} concentrations in cattle are not indicative of the functional cobalt status in cattle. Alternative methods for determining cobalt status are analysis of urine for formimino-glutamic acid [8,9] and MMA [3,10,11]. Low concentrations of formimino-glutamic acid have been reported to indicate low vitamin B_{12} concentrations and hence cobalt deficiency in sheep [8]; however Stebbings and Lewis [9] concluded that formimino-glutamic acid concentrations were not a reliable indicator of cobalt deficiency. A MMA serum assay using capillary gas chromatography (GC) has been reported [12] for determining cobalt deficiency in sheep. This method has been used by a number of authors; however, there is only one report of this method being applied to cattle [13], but without evidence of method validation. Paterson and McPherson [13] concluded that bovine serum MMA concentrations of greater than 2 μmol/l indicate cobalt deficiency. This report describes several modifications to the GC method [12] and its application and validation for the clinical determination of cobalt status in bovine samples.

EXPERIMENTAL

Chemicals

Acetone, ethyl acetate, butanol and *n*-hexanes were all HPLC grade obtained from Waters Millipore (Sydney, Australia). The organic acid standards methylmalonic, ethylmalonic, malonic, oxalic, benzoic, phenylacetic, succinic, hydrocinnamic, glutaric and adipic acids were all obtained from Sigma (St. Louis, MO, U.S.A.). The derivatising reagent, 14% BF_3 in propanol, was obtained from Applied Science (State College, PA, U.S.A.), acetyl chloride from BDH (Poole, U.K.) and sodium chloride from BDH (Melbourne, Australia).

Sample preparation

A 500-μl sample of either serum or plasma was pippetted into a glass test tube and 100 μl of the internal standard [1000 μmol/l ethylmalonic acid (EMA) in ethyl acetate] were added. Serum proteins were precipitated by adding 0.5 ml of acetone while vortex-mixing. Ethyl acetate (1 ml) was added followed by 2 ml of 0.5 mol/l H_2SO_4 in saturated NaCl and the mixture was vortex-mixed. Following centrifugation the upper ethyl acetate layer was transferred to a screw-capped test tube using a glass pasteur pippette. A further 1 ml of ethyl acetate was added to the sample and the extraction repeated. Both ethyl acetate fractions were collected into the same test tube.

Derivatisation

To prepare MMA and EMA propyl esters the ethyl acetate was first evaporated using a stream of nitrogen at room temperature; then 200 μl of 14% BF_3–propanol were added to the test tubes which were then tightly capped and heated at 95°C for 60 min in a dry block heater. When the test tubes had cooled, 100 μl of

hexane and 1 ml of distilled water were added and the test tube contents mixed well. When the layers had separated approximately 60–80 μl of the upper hexane layer was transferred to an autosampler vial to which approximately 100 mg of anhydrous Na_2SO_4 had already been added.

Gas chromatography–flame ionisation detection (GC–FID)

A Packard Model 427 gas chromatograph equippped with a split capillary injector was used for all analyses. The capillary columns used for the separation were either a J & W DB-17 (30 m × 0.25 mm I.D.) or an Alltech Econocap SE-54 (30 m × 0.25 mm I.D.) both with a linear nitrogen gas velocity of 25 cm/s. The injector split ratio was 1:20. The oven temperature programme was as follows: the oven temperature was initially held at 100°C for 1 min and then increased at 10°C/min to 280°C and held for 20 min before the oven temperature was returned to 100°C. Injections of 3 μl for both standards and samples were made, with the mixture containing the standards injected every five to six samples.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were carried out on a Hewlett Packard Model 5970B MSD instrument. A DB-5 30 m × 0.25 mm I.D. column was used for the separation. The ion source conditions were: temperature 200°C, electron energy 70 eV, and beam current 300 μA. The transfer line between the gas chromatograph and mass spectrometer was held at 280°C. The GC–MS instrument was operated in either full-scanning mode (range m/z 500–50) or selected-ion monitoring (SIM) mode; mass m/z 143 was monitored for MMA and mass m/z 157 for EMA propyl esters.

RESULTS AND DISCUSSION

The extraction method used was essentially the standard ethyl acetate extraction method used by McMurray *et al.* [12] and others [14,15]. This analytical approach utilises a relatively non-specific extraction method with minimal clean-up and hence the specificity of the method is almost entirely dependent on the separating power of the capillary column used. The retention data of organic acid derivatives on several different columns have been reported [16] and indicate the possibility that other endogenous organic acids may interfere with the measurement of MMA and EMA propyl esters. Table I compares retention data obtained for a number of organic acids on two different capillary columns for both propyl and butyl esters. Initially we had intended to use a non-polar column as recommended by McMurray *et al.* [12], however, as shown in the first column of Table I, the propyl esters of MMA and benzoic acid were not separated on the SE-54 column. Also separation of these two compounds could not be obtained on other similar non-polar columns such as DB-5 or BP-1. The best separation of organic acid propyl esters was obtained on a DB-17 column (Table I). Thus this column was used for all further assays. The optimisation of the benzoic acid and MMA

TABLE I

RELATIVE RETENTION DATA OF PROPYL AND BUTYL ORGANIC ACID ESTERS ON DB-17 AND SE-54 CAPILLARY COLUMNS

Organic acid	Propyl ester		Butyl ester	
	DB-17[a]	SE-54[b]	DB-17[a]	SE-54[b]
Oxalic	0.755	0.678	0.837	0.780
Malonic	0.881	0.826	0.926	0.887
Methylmalonic	0.895	0.870	0.880	0.896
Benzoic	0.941	0.870	0.927	0.909
Ethylmalonic	1.000	1.000	1.000	1.000
Succinic	1.057	1.033	1.058	1.034
Phenylacetic	1.076	0.990	0.974	1.043
Glutaric	1.204	1.204	1.162	1.166
Hydrocinnamic	1.228	1.183	1.089	1.291
Adipic	1.361	1.388	1.273	1.421

[a] DB-17 = J & W DB-17 column (30 m × 0.25 mm I.D.).
[b] SE-54 = Alltech Econocap SE-54 column (30 m × 0.25 mm I.D.).

separation proved to be a crucial step in the method development as many bovine serum samples were subsequently found to contain benzoic acid which would have produced false MMA results.

Although a variety of reagents have been reported for the preparation of organic acid esters for use in analytical methods [12,16,17] 14% BF_3–propanol was chosen to prepare propyl esters. Even though McMurray *et al.* [12] prepared butyl esters, preparation of propyl esters is advantageous because the derivatives are more volatile and therefore the compounds of interest have shorter retention times. More importantly the retention times of coextracting compounds which elute with long retention time can be kept to a minimum allowing for faster analysis times. The 14% BF_3–propanol reagent is also convenient to use in a routine laboratory as it has a simple derivatisation procedure, a long shelf life and is relatively inexpensive.

A further consequence of the non-specific extraction method was the presence of relatively large amounts of coextractives. On average approximately 1200 μg of ethyl acetate-soluble material was extracted per 0.5 ml of serum. Most of this material is dissolved in the final 100 μl of hexane. When full GC–FID chromatograms were recorded at high column temperatures many large peaks were found, which indicated the presence of a considerable amount of high-boiling-point coextractives. To allow these later-eluting compounds to elute it was necessary to hold the column at a high temperature (280°C) for 20 min between samples which resulted in an analysis time of 40 min per injection. Large amounts of coextractives often contaminate capillary columns reducing performance espe-

cially when "cold on-column" and split–splitless injection techniques are used. After the analysis of approximately 200 samples with split injection, column performance, with respect to increased peak widths, had degraded slightly, however, the original performance was restored by removing 15 cm from the head of the column. As the column contained an immobilized stationary phase, washing the column with solvent would probably have also restored column performance.

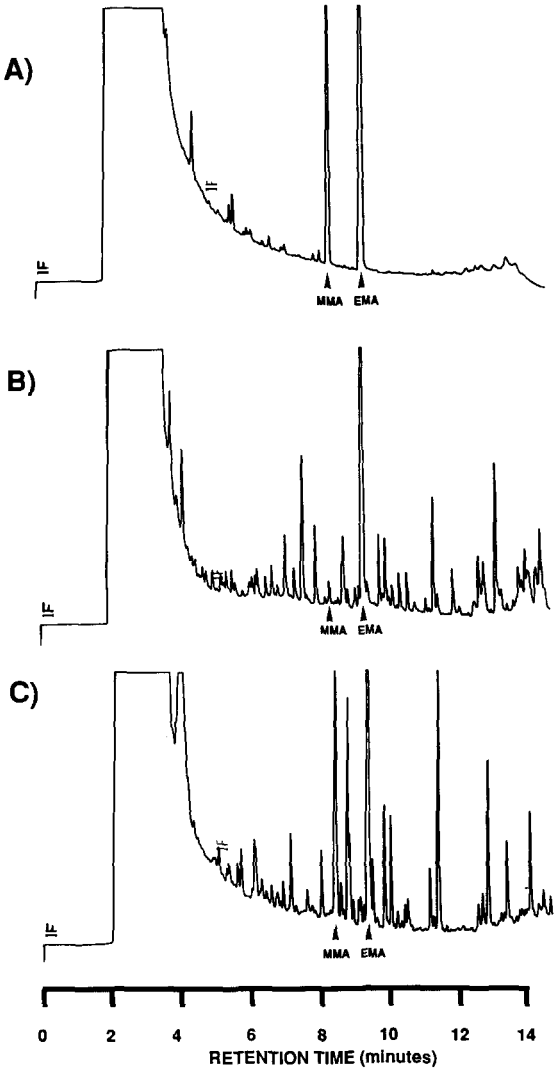

Fig. 1. Typical GC–FID chromatograms obtained for (A) standards (40 μmol/l MMA, 200 μmol/l EMA), (B) cattle serum sample, normal MMA concentration (1.2 μmol/l) and (C) cattle serum sample, elevated MMA concentration (47 μmol/l).

Typical chromatograms obtained from the analysis of bovine serum are shown in Fig. 1. The propyl ester derivatives of MMA and EMA elute at 8.1 and 9.1 min, respectively, and although the chromatograms were relatively complicated there was no obvious signs of interference for either compound. The detection limit of the method was calculated at 0.5 μmol/l (three times signal-to-noise ratio). This detection limit was adequate as Paterson and McPherson [13] have shown that cobalt deficiency is indicated by serum MMA concentrations of greater than 2 μmol/l in cattle. Table II shows data obtained from recovery and reproducibility experiments. The analytical procedure resulted in satisfactory recovery for both MMA and EMA; good between-sample precision was obtained through use of the internal standard.

To verify the validity of results obtained using this method, six samples found to have a range of MMA concentrations by GC–FID, were also analysed by GC–MS using SIM. Table III shows the results obtained and the statistical comparision. The correlation coefficient of 0.979 indicates a strong linear relationship between the GC–MS and GC–FID results. The intercept value of 0.416 and slope of 0.997 shows there was little systematic bias between the two methods of analysis. These results show that analysis by GC–FID gives an accurate quantitation of MMA concentration in bovine serum samples. Full-spectrum GC–MS analyses was also carried out on several samples to identify major peaks in the GC–FID chromatograms. Compounds identified by GC–MS included benzoic, phenylacetic, succinic and hydrocinnamic acids. These identifications were also confirmed by comparision of retention times with organic acid standards. As high concentrations of benzoic acid were found in some bovine serum samples investigations are underway to determine the suitability and clinical relevance of using this method for the analysis of a wide range of metabolic organic acids in bovine serum.

Serum samples were obtained from cattle which were showing typical symptoms of cobalt deficiency, namely lowered weight gains and pale, dry ("coasty") coats. The property on which these animals were grazing also had a history of cobalt deficiency in sheep. Analysis for MMA and vitamin B_{12} revealed that all

TABLE II

REPRODUCIBILITY AND RECOVERY DATA OBTAINED FROM REPLICATE ANALYSES OF BOVINE SERUM SAMPLES SPIKED AND UNSPIKED WITH MMA (n = 3)

MMA added (μmol/l)	MMA found (μmol/l)	Recovery (%)	S.D. (MMA found)	Relative standard deviation (%)
0	5.95	–	0.08	1.3
10	13.9	79.5	0.226	1.6
20	25.3	96.8	0.331	1.3

TABLE III

COMPARISON OF MMA RESULTS OBTAINED FROM ANALYSIS BY GC-FID AND GC–MS

GC–FID (μmol/l)	GC–MS (μmol/l)
49.4	56.4
20.1	17.2
96.6	94.6
42.9	43.9
67.0	63.0
31.8	31.0

Correlation (r^2) = 0.979.
Regression: slope = 0.997; intercept = 0.416.

animals had low vitamin B_{12} concentrations (range 4–48 pmol/l, normal >100 pmol/l) and elevated MMA concentrations (range 20.1–96.6 mmol/l, normal <2 μmol/l). These results are in agreement with previous reports which found that in sheep elevated MMA concentrations in serum indicate cobalt deficiency [10,11].

CONCLUSIONS

It can be concluded that this improved method is specific and suitable for the routine determination of MMA concentrations in cattle. Use of 14% BF_3–propanol as derivatising reagent is economical, convenient and allowed the assay to be used in a diagnostic laboratory. Further work is required to accurately determine the relationship between serum MMA concentrations and production and weight gain indices in cattle. The availibility of a validated routine MMA assay in bovine serum will facilitate this.

ACKNOWLEDGEMENTS

The author is grateful to Ms. Donna Waldron who provided diligent technical assistance and to Dr. David Allen and Ms. Karina Watkins of the Victorian State Chemistry Laboratory for providing the GC–MS analysis.

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