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Note

A gas chromatographic method for the determination of methylmalonic acid in urine

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Increased levels of methylmalonic acid (MMA) in the urine have been used as an indication of vitamin B_{12} deficiency in man[1–3] and sheep[4,5].

Paper chromatography[2] and thin-layer chromatography (TLC)[6,7] have been used to screen urine samples for MMA, but, as these methods are only semi-quantitative, they are unsuitable for detecting small changes in MMA concentration. Although the colorimetric method of Giorgio and Plaut[8] appeared initially to be suitable, some human (and most sheep) urines contain substances other than MMA that react with the diazonium reagent, producing a brown interfering colour that masks the green MMA complex. This interference was overcome in the analysis of MMA in human urine by the use of a three-wavelength correction[9]. The brown colour produced with sheep urine has been shown by TLC to be due to a large number of unidentified compounds, and, in most samples, the absorbance is too great to allow the wavelength-correction method to be used[10]. These compounds may be removed by a second ion-exchange step[11] or by the inclusion of a Sephadex gel filtration step[12]; however, these techniques are too slow and complex for routine use. Two-stage methods involving either ion-exchange chromatography or TLC followed by gas chromatography[1,13] are not suitable for the analysis of a large number of samples, and the over-all recoveries of MMA are low.

This paper describes an improved method based on gas–liquid chromatography (GLC) of the butyl ester of MMA, which is produced by reacting a diethyl ether extract of urine or freeze-dried urine with a mixture of boron trifluoride and butanol.

EXPERIMENTAL

Standard and reagents

Methylmalonic acid was obtained from J. T. Baker Chemicals (Phillipsburg, N.J., U.S.A.). 2-[^{14}C]Methylmalonic acid (New England Nuclear, Boston, Mass., U.S.A.) was found by TLC[14] to be at least 98% pure. The following analytical-grade reagents were used: diethyl ether (BDH, Poole, Great Britain), 14% (w/v) boron trifluoride in butanol (Applied Science Labs., State College, Pa., U.S.A.), sodium chloride (BDH), sulphuric acid (BDH), methyl palmitate (Sigma, St. Louis, Mo., U.S.A.). Commercial-grade *n*-hexane was distilled before use.

Apparatus

For GLC, a Varian Aerograph 1740 gas chromatograph (Palo Alto, Calif., U.S.A) equipped with a hydrogen flame ionization detector was used. The glass column (1.83 m × 2.5 mm I.D.) was packed with Chromosorb W AW DMCS (60–80 mesh) coated with 10% of EGSS-X (Varian Aerograph) and conditioned at 210° for 24 h with a flow-rate of nitrogen of 25 ml/min. The operating conditions were: column temperature, 155°; injector temperature, 205°; detector temperature, 250°; carrier gas (nitrogen) flow-rate, 25 ml/min; hydrogen flow-rate, 25 ml/min; air flow-rate, 300 ml/min.

Samples were dissolved in toluene–Triton X-100 phosphor (1:1)[15] and counted in a Packard Tri-Carb liquid scintillation spectrometer equipped with external standardization.

Determination of standard graphs and quantitative analysis of the butyl ester of MMA

MMA was dissolved in ethanol (1 mg/ml), and different volumes of this solution covering the range 5–2000 μg of MMA were evaporated to dryness at 45° in a gentle stream of nitrogen. To the residue was added 1 ml of boron trifluoride–butanol; the reaction vessel was tightly capped and placed in a boiling-water bath for 2 min. After cooling, 5 ml of water were added and the butyl ester was extracted into 2 ml of *n*-hexane by gentle shaking for 1 min. A 2-ml aliquot of the upper organic phase was removed, 100 μl of a *n*-hexane solution of methyl palmitate (0.5 mg/ml) were added as an internal standard, and 1 μl of the mixture was injected on to the column. The corrected peak heights were measured at different instrument attenuations.

When 2 μg (0.17 μCi) of 2-[^{14}C]methylmalonic acid were taken through this procedure, the recovery into the *n*-hexane layer was quantitative.

Analysis of MMA in urine

Urine was stored at −15°. To 1-ml samples of urine in 15-ml glass-stoppered test tubes were added 1.5 ml of 0.1 *M* sulphuric acid, 0.1 ml of aqueous 2-[^{14}C]MMA (1.9 μg, 0.17 μCi) and 2 g of sodium chloride. Each mixture was extracted three times with 5-ml volumes of diethyl ether, and the combined extracts were evaporated to dryness at 45° with nitrogen. As an alternative to diethyl ether extraction, 1-ml samples of urine were freeze-dried after the addition of labelled MMA.

In both instances, the residue was caused to react with boron trifluoride–butanol and processed as described above. A 0.1-ml volume of the *n*-hexane layer was counted, and recovery corrections were applied. Volumes of 2 and 3 ml of urine were also analysed by diethyl ether extraction.

The reproducibility of the method was assessed by analysing a sheep urine sample five times. The recovery of MMA from urine was measured by adding 30, 60, 90 and 120 μg of MMA to 1-ml aliquots of sheep urine. The diethyl ether extraction procedure was used for these experiments and was also applied to the analysis of MMA in samples of human urine, and urine from cobalt-deficient and cobalt-supplemented sheep.

RESULTS

In Fig. 1 are shown typical chromatograms obtained with an MMA standard, diethyl ether extracts of human and sheep urine and freeze-dried sheep urine. The *n*-

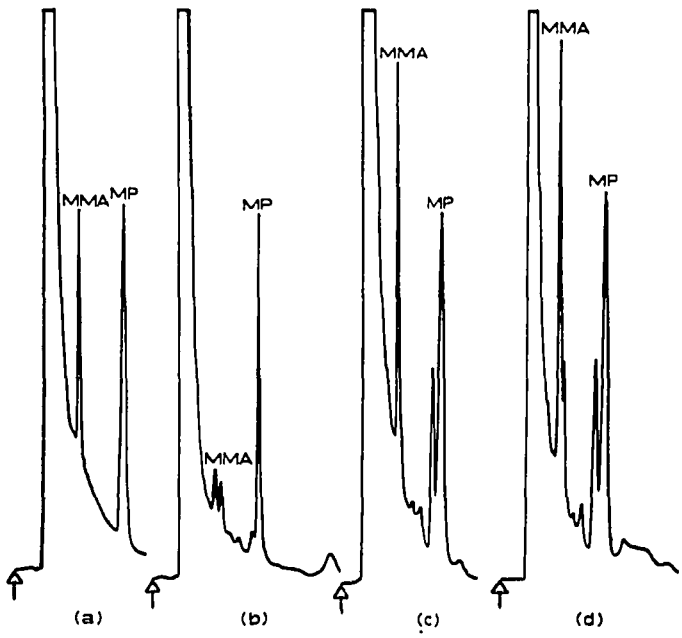

Fig. 1. Gas chromatograms of 1 μl of the *n*-hexane extract following the reaction of boron trifluoride–butanol with (a) 200 μg of MMA, (b) diethyl ether extract of 1 ml of human urine, (c) diethyl ether extract of 1 ml of sheep urine, and (d) 1 ml of freeze-dried sheep urine. Methyl palmitate (MP) was the internal standard. Attenuation 16×10^{-11}.

hexane solution from the human sample was concentrated five times before injection. The butyl ester of MMA had a retention time (R_t) of 3 min 25 sec. The other major peak in sheep urine extracts is butyl succinate (R_t 7 min 17 sec). No interfering peaks in the region of butyl methylmalonate or methyl palmitate have been observed in over 50 samples examined by both methods. A calibration graph covering the normal range of MMA levels in urine (0–200 μg) is illustrated in Fig. 2 (instrument attenuation 16×10^{-11}). The range of concentrations can be extended to 500 μg

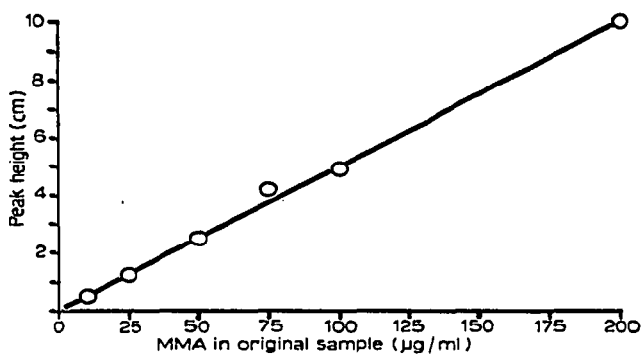

Fig. 2. Standard calibration graph for the butyl ester of MMA (attenuation 16×10^{-11}).

(attenuation 32 × 10^{-11}), 1000 μg (attenuation 64 × 10^{-11}) or 2000 μg (attenuation 128 × 10^{-11}) without loss of sensitivity or resolution. Linear calibration graphs were obtained in all instances.

The recovery of MMA added to sheep urine is shown in Table I. The concentration of MMA found in the urine sample analysed five times was 273.8 ± 11.9 μg/ml. When 2- or 3-ml portions of sheep urine were each extracted with a total volume of 15 ml of diethyl ether, the concentrations of MMA found (393 and 370 μg/ml, respectively) were in close agreement with the result obtained with a 1-ml sample (379 μg/ml). For urine samples containing less than 50 μg of MMA per ml, the sensitivity of detection can be enhanced either by concentrating the *n*-hexane extract of the ester before injection or by extracting larger volumes of urine. Provided that the *n*-hexane solution is not taken to dryness, no loss of the butyl ester occurs.

In Table II, results for MMA in diethyl ether extracts of urine and in freeze-dried urine are compared. The diethyl ether extraction method is recommended for the analysis of large numbers of samples, as peaks having long retention times are sometimes found with freeze-dried extracts.

TABLE I

RECOVERY OF MMA ADDED TO 1 ml OF SHEEP URINE (ETHER EXTRACTION METHOD)

MMA added (μg)	*% Recovery of [^{14}C]MMA*	*MMA found (μg)*	*MMA recovered (μg)*
0	90	35.5	0
30	85	66.2	30.7
60	83	96.6	61.1
90	86	126.0	90.5
120	85	158.5	123

TABLE II

COMPARISON OF THE METHOD OF ETHER EXTRACTION AND THE METHOD OF FREEZE-DRYING

Sample No.	*Ether extract MMA (μg/ml)*	*Freeze-dried MMA (μg/ml)*
1	349	323
2	38	23
3	220	215
4	83	91
5	280	272
6	1198	1175

The amounts of MMA found in human urine and in urine from cobalt-deficient and cobalt-supplemented sheep are shown in Table III. Unfortunately, no samples were available from humans suffering from pernicious anaemia, in which an elevation of MMA concentration would be expected.

TABLE III

MMA FOUND IN HUMAN AND SHEEP URINE (μg/ml)

	Human	*Sheep*	
		Cobalt-deficient	*Cobalt-dosed*
	3.9	89	13
	3.2	321	21
	2.9	277	30
	3.5	1173	34
	2.7	45	17
	4.9	51	30
	2.3	1415	49
Mean ± S.D.	3.3 ± 0.8	482 ± 527*	28 ± 12

* Significance, $p < 0.05$.

CONCLUSION

The GLC method described is more selective, more rapid and more sensitive than other methods currently available for the analysis of MMA in urine. By concentrating the *n*-hexane extract it should be possible to detect MMA in urine at a concentration of 0.2–0.4 μg/ml. Because of the reproducibility of the extraction procedure, as evidenced by the recovery of labelled MMA, it is not necessary to monitor the recovery of MMA by this method.

Results obtained with sheep urine confirm previous observations that the concentration of MMA increases in the urine of cobalt-deficient animals.

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