Journal of Chromatography, 81 (1973) 65–69

CHROM. 6714

A RAPID GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF VOLATILE FATTY ACIDS IN URINE

PROPIONIC ACID EXCRETION IN VITAMIN B_{12} DEFICIENCY

B. F. GIBBS, K. ITIABA, J. C. CRAWHALL, B. A. COOPER and O. A. MAMER
McGill University Clinic, Royal Victoria Hospital, Montreal, P.Q. (Canada)
(Received February 28th, 1973)

SUMMARY

A method for the isolation and quantitation of urinary volatile fatty acids is described. The method involves a simple clean-up procedure to eliminate neutral substances (*e.g.* ketones) and subsequent gas chromatographic analysis of the free acids. Analysis time is shorter, recoveries are excellent, and separation of the acids has been improved over previously reported methods. The method is used to study propionic acid excretion in vitamin B_{12} deficient patients.

INTRODUCTION

The analysis of urinary volatile fatty acids (VFA) is assuming an important role in the diagnosis of inherited metabolic diseases. In recent years, several disorders in VFA metabolism have been reported, including isovaleric acidemia[1] and propionic acidemia[2].

The free fatty acids in physiological fluids have usually been identified by gas chromatographic (GC) methods[3–6] but the difficulties reported by several authors[5,6] prevented the development of a standard isolation and quantitation method. One of the main drawbacks reported was the severe tailing of the free acid peaks that occurred when chromatographed on conventional column packings. The problem was analysed by Robinson[5] in a detailed study on the effect of column variables in the analysis of VFA. In our laboratory, a conventional phase was used and found adequate in conjunction with our extractive procedure. Previously reported methods are either time-consuming or do not exclude neutral substances (*e.g.* ketones) which may be confused with acids on GC.

We describe here an analytical procedure for the C_2–C_5 biologically significant fatty acids which is both rapid and accurate and simple enough to find application in clinical situations. Our method has been used to study the elevated propionate excretion in vitamin B_{12} deficient patients and the results support the findings of Cox *et al.*[7].

EXPERIMENTAL

Materials

The authentic acids and solvents in their highest available purities were obtained from Fisher Scientific Co. and used as received. The GC column packing was obtained from Chromatographic Specialities Co., Brockville, Ontario.

Naphthalene (zone refined) was obtained from Aldrich Chemical Co. and was also used as received. It was chosen as the internal standard because of its stability and suitable retention time.

Standards preparation and GC conditions

Accurately weighed quantities of pure authentic short-chain fatty acids (C_2–C_5) and naphthalene were mixed in varying ratios. They were dissolved in carbon disulphide, and small quantities (1–3 μl) were injected into a Hewlett-Packard Model 5750 gas chromatograph equipped with a flame ionization detector operated in single-column mode under the following conditions: column, 1/8 × 72 in. stainless steel packed with 25% neopentyl glycol adipate and 2% phosphoric acid on 100–120 mesh Chromosorb W AW-DMCS; temperature, programmed from 80° at 8°/min to 175° with a post-injection interval of 2 min, and an upper-limit interval of 8 min; detector temperature, 200°; injection port temperature, 190°; flow-rate of helium, approx. 15 ml/min. The peak areas were measured with a Hewlett-Packard Model 3373B electronic integrator. Calibration curves were plotted using an earlier reported method[8]. The relative responses of the detector varied linearly with the ratio of the weight of the acids of interest to the internal standard.

Preparation of samples for analysis

Urine samples were collected into thoroughly cleaned glassware and an aliquot was stored (at −20°) for possible further investigation. A 2-ml aliquot contained in a centrifuge tube was saturated with NaCl (approx. 0.5 g), and a few drops of 1 *N* NaOH were added until the pH was 11–12. An equal volume of ether was added and the tube was tightly capped and shaken on a small Vortex agitator at high speed for 40 sec. The ether layer was discarded and the aqueous phase was brought to pH 2–3 with 1 *N* HCl. The basic extraction was done to eliminate neutral metabolites which usually occur in urine. Approx. 2 ml of ethyl acetate were then added to the acidic aqueous phase and the tube was thoroughly shaken. To ensure two distinct phases slight centrifugation was often necessary. The ethyl acetate phase was removed and transferred to a 10-ml erlenmeyer flask. Traces of water were removed by drying briefly over anhydrous sodium sulphate (2 min). The extract was then carefully evaporated in a slow stream of dry nitrogen, taking care not to lose any of the volatile fatty acids. A known amount of naphthalene was added and the residue was dissolved in 25 μl of carbon disulphide. A convenient aliquot (1–3 μl) was then injected into the gas chromatograph.

RESULTS

Separation of five authentic saturated VFA compounds is shown in Fig. 1. As an example of the utility of this method, the propionic acid excretion was

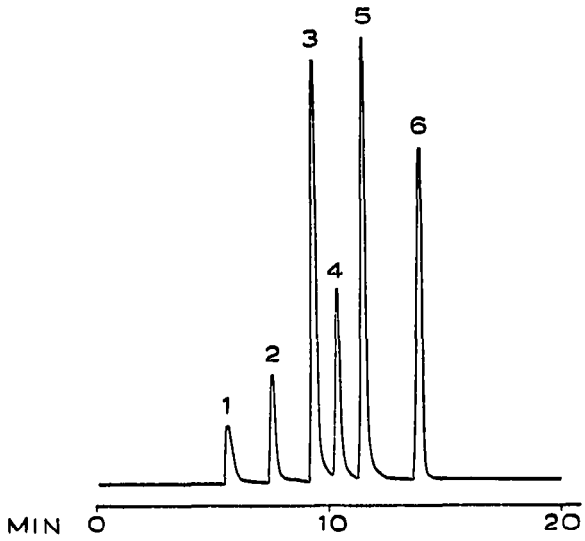

Fig. 1. Separation of five authentic saturated VFA compounds. GC conditions described in text. Peaks: (1) Acetic acid; (2) propionic acid; (3) butyric acid; (4) isovaleric acid; (5) valeric acid; (6) naphthalene (internal standard).

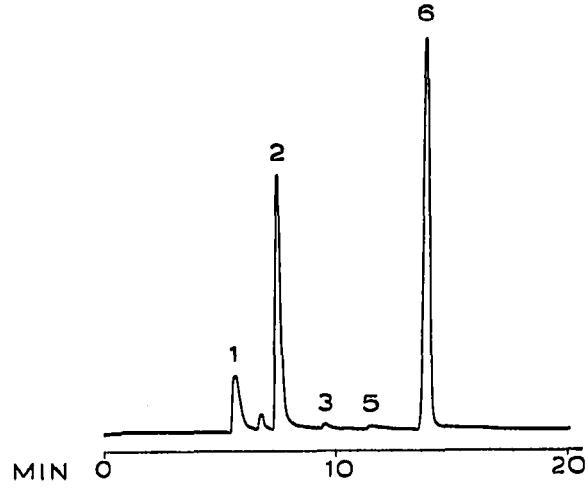

Fig. 2. Chromatogram obtained from a pernicious anemia patient. GC conditions and numbering of peaks as in Fig. 1.

studied in patients with vitamin B_{12} deficiencies. Fig. 2 illustrates the elevated level of propionate (not detected in normals) found in the urine of a pernicious anaemia patient. Table I summarizes the levels of urinary propionate and methylmalonate for three patients. The use of propionate as an index of vitamin B_{12} deficiency is evident.

The extraction efficiencies for these acids were checked by extracting normal urines before and after known weights of VFA were added to them. For acetic acid, 82% was recovered, the other acids showing recoveries in excess of 90%.

TABLE I

URINARY PROPIONIC AND METHYLMALONIC ACIDS IN PATIENTS WITH PERNICIOUS ANAEMIA

Patient (No.)	*Propionic / methylmalonic acids (mg per 24-h urine collection)*		
	Before valine	*After valine**	*After** vit. B_{12}*
1	10.2 / 12.8	48.1 / 109.3	14.3 / 35.0
2	21.1 / 60.9	37.7 / 265.0	4.3 / 10.3
3	nil / 8.7	6.9 / 20.0	0.4 / 6.7

* 5-g oral valine load.
** 1000 μg cyanocobalamin derivative *i.m.*

DISCUSSION

Various procedures for the analysis of VFA in physiological fluids have been reported. Recently Robinson[5] studied the effect of column length, conventional column packings, and modified stationary phases, in an effort to correct existing problems. His preliminary extraction was done by ultrafiltration. Most current methods involve steam-distillation followed by GC. Earlier methods of partition and adsorption chromatography[9] are insensitive and yield poor recoveries. Gas–liquid chromatography of the methyl and butyl esters of VFA have also been reported[10] but require the addition of a derivatization step.

We have developed a simple and accurate method which has several advantages over the previously published methods. The first ether extraction (from alkalinized urine) serves to eliminate neutral substances that may subsequently extract with the acids and confuse the GC analysis. We propose this method for screening short-chained acidurias on a routine clinical basis. Though steam-distillation has been avoided, recoveries are comparable to existing methods, analysis time is shorter, and separation of the acids has been improved. The modified phase used is suitable in conjunction with our extractive procedure. Before packing, the column should be washed with acetone and dried with dry compressed air. The packed column should be conditioned at 180° overnight, with the detector end disconnected. Carbon disulphide is useful as a solvent[3] for sample injection as it does not yield significantly to flame ionization detection, and as a result early eluting peaks are on baseline and not on a solvent tail.

We have used this method to study propionic acid excretion in vitamin B_{12} deficient patients. Urinary methylmalonic acid (MMA) is known to be excreted in established cases of pernicious anaemia[11]. In borderline cases, oral administration of valine, a precursor of methylmalonyl CoA, increases the excretion of MMA in the urine[12]. On the other hand, administration of 5′-deoxyadenosyl-B_{12}, the deficient coenzyme in this disorder, reduces the accumulation of MMA[11]. Since propionyl CoA is an important precursor of methylmalonyl CoA, it seemed probable that elevated excretion of urinary propionic acid might also occur in pernicious anemia. The results reported here verify the findings of Cox and White[11], who estimated propionate using an ion-exchange resin as a purification step. Table I shows the MMA and propionic acid levels before and after treatment. MMA quantitation was

done by an earlier reported method[8]. A marked increase of both of these acids occurred when valine was administered and a decrease was noted upon the administration of the B_{12} derivative.

GC identification of MMA may be difficult in situations involving borderline cases when the MMA peak is small and the chromatogram becomes cluttered with other acids. Since propionic acid elutes under these conditions in an uncluttered section of the gas chromatogram, its presence in urine could easily be detected with confidence and used as a second diagnostic procedure coupled with a valine load.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to the Medical Research Council of Canada for support (Grant No. MA 3331 and MA 3719). They also wish to thank Dr. E. Gibbs for her editorial assistance.

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