Optimizing Methylation Conditions for Gas Liquid Chromatography Assay of Lactic and Succinic Acid in Biological Samples

K. S. Bricknell / I. Brook* / S. M. Finegold

Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024, and Medical Service, VA Wadsworth Hospital Center, Los Angeles, California 90073, USA

Summary

Aqueous standard solutions of lactic and succinic acids were methylated for assay by gas-liquid chromatography. The efficiency of methylation using boron trifluoride-methanol reagent was compared to methylation using a sulfuric acid-methanol mixture; the ratio of sulfuric acid and methanol was also varied. Reactions were done at 100 °C/5 min and at room temperature/20 hours. We found the sulfuric acid-methanol method was most efficient for a rapid assay; however, the ratio of sulfuric acid and methanol used is critical in the assay of lactic acid and would be especially important in the clinical laboratory using gas-liquid chromatography for analysis of cerebrospinal fluid from suspect meningitis patients or other body fluids.

Introduction

Gas-liquid chromatography (GLC) assay of biological samples may produce profiles which relate to particular clinical disorders. An example of this is the profile for lactic acid (LA) in cerebrospinal fluid (CSF). Normal CSF-LA is in the range of 10–20 mg per 100 cm^3 and elevated values are found in cases of bacterial meningitis [1–6].

The conventional assay procedure involved enzymatic spectrophotometry and required careful control of temperature, pH and timing. A recent study by Controni and co-workers [7] showed that GLC assay of LA as the methyl ester was possible, the procedure being quite simple and results obtained in much less time; this allowed appropriate patient treatment to commence earlier.

During our study of CSF and serum from 67 patients previously reported [8] we investigated the methylation procedure in order to find the optimum conditions for maximum esterification efficiency. For this purpose we used a standard aqueous solution of lactic acid and succinic acid (SA).

We found that increased sulfuric acid in the reaction mixture decreased the conversion of LA to its methyl ester and conversely gave higher yields of dimethylsuccinate.

Materials and Methods

Reagents and chemical were: lactic acid and succinic acid (Eastman Organic Chemicals, Rochester, N.Y. 14560); sulfuric acid, methanol and chloroform (Mallinckrodt Chemical Works, St. Louis, Mo. 63160); boron trifluoride-methanol reagent (Applied Science Labs., State College, Pa. 16802).

Experimental

A single stock aqueous solution containing LA (10 μmoles/cm^3) and SA (10 μmoles/cm^3) was diluted to make standard solutions containing 1, 2, 3, 4, 5, 6, 7, 8, 9 μmoles/cm^3 of each acid. 1.0 cm^3 aliquots of these dilutions were methylated by the following methods:

Method I	Boron trifluoride-methanol reagent	1.0 cm^3
Method II	Sulfuric acid (50%) 0.1 cm^3-methanol	1.0 cm^3
Method III	Sulfuric acid (50%) 0.4 cm^3-methanol	2.0 cm^3

The methylation reaction was carried out using 100 X 13 mm test tubes with teflon-lined screw caps. Upon completion of the reaction 0.2 cm^3 of chloroform was added and the mixture briefly vortexed and centrifuged. Longer reaction times than 100 °C/5 min and room temperature/20 hours did not increase the yield of methyl ester significantly.

Variations in conditions of methylation using boron trifluoride-methanol or sulfuric acid-methanol were made as indicated in Figs. 1 and 2.

* Present address: Children's Hospital National Medical Center, Clinical Laboratory, Washington, D.C. 20010, USA

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Gas Chromatography

A model 1420 chromatograph (Varian Instruments, Sunnyvale, Calif. 94086) fitted with thermal conductivity detector was used. Stainless steel columns, 6 ft X 1/8" o.d., were packed with Resoflex (Burrell Corporation, 2223 Fifth Avenue, Pittsburg, Pa. 15219). Helium (zero grade; Liquid Carbonic Corporation, Chicago, Ill. 60603) was used as a carrier gas at a flow rate of 60 cm^3/min.

Injector port, oven and detector block temperatures were 155 °C, 145 °C, and 165 °C respectively. Filament current was 200 ma and attenuation of lx used. The output signal was connected to a model A25 recorder (Varian Instruments) with a span of 1 mv and a chart speed of 1/2"/min.

10 µliter aliquots of the chloroform extract were injected. Retention times for methyl lactate and dimethyl succinate were 1.5 and 3.0 min respectively.

Results

Esterification was more efficient with sulfuric acid-methanol (ratio 0.1:1.0) than with boron trifluoride-methanol if reactions were carried out at 100 °C/5 min, but it was more efficient with boron trifluoride-methanol than with sulfuric acid-methanol if reactions were carried out at room temperature/20 hrs (Figs. 1 and 2).

With variation of the sulfuric acid-methanol ratio, esterification of LA as well as SA was altered (Fig. 2).

Method II, using 0.1 cm^3 sulfuric acid was more efficient for LA than SA. Method III, with increased sulfuric acid ratio, was more efficient for SA than LA.

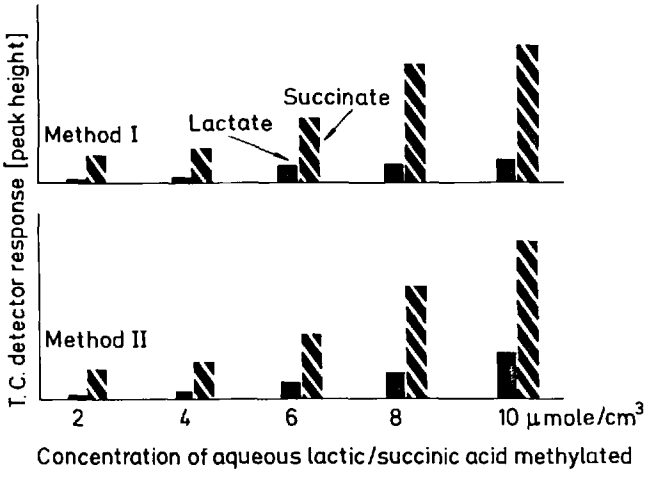

Fig. 1

- Comparison of BFI_3-MeOH reagent versus H_2SO_4-MeOH.

Method I:
BFI_3-MeOH reagent 1.0 cm^3
Method II:
H_2SO_4 (50 %) 0.1 cm^3 MeOH 1.0 cm^3
} 100 °C/5 min

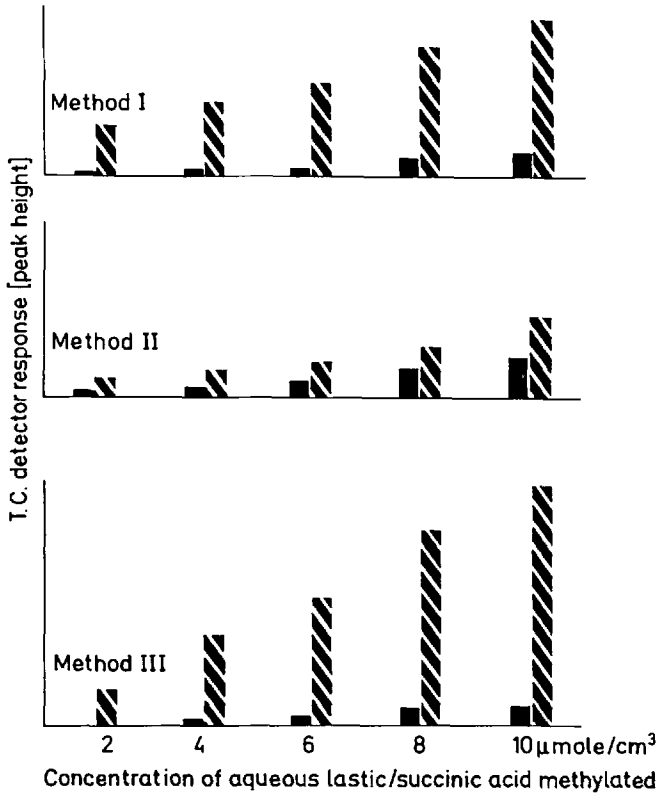

Fig. 2

- Comparison of BFI_3-MeOH reagent versus H_2SO_4-MeOH.

Method I:
BFI_3-MeOH reagent 1.0 cm^3
Method II:
H_2SO_4 (50 %) 0.1 cm^3 MeOH 1.0 cm^3
Method III:
H_2SO_4 (50 %) 0.4 cm^3 MeOH 2.0 cm^3
} Room temp./20 hrs

Discussion

The main purpose of this study was to find the most efficient methylation procedure to assay LA in CSF and serum using GLC. We have previously confirmed the findings of Harmon and Doelle who reported [9] that conversion of lactic acid to its methyl ester is only 50–55 % complete. We have also reported [10] that it is not possible to extract all the ester into chloroform with a single extraction.

It is obvious from these results that increasing the amount of sulfuric acid adversely affects the conversion of LA to its methyl ester. We therefore used Method II for our CSF/serum study. Stoppered tubes containing 0.1 cm^3 50 % v/v aqueous sulfuric acid were kept available and freshly obtained samples ($\sim$ 1.0) of CSF or serum added to the tube which was then mixed and immediately frozen and held at − 40 °C until assayed. Total volume was determined and correction for the dilution effect made in the final calculations.

The procedure outlined is quite simple and the early results would be invaluable to the physician in the diagnosis and treatment of the patient, particularly since it permits differentiation between partially treated bacterial meningitis and viral meningitis. Effect of therapy could also be followed by subsequent assays.

A disadvantage of the assay procedure is the volume of CSF (1.0 cm^3) required. It is difficult to obtain this quantity from infants. We have done the assay using much less volume (i.e. 0.2 cm^3) of sample; however, the accuracy would tend to decrease in proportion to the decrease of sample volume.

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