*Journal of Chromatography*, 97 (1974) 39–45


CHROM. 7577

# DIRECT DETERMINATION OF MOLAR RATIOS OF VARIOUS CHEMICAL CONSTITUENTS IN ENDOTOXIC GLYCOLIPIDS IN SILICIC ACID SCRAPINGS FROM THIN-LAYER CHROMATOGRAPHIC PLATES

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(Received March 26th, 1974)

## SUMMARY

Analytical procedures are described for the determination of the ratios of nitrogen, phosphorus, hexosamine, carbohydrates, fatty acids and 2-keto-3-deoxy-octonate (KDO) in bacterial endotoxic glycolipids separated by thin-layer chromatography (TLC). The methods make it possible to carry out analyses in the presence of silicic acid to obtain the molar ratios of the chemical constituents of natural products. For some micro-determinations, such as nitrogen, phosphorus or KDO, scrapings from a single TLC plate are sufficient for carrying out the quantitative analyses.

## INTRODUCTION

Thin-layer chromatography (TLC) at present can be considered to be one of the most effective and useful techniques for the separation of lipids. The advantages of TLC include simplicity, speed and high resolution. Preparative TLC usually effects better separations than silicic acid column chromatography. Some natural products can be separated to form a homogeneous single band and several milligrams of purified material can be recovered by collecting scrapings from five or more plates. The disadvantages of preparative TLC are two-fold. Firstly, the eluate is frequently contaminated with fine silicic acid particles[1]. Filtration of the eluate through ultrafine glass filters does not eliminate all the contamination. Centrifugation and partition in two-phase solvents have been designed to eliminate the fine particles[2], but the procedures are tedious and removal of the silicic acid is not complete. Secondly, recovery of the material from the scrapings is usually low, especially when the lipids must be developed in a rather polar solvent system[3].

Bacterial endotoxic glycolipids are heterogeneous. The purification and isolation of purified material is necessary before the structure–function relationship can be verified. The endotoxic glycolipids from *Salmonella minnesota* R595 have been separated by TLC[4]. In order to avoid the difficulties in the elution of glycolipids from silicic acid scrapings, quantitative analytical procedures have been developed in our laboratory for the determination of the molecular ratios in these glycolipids

in the presence of silicic acid. Scrapings from TLC plates were taken and subjected to quantitative chemical microanalyses, without attempting the elution of the components from the scrapings.

Chemical analyses included the measurement of total nitrogen, phosphorus, hexosamine, total carbohydrates, long-chain carboxylic acids and 2-keto-3-deoxy-octonate (KDO). The molecular ratios could be calculated from these data.

## MATERIALS AND METHODS

### *Endotoxic glycolipid*

Chloroform–methanol (4:1) soluble glycolipids were directly extracted from the lyophilized cells of a rough mutant, *Salmonella minnesota* R595, as reported elsewhere[4].

### *Thin-layer chromatography*

All TLC was carried out with silicic acid Bio-Sil A (2–10 μm; Bio-Rad Labs., Richmond, Calif., U.S.A.) and with plates 20 × 20 cm in size and 0.25 cm in thickness. To 30 g of silicic acid, 56 ml of water containing 4 ml of concentrated ammonia solution were added. The plates were air-dried, activated at 120° for 1 h in a vacuum oven, then cooled in a vacuum desiccator. The solvent system used was chloroform–methanol–water–ammonia (100:50:8:4).

As these plates could not be used for the determination of nitrogen, other plates were prepared in which the slurry was made in 0.1 *M* phosphate buffer[5], pH 7.8, and the pH of the slurry was again adjusted to 7.8 with 10 *N* sodium hydroxide solution. In order to obtain reasonably good separations, the solvent systems used for these plates were either chloroform–methanol–water (3:3:1) or *n*-propanol–water (1:1).

The spray reagents used were either chromic acid–sulfuric acid (5% potassium dichromate in 40% sulfuric acid) or water. For preparative TLC, about 1 mg of glycolipid per plate was applied with a sample applicator (Applied Science Labs., State College, Pa., U.S.A.). The plates were developed, dried and sprayed with distilled water; those parts of the silicic acid which contained lipids remained white, while other zones became translucent. The outlines of the visible bands were marked and the plates were dried at room temperature. The bands were scraped off and the identical bands or zones from several TLC plates were pooled. The scraped silicic acid was dried at 70° in a vacuum oven over phosphorus pentoxide, then stored in vials in a vacuum desiccator over phosphorus pentoxide at room temperature. A blank zone was also scraped and treated in the same manner.

### *Determination of nitrogen*

Before all quantitative chemical analyses, the pooled scrapings were thoroughly mixed. A 500-mg amount (the weighing should be carried out as rapidly as possible) of the scrapings was transferred into a 100-ml Kjeldahl digestion flask. Concentrated sulfuric acid (2 ml) saturated with anhydrous copper(II) sulphate was added and the digestion was carried out in the presence of alundum particles, as described elsewhere[6].

The distillation was performed with a Labconco micro-distillation apparatus (Labconco Corp., Kansas City, Mo., U.S.A.). A 5-ml volume of 10 *N* sodium hydroxide solution was added to the sample and the ammonia liberated was steam-distilled into a recipient 25-ml graduated cylinder containing 10 ml of 0.02 *N* sulfuric acid. After distillation, the cylinder was filled to 20 ml with water and its ammonia content was determined by Nesslerization. To a 5-ml aliquot, Nessler reagent (5 ml; Hartman–Leddon Co., Philadelphia, Pa., U.S.A.) was added and the color was measured at 440 nm. A calibration curve was prepared with ammonium sulphate.

*Determination of phosphorus*

The determination of phosphorus was carried out using a modified method of Chen *et al.*[7]. Liberation of organic phosphorus was achieved by heating 50-mg aliquots of silicic acid scrapings in 0.8 ml of 6 *N* sulfuric acid at 100° for 16 h in a glass-stoppered tube. A 4-ml volume of water was added and mixed well. After cooling, 0.8 ml of 2.5% ammonium molybdate mixed with 1.6 ml of water and 0.8 ml of 10% ascorbic acid was added. The tubes were thoroughly mixed and incubated at 37° for 1½ h. The mixture was centrifuged at 1000 *g* for 15 min, and the color intensity of the supernatant was determined at 820 nm. A calibration curve was prepared with potassium dihydrogen orthophosphate.

*Determination of hexosamine*

A 300-mg amount of silicic acid scrapings was hydrolyzed with 2 ml of 4 *N* hydrochloric acid in a glass-stoppered test-tube at 100° for 18 h. The contents of the tubes were filtered into a 100-ml round-bottomed flask and washed about five times with 3 ml of water. The filtrate was dried thoroughly on a Büchi evaporator. The flask with the dried hydrolyzate was placed in a vacuum desiccator and dried overnight over potassium hydroxide pellets. A 3-ml volume of water was added to dissolve the residue and 0.5- and 1.5-ml aliquots were taken for the determination of hexosamine. The procedure for color development was essentially the same as that described by Rondle and Morgan[8]. A calibration curve was prepared with glucosamine.

*Determination of carbohydrate*

The carbohydrate content was determined according to the method of Dubois *et al.*[9]. To 200 mg of silicic acid scrapings and 0.5 ml of water in a test-tube, 1.0 ml of 5% phenol solution was added and mixed well. After the tubes had been chilled in an ice-bath, 5 ml of conc. sulfuric acid were added, mixed well and the tubes were returned to the ice-bath. The tubes were then immersed in a boiling water-bath for 15 min, after which they were cooled in an ice-bath. The sample was then centrifuged at 1000 *g* for 20 min and the supernatant was read at 490 nm. A calibration curve with standard dextran solution was used for the determination.

*Determination of long-chain carboxylic acids (fatty acids)*

A 2-ml amount of boron trifluoride reagent (Applied Science Labs.) was added to a test-tube with a standard-taper-joint which contained 500 mg of silicic acid scrapings and granules of alundum. The tubes were firmly attached to well cooled reflux condensers and the lower end (*ca.* ½ in.) was immersed in an 80–90° oil-bath for 5 h in order to transesterify the fatty acids[10]. The tubes attached to their condensers were cooled,

and 2 ml of redistilled methanol were added through the reflux condenser into the tubes. Water (2 ml) and *n*-hexane (2 ml) were added to the disconnected tubes, which were immediately closed with glass stoppers. The tubes were shaken vigorously for 30 sec and allowed to stand at room temperature for approximately 1 h until the silicic acid had settled and the two phases were clearly separated. The *n*-hexane phase was retained and 2 ml of fresh *n*-hexane were added for extraction. A total of four such extractions were repeated. Any contaminating water was removed with anhydrous sodium sulfate. The pooled *n*-hexane was then evaporated until completely dry with nitrogen gas at room temperature. The fatty acid methyl esters were quantitatively determined by the hydroxylamine method[11]. A calibration curve was made with glucose pentaacetate.

*Gas–liquid chromatography (GLC)*

The methyl esters used for quantitative fatty acid analysis were also examined by GLC using an F & M 609 instrument equipped with a hydrogen flame ionization detector. The components on the chromatograms were identified by co-chromatography with the authentic fatty acid methyl esters.

*Determination of KDO*

The hydrolysis of KDO is usually destructive and a hydrolysis curve is needed for each sample to obtain the value at zero time. When the sample is coated on silicic acid, however, KDO is protected against destruction by acid and a 20-min hydrolysis was found to be optimal.

A 100-mg amount of silicic acid scrapings was hydrolyzed with 1.0 ml of 0.025 *N* sulfuric acid in a boiling water-bath for 20 min. The cooled hydrolyzates were centrifuged at approximately 1000 *g* for 15 min. A 0.4-ml amount of the supernatant was used for the determination of KDO. The method was essentially the same as that described by Weissbach and Hurwitz[12] and modified by Osborn[13], except that the amounts of all the reagents were doubled. A calibration curve was prepared with ammonium KDO (a gift from Dr. O. Lüderitz, which the authors greatly appreciate) in the range 1–10 $\mu$g.

## RESULTS AND DISCUSSION

The separation of endotoxic glycolipids from *S. minnesota* R595 by TLC with solvent system chloroform–methanol–water–ammonia (100:50:8:4) is shown in Fig. 1. Four major bands were obtained and all were found to be biologically active[1]. The KDO, hexosamine, phosphorus and fatty acid contents of each band were determined by the procedure described above. It was found that these four bands (a, b, c and d) had similar molar ratios, namely KDO:hexosamine:phosphorus:fatty acid = 2:2:3:6 (Table I). This ratio was in the range of reported data[11,15] for glycolipids from *S. minnesota* R595. In addition, the *n*-hexane extract of the transesterified methyl esters was also analyzed by GLC. Fig. 2 shows that the four biologically active bands revealed similar fatty acid compositions. Thus, by determining the amounts of the constituents of the silicic acid scrapings, it is possible to calculate the molecular ratios of various components in a natural product without knowing the exact organic material content of the silicic acid scrapings.

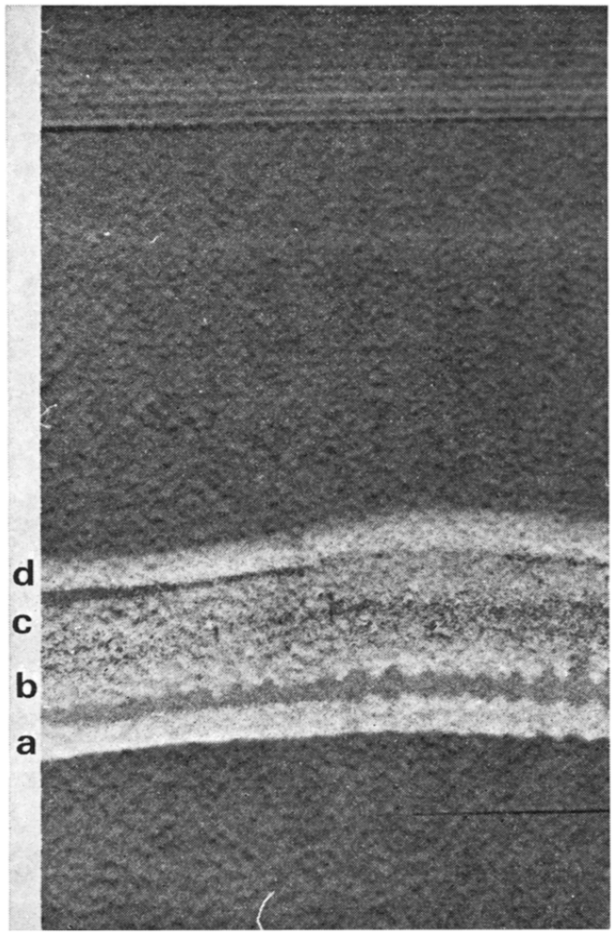


Fig. 1. Thin-layer chromatography of purified glycolipids with silicic acid Bio-Sil A (2–10 μm) and solvent system chloroform–methanol–water–ammonia (100:50:8:4). Silicic acid slurry was prepared with distilled water–ammonia. The plate was sprayed with water.

All the methods were tested with glycolipid or other known samples with blank silicic acid added. The results obtained from the known materials in the presence or absence of silicic acid were identical. The batches of silicic acid used in these experiments were free from contaminants which would interfere with the reac-

TABLE I

MOLAR RATIOS OF THE GLYCOLIPID FRACTIONS

| *Glycolipid fraction* | *Hexosamine* | *Fatty acid ester* | *P* | *KDO* |
|---|---|---|---|---|
| a | 1 | 2.90 | 1.69 | 1.07 |
| b | 1 | 2.75 | 1.60 | 0.93 |
| c | 1 | 3.10 | 1.36 | 0.83 |
| d | 1 | 2.67 | 1.34 | 1.08 |

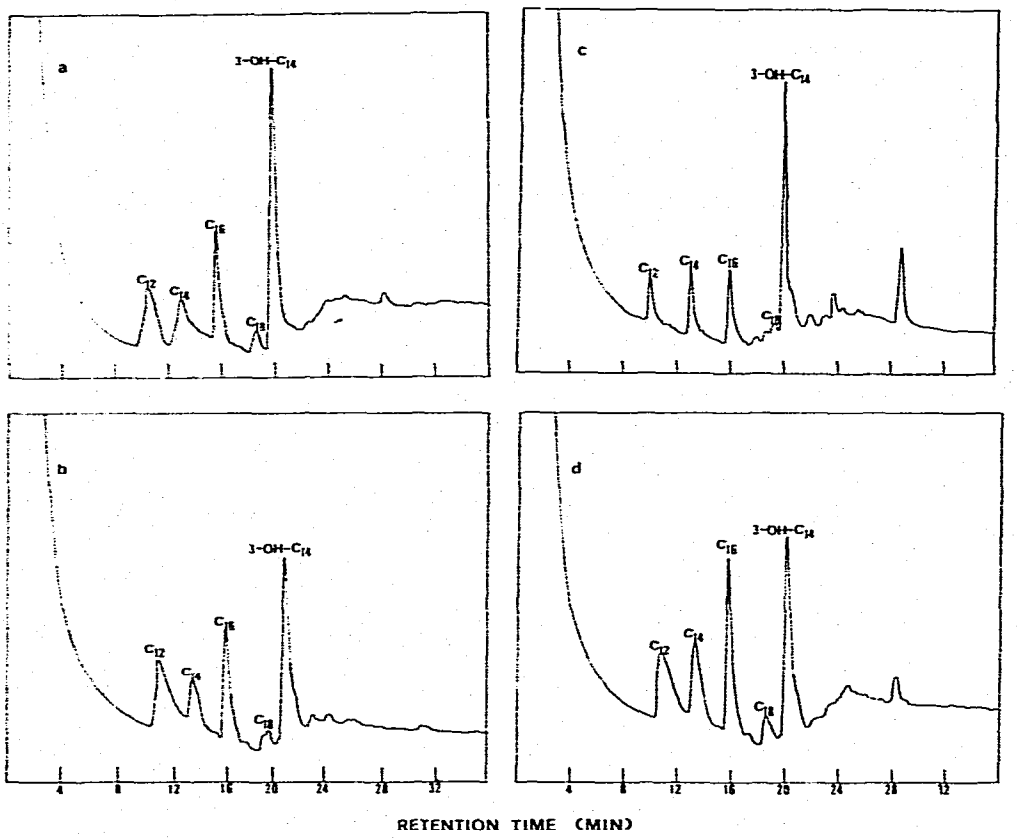


Fig. 2. Gas–liquid chromatogram of fatty acids present in glycolipid fractions. Column: 3% ECNSS-M on 100–120 mesh Gas-Chrom Q, 12 ft. Temperature programmed from 50° to 185° at 6.4°/min. a, b, c and d = fractions obtained from glycolipids by TLC. $C_{12}$ = Lauric acid; $C_{14}$ = myristic acid; $C_{16}$ = palmitic acid; $C_{18}$ = stearic acid; 3-OH-$C_{14}$ = 3-hydroxymyristic acid.

tion. The silicic acid obtained from other sources may have a significant amount of contamination, in which case it should be pre-washed or a calibration curve with blank silicic acid plus known amounts of standard material should be used.

The amount of silicic acid scrapings needed for each analysis depends on the total organic material as well as the percentage of the particular component in the isolated band. The optimal ranges for the components to be determined are shown in Table II. Thus, if 1–3 mg of the total substance can be separated on one TLC plate, scrapings from a single band may be sufficient to determine nitrogen, phosphorus and KDO, and to calculate the molecular ratios if the compound in the band is relatively rich in these components.

The major source of error in these analyses is the variation in the water content of the silicic acid scrapings. The scrapings should be mixed and dried thoroughly and all samples should be kept constantly under vacuum (less than 0.1 mm Hg at room temperature over phosphorus pentoxide). The weighing procedure should be carried out as rapidly as possible.

TABLE II

RANGE OF THE OPTIMAL AMOUNTS OF THE COMPONENTS FOR DETERMINATION

| *Compenent* | *Optimal range (μg)* |
|---|---|
| Nitrogen | 5–25 |
| Phosphorus | 1–10 |
| Hexosamine | 20–80 |
| Fatty acid | 150–1250 |
| Carbohydrate | 20–100 |
| KDO | 2–10 |

While our procedures were developed for an endotoxic glycolipid, other natural products which can be separated by TLC may be subjected to similar analyses in order to obtain the chemical molar ratios without the difficulties of elution and contamination of silicic acid.

A similar TLC pattern to that of *S. minnesota* R595 glycolipids has been observed in our laboratories for glycolipids extracted from another rough mutant, *Salmonella typhimurium* SL1102. The molar ratios of KDO:hexosamine:phosphorus:fatty acid in pooled glycolipids was found[16] to be 2:2:4:7.

ACKNOWLEDGEMENTS

The excellent assistance of Mrs. Anne Nowotny is gratefully acknowledged.

This investigation was supported by Public Health Service grant AI-05591 from the National Institute of Allergy and Infectious Diseases.

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