Quantitative gas-liquid chromatographic determination of free glycerol in blood serum

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SUMMARY Quantitative gas–liquid chromatographic determination of free glycerol in blood serum was accomplished through the use of butane-1,4-diol as internal standard. After removal of proteins with phosphotungstic acid, the glycerol and butanediol were acetylated for 1 hr with acetic anhydride. The acetates thus formed were extracted with diethyl ether, the solvent was removed, and the butanediol diacetate and glycerol triacetate were separated on a column with butanediol succinate as stationary phase at temperatures programmed from 150 to 190°. Normal sera were found to contain 0.4–1.2 mg of glycerol per 100 ml, the estimation having a range of ±5–10%. The method can be exploited to determine as little as 0.01 mg of glycerol per 100 ml when a sensitive flame ionization detector is used.

THERE IS AT PRESENT considerable interest in the determination of free glycerol in blood and biological materials (1–3), which is one of the more difficult analytical problems in lipid chemistry (4). The most widely used methods for assay of glycerol appear to be those based on periodic acid oxidation (5–7), as well as various enzymic procedures employing glycerokinase and glycerophosphate dehydrogenase (1, 8). Methods of the first type suffer from interference by biological compounds like glucose, glycerophosphate, and ethanolamine (4).

During our studies on lipolysis and glycerol metabolism, we have developed a procedure for the determination of free glycerol in blood serum or in other biological materials by gas–liquid chromatography (GLC). The method is highly specific and takes advantage of the great sensitivity of GLC.

EXPERIMENTAL METHODS

Principle

To the serum is added a known amount of the internal standard, butane-1,4-diol, which is normally not present in blood. The serum proteins are precipitated with phosphotungstic acid and removed. The acidic solution, which contains the glycerol and the butanediol, is shaken with petroleum ether to remove traces of triglycerides, and then acetylated with acetic anhydride. The glycerol triacetate and butanediol diacetate thus formed are extracted with diethyl ether and the solution is concentrated to a small volume before injection into the gas chromatograph. A comparison of the two peak areas gives the amount of free glycerol.

Materials

Serum was obtained from healthy blood donors. Glycerol triacetate, butanediol diacetate, and diethyleneglycol diacetate were synthesized from the respective alcohols by acetylation with acetic anhydride, followed by ethyl ether extraction and distillation. Glycerol and butane-1,4-diol were distilled under reduced pressure to remove traces of water. The ether used for extraction purposes was redistilled. All other chemicals used in this investigation were of analytical grade.

Gas–Liquid Chromatography (GLC)

The analyses were obtained with a dual column, temperature-programmed gas chromatograph with differential flame ionization detector (Perkin-Elmer, Model 800).[1] Two 6 ft stainless steel columns, $^1/_8$ in. o.d., containing 8% butanediol succinate (BDS), on 80–100

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[1] Presented to Professor L. Eldjarn by A/S Apotekernes Laboratorium, Norway.

mesh Chromosorb W, were used in the chromatographic separations at temperatures programmed from 150 to 190° at the rate of 13.3°/min. Nitrogen, at a flow rate of 20 ml/min, was the carrier gas.

Alternatively the analyses may be run isothermally at 165°. At this temperature the retention time of the glycerol triacetate is increased by several minutes. Higher temperatures are, however, not recommended because the internal standard is then eluted too early, and may thus be disturbed by solvent tailing. For this reason and to save time, temperature programming is of advantage.

Procedure

To freshly obtained serum (1 ml) was added 100 μl of a 0.16% (w/v) aqueous solution of butanediol as internal standard. The proteins were precipitated with phosphotungstic acid (2.0 ml of a 2% solution in 2 N HCl) and centrifuged down. The acidic supernatant solution was filtered into a test tube of about 55 ml capacity fitted with a ground glass stopper, and extracted once with 10 volumes of petroleum ether. The organic phase was removed and discarded.

To the aqueous phase, usually about 1.5 ml, was added 11 ml (i.e., about 30–40% excess) of acetic anhydride. The test tube was allowed to stand for 1 hr on an electrically heated plate to keep the acetylating mixture simmering. A reflux condenser was not required. Excess ethanol (8–10 ml) was added, the tubes were kept at 70° for 20 min, and the ethyl acetate and water thus formed were carefully boiled off until the volume was reduced to about 3–4 ml. The residual acetic acid was just neutralized with 15% NaOH solution using phenolphthalein as indicator. During this process the tubes were kept at 0° to prevent hydrolysis of the acetates. The glycerol triacetate and butanediol diacetate were then immediately extracted with 3–4 volumes of diethyl ether. The ether extract was shaken with anhydrous sodium sulfate to remove water, filtered, and evaporated to a very small volume (about 20–25 μl) using a conical test tube. Of this solution 1 μl was injected into the gas chromatograph attenuated at × 200. When the internal standard peak had appeared on the chromatogram, the sensitivity was increased by a factor of 10 for recording of the glycerol triacetate peak. Figure 1 shows a typical gas chromatogram obtained from a normal serum. Peak areas were determined by triangulation.

From a comparison of the two peak areas (appropriately adjusted according to the attenuation), a knowledge of the amount of internal standard added to the serum, and the relative response of the two compounds in the gas chromatograph, the amount of glycerol was readily obtained.

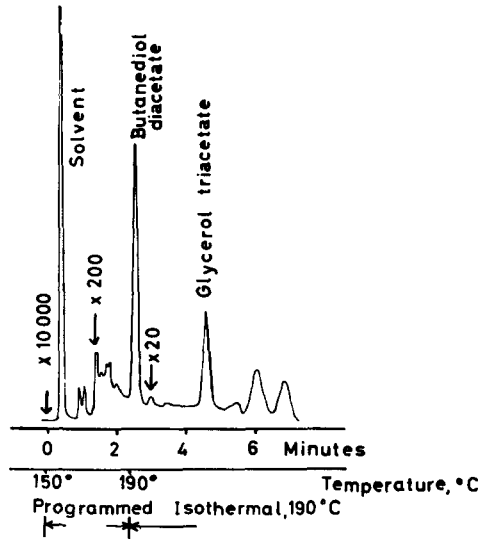

FIG. 1. A typical gas chromatogram as obtained with the present method applied to a normal serum. Stainless steel columns, 6 ft x 1/8 in. o.d., 8% butanediol succinate on Chromosorb W. Column temperature programmed from 150 to 190° at the rate of 13.3° per min. Injection block at 240°; detector 190°.

As seen from the chromatogram, several irrelevant peaks were also produced. The compounds responsible for these have not been identified. The reagents contained no such impurities, hence the peaks must be related to compounds originally present in the serum.

DISCUSSION OF THE METHOD

Choice of Internal Standard

Free glycerol is difficult to determine directly by GLC. However, its triacetyl derivative is readily volatilized and gives no tailing in the gas chromatograms. A procedure for detection by GLC of the minute amounts of free glycerol in serum must necessarily involve conversion into a volatile derivative such as the triacetate, extraction, and evaporation of solvent to very small volumes. The latter operation in particular renders quantitative analysis of glycerol extremely difficult unless the well-known "internal standard" method (9) is used.

The reference compound should fulfill certain conditions. It should normally be absent from blood serum, and physically and chemically it should resemble glycerol as closely as possible. Three compounds have been examined for this purpose. Ethylene glycol was rejected because it was too volatile, and diethylene glycol was discarded because the diacetate was too soluble in water. We have chosen butane-1,4-diol as the reference compound, although other alcohols such as penta- and hexamethylene glycols are also likely to be suitable. Butanediol has a relatively high boiling point (230°),

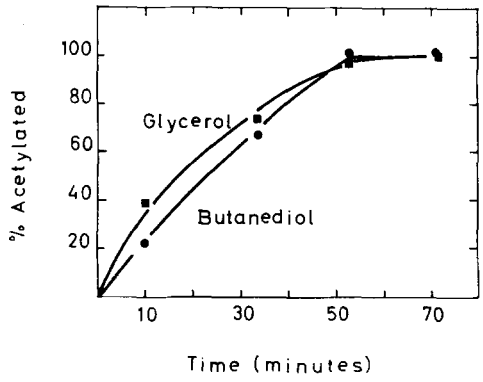

FIG. 2. Acetylation of glycerol and butane-1,4-diol as a function of time. Acetic acid–acetic anhydride 7:3 at 120° was used as the acetylating mixture. The amount of glycerol triacetate and butanediol diacetate formed were measured by GLC.

is miscible with water, and like glycerol is readily acetylated to yield a diacetate, bp 229–230°, suitable for GLC. Butanediol diacetate was eluted well ahead of the glycerol triacetate (bp 258°) under the gas chromatographic conditions described. The retention times were 2.50 and 4.50 min, respectively; i.e., relative retention time of glycerol triacetate = 1.8 (butanediol diacetate = 1).

The relative response is of importance in a method such as this. It was found that when 1 g of pure butanediol (mol wt 90.12) and 1 g of pure glycerol (mol wt 92.09) were mixed, acetylated with excess acetic anhydride for several hours, and injected into the gas chromatograph, the peak areas differed only slightly:

$$\frac{\text{Peak area of "glycerol"}}{\text{Peak area of "butanediol"}} = 0.94\text{–}0.96.$$

This factor should therefore be taken into account.

The amount of internal standard added to the serum was about 10–20 times greater than the amount of free glycerol present, in order to facilitate the peak area measurement. If instead considerably smaller amounts of butanediol were added to the serum and the gas chromatograph operated at 10–20 times higher sensitivity than above, the measurement of the butanediol diacetate peak was disturbed by a number of unidentified peaks constantly present. This interference was overcome by increasing the amount of internal standard and decreasing the sensitivity of the instrument. The attenuator was linear to within ±1–2% in the sensitivity range required.

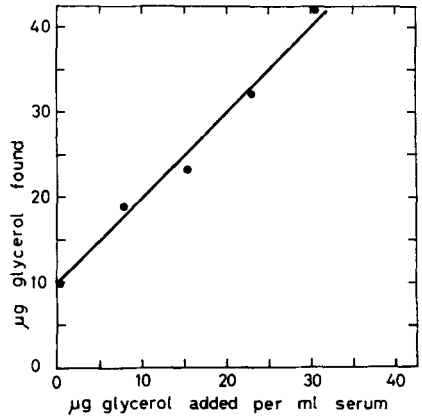

FIG. 3. Recovery of glycerol added to a normal serum (originally containing 10 μg free glycerol per ml).

Protein Precipitation

Complete removal of the proteins, which is best accomplished with phosphotungstic acid, seemed to reduce the number of several unidentified irrelevant peaks in the final gas chromatograms. No evidence for adsorption of glycerol and butanediol to the protein–phosphotungstic acid precipitate has been found.

Petroleum Ether Extraction

This was included in order to remove traces of triglycerides which may undergo transesterification with acetic anhydride. Glycerol and butanediol are insoluble in petroleum ether and were not extracted.

Acetylation Process

In order to determine the time required for complete acetylation the following experiment was performed. Equal amounts of glycerol and butanediol (28 mg of each) were acetylated with a mixture of acetic acid and acetic anhydride 7:3 at the boiling point of the mixture. Samples were withdrawn at intervals, neutralized, and extracted with diethyl ether. The aqueous phase was removed, and previously synthesized diethylene glycol diacetate was added to the ether phase as a "second" internal reference compound. The mixture was reduced in volume and separated in the gas chromatograph as before. The retention time of diethyleneglycol diacetate relative to that of butanediol diacetate was 1.48. The amounts of glycerol and butanediol which had been acetylated were calculated relative to the area of the diethyleneglycol diacetate peak. The results (Fig. 2) clearly indicate that complete acetylation of butanediol as well as glycerol was accomplished in 1 hr. Horrocks and Cornwell (10) also found complete acetylation of glycerol after 1 hr under comparable conditions. (They examined the products by thin-layer chromatography.)

Glycerol, butanediol, and their respective acetates are volatile to a certain extent when aqueous or acid extracts are evaporated to dryness (4). However, when excess ethanol, ethyl acetate, and water were boiled off as described in the procedure, only negligible loss of

butanediol diacetate and glycerol triacetate occurred. Gas chromatographic analyses proved that under these conditions less than 0.5% butanediol diacetate and less than 0.1% glycerol triacetate were lost.

Neutralization and Extraction Procedure

Acetic acid is soluble in diethyl ether and will be partly extracted by this solvent from an aqueous phase. Neutralization before extraction was therefore included to avoid having acetic acid in the ether phase. It was soon realized that unless special precautions were taken, partial hydrolysis of the butanediol diacetate and glycerol triacetate would take place at alkaline pH. However, if the neutralization was carried out at 0°, NaOH added until the phenolphthalein just turned pink, and ether extraction performed immediately, no loss due to hydrolysis could be detected by GLC.

Butanediol diacetate and glycerol triacetate are relatively insoluble in water as well as in sodium acetate solution, and the extraction of both organic acetates with 3–4 volumes of ether was found to be quantitative.

RESULTS AND CONCLUSION

In order to establish the recovery of glycerol, both internal standard and known amounts of glycerol were added to aliquots (1 ml) of a serum sample and carried through the analytical procedure as described. The results, shown in Fig. 3, indicate a satisfactory recovery.

The amount of free glycerol in several freshly obtained normal sera was assayed with the gas chromatographic method. Table 1 shows that values ranging from 0.4 to 1.2 mg of glycerol per 100 ml serum were found. These values are in agreement with those obtained for normal sera by the enzymic method of Wieland (1).

The advantages of the gas chromatographic method are in particular its high specificity, due to the clean separation obtained in the gas chromatographic column, the extreme sensitivity, and the wide range of concentrations that may be determined. With the present instrument operated at maximum sensitivity as little as 0.002 μg of glycerol triacetate could be quantitatively determined. It is thus possible using ionization detectors to determine concentrations of glycerol as small as 0.01 mg/100 ml. No expensive or unstable reagents are required. However, the precision of the method is not better than ±5–10% (Table 1). This is partly due to errors in measuring the peak area.

TABLE 1 ANALYSES OF FREE GLYCEROL IN NORMAL SERA BY GLC

Serum No.	Duplicate Analyses of Freshly Obtained Serum	
	mg of glycerol per 100 *ml*	
1	0.77	0.70
2	0.55	0.60
3	0.80	0.86
4	0.73	0.68
5	0.60	0.65
6	0.35	0.45
7	1.11	1.16
8	0.91	0.98
9	1.0	1.10

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REFERENCES

1. Wieland, O. *Biochem. Z.* **329:** 313, 1957.
2. Vaughan, M. *J. Biol. Chem.* **237:** 3354, 1962.
3. Hagen, J. H., and P. B. Hagen. *Can. J. Biochem. Physiol.* **40:** 1129, 1962.
4. Hanahan, D. J. *Lipide Chemistry*. John Wiley and Sons Inc., New York, 1960, p. 187.
5. Lambert, M., and A. C. Neish. *Can. J. Research* **Sect. B, 28:** 83, 1950.
6. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50:** 152, 1957.
7. Hanahan, D. J., and J. N. Olley. *J. Biol. Chem.* **231:** 813, 1958.
8. Boltralik, J. J., and H. Noll. *Anal. Biochem.* **1:** 269, 1960.
9. Chovin, P. In *Comprehensive Biochemistry*, edited by M Florkin and E. H. Stotz. Elsevier Publishing Company, Amsterdam, 1962, Vol. 4, p. 249.
10. Horrocks, L. A., and D. G. Cornwell. *J. Lipid Res.* **3:** 165, 1962.