Acetic anhydride–trifluoroacetic acid acetolysis for the estimation of glycerol in phosphatidyl choline by gas–liquid chromatography

K. S. Holla and David G. Cornwell

Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio

SUMMARY Low glycerol recoveries are obtained when acetic anhydride–acetic acid acetolysis is used to dephosphorylate lecithin prior to glycerol (and fatty acid) analysis by saponification–acetylation and gas–liquid chromatography.

Quantitative glycerol recoveries are obtained for both phosphatidyl choline and phosphatidyl ethanolamine when a modified acetolysis using acetic anhydride–trifluoroacetic acid is employed. Modified acetolysis is limited to the analysis of glycerol alone since unsaturated fatty acids are destroyed.

KEY WORDS glycerol estimation · phosphatidyl choline · phosphatidyl ethanolamine · acetolysis · trifluoroacetic acid · glyceryl triacetate · gas–liquid chromatography

Hydrogenolysis–acetylation and saponification–acetylation have been used to synthesize glyceryl triacetate for the quantitative estimation of glycerol in neutral lipids by gas–liquid chromatography (GLC) (1, 2). These procedures yield low glycerol recoveries when they are applied in the analysis of phospholipids. The glycerol content of ethanolamine phosphatides was readily estimated by saponification–acetylation if the samples were first dephosphorylated by acetolysis (2). However, preliminary experiments suggested that acetolysis did not improve the analysis of glycerol in lecithin. In this study, an acetolysis procedure using acetic anhydride–acetic acid (3, 4) and a modified acetolysis procedure using acetic anhydride–trifluoroacetic acid are compared for the estimation of glycerol in both phosphatidyl ethanolamine and phosphatidyl choline. Furthermore, the effect of acetic anhydride–trifluoroacetic acid on fatty acid components of the phosphatides has been investigated.

Materials. "L-α-Cephalin (chromatographically pure)" and "L-α-lecithin (chromatographically pure)" were purchased from General Biochemicals Inc. (Chagrin Falls, Ohio). Trifluoroacetic acid was purchased from Eastman Organic Chemicals (Rochester, New York). All solvents were reagent grade. Other materials have been described previously (1, 2). Crude phospholipids were prepared from fresh egg yolk as the acetone-insoluble and petroleum ether-soluble fraction (5). These phospholipids were then separated by chromatography (5, 6) on basic alumina (M. Woelm, Eschwege, Germany). Phosphatidyl choline (PC) was eluted with chloroform–methanol 1:1 (v/v). The solvent system was changed to ethanol–chloroform–water 5:2:2, and phosphatidyl ethanolamine (PE) was eluted. Fractions were identified by thin-layer chromatography (TLC) and pooled. TLC analyses were obtained with chloroform–methanol–water 65:25:4 (7, 8). The two phospholipid fractions were purified further by chromatography on Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pa.) with chloroform–methanol 68:32, a solvent system described by Rhodes and Lea (5). These purified fractions and the commercial phospholipid samples were judged homogeneous by TLC. Fatty acid ester (9) and phosphorus (10) were determined. Ester-to-phosphorus ratios were: commercial PC, 1.99; egg yolk PC, 2.01; commercial cephalin, 2.08; egg yolk PE, 2.00.

Gas–Liquid Chromatography. Analyses were obtained with an Aerograph A-350-B instrument (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) equipped with a Wheelco Type A electronic integrator (Barber-Colman Co., Rockford, Ill.). Ten-foot stainless steel columns, 0.25 inch i.d., containing 10–13% ethylene glycol succinate polyester on 60–80 mesh Gas Chrom P were purchased from Applied Science Laboratories (State College, Pa.). Operating conditions were described previously (2). The peak area for glyceryl triacetate was

corrected for molar response relative to hexadecanyl acetate (2, 11).

Acetolysis with Acetic Anhydride–Acetic Acid. From 60 to 100 mg of phospholipid and a known amount of hexadecanyl acetate were refluxed with 10 ml of acetic anhydride–acetic acid 1:4 for 8–10 hr. The acetolysis mixture was evaporated using first a water-pump and then high vacuum. The residue was refluxed with sodium methoxide (about 50 mg of sodium dissolved in 10 ml of methanol) for 2 hr. Methanol was evaporated and 5 ml of water was added for saponification. This mixture was refluxed for 2 hr. Glyceryl triacetate was then prepared as described previously (2). Although in that study quantitative glycerol recoveries were obtained with PE, the glyceryl triacetate yield with commercial and egg yolk PC varied from 35 to 51% (Table 1). These recoveries increased to 63–73% when 10 ml of acetic anhydride–acetic acid was added after the first acetolysis reaction and this mixture was refluxed for an additional 10 hr. Bevan et al. (3) reported that acetyl dipalmitin was prepared in practically quantitative yield by the acetolysis of phosphatidyl (dipalmitoyl) choline. Renkonen (12) recently stated that nearly quantitative acetolysis yields were obtained with egg PC. However, the actual yields were not reported in these studies. Ansell and Spanner (13) have suggested that acetolysis is not quantitative. Our glycerol recovery data indicate that this acetolysis procedure is not quantitative when applied to PC.

Acetolysis with Acetic Anhydride–Trifluoroacetic Acid. From 60 to 100 mg of phospholipid and a known amount of hexadecanyl acetate were refluxed with 10 ml of acetic anhydride–trifluoroacetic acid 4:1 for 10 hr. Extensive charring occurred even in the absence of lipid. The acetolysis mixture was evaporated using first a water-pump and then high vacuum. The flask was flushed with nitrogen to remove traces of the acetolysis reagents. The residue was dissolved in 20 ml of methanol. This solution was refluxed with sodium methoxide (about 70 mg dissolved in 10 ml of methanol) for 2 hr. Methanol was evaporated and 3 ml of water and 2 ml of 1 N sodium hydroxide were added to insure complete saponification. The mixture was refluxed with occasional swirling for 2.5 hr. Glyceryl triacetate was then prepared as described (2). The glycerol content of both PC and PE was estimated accurately by this procedure (Table 2).

Acetolysis was also measured by the phosphorus partition between aqueous and chloroform phases. A PC sample was hydrogenated, and aliquots were refluxed with the two acetolysis mixtures. The acetolysis mixture was evaporated and the products were dissolved in chloroform–methanol 2:1. Water was added and the phosphorus content of the aqueous and chloroform phases was determined. Acetic anhydride–acetic acid acetolysis yielded 44.7 and 53.9% phosphorus in the chloroform phase and 55.3 and 46.1% phosphorus in the aqueous phase. Acetic anhydride–trifluoroacetic acid acetolysis yielded 2.3 and 2.9% phosphorus in the chloro-

TABLE 1 GLYCEROL CONTENT OF PHOSPHATIDYL CHOLINE ESTIMATED BY ACETOLYSIS WITH ACETIC ANHYDRIDE–ACETIC ACID FOLLOWED BY SAPONIFICATION–ACETYLATION AND GLC*

Sample	Glycerol		Recovery
	Known	Found	
	μmoles	*μmoles*	%
PC (egg yolk)	93†	41 ± 1 (5)‡	44
	93	41 ± 1 (5)	44
	93	47 ± 3 (3)	51
	79	39 ± 2 (5)	49
	79	38 ± 3 (5)	48
	79	40 ± 2 (5)	51
	171	72 ± 2 (5)	42
	171	85 ± 2 (5)	50
	171	59 ± 1 (4)	35
	157	115 ± 3 (5)	73§
	157	99 ± 3 (6)	63§
PC (commercial)	59	27 ± 1 (6)	46
	59	39 ± 3 (11)	66§
	59	38 ± 2 (12)	64§

* Hexadecanyl acetate was the internal standard.
† Estimated from phosphorus content.
‡ Mean ± SD. Number of tracings are in parenthesis.
§ Additional acetolysis reagent and increased reflux time.

TABLE 2 GLYCEROL CONTENT OF PHOSPHATIDYL CHOLINE AND PHOSPHATIDYL ETHANOLAMINE ESTIMATED BY ACETOLYSIS WITH ACETIC ANHYDRIDE–TRIFLUOROACETIC ACID FOLLOWED BY SAPONIFICATION–ACETYLATION AND GLC*

Sample	Glycerol		Recovery
	Known	Found	
	μmoles	*μmoles*	%
PC (egg yolk)	114†	103 ± 3 (5)‡	90
	114	116 ± 4 (5)	102
	114	113 ± 1 (6)	99
	114	108 ± 8 (4)	95
	79	71 ± 1 (7)	90
	79	72 ± 2 (6)	91
	157	156 ± 3 (6)	99
	157	150 ± 4 (12)	96
	157	155 ± 5 (7)	99
PC (commercial)	59	54 ± 2 (5)	92
	80	79 ± 3 (5)	99
	118	116 ± 5 (8)	98
	118	109 ± 4 (5)	92
PE (egg yolk)	127	118 ± 2 (4)	93
	127	116 ± 4 (5)	91
Cephalin (commercial)	96	92 ± 1 (6)	96
	96	94 ± 1 (5)	98
	96	96 ± 2 (6)	100
	84	83 ± 0 (4)	99

* Hexadecanyl acetate was the internal standard.
† Estimated from phosphorus content.
‡ Mean ± SD. Number of tracings are in parenthesis.

form phase and 97.7 and 97.1% phosphorus in the aqueous phase. These results confirm the glycerol recovery data reported in Tables 1 and 2.

Since hexadecanyl acetate was used as the internal standard, it appeared that saturated hydrocarbon chains were not degraded by acetolysis with acetic anhydride–trifluoroacetic acid. Tripalmitin gave the theoretical ester-to-glycerol ratio after acetolysis–hydrogenolysis–acetylation. However, unsaturated fatty acids were destroyed. For example, only trace peaks corresponding to unsaturated fatty alcohol acetates were observed on GLC when egg yolk phospholipid was subjected to acetolysis with acetic anhydride–trifluoroacetic acid followed by hydrogenolysis–acetylation. Degradation was also noted on TLC. Acetyl diglycerides, synthesized from egg yolk phospholipids by acetolysis with acetic anhydride-acetic acid, have nearly the same R_F as tripalmitin in hexane–ether solvents. The product obtained with egg yolk phospholipid by acetolysis with acetic anhydride–trifluoroacetic acid streaked from the origin and showed no distinct glyceride spots. A streak was also found with the charred acetolysis mixture after it was refluxed without lipid. Model compounds such as dipalmitin and monopalmitin were, therefore, refluxed with acetic anhydride–trifluoroacetic acid. Products corresponding to the mono- and diacetyl derivatives of the saturated glycerides were visible on TLC. Since glyceride derivatives were detected after reacting saturated glycerides with the acetolysis mixture and since no glyceride derivatives were detected after the acetolysis of highly unsaturated egg yolk phospholipid, it appeared that unsaturated fatty acid moieties were destroyed. Meade and Walder (14) found in their acetolysis studies that side reactions at double bonds were enhanced with strong acid catalysts. These side reactions limit the application of acetolysis with acetic anhydride–trifluoroacetic acid to the analysis of glycerol alone.

We are indebted to Dr. J. B. Brown and Dr. L. A. Horrocks for discussions and to Mrs. Vallya Freimanis for technical assistance.

This study was supported in part by PHS Research Grant GM-09506 from the National Institutes of Health, U. S. Public Health Service.

Manuscript received August 18, 1964; accepted November 12, 1964.

References

1. Horrocks, L. A., and D. G. Cornwell. *J. Lipid Res.* **3:** 165, 1962.
2. Holla, K. S., L. A. Horrocks, and D. G. Cornwell. *J. Lipid Res.* **5:** 263, 1964.
3. Bevan, T. H., D. A. Brown, G. I. Gregory, and T. Malkin. *J. Chem. Soc.* 127, 1953.
4. Hoefnagel, M. A., A. Van Veen, and P. E. Verkade. *Rec. Trav. Chim.* **81:** 461, 1962.
5. Rhodes, D. N., and C. H. Lea. *Biochem. J.* **65:** 526, 1957.
6. Lea, C. H., D. N. Rhodes, and R. D. Stoll. *Biochem. J.* **60:** 353, 1955.
7. Malins, D. C., and H. K. Mangold. *J. Am. Oil Chemists' Soc.* **37:** 576, 1960.
8. Horrocks, L. A. *J. Am. Oil Chemists' Soc.* **40:** 235, 1963.
9. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6:** 158, 1953.
10. Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr. *J. Biol. Chem.* **207:** 1, 1954.
11. Horrocks, L. A., D. G. Cornwell, and J. B. Brown. *J. Lipid Res.* **2:** 92, 1961.
12. Renkonen, O. *Acta Chem. Scand.* **18:** 271, 1964.
13. Ansell, G. B., and S. Spanner. *Biochem. J.* **88:** 56, 1963.
14. Meade, E. M., and D. M. Walder. *J. Am. Oil Chemists' Soc.* **39:** 1, 1962.