# Studies with Clearing Factor III. Application of Column and Thin Layer Chromatography for Separation of Lipid Hydrolysis Products By Clearing Factor.* (31175)

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Many researchers have employed column and thin layer chromatography for the separation of lipids. Qualitative thin layer chromatographic separation of cholesterol esters, triglycerides, fatty acids, cholesterol, di- and monoglycerides, and phospholipids on silica gel plates of normal plasma and of patients with lipid disorders has been reported(1). Also, separation of 1,3- and 1,2-diglycerides and $\alpha$- and $\beta$-monoglycerides by thin layer chromatography, after pancreatic lipase digestion of fats, has been demonstrated(2).

Silicic acid column chromatography was used for a similar purpose by Borgstrom(3), Fillerup and Mead(4), and Hirsch and Ahrens(5).

In this report, the digestion products of the action of Clearing Factor on coconut oil were studied. The presence of $\alpha$-, $\beta$-monoglycerides and 1,2- and 1,3-diglycerides intermediates were indicated by various thin layer and column chromatographic procedures. The free fatty acids were the major end product of hydrolysis.

Among several separation methods of complex lipid mixtures, column chromatography in combination with thin layer chromatography produced the best information and is recommended.

*Materials and methods.* Post-heparin Clearing Factor was prepared as reported previously(10). Tripalmitin, triolein, and cholesterol were purchased from Mann Biochemical Laboratories. Monoolein, monopalmitin, 1,2- and 1,3-dipalmitin were supplied by Dr. J. Hirsch.

Conditions of hydrolysis of Ediol with Clearing Factor was the same as previously reported(9,10). Five ml aliquots were taken at zero time, 1 hr, 3 hr, and overnight from the incubation mixtures. Each aliquot was extracted with 50 ml of Bloor solution (3 parts ethanol:1 part diethyl ether).

Thin layer plates were (20 × 20 cm glass) coated with a 225-250 layer of silica gel G and air dried(6). The plates were then activated for one hour at 160° and placed in a desiccator.

For 2-dimensional chromatography, petroleum ether (b.p. 40-60°):ether:acetic acid (80:20:1) for the first dimension and chloroform:benzene (3:2) for the second dimension was used. Plates were (in the corner area, corresponding to monoglyceride spot) sprayed with 0.1 M potassium metaperiodate at pH 4, after developing with first solvent. During this procedure the diol portion of the glycerol moiety of the $\alpha$-monoglyceride was oxidized and became less polar. The mobility of this derivative of the $\alpha$-monoglyceride was similar to those of diglycerides, and thus was separated from the $\beta$-monoglycerides (Fig. 2).

Methods of detection. Developed plates were placed in iodine vapor to make the spots visible. Other detection methods used were phosphomolybdic acid (10% in ethanol), 5% chromic acid in 50% (V:V) sulfuric acid, bromocresol green (0.04% in ethanol, titrated to a blue color with 0.1 N NaOH), bromothymol blue (0.04% in distilled water titrated to a blue color with 0.1 N NaOH), acetic anhydride:sulfuric acid:ethanol (5:5:50), dichlorofluorescein (0.2% in 96% ethanol), rhodamine B (0.2% in 96% ethanol), and hydroxylamine-ferric chloride(6).

Bromothymol blue was sprayed after allowing the acetic acid to evaporate. On drying, fatty acids and monoglycerides appeared as bright yellow spots, and diglycerides and cholesterol as orange brown spots on an orange background. With bromocresol green, only free fatty acids appeared as yellow spots on a blue background.

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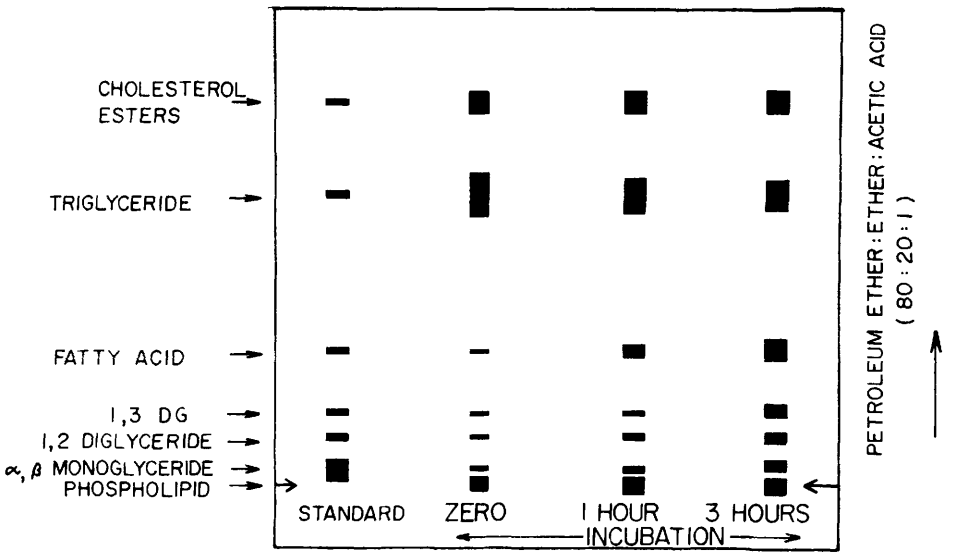


FIG. 1. Thin layer chromatographic separation of lipids from incubation mixtures of Clearing Factor with Ediol. Absorbant: Silica gel G 250 $\mu$ thick on plates. Developer: (diethyl ether: petroleum ether: acetic acid 20:80:1). Developing time: 40 min. Detector: iodine vapor and phosphomolybdic acid spray.

Unsaturated compounds absorbed iodine vapors more strongly than saturated compounds.

Spots were then outlined and copied on thin paper.

Elution. Spots made visible by iodine vapor were marked and the iodine allowed to evaporate. Each band or spot was then scraped off the plate into a conical centrifuge tube. Lipids, extracted with methanol, were used after evaporation of solvent for specific assays or rechromatogrammed to test the degree of purity.

Preparation of columns. Columns (1.8 $\times$ 30 cm) were made with 13 or 18 g silicic acid activated at 160°. The solvents used were reagent grade and were not purified further. The samples (80 mg lipids) to be assayed were dissolved in petroleum ether (10 ml) and placed on the column.

Samples of the eluate (4 ml) were collected in a fraction collector, and the solvent evaporated. Each sample was redissolved in a small volume of chloroform (40 $\mu$g in 5 $\mu$l), spotted on thin layer plates and developed as described above.

*Results and discussion.* Separations of lipids from the incubation mixtures of Clearing Factor with Ediol at zero time and 1 and 2 hours are presented in Fig. 1(2). The results indicated that the triglyceride spot was decreased but the spots of free fatty acids, 1,2- and 1,3-diglycerides and monoglycerides were increased in size and after 3 hours' incubation as compared to the spots at zero time.

Free cholesterol, in this solvent system, was not separated from 1,3-diglycerides. Also separation of monoglycerides ($\alpha$ and $\beta$) was not observed. Saturated and unsaturated triglyceride, fatty acids, and triglycerides of short or long chain fatty acids had similar mobility.

Fig. 2 demonstrates that the size of $\alpha$- and $\beta$-monoglycerides spots were increased in size several-fold for the extracts of 3-hour incubation mixtures of Ediol treated with Clearing Factor. Zero time extracts had only a trace or no monoglycerides.

The isolation of these lipid components at a preparative scale with one-dimensional chromatography was not successful because of the complexity of these mixtures as well as some degree of absorption of lipids on each other. Therefore, preparation of less complex lipid mixtures was tried by column chro-

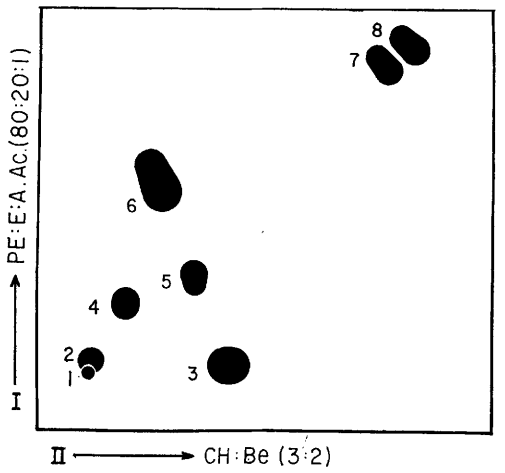


FIG. 2. Two dimensional, thin layer chromatographic separation of hydrolysis products of Ediol incubated 3 hr with Clearing Factor. I. Petroleum ether:ether:acetic acid (80:20:1). II. Chloroform: benzene (3:2). See periodate spray before developing with second solvent as described in *Methods*. 1—phospholipid, 2—β-monoglycerides, 3—α-monoglycerides, 4—1,2-diglycerides, 5—1,3-diglycerides, 6—fatty acids, 7—triglycerides, 8—chloresterol esters.

matography. Then these simpler mixtures were separated by thin layer chromatography.

Column chromatography on silicic acid was performed on lipid mixtures before and after digestion by Clearing Factor (Fig. 3). In this system, 80 mg of sample was chromatographed on a silicic acid column as described in *Methods*.

Extracts obtained from zero time incubation mixture have shown low concentration of fatty acids and no or trace amounts of mono- and diglycerides. Samples at 2 and 3 hours have shown highest amounts of diglycerides and monoglycerides. Larger spots for 1,3-diglycerides than for 1,2-diglycerides were observed at all times. Smaller spots of monoglycerides and larger spots of fatty acids were detected, however, in overnight samples.

Although cholesterol was not separated from 1,3-diglycerides on thin layer with petroleum ether:ether:acetic acid solvent system, column chromatography made the separation possible. Also by column chromatography it was possible to separate 1,2- and 1,3-diglyceride or α- and β-monoglycerides. Thin layer chromatography contributed to the separation of these compounds from each other.

With 25% methanol in diethyl ether whole new spots were eluted, with mobility and reactions similar to those of cholesterol esters, triglycerides, fatty acids and diglycerides and

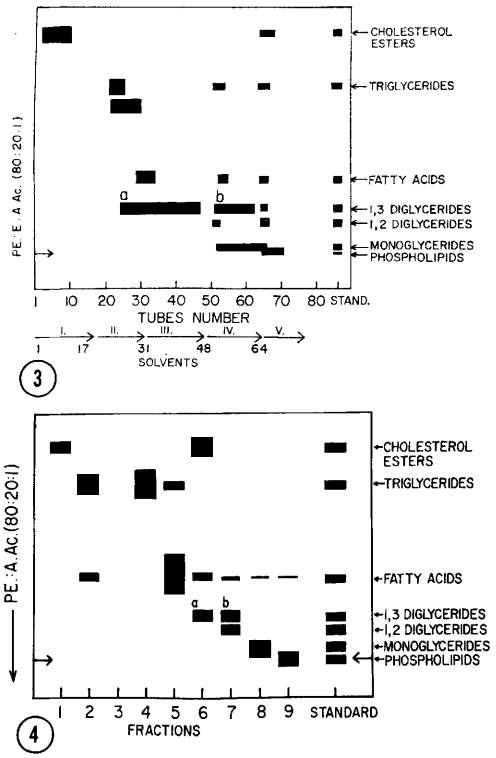


FIG. 3. Thin layer chromatography of fractions obtained by column chromatoraphy of lipids according to Fillerup and Mead(4). Extracts of 3 hr incubation mixture of Ediol with Clearing Factor were fractionated on column as described in the text. Fractions shown were prepared by column chromatography. Solvents (200 ml each) used were: I. 1% diethyl ether in petroleum ether (DE:PE), II. 4% DE:PE, III. 10% DE:PE, IV 50% DE:PE, and V. 25% methanol in DE.

FIG. 4. Thin layer chromatography of fractions obtained by column chromatography of lipids according to Hirsch and Ahrens(5). Extracts of 3 hr incubation mixture of Ediol with Clearing Factor were fractionated on column as described in the text. a—cholesterol. b—1,3-diglycerides. Fractions were prepared by silicic acid column chromatography. Following solvents (200 ml each) were used: (1) 350 ml of 1% ether in petroleum ether, (2) 60 ml of 4% ether:petroleum ether, (3) 120 ml of 4% ether: petroleum ether, (4) 140 ml of 4% ether:petroleum ether, (5) 200 ml of 8% ether:petroleum ether, (6) 450 ml of 8% ether:petroleum ether, (7) 250 ml of 25% ether:petroleum ether, (8) 300 ml ether, (9) 400 ml of methanol.

the rest of monoglycerides. These may be entirely new compounds or the tailing of the ones eluted in previous solvents. Modification of the ether:petroleum ether ratios did not help to correct this tailing effect during the column-chromatography procedure.

With a method based on procedures used by Borgstrom(3), separation of the complex lipid mixtures were tried. Solvents used were (200 ml each): (a) benzene:petroleum ether (2:8), (b) benzene, (c) chloroform:benzene (2:98), (d) chloroform:benzene (1:4), (e) chloroform, (f) methanol.

In this procedure tailing was not observed, and chloroform did not elute any remaining compounds despite the sudden change in the composition of the solvent mixture. However, the overall separation was less efficient.

The most efficient of these procedures was the one described by Hirsch and Ahrens(5), in combination with thin layer chromatography. In this procedure no tailing effect was observed during elution with the last solvent, as was observed with the method shown in Fig. 3(4). Therefore, a combination of this method with thin layer chromatography made this procedure more recommendable than others.

As was demonstrated with previous procedures, this method also showed none or trace amounts of mono- or diglycerides for zero time extracts of incubation mixtures of Ediol with Clearing Factor. They were present in large spots at the 2- and 3-hour samples but monoglycerides decreased in overnight samples. 1,3-diglycerides were found in greater quantities than 1,2-diglycerides as shown by the size of their spots.

It has been reported that the diglycerides (3,5) and monoglycerides(7,5), accumulate during the hydrolysis of triglycerides by Clearing Factor. This was considered as an indicator of position specificity of this enzyme. However, no attempt was made in the earlier work to identify the type of di- or monoglyceride that was accumulated. On the other hand, Korn claimed that Clearing Factor had no fatty acid and position specificity and that the free fatty acids which accumulated were hydrolyzed randomly; hydrolysis was considered as total without accumulation of any mono- and diglycerides, a concept not consistent with the accumulation of random hydrolysis products(8). Our previous observation indicated that unsaturated triglycerides with high dienoic and trienoic acid content, *i.e.*, corn and soya bean oils, were hydrolyzed by Clearing Factor at a rate slower than that for coconut oil(9). Also the present report demonstrates an accumulation of mono- and diglyceride intermediates similar to the observations made by others(3,5,7). Selective hydrolysis of fatty acids from $\alpha$- and $\beta$-positions may be affected by the presence of fatty acids with dienoic and trienoic acids. Studies to elucidate this point are under way, using the fractionation procedure modified in this laboratory.

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