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Quantitative Analysis by Liquid Chromatography*,†

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Summary

A method has been developed for performing rapid, quantitative analyses by liquid chromatography. Microgram quantities of lipids were separated on semimicro silicic acid columns by eluting them with a succession of solvents. Separations similar to those performed by thin-layer chromatography were accomplished in similar time periods. The analyses were quantified using a liquid-chromatography detector based on the difference in volatility between the compounds to be detected and the eluting solvents. The column effluent was deposited on a continuously moving metal chain. The solvent was evaporated at a controlled temperature. The residue was then carried into a heated tube in which it was volatilized or pyrolyzed in an atmosphere of nitrogen. The resulting vapors and pyrolysis products were aspirated into a hydrogen flame-ionization detector. Both high-boiling and nonvolatile lipids were detected quantitatively with fairly uniform sensitivity.

A variety of separations of both volatile and nonvolatile compounds can be performed by liquid-liquid chromatography and liquid-solid chromatography (LLC and LSC) that would be difficult or impossible by gas-liquid chromatography (GLC). Since many of the advantages of GLC stem from the high sensitivity of the GLC detector, the possibility was studied of using a comparable detection technique with these other forms of chromatography.

Most GLC detectors continuously monitor the concentration of a vapor in a carrier gas with very different physical or chemical

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properties. In liquid chromatography a similar approach may not be practical, since the optimal solvents for eluting a given compound often are chemically related to it. Furthermore, separations can often be performed more easily by changing the composition of the solvent during the course of the analysis. One of the few generally applicable approaches is to use eluting solvents that are appreciably more volatile than the compounds to be analyzed and to detect these compounds after evaporating the solvents. A sensitive method for accomplishing this assay is to vaporize the residue and deliver the vapors to a gas-chromatography detector. Several techniques for accomplishing this have now been described (1–6).

A system has been developed for detecting compounds that range in volatility from methyl esters of long-chain fatty acids through much less volatile sterol esters and triglycerides (7). This paper describes the use of this system for the analysis of lipids, such as those found in blood plasma, on semimicro silicic acid chromatographic columns. A primary objective was to determine if analyses comparable to those usually performed by TLC could be made quantitative.

EXPERIMENTAL

Flame Detector for LLC

The detection system has been described in detail previously (7). The effluent of the LLC column is deposited on a continuously moving, endless platinum chain. The solvent is removed by a stream of heated air. The residue is then carried by the chain into a quartz tube filled with nitrogen in which it is subjected to increasingly high temperatures. Volatile materials are evaporated and nonvolatiles are pyrolyzed. The resulting vapors are aspirated into a hydrogen flame-ionization detector. Because of the geometry of the pyrolysis tube, the vapors leaving the chain flow toward the detector inlet and are aspirated quantitatively. Each compound is subjected to temperatures only high enough to volatilize or pyrolyze them and losses due to carbonization are minimized. As a result, both high-boiling volatile compounds and nonvolatile compounds are detected with close to the same sensitivity. A similar device is now available commercially (Packard Instrument Company, Inc., Downers Grove, Ill.).

The electrical conductivity of the flame was monitored in the usual way except that the time constant of the measuring circuit

was increased to approximately 8 seconds. During the elution of a compound from the column, the conductivity of the flame increases in spurts, as the detector responds to each drop of effluent. The longer time constant smoothed the curves without causing any significant loss of resolution and made measurement of peak areas easier.

Micro Columns

The columns consisted of two sections of borosilicate glass tubing fused together. The upper section, the solvent reservoir, was 7 mm I.D. and 50 mm long. The lower section, which contained the chromatographic packing, was thick-walled capillary tubing, 2 mm I.D. and from 25 to 100 mm long.

The column packing was Unisil-activated silicic acid (Clarkson Chemical Co., Williamsport, Pa.), either 100–200 mesh or 200–325 mesh. The solvents consisted of various mixtures of the following reagents: petroleum ether, diethyl ether, methanol, formic acid, benzene, and ethyl acetate. Each reagent was redistilled in glass prior to use.

The columns were packed by filling the reservoir with a slurry of silicic acid in diethyl ether. When the silicic acid settled and filled the narrow-bore tubing, the excess silicic acid and ether were removed and two reservoir volumes, approximately 5 ml of petroleum ether, were passed through the column. The sample was then injected below the surface of the silicic acid with a Hamilton 10-μliter syringe. Elution was then carried out by either of two methods. In the first, the petroleum ether remaining in the reservoir was removed almost completely and replaced by the first eluting solvent. When sufficient time had elapsed for the elution of the first compound or class of compounds from the column, the solvent remaining in the reservoir was removed and replaced by the second eluting solvent. This process was repeated until the desired number of solvents had been added. In the second method, the addition of solvents to the reservoir was automated. A constant head of solvent was obtained by mounting a 250-ml reservoir filled with petroleum ether approximately 1 m above the bench top. This reservoir drained through a length of 0.010-inch stainless-steel capillary tubing which was crimped to provide a flow of approximately 0.4 ml/min of petroleum ether. To the end of this capillary tubing were attached, in sequence: a short length of 1.5-mm-I.D. Teflon tubing, a three-way stopcock fitted with 1/16-inch-diameter

stainless-steel tubes at two of the ports and a 10-ml syringe at the third, and a 10-ft length of 1.5-mm-I.D. Teflon tubing. The entire length of Teflon tubing was filled with petroleum ether from the reservoir. The stopcock was then turned, and the desired volumes of each of the eluting solvents, in the reverse order of their use, were drawn up into the tubing with the syringe. Each solvent was separated from the next by approximately 0.5 inch of air. When the solvents had been drawn up, the syringe was excluded from the flowing stream, and the reservoir was reconnected. Solvents then drained into the small solvent reservoir of the micro column at a rate preset to equal the rate at which solvents pass through the columns (0.4 ml/min). Elution with a continuous flow of a succession of different solvents each flowing at a predetermined rate, with a minimum of mixing of one with the next, was thus accomplished.

RESULTS

Separation of Cholesterol Esters, Triglycerides, Free Cholesterol, and Phospholipids

Analysis of approximately 20 μg each of cholesteryl palmitate, triolein, and cholesterol on a 120-mm-long silicic acid column took

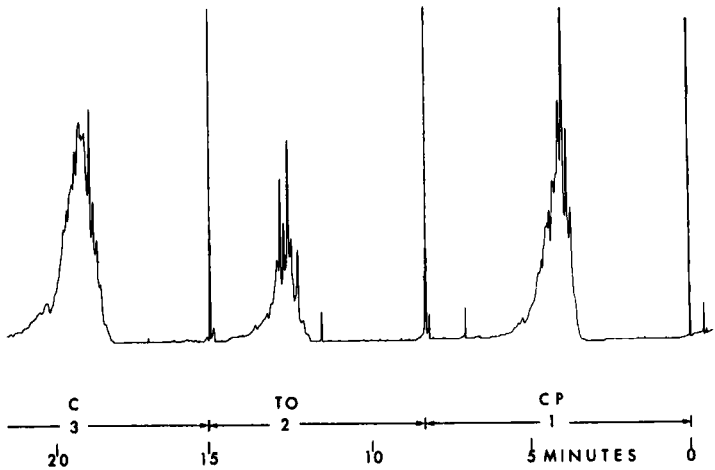

FIG. 1. Analysis of approximately 20 μg each of cholesteryl palmitate (CP), triolein (TO), and cholesterol (C) on a 120-mm-long, 2-mm-I.D. silicic acid column. Solvent changed at times indicated by arrows. Solvent 1, 95% petroleum ether, 5% diethyl ether; solvent 2, 75% petroleum ether, 25% diethyl ether; solvent 3, diethyl ether. Full scale sensitivity, 10^{-8} A.

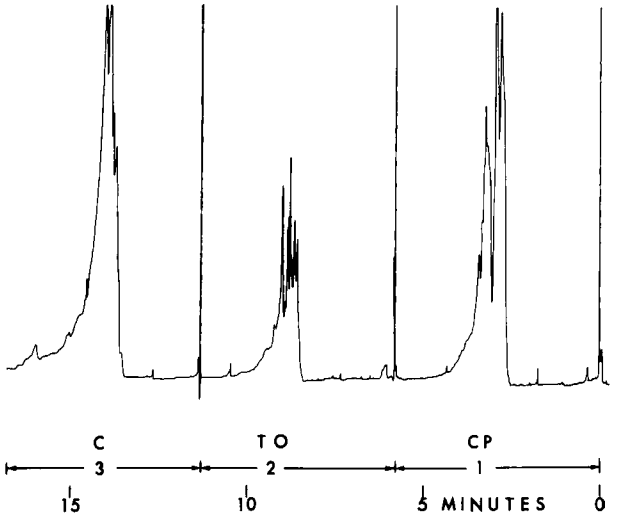

FIG. 2. Analysis of a mixture similar to that in Fig. 1 using an 85-mm-long, 2-mm-I.D. column and the same sequence of solvents. Solvent changes shown by arrows. Full scale sensitivity, 10^{-8} A.

approximately 20 minutes if each solvent was permitted to flow until the compound to be eluted had emerged completely from the column and the detector had returned to its base-line level (Fig. 1). Analysis of a similar mixture on a column 85 mm long took 15 minutes (Fig. 2); analysis on a 77-mm-long column, 10 minutes (Fig. 3). No difference in resolution in these three analyses is apparent.

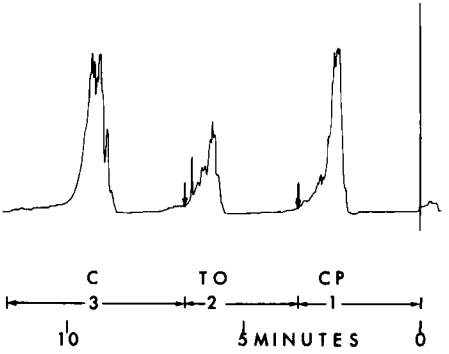

FIG. 3. Analysis of a mixture similar to that in Fig. 1 and 2 using a 77-mm-long column, 2-mm-I.D. and the same sequence of solvents changed as indicated by the arrows. Full scale sensitivity, 3×10^{-8} A.

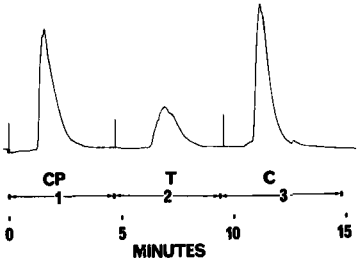

FIG. 4. Separation of the same mixtures as in Fig. 1, done with automatic delivery of the succession of solvents.

The analysis of a mixture of cholesteryl palmitate, triolein, and cholesterol by successive solvents added automatically by the system described is shown in Fig. 4. Separation of a mixture containing lecithin as well as the above three lipids required only slightly more time (Fig. 5). The reproducibility of repeated analyses is shown in Table 1.

In these analyses, free fatty acids emerged from the column slightly after the triglyceride peak but were not completely separated from it. Complete separation of free fatty acid from triglyceride was accomplished with either a 100-mm column filled with 100–200 mesh Unisil or a 50-mm column filled with 200–325 mesh Unisil. Even though the solvent flow through these columns was slower, it was still possible to separate cholesterol esters, triglycerides, free fatty acids, free cholesterol, and phospholipids in a single analysis in only slightly more than 30 minutes.

Separation of Steroids and Steroid Esters

The same approach was used to separate testosterone from testosterone acetate (Fig. 6). Separation of this mixture was complete in less than 15 minutes on a 70-mm-long column, even though the

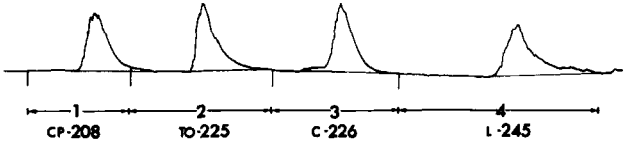

FIG. 5. Separation of approximately 20 μg each of cholesteryl palmitate, triolein, cholesterol, and lecithin; the first three solvents were as in Fig. 1. The fourth was methanol/formic acid, 1:1. Areas are given beneath each peak.

TABLE 1

Reproducibility of Analyses of Lipid Mixtures
(each contains 4 lipids, 15 μg each)

Sample	Cholesteryl palmitate	Triolein	Cholesterol	Lecithin
1	382	365	364	220
2	382	389	368	114
3	444	375	372	179
4	413	360	408	230
5	398	396	405	121
Mean	404	377	383	173

second solvent was not added until the testosterone acetate had completely emerged from the column.

This separation could be accomplished either with two successive solvents or with a single solvent of intermediate polarity. A single solvent (benzene/acetone 90:10) just polar enough to

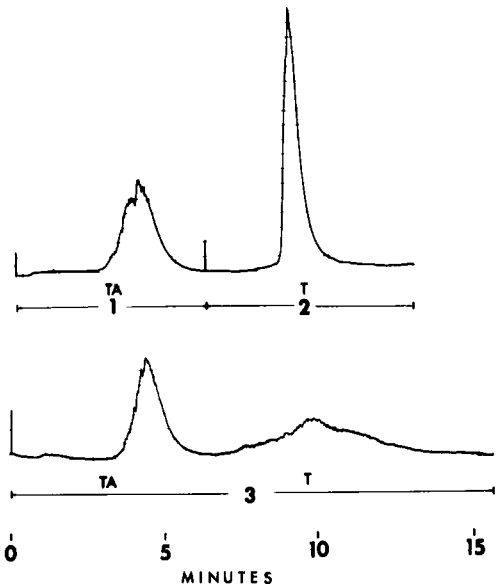

FIG. 6. The separation of testosterone acetate and testosterone with successive solvents compared to the separation with a single solvent of intermediate polarity. Solvent 1: benzene/acetone 90:10; solvent 2: acetone; solvent 3: benzene/acetone 85:15.

elute the acetate as a sharp peak also eluted the free sterol if given sufficient time, but the second peak was greatly spread out. As the polarity of the solvent was increased, the testosterone emerged as a sharper peak, but the separation between the two was compromised. The lower record in Fig. 6 was obtained using a single solvent, the most polar solvent that would give a complete separation between the two peaks. The upper record shows the more easily quantifiable record obtained by using two successive, different solvents.

This separation of testosterone from its ester was of interest because of the possibility of using it as part of an ultramicro analytical method for serum testosterone involving synthesis, isolation, and detection of the haloacetate. To substitute for the TLC in this procedure, however, the micro column had to do more than simply separate sterol ester from free sterol. It had to isolate these compounds from other kinds of compounds in the mixture. A five-solvent system was therefore used. The first solvent eluted all compounds less polar than the sterol acetate; the second, the sterol acetate; the third, all compounds intermediate in polarity between the sterol acetate and the free sterol; the fourth, the free sterol; and the fifth, more polar material. Separation of a synthetic mixture of testosterone acetate and testosterone using this system of solvents is shown in Fig. 7. This chromatographic system was used for assessing the yield of the acylation reaction. Testosterone labeled with carbon-14 was added to the serum as an internal standard. Chromatography of the original extract showed that the radioactivity was all eluted with the free sterol by solvent 4 (Table 2, column 1). Following acylation, most of the radioactivity was eluted by solvent 2, indicating that the conversion of the steroid to the haloacetate was almost quantitative (reaction mixture I, Table 2).

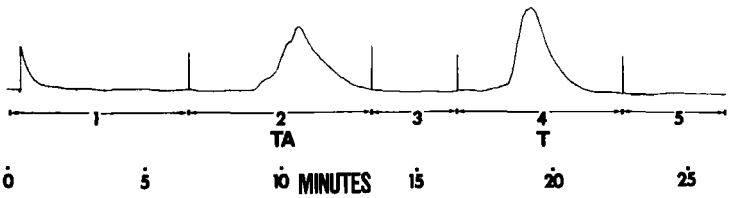

FIG. 7. Isolation of testosterone acetate from testosterone with five successive solvents added automatically. Solvent 1: benzene; solvent 2: benzene/ethyl acetate 90:10; solvent 3: benzene/ethyl acetate 90:10; solvent 4; ethyl acetate; solvent 5: methanol/formic acid 50:50.

TABLE 2

Acylation of ^{14}C-Testosterone Studied Using Micro Silicic Acid Column Chromatography (^{14}C in each sample eluted from the column, cpm)

Sample	Starting material ^{14}C-testosterone	Reaction mixture I	II	III	IV
Total cpm applied:	18,094	10,172	10,010	9,132	7,990
Fraction 1 (benzene 100, 1.2 ml)	28	94	87	632	52
Fraction 2 (benzene/ethyl acetate 80:20, 1.2 ml)	24	10,715	9,800	6,745	7,160
Fraction 3 (benzene/ethyl acetate 80:20, 0.6 ml)	75	190	209	31	82
Fraction 4 (benzene/ethyl acetate 80:20, 1.2 ml)	17,835	168	369	29	229
Fraction 5 (ethyl acetate 100, 1.2 ml)	318	46	642	36	52
Fraction 6 (methanol/formic acid 50:50, 1.2 ml)	113	357	1,306	945	355

In other reaction mixtures (II, III, IV), a small fraction of the ^{14}C-testosterone was converted to a highly polar decomposition product that was eluted by the last solvent. Analysis by GLC of a concentrate of the material eluted by solvent 2 revealed only very small quantities of compounds other than the haloacetate, less than when the same separations were done by TLC.

DISCUSSION

The liquid-chromatography detection system used here detects compounds that range in volatility from those that can be analyzed by medium-high-temperature GLC to those that are volatile only if pyrolyzed. The sensitivity to all these compounds is fairly uniform, partly because of the nonspecificity of the flame-ionization detector but also because both vapors and pyrolysis products are aspirated

into the detector quantitatively. The sensitivity is almost as high as that of the flame detector used with a GLC column.

This detection system responds almost as rapidly as the usual GLC detector. Since compounds generally emerge from a liquid column more slowly than from a GLC column, a detection system that responds so rapidly is often not required. The analyses described were an attempt to accomplish the same kinds of rapid separations by column chromatography as are usually done by TLC and to take advantage of the rapid response of the detector.

The detection system can also be used to monitor a more conventional LLC and LSC column from which compounds emerge over extended time periods. In these analyses, however, it may be useful to concentrate the effluent before delivering it to the detector so that many minutes worth of residue is delivered all at once. Since the flame responds in proportion to the rate of delivery of organic compound, this would increase the sensitivity of the system.

In TLC, all compounds in a mixture do not travel to the end of the TLC plate. Solvents therefore can be used that move one compound appreciably while moving another hardly at all. Similar separations can be accomplished in column chromatography if the compounds are eluted with a succession of solvents. Since the sample quantities, the chemical nature of the stationary and moving phases, the lengths of stationary phases, and the flow rates of solvent are all similar in TLC and in the micro columns, one might reasonably predict that similar separations could be obtained in comparable time periods. The validity of this prediction was borne out by the experimental results.

Resolution is difficult to specify in column chromatography when different solvents are put through the column in succession. The width of a peak does not seem to be increased appreciably during the passage of any of the solvents up to the one that finally elutes the compound. Peak width is thus a poor measure of the resolving power of the column. The separation of compounds on the columns may also be less complete than is indicated on the record. For example, if a compound "trails" excessively, the succeeding solvent can gather it up on the column and elute it as a peak that is indistinguishable from that of a second compound. This uncertainty can be reduced by passing a second solvent through the column after a compound is apparently completely eluted. This solvent should be more polar than the first but not polar enough to elute the next compound of interest. If the second solvent does not elute appre-

ciable material from the column, one can reasonably assume that the elution of the previous compound is adequate and that the separation of the two compounds is complete. The accuracy of the identification of peaks depends on the proper choice of these solvents.

When the addition of solvents is made automatic, the elution of a given compound is taken away from the direct control of the operator and retention times measured on the record are somewhat more useful for identifying compounds. On the other hand, the retention volumes of various compounds, if properly noted, may serve as an equally good index.

Since detection of microgram quantities is not difficult for a hydrogen flame-ionization detector, it is theoretically possible to extend the sensitivity of the liquid detector well into the submicrogram range. One factor that limits the sensitivity is the level of impurities in the solvents. As each solvent passes through the column, its polar impurities are retained on the silicic acid. Succeeding solvents elute these impurities as peaks that are not distinguishable from those of the compounds of interest. Difficulties from this source are reduced if the solvents are prepurified by distillation.

Micro-column chromatography is more convenient than TLC in several ways other than that quantification by the system described is easier. Larger volumes of samples can be injected into a micro column so that the preparation of the sample is simplified. Compounds emerge from the column dissolved in a volatile solvent ready for GLC or for liquid-scintillation counting. Much of the effort involved in applying the sample to the TLC plate and in eluting and recovering the compounds from the plate is therefore avoided.

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