Microdetermination of Long-chain Fatty Acids in Plasma and Tissues*

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The ternary mixture, heptane-isopropyl alcohol-water, provides a convenient two-phase system for extraction of long-chain fatty acids (1). When the solvent components are taken in suitable proportions, the phases separate rapidly without centrifugation. The long-chain fatty acids distribute predominantly into the upper, nonpolar phase, whereas the more polar acids remain below.

This method of extraction has been used mainly for studies of changes in the concentration of fatty acids in blood plasma (1) and for measuring the output of fatty acids from isolated pieces of adipose tissue (2). The need in studies of this kind is for a method that is sensitive, reproducible, and rapid. Absolute specificity is not required providing that the interfering materials are constant and relatively low in concentration, and that the interpretations depend only on changes in concentration of the fatty acids. These conditions are satisfied in the usual analyses of blood plasma and of nutrient media; for these studies, a single extraction method is ideally simple and precise.

It must be emphasized, however, that the single extraction procedure may not be adequate for study of tissue lipids. Two or more distributions will be required when only traces of long chain fatty acids are mixed with larger amounts of other organic acids and acidic phospholipids. The extraction necessarily becomes more elaborate, but even with complex mixtures a practical method of separating fatty acids from acidic contaminants usually can be worked out if one knows the kinds and relative proportions of materials present, and their partition coefficients in the two-phase extraction system.

Other two-phase systems containing the same solvents in different proportions are also available for extraction, and some of these may provide greater selectivity for special purposes. To make efficient use of the many possibilities for differential extraction one needs a general map of the ternary system and a table of partition coefficients. The present paper outlines the phase relations of the ternary system at room temperature, gives partition coefficients of fatty acids and other materials in a standard two-phase extraction mixture, discusses use of the system in countercurrent extraction, and outlines some modifications of the original procedure that have developed during five years of analytical work in this laboratory.

EXPERIMENTAL PROCEDURES AND RESULTS

Phase Relations—The phase relations as a function of composition are shown in Fig. 1, the composition being specified by the relative volumes of the three liquid components entering into any given mixture. This representation, which differs from the more usual mapping in terms of weight fractions, is convenient for the analyst who usually measures his solvents by volume. The composition by weight can of course be computed from the volume fractions by taking account of the densities.

The curved line in the figure shows the transition from one-phase to two-phase systems. Mixtures represented by points in the area below the curve form two phases with compositions indicated by intersections of the straight tie line containing the given point and the curved saturation line. A family of mixtures represented by points on the same tie line are composed of the same two phases, but with different relative volumes. Above the line all mixtures form a single phase.

This diagram, which is only an approximate mapping, brings out an important practical point. The advantages of rapid and clean separation of phases are realized only in the upper part of the two-phase area (indicated by the background shading). Below this area pure ternary systems separate more slowly, and in the presence of biological materials the systems tend to form stubborn emulsions.

The differences in ease of phase separation may be explained in part by the variation in interfacial tension, which ranges from 51 dynes per cm at a heptane-water interface (3) to zero at the plait point. The interfacial tensions of phase pairs corresponding to tie lines of intermediate position were estimated by measuring the relative drop weights of lower phases delivered slowly from a pipette into the equilibrated upper phase. It can be seen that the favorable region for extraction corresponds to phase pairs with low interfacial tension, roughly, those with tensions less than 2 dynes per cm. At these low tensions the surface active lipids and proteins seem to be excluded from the interface, and the systems separate rapidly.

The standard extraction system lies near the center of the favorable region. The upper point in Fig. 1 shows the one-phase system obtained by adding plasma, 1 volume, to 5 volumes of the extraction mixture: 20% water (1 N H₂SO₄), 78% isopropyl alcohol, 20% heptane. Further additions of water, 2 volumes, and heptane, 3 volumes, shift the mixture to the two-phase system: 28% water (0.03 N H₂SO₄), 36% isopropyl alcohol, 36% heptane.

Effect of pH—The presence of a strong acid in low concentration has no significant effect on the phase volume relations, but it does markedly influence the partition of weak acids. If the aqueous phase of an extraction mixture is neutral, no significant amount of long-chain fatty acid appears in the upper phase; when enough strong acid is added to bring the lower phase to pH 2.5, a maximal amount of fatty acid distributes to the sol-
Preparations differ in buffer capacity largely because of differences in content of protein. If the buffering in a series of extractions is variable, it may be convenient to acclify with H$_2$PO$_4$ which dissociates with $pK_a = 2.1$ and thus provides a suitable buffer.

**Partition of Fatty Acids and Some Metabolites in System 28% Water (0.033 x H$_2$SO$_4$), 36% Isopropyl Alcohol, 36% Heptane**—Table I gives the partition ratios of fatty acids and some metabolic acids in the standard two-phase system. The "recovery factor," $f$, in this table shows the fraction of each acid that distributes to the heptane phase, relative to the fraction of palmitic acid distributed there.

$$f = \frac{r_0(4r + 7)}{r(4r + 7)} = \frac{4.63r}{4 + 7}$$

The coefficients 4 and 7 indicate the relative volumes of heptane and aqueous phase, respectively; $r_0 = 11.0$ is the partition ratio of palmitic acid, and $r$ is that of the acid being determined. As can be seen in Table I this factor is close to unity for fatty acids longer than lauric (12:0). The mean recovery factor for fatty acid mixtures usually encountered in biological studies can be taken as 1.00 without significant error.

The recovery factor is useful in evaluating the interference due to polar organic acids and to cephalins in single extraction analysis. For example, lactic acid has a recovery factor of 0.014. This means that the lactic acid in a preparation will register in single extraction analysis as 1.4% of its actual concentration. If a sample of plasma contains lactic acid in concentration of 1 meq per liter, the apparent concentration of long-chain acids will be increased by 14 meq per liter, an insignificant amount. If, however, the sample contains 10 to 20 meq per liter (as it might under some conditions) the interference would be excessive.

The interference can be easily eliminated by a second extraction. If an aliquot of upper phase is transferred to a second tube and shaken with blank lower phase (maintaining the 4:7 volume ratio), and a palmitic acid standard is treated similarly, the recovery factor becomes $f'$. As can be seen in Table II, this second distribution greatly increases the specificity of analysis. With a single extraction, the long-chain fatty acids of normal plasma account for 80 to 90% of the measured acidity; the remainder is divided between cephalins, lactic acid, and acetic acid. A second distribution increases the specificity. Interference from lactic, acetic, and $\beta$-hydroxybutyric acids is virtually eliminated even under the most extreme conditions likely to be encountered in biological material; the high values in Table II represent possible concentrations of these acids in plasma after vigorous muscular work (lactic acid), in ruminants (acetic acid), and in diabetic coma (\(\beta\)-hydroxybutyric and acetocetic acids).

A second distribution of the plasma extract should be made whenever interference from polar acids is suspected; double extraction reveals the presence of polar contaminants (by comparison of aliquots taken from upper phase after the first and second distributions) and eliminates their interference. It also makes the cephalin interference relatively unimportant, but in this instance the success of the method depends partly on the low initial concentration of cephalins in normal blood plasma (4). Some tissues contain huge amounts of cephalins relative to non-esterified fatty acids, and for these the double extraction method fails. Multiple washing of the extract with blank lower phase
TABLE II

Specificity of single and double extraction methods in analysis of plasma

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| Long-chain fatty acids | 1.00 | 700 | 700 | 700 | 1500 | 1200 | 10 |
| Acetic (plus formic and propionic) | 0.08 | 300 | 24 | 2 | 20,000 | 250 | 4 |
| Lactic                | 0.014 | 1200 | 17 | 2 | 20,000 | 250 | 4 |
| Pyruvic               | 0.022 | 100 | 2 | 2 | 20,000 | 250 | 4 |
| β-hydroxybutyric (plus acetoacetic) | 0.025 | 25 | 110 | 49 | 250 | 250 | 13 |
| Cephalins             | 0.44 | 250 | 110 | 49 | 250 | 250 | 49 |
| Total organic acidity | 2550 | 853 | 751 | 42,550 | 1712 | 776 |
| Percentage of long-chain fatty acids | 27 | 82 | 93 | 31 |

would remove the interference, but an appreciable part of the fatty acids would be lost and the fatty acid composition of the remainder would be distorted. Countercurrent distribution, as described in the next section, avoids this difficulty, and at the same time discloses the relative proportions of fatty acids and acidic contaminants.

Phospholipids in blood and tissues may cause additional difficulty by their tendency to hydrolysis (5). The aliquots of upper phase should always be removed promptly after formation of the two-phase extraction system, since additional acid may be released from the lower phase. The amount of acid thus generated in the usual extraction of plasma appears to be quite small, but splitting of phospholipid can cause considerable error in preparative work. The proportions of different fatty acids contained in phospholipids differ markedly from those in the nonesterified fatty acids of plasma, and the turnover rates of these fractions are of different magnitude.

Countercurrent Distribution—The two-phase extraction system is well suited to countercurrent analysis (6). The phases separate rapidly, and fatty acids contained in tissue extracts distribute theoretically, without appreciable interaction. The independence of distribution was shown by experiments in which the fatty acids present in extracts of plasma, red blood cells, and liver migrated at the theoretical rate calculated from partition coefficients of pure acids (Figs. 2, 3, 4). A further test, even more sensitive to interaction, confirmed this result. A tracer amount of C¹⁴-palmitic acid was added to extracts of plasma and liver, and to carrier palmitic acid dissolved in the extraction system. The extract represented by the continuous line was made immediately after sacrifice. A portion of the same liver (dotted line) was incubated 2 hrs. at 37°, then extracted and fractionated. Eight transfers all three preparations showed essentially the same distribution of radioactivity, and this distribution conformed to theory.

Eight transfers produce an appreciable fractionation of fatty acids in the leading tubes. This effect was utilized to determine the partition coefficients of fatty acids not available in pure form. A mixture of naturally occurring fatty acids was obtained by extracting liver with chloroform-methanol (7) and saponifying the esters. The mixture was fractionated by eight-transfer coun-
tercurrent distribution and the fatty acid compositions of solvent phases in the leading tubes were determined by gas-liquid chromatography (8). The relative partition coefficients could be calculated from these data since the amount of any fatty acid relative to the amount of palmitic acid in each tube varies from tube to tube as a linear function of the relative partition ratio. The ratio of concentrations plotted against tube position on semilogarithmic paper yields a straight line; the slope gives the relative partition ratio and the constancy of the ratio is shown by absence of curvature.

The values thus estimated for saturated fatty acids were essentially the same as the values found on partition of pure acids. The values obtained for unsaturated acids showed the expected increase in polarity associated with the presence of double bonds (Table I).

**Recommended Procedures**

**Single Extraction Method**

**Extraction**—To 2 cc of fresh (9) plasma in a glass-stoppered tube add 10 cc of the extraction mixture: 1 cc H$_2$SO$_4$ (0.1 vol.), heptane (1 vol.), isopropyl alcohol (4 vol.). Shake; allow to stand at room temperature about 5 minutes (the exact time not critical), and then add water (4 cc) and heptane (6 cc). Shake again. The system should separate cleanly into two phases within 5 minutes. Take duplicate 3-cc aliquots of upper phase without delay and deliver them into 15-cc conical bottomed centrifuge tubes.

If material other than plasma is being extracted, the pH of the aqueous phase should be checked and if necessary adjusted to pH 2.0 to 2.5.

**Standard and Blank**—Recrystallized palmitic acid is weighed and dissolved in heptane to make a reference solution with known concentration. It should be distributed in the extraction system with every set of analyses.

The blank extract contains 2 cc of water instead of plasma; the standard, 6 cc of palmitic acid solution rather than 6 cc of heptane. It is convenient to make the concentration of the palmitic acid reference solution about 50 mg per liter, which is about one-third the concentration of nonesterified fatty acids in plasma, and to assign to this solution a nominal value (in meq per liter) three times its actual concentration, so as to allow for the greater volume of reference solution in the standard. The concentration of fatty acids in plasma is the product of the nominal value and the ratio of titrations of unknown and standard extracts.

**Titration**—Fig. 5 shows a Gilmont microburette, slightly modified. In order to provide access for the centrifuge tubes used in titration, it is necessary to cut a scallop from the base under the pipette tip. A movable clamp is needed to hold the tube. The microburette should be mounted at eye level on a heavy stand, and apparatus arranged so that the operator can sit comfortably with elbows on bench top while titrating. Behind the tube is a sheet of white paper. In front of it and slightly above is a movable fluorescent lamp which can be adjusted by the operator to give optimal lighting.

Nitrogen is washed through a column containing dilute alkali and an indicator, and is delivered to the bottom of the titration tube through a capillary tube. Fig. 5 shows the stopcock used to divert the gas or deliver it into the tube. The alkali wash is necessary, even with the purest commercial gas.

For a sharp end point the alkali used for titration should contain a minimal amount of carbonate. A suitable reagent can be made by taking the clear supernatant of saturated NaOH (which has stood quietly for two weeks or longer to settle out insoluble carbonates) and diluting it 1:1000 with freshly distilled water. Deliver approximately 0.05 cc of the supernatant into a 50-cc glass-stoppered cylinder; fill immediately to the mark with the CO$_2$-free water; stopper and mix. In general the exact normality of the reagent is unimportant since unknown extracts are determined by comparison with a standard. If, however, absolute rather than relative values are needed the alkali can be standardized by titrating a suitable amount of standard HCl or H$_2$SO$_4$.

The indicator solution is made by diluting 10 volumes of stock solution (0.1% thymol blue in water) (10) to 100 volumes with freshly redistilled ethanol. The acidity of this solution is important. If it is too acid, the blank titration will be excessive; if neutral, the solution will take up CO$_2$. In the latter case the net titration values for extracts of fatty acids will be too low since part of the acidity in the extract will be neutralized by the bicarbonate buffer in the indicator solution. It has proven convenient to adjust the acidity (with dilute HCl or with the titration alkali) so that 1 cc of indicator solution requires about 10 ul of titration alkali (about 0.018 N) for neutralization.

A 3-cc aliquot of upper phase is transferred into a 15-cc conical-bottomed centrifuge tube, and 1 cc of indicator solution is added to it. The tube is mounted so that the burette tip stands just above the surface and the gas capillary reaches the bottom. When the nitrogen is driven through the tube the burette tip is washed by splashing but the tip should not be immersed continuously in the solution. The end point (a mustard yellow color) is read by stopping the nitrogen flow momentarily to permit separation of phases. It is quite sharp, and with practice should be reproducible to about 1 uliter of alkali in the usual titration of plasma.

**Double Extraction Procedure**—Extract 4 cc of plasma or aqueous extract with double the quantity of reagents used in the single
extraction method. Take duplicate 3-cc aliquots of upper phase for titration of single extraction acidity, and transfer an 8-cc aliquot to another extraction tube. Add 14 cc of blank lower phase (the lower phase taken from a blank extraction); mix by inverting about 25 times, and after separation of phases take duplicate 3-cc aliquots for titration.

Titrate extracts of standard palmitic acid treated similarly, and calculate the apparent fatty acid concentrations given by single and double extractions. The re-extraction will decrease the concentration of standard and of long-chain fatty acids by about 15%. A greater drop in acidity shows the presence of interfering polar acid. If its chemical nature is known, its concentration in plasma can be estimated by dividing its recovery factor into the change in apparent fatty acid concentration.

Determination of Total Esterified and Nonesterified Fatty Acids—Extract the total lipids with chloroform-methanol (6), or other suitable solvent. Take an aliquot containing 2 to 20 amoles of esterified fatty acid and remove the solvent by evaporation under a stream of nitrogen. Add 0.5 cc of 0.5 N KOH in methanol, and reflux in 80° bath for 2 hours. Neutralize with 2 cc of 0.15 N HNO₃ and extract with the reagents and volumes that would be used for determination of nonesterified fatty acids in 2 cc of plasma. To calculate the total amount of fatty acid yielded by the sample use the same standard as in the determination of nonesterified fatty acids; calculate as usual to obtain the concentration of fatty acids in the 2 cc solution after saponification, and multiply by 0.002.

Alternatively, if the lipids are present in dilute aqueous solution (e.g. a dilute solution of lipoproteins), it might be more convenient to add alkali and isopropyl alcohol, saponify in the dilute solution, then acidify and shift the system to the two-phase region by adding a suitable amount of heptane.

Countercurrent Analysis—For routine examination of unknown extracts it has been convenient to use a 10-tube, all glass, manually operated Craig apparatus (6), with 5 cc of upper phase and 10 cc of lower. Loading the machine, making eight transfers and sampling the upper phases (the tenth tube provides a blank) requires less than 30 minutes. This procedure divides the organic acidity of the extract into three general classes: fatty acids, cephalins, and more polar acids. Titration of 3-cc aliquots from upper phases and estimation of partition ratios from the position of the peaks provides a measure of the quantities present in the original extract.

SUMMARY

A single extraction of blood plasma with a two-phase heptane-isopropyl alcohol-water system provides sufficient analytical specificity for determination of long-chain nonesterified fatty acids under usual conditions. If exceptional quantities of lactic, acetic, acetoacetic, or β-hydroxybutyric acids are present, a second extraction eliminates their interference.

The system is also useful for countercurrent distribution. The table of partition coefficients of fatty acids and various organic acids, given in the present paper, permits one to calculate the number of transfers needed for analytical or preparative work. As an initial step in studying unknown mixtures, a simple 8-transfer procedure, requiring less than one-half hour, separates the acidity of an extract into three classes: long-chain fatty acids, cephalins, and polar organic acids.

The phase volume relations and interfacial tensions of the ternary system are given. These data map out a family of two-phase systems which, like the standard system employed in the present work, separate rapidly and cleanly without centrifugation.

REFERENCES
