Quantitative Radiometric Analyses of ^{14}C-Labelled Fatty Acids by Gaschromatography

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In connection with metabolic studies in humans on the incorporation of labelled fatty acids into the chylomicron lipids the necessity arose of using several labelled fatty acids in order to follow the ratio of specific activity of the different compounds.[1] Gas liquid chromatography has proved a very useful technique both for the analysis and purification of labelled compounds in these studies.[2] In order to avoid the misleading results that may be obtained by condensing fractions and measuring their activities individually it is desirable to measure continuously the activity of the gas coming from the column of the gas chromatograph.[3,4]

In the technique described by James and Piper[3] substances emerging from a gas-liquid partition column are quantitatively oxidized on copper oxide to form carbon dioxide and water. The water is reduced to hydrogen by finely divided iron. In the present work the quantitative aspects of the radioassay of higher labelled fatty acids has been investigated using gas chromatography in combination with a proportional counter.[3]

Apparatus and methods. The gas chromatograph employed is a preparative "Pye" chromatograph equipped with a ionization chamber. Instead of the stainless steel splitter equipped to the apparatus a glass splitter of pyrex glass was constructed. In the conventional splitter condensed radioactive material was very easily trapped and it was difficult to clean the splitter. The glass splitter used in our experiments was not contaminated. The glass splitter may be easily dismantled for decontamination without affecting its calibration.

The glass splitter has significantly improved the performance of the apparatus. It is connected by means of silicon rubber fitting to a stainless tube 12 cm long and having an internal diameter of 10 mm. This tube is filled in the first section with copper oxide and in the second one with reduced iron on inert support and is heated to 800 ± 10°C by means of an electronically controlled furnace. The radioactivity of the CO_2 peak is assayed by an 10 ml internal flow proportional counter at room temperature.

The sample is introduced with the aid of a spiral of platinum direct on the top of the column as described earlier.[5] The main advantage with this technique is that an exact amount of labelled material can be introduced into the gas chromatograph. The length and size of the column, its stationary phase, the operating temperature and the carrier gas flow rate are chosen according to the sample being analyzed. In the experiments described the separation is carried out by means of a glass column (1200 mm long, 10 mm I.D.) packed with 14 % poly-ethylene-glycol-succinate on acid washed silanized Gas-chrom P 100–120 mesh. The output of both the ionization chamber and the proportional counter is fed into a two-channel potentiometric recorder (Texas Model PWS). The argon flow through the column was 140 ml/min and 1/50 of this flow is led through the ionization chamber and is diluted 10 times with Argon. When the proportional counter is employed inactive CO_2 is used to dilute

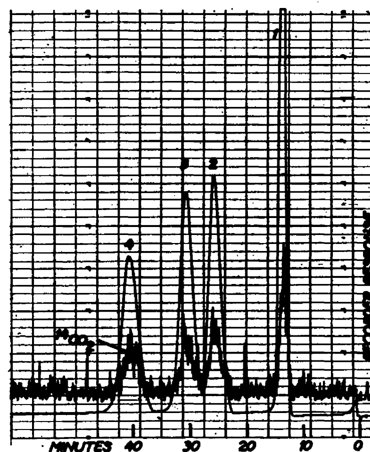

Fig. 1. Gas chromatographic separation of a four-component mixture containing methyl ester of 1. Palmitic acid-1-^{14}C; 2. Stearic acid-1-^{14}C; 3. Oleic acid-1-^{14}C; 4. Linoleic acid-1-^{14}C. An ionization chamber was used for determination of the mass. The effluents of the gas chromatograph are simultaneously assayed for radioactivity with a proportional counter after combustion to CO_2.

Table 1. Analyses of mixture of ^{14}C-labelled palmitic, stearic, oleic, and linoleic acid with known specific radioactivity. An ionization chamber was used for determination of the mass, and a flow proportional counter for determination of $^{14}CO_2$.

Fatty acid Me-ester	Calc. % activity	Found activity %			Average	Calc. spec. act. cps/mg	Found spec. act. cps/mg
		I	II	III			
16:0	30.3	31.8	33.1	31.4	32.3	167	162
18:0	25.0	25.2	26.7	26.3	25.9	202	197
18:1	26.0	24.6	23.2	24.2	24.0	137	137
18:2	18.7	18.4	17.0	18.1	17.8	106	106

the effluents from the gas chromatograph so that CO_2 is about 4 % of the gas flow through the counter. Small changes in this ratio in a series of analyses do not affect the plateau of the counter. However, since the efficiency of the radioactivity detector and the performance of the column depends on the flow rate, a soap film flowmeter is employed to determine the flow rate. The total activity of a compound is calculated from the area of the corresponding peak just as the area under the mass peak was found to be proportional to the amount of mass present.

Radioassay of fatty acid fractions other than the GLC effluents was performed by liquid scintillation counting using an Eccho liquid scintillation Counter Model 610. Samples were taken to dryness in the counting vials and 5 ml of a solution of 3 % diphenyloxazole in toluene was added.

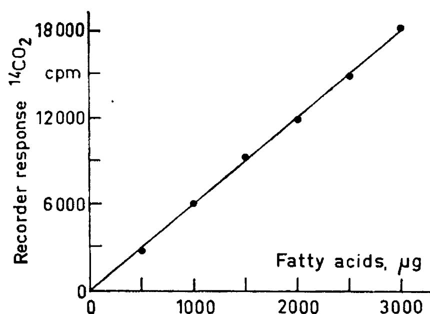

Fig. 2. Radio-gaschromatography of palmitic-1-^{14}C acid with different amount of labelled material put on the column showing linear response of the proportional counter.

Comments. A typical analysis of a mixture of carbon-14 labelled palmitic, stearic, oleic, and linoleic acid is shown in Fig. 1. This mixture has previously been analysed by gas-liquid chromatography and purified as methylester on silicic acid and hence free of radioactive impurities. The sensitivity of the apparatus employed allows detection of a single carbon-labelled compound having a minimum activity of about 1 mμC.

Quantitative analyses have been carried out with the use of weighed quantities of fatty acids having known specific activities. In Fig. 2 is shown analysis of ^{14}C-labelled palmitic acid with increasing amount of material put on the column. It was found that in range of 500—3000 μg the proportional counter gave a linear response although the shape of the peak differed. In order to get reproducible results it was found that the most reliable technique was to cut out the peaks from the recorder chart and to weigh the paper.

In Table 1 the results are given and a comparison made between the theoretical value of the specific activity and the found specific activity after separation of a test mixture containing ^{14}C-labelled palmitic, stearic, oleic, and linoleic acid. The technique has been tested in biochemical experiments where the amount of radioactivity is small so that several mg of the material has been loaded on the column.

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Gas Chromatography of β-Hydroxybutyric Acid and Biochemically Related Compounds

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In metabolic studies of diabetes mellitus there is a great need for specific methods for the quantitative determination of the ketone bodies (acetone, β-hydroxybutyric acid, and acetoacetic acid) and related metabolic intermediates[1] (*e.g.* pyruvic and lactic acids and the components of the tricarboxylic acid cycle). The available biochemical methods for the determination of the ketone bodies are cumbersome and unsatisfactory recoveries are obtained, especially with regard to β-hydroxybutyric acid.[2]

No satisfactory gas chromatographic technique has yet been published for separation of the ketone bodies and related compounds. During the last years there has, however, appeared a few reports on the separation of the biologically important di- and tricarboxylic acids.[3-5] In the present report a study has been made of the gas chromatographic separation of β-hydroxybutyric, acetoacetic and related acids, and the quantitative aspects of this analysis has been investigated.

Experimental. An Aerograph Hi—Fi 600 Gas Chromatograph with a flame ionization detector was used together with a 1 mV potentiometric recorder (Servo/riter, Texas Instruments Inc.). Coiled glass columns, 1500 mm long and with 2 mm I.D., were packed with 25 % by weight of Castorwax (Wilkens Instrument Inc.) on 100 to 120 mesh acid-washed and dimethyldichlorosilane-treated Chromosorb W. The columns were preconditioned overnight at 180°C. The following gas chromatographic conditions were employed: injection port temperature 250°C, oven temperature 125°C, flow rate of carrier gas (nitrogen) 25 ml/min and flow rate of hydrogen 25 ml/min.

The acids to be analyzed were dissolved in ether and converted to their methyl esters through the addition of an ethereal solution of diazomethane. A Hamilton microsyringe was used for the injection of the samples. The reference compounds used were of the highest purity commercially available and were in most cases gas chromatographically pure within reasonable limits.

Results. In Table 1 are listed the retention times of the methyl esters of some organic acids relative to the ester of β-hydroxybutyric acid. A good separation between the different acids can be achieved as illustrated in Fig. 1, which shows a chromatogram of four biologically important acids.

Table 1. Relative retention times of the methyl esters of some organic acids.
(β-Hydroxybutyric acid methyl ester = 1.00, 5.3 min)

Acetic 0.07; propionic 0.11; butyric 0.19; valeric 0.37; caproic 0.69; enanthic 1.30; octanoic 2.45.
Lactic 0.42; β-hydroxybutyric 1.00.
Pyruvic 0.30; α-ketobutyric 0.53; acetoacetic 0.76; α-ketocaproic 1.69.
Oxalic 0.57; malonic 0.95; succinic 1.97.

These four acids, pyruvic, lactic, acetoacetic, and β-hydroxybutyric acids, were also chosen for a quantitative study of the method. There is a linear relation between the amount of ester injected and the peak area recorded, which is illustrated for β-hydroxybutyric acid in Fig. 2. In Table 2 the results of three gas chromatographic analyses of a mixture of the four acids are presented compared to the weight composition of the mixture. In the quantitative experiments the peak areas were either compared to that of octanoic acid methyl ester as an internal standard or factors for the individual acids determined from a known standard mixture. Both methods gave satisfactory results.

Comments. These preliminary results indicate that it is possible to separate and quantitate a variety of low molecular