notes on methodology

Determination of volatile free fatty acids of human blood

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SUMMARY A simple and accurate method for the extraction and quantitative estimation of individual, volatile free fatty acids of human blood is described. Values in normal serum, plasma, and red cells are given. Besides acetic and propionic, normal and isobutyric and isovaleric acids have been found.

SUPPLEMENTARY KEY WORDS extraction · gas–liquid chromatography · aqueous solutions · polymer beads · butyric · valeric

THE NUTRITIONAL importance of volatile fatty acids (VFA) in the ruminant has led to many investigations concerning these compounds of low molecular weight, including their estimation in the blood (1–4). However, the significance of these short-chain acids in the blood of nonruminants, especially man, has not been properly studied and methods for their extraction, identification, and quantification have not been standardized.

Various procedures have been described in the literature for obtaining VFA concentrates from ruminant blood. Erwin, Marco, and Emery (5) lyophilized the deproteinized blood filtrate after neutralization with alkali and extracted the VFA with acidified ether or methanol. Ramsey (6) found that the deproteinized filtrate contained considerable amounts of substances other than the organic acids and deemed it necessary to remove them before quantifying the VFA by gas–liquid chromatography (GLC). Scarisbrick (7) recommended estimation of blood VFA by steam distillation of whole blood in the presence of potassium oxalate and oxalic acid (pH 4.0), but recoveries and values for blood VFA were not given.

McClendon (8) steam-distilled deproteinized human blood in the presence of phosphoric acid and reported a titer of 0.0003 N for total blood VFA. Annison (4) steam-distilled deproteinized blood filtrates at pH 3.0 and reported 0.28–0.3 mmole of total VFA per liter. Formic, acetic, and propionic acids constituted 23–34, 64–77, and 0–4 moles%, respectively. No other VFA was identified. Friedemann (9) conducted steam distillation of blood in the presence of magnesium sulfate, since high concentrations of $MgSO_4$ were shown to increase the rate of distillation of VFA (10). McAnnally (11) found that the VFA content of a constant volume of distillate obtained by the Friedemann procedure increased with the time taken for distillation, because of the decomposition of labile substances.

Recently, Tanaka, Budd, Efron, and Isselbacher (12) removed total free fatty acids from a chloroform–methanol extract of the total lipids of serum by means of 0.1 N NaOH. The alkaline extract was acidified with phosphoric acid and steam-distilled to obtain VFA.

The methods of estimation of VFA described in the literature have been just as varied as the methods described above for their extraction from blood. Earlier methods of partition and adsorption chromatography used to separate individual short-chain fatty acids (13, 14) have been supplanted by GLC. Because of the many difficulties encountered during GLC analyses of aqueous solutions of VFA, such as tailing and incomplete separations, methods were devised to replace the aqueous medium with organic solvents (15). The use of the flame-ionization detector, which is insensitive to water, has obviated these difficulties and aqueous solutions of VFA can now be analyzed directly.

This paper describes a simple procedure for the extraction of circulating VFA from human serum in good yields and the quantification of the normal and iso acids by GLC on polymer beads. The distribution of the various VFA between the red cells and plasma of normal human blood is also reported.

Materials. All solvents used for extraction purposes were AR grade and distilled. Glass-distilled water was used wherever necessary.

Venous blood from fasting humans was collected in Vacutainer tubes containing oxalate and fluoride. Plasma was separated from the red cells by centrifugation at 750 *g* for 30 min. The red cells, free from "buffy coat," were washed three times with cold 0.15 M saline. To obtain serum, we collected blood in ordinary glass tubes, which we kept at 37°C for 1 hr and then centrifuged.

Extraction of VFA. A satisfactory recovery of VFA from blood is an important prerequisite to their subsequent separation and quantification by GLC. The following method gave a higher yield of VFA than the procedures described by Friedemann (9) and Scarisbrick (7), and in addition revealed hitherto unreported VFA. The procedure used to prepare VFA concentrate from

Abbreviations: VFA, volatile fatty acid; GLC, gas–liquid chromatography.

TABLE 1 DETERMINATION OF ACIDS IN TWO STANDARD MIXTURES OF ACIDS

	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	*n*-Valeric
Mixture 1						
Amount present, μg	40.9	39.2	37.6	37.2	37.3	36.1
Amount estimated (mean ± SD, n = 6)	38.8 ± 1.7	38.9 ± 0.9	37.9 ± 1.0	37.1 ± 1.3	38.1 ± 0.8	36.1
% of expected	94.9	99.3	100.7	99.7	102.2	100.0
*Mixture 2**						
Amount present, μg	82.2	8.63	8.24	1.31	4.12	30.0
Amount estimated (mean ± SD, n = 6)	79.6 ± 0.7	8.46 ± 0.08	8.01 ± 0.28	1.28 ± 0.03	4.06 ± 0.05	30.0
% of expected	96.9	98.0	97.2	97.7	98.3	100.0

* Contained amounts of the acids normally found in 5 ml of plasma.

TABLE 2 RECOVERY OF PURE ACIDS ADDED TO SERUM

	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	*n*-Valeric
Acid added, μg	40.9	39.2	37.6	37.2	37.3	36.1
Acid recovered, μg	41.3	39.3	37.7	38.5	36.7	36.1
Recovery %	100.9	100.2	100.2	103.0	98.4	100.0
Acid added, μg	32.0	31.0	29.0	29.0	29.0	28.0
Acid recovered, μg	32.2	30.3	28.2	29.7	28.6	28.0
Recovery %	100.7	97.7	97.4	102.4	99.2	100.0

plasma described below is applicable also to serum and red cell fractions.

Plasma (5 ml) was extracted with 20 volumes of chloroform–methanol 2:1 in the presence of 0.2 volume of 1.0 N H_2SO_4 in a Servall Omnimixer and filtered. The residue was washed thrice with 10 ml portions of the same solvent mixture. The filtrates were combined and mixed with a volume of water equal to 0.2 the total volume of the chloroform–methanol extract, and the two phases were allowed to separate. After the interface had become sharply defined, the lower (chloroform) phase was removed and extracted with 50 ml of methanol–water 1:1 that had been equilibrated with 10 ml of chloroform. The aqueous-methanolic fractions were combined, neutralized with 1 N KOH to pH 8.0–9.0, and evaporated to dryness. The residue was acidified with 25% aqueous metaphosphoric acid to pH 3.0, 5.0 g of $MgSO_4$ was added, and the mixture was steam-distilled in a micro-Kjeldahl distillation unit (Labconco, Kansas City, Mo.). At this pH pyruvic, lactic, and β-hydroxybutyric acids, at concentrations normally encountered in blood, are not distilled off (4) and hence do not interfere with the determinations of VFA. 200 ml of distillate was collected in 0.3 ml of 1 N NaOH (pH 9.0–10.0) and evaporated to dryness in vacuo. The residue was made up to a known volume with 25% aqueous metaphosphoric acid and the VFA were analyzed by GLC.

GLC. The aqueous phosphoric acid solutions of the VFA concentrates were chromatographed on a Beckman GC-4 dual-column instrument equipped with flame-ionization detectors. The aluminum columns were 8 ft × 1/16 inch (I.D.) and were packed with 150–200 mesh Porapak Q [beads of ethylvinylbenzene–divinylbenzene polymer (Waters Associates, Framingham, Mass.)] coated with 4% phosphoric acid. Details of quantitative analysis of VFA, including column preparation, separation, and area-response factors for individual VFA with respect to *n*-valeric acid have been described previously (16). Since *n*-valeric acid was not found in blood, all quantitative analyses were performed with *n*-valeric acid as the internal standard.

Results. The over-all accuracy of the extraction and separation of the acids was tested with standard solutions of the mixed acids. Table 1 shows the results obtained in the analysis of two synthetic mixtures containing different concentrations of the acids. Mixture 1 contained roughly equal amounts of the acids and mixture 2 was made up to approximate the amounts of these acids normally found in 5 ml of plasma. Values obtained were close to 100% of expected for all the acids studied.

Table 2 shows recoveries for pure acids added to serum. Two mixtures containing different amounts of the acids were used; recovery ranged form 97 to 102%.

We checked the reliability of the method for blood VFA analysis by performing six analyses on a pooled sample of human serum (Table 3).

The mean concentrations of five VFA present in the

TABLE 3 REPRODUCIBILITY OF VFA ANALYSIS OF HUMAN SERUM

Acid	Concentration*
	μg/100 ml
Acetic	1150 ± 16
Propionic	40.5 ± 1.41
Isobutyric	31.5 ± 0.93
n-Butyric	7.9 ± 0.17
Isovaleric	61.0 ± 1.27
Total	1290 ± 14.5

* Mean ± SD (n = 6).

TABLE 4 VFA CONTENT OF NORMAL HUMAN SERUM, RED CELLS, AND PLASMA

Acid	Serum	Plasma	Red Cells
	μg/100 ml of fluid or packed cells		
Acetic	1170 ± 136*	1640 ± 378	1400 ± 263
Propionic	42.7 ± 8.9	163 ± 25	188 ± 28
Isobutyric	30.7 ± 7.9	143 ± 30	96 ± 21
n-Butyric	9.7 ± 5.5	25 ± 5	16 ± 4
Isovaleric	58.3 ± 35	194 ± 80	0
Total	1320 ± 170	2170 ± 472	1700 ± 258

* Mean ± SD (n = 6 subjects).

serum of six normal subjects selected at random and the distribution of blood VFA between plasma and red cells of the same subjects are shown in Table 4. Acetic acid constitutes 89% of the total VFA in serum and isovaleric and propionic acids, 4.4 and 3.2%, respectively. Isobutyric and butyric acids are also present in minor amounts. A significant amount of VFA is present in the washed red cells. Acetic and propionic acids of blood are approximately equally distributed between the plasma and the red cells (hematocrits were 45–47.5%). The plasma contains more isobutyric and butyric acids than the red cells. All the isovaleric acid is present in the plasma and none in the red cells. The total VFA content of serum is less than that of either plasma or red cells. Presumably some VFA is adsorbed onto the clot and not completely released in the serum.

The washings obtained during the preparation of red cells were saved and analyzed. Acetic and propionic acids were present in the washings to the extent of 15% of the amounts in the washed red cells. However, it was not possible to conclude whether these acids were derived from the surface of the cells or by transport through the cell membrane. No isobutyric or butyric acid was detected in the washings.

Discussion. The determination of VFA in blood entails essentially two steps: (*a*) extraction and concentration; and (*b*) separation by GLC and quantification. It is imperative that a suitable method for extraction and preparation of a VFA concentrate be employed, not only to obtain the maximum yield but also to permit proper resolution during GLC. Direct injection of as much as 10 μl of serum into the inlet system of the gas chromatograph failed to yield any peaks corresponding to VFA. Direct steam distillation of acidified plasma yielded much less VFA than the steam distillation of the aqueous-methanolic extract. GLC of the extract without steam distillation also yielded much less VFA. It is reasonable to assume, from the above results, that the VFA is bound to a protein or some other constituents of blood and not readily released. A combination of extraction, partition, and steam distillation seems to yield the maximum amount of VFA, since no VFA was detectable in the residue after distillation or in the chloroform phase. In addition, the procedures adopted here yield a VFA concentrate devoid of other substances which interfere with their subsequent determination by GLC. To test the reliability and reproducibility of the method, we have analyzed pooled frozen human plasma at regular intervals. Statistically indistinguishable results were obtained; storage of plasma had no effect on the VFA values.

Many investigators have resorted to the use of methyl esters and butyl esters rather than the free acids for separation by GLC (17–19) because of the troublesome tailing of the free acids on the GLC columns commonly used. The successful separation of aqueous solutions of the free acids on Porapak Q has obviated many of the chores involved in dehydrating aqueous media and esterification reactions.

VFA were identified by comparison of their retention times with those of standards. However, isovaleric and α-methyl butyric acids did not differ in retention time sufficiently to allow them to be separated and unequivocally identified by GLC. It is hoped that this difficulty can be resolved by the use of combined GLC–mass spectrometry.

Although formic acid is present in blood (4) and appears in the steam distillate, it was not detected by the flame-ionization detector. The extraction procedure described afforded a fortuitous and selective separation of the C_2–C_5 short-chain acids from the remaining free fatty acids. We established this by using standard mixtures containing C_2–C_{10} acids; details of these results will be reported later.

Annison (4) did not report the presence of any VFA with longer chains than propionic acid in human blood. The present work has shown that isobutyric and isovaleric acids are also present in significant amounts. *n*-Butyric acid is also present in small but detectable amounts, but we did not detect *n*-valeric acid. This is consistent with the observations of Gerson, Hawke, and Shorland (20), who showed that sodium valerate was degraded in vivo to acetate and propionate when administered to cows intravenously. A similar degrada-

tive phenomenon might account for its absence from human blood.

A detailed study of the blood VFA levels in the presence of liver disease is underway in these laboratories.

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