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AN ACCURATE, SENSITIVE AND REPRODUCIBLE METHOD FOR THE COLORIMETRIC ESTIMATION OF FREE FATTY ACIDS IN PLASMA

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ABSTRACT

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A sensitive and reproducible method permitting the estimation of 0,030–3,000 mM of free fatty acids (FFA) in plasma with a standard error of 0,006 5 was developed. The method is based on the extraction of phosphate-buffered plasma with a chloroform-heptane-methanol mixture, formation of the cobalt complex and on the subsequent determination of the metal with 1-nitroso-2-naphthol. The method was used to obtain normal values (mean 0,547 mM range 0,147–1,197 mM) for Merino wethers fed lucerne hay.

Résumé

SUR UNE MÉTHODE PRÉCISE, SENSIBLE ET REPRODUCTIBLE POUR LE DOSAGE COLORIMÉTRIQUE DES ACIDES GRAS LIBRES DANS LE PLASMA

On a mis au point une méthode sensible et reproductible qui permet d'estimer de 0,030 *à* 3,000 *mM d'acides gras libres (FFA) dans le plasma avec une erreur standard de* 0,0065. *La méthode repose sur l'extraction du plasma tamponné au phosphate par un mélange chloroforme-heptane-méthanol, la formation du complexe cobaltique et la détermination subséquente du métal par le 1-nitroso-2-naphthol. La méthode a été employée pour obtenir les valeurs normales (moyenne* 0,547 *mM, extrêmes* 0,147–1,197 *mM) chez des béliers mérinos châtrés alimentés au fourrage de luzerne.*

INTRODUCTION

Numerous methods have been developed for the determination of the concentration of free fatty acids (FFA) in biological fluids. Depending on the technique used, these may be divided into the 2 categories, namely titrimetric and colorimetric. The methods of the first type rely on the extraction of the FFA with a mixture of organic solvents and their subsequent microtitration with a weak alkali (Dole, 1956; Gordon, 1957; Trout, Estes & Friedberg, 1960). The colorimetric methods on the other hand are based on the property of the FFA to form complexes with copper or cobalt which are soluble in chloroform (Ayers, 1956). The metal content of the complexes is then determined colorimetrically with the aid of specific colour reagents such as diethyldithiocarbamate (DDC) (Duncombe, 1964), 1-nitroso-2-naphthol (Novak, 1965), diphenylcarbazide (DPC) (Laurell & Tibbling, 1967) or bathocuproine (Fukui, Kushira, Takano & Soyama, 1972).

During the course of our investigation of the fat and carbohydrate metabolism of sheep we utilized several FFA determination methods with varying degree of success. The titrimetric technique of Dole (1956) was too laborious and required the presence of 2 separate observers in order to obviate possible misinterpretations of the end-point colour. Of the colorimetric methods used, those of Lappin (1971) and Mikac-Dević, Stanković & Bosković (1973) proved of limited value since, despite much effort, we were unable to reproduce the results of these workers. Although the methods of Duncombe (1964), Novak (1965) and Falholt, Lund & Falholt (1973) proved to be more reliable, they were still often subject to sudden and inexplicable errors. In view of this we decided to combine the extraction procedure of Falholt *et al.* (1973) with our own unpublished modification of the colorimetric technique of Novak (1965). We were thus able to develop a method which was sensitive, accurate and gave consistently reproducible results. The new method was used in the present investigation to determine the normal FFA levels of young Merino wethers.

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MATERIALS AND METHODS

Animals

Ten two-year-old Merino wethers were used for the determination of the normal FFA values. The animals had a mean mass of 45–50 kg and were each fed a daily ration of 1 200 g lucerne hay supplemented with 30 g of a salt and trace element mixture* and 1 g vitamin A† weekly. They were housed in individual pens and had free access to water.

Reagents

All reagents are of AR quality.

Extraction solution (CHM): Chloroform and heptane were mixed in a 1:1 (v/v) ratio and 2% methyl alcohol was then added.

Phosphate buffer (pH 6,4): M/30 potassium dihydrogen phosphate was mixed with M/30 disodium-hydrogen phosphate in a 2:1 (v/v) ratio.

Cobalt reagent

Solution A consisted of cobalt nitrate (6 g) and glacial acetic acid (0,8 ml) added to a saturated and filtered solution of potassium sulphate to give a final volume of 100 ml at 37 °C. The solution was then maintained at this temperature.

Solution B was a saturated solution of sodium sulphate, also maintained at 37 °C.

Triethanolamine (1,35 volumes) was made up to 10 volumes with solution A, following which 7 volumes of solution B were added and the mixture shaken well. This reagent, being unstable, was prepared just before use.

Colour reagent

The stock reagent consisted of a 0,4% (m/v) 1-nitroso-2-naphthol solution in 96% ethyl alcohol. Just before use, the stock reagent was diluted by a factor of 12,5 with ethyl alcohol.

Standard solutions were made by dissolving the appropriate amounts of palmitic acid in the chloroform-heptane-methanol extraction solution.

* Kimtrafos 25, Kynoch Feeds, Kimberley, RSA
† Vitamin A, Peter Hand Panvet, Johannesburg, RSA

Collection of blood: Jugular blood was collected every morning at 08h00 just before feeding. Within 10 min of extraction plasma was separated from the cells by centrifugation and the determination of FFA in the plasma started immediately.

Procedure

The addition of 250 μl of plasma to glass-stoppered centrifuge tubes was followed by 1,0 ml phosphate buffer and 6,0 ml CHM. The mixture was shaken for 15 min on a reciprocating shaking machine* at a rate of 100 cycles/min and then centrifuged for 15 min at 826 *g*. The buffer was removed by suction and 3,0 ml of the organic phase was added to 2,5 ml of cobalt reagent, the mixture being shaken for 10 min and centrifuged for 5 min at 826 *g*. Finally, a 2 ml aliquot of the organic phase was added to 2,5 ml of colour reagent and shaken. After 30 min, the absorbance was read in a spectrophotometer at either 500 nm or 450 nm. The colour was stable for at least 3 h. A blank and 3 standards were also run through the complete procedure.

Results

Standard curve

A standard curve was obtained from the procedure described above (Fig. 1), and gave a straight line for concentrations ranging from 0,125–3,000 mM. The line did not pass through the origin and, as a result, FFA levels below 0,125 mM could not be determined. This drawback was overcome by reading low FFA concentrations (0,030–0,100 mM) at 450 nm instead of at 500 nm. Fig. 2 demonstrates clearly the effect of the change in wavelength. Thus, by making use of the second standard curve, FFA concentrations as low as 0,030 mM could be determined with ease.

Recoveries

Plasma obtained from a sheep starved for 2 days, was diluted in stepwise fashion with plasma from a well-fed sheep. These plasma standards were equilibrated against stearic acid-1-^{14}C† for 30 min before they were extracted and analysed. The FFA concencentrations varied linearly with the dilution factor over the range 0,05–2,25 mM, with a 2% standard error. Despite this wide range of concentrations, 95± 0,8% of the labelled stearic acid was recovered from the extraction step. However, further label was lost exponentially with increasing concentration during the cobalt complexing step ($y=47,89e^{-0,476x}$, $r=0,96$ $P>0,95$, with y=% of labelled stearic acid lost and x=total FFA concentration in mM). Despite this, the reaction of the Co-FFA complex in the organic phase with the aqueous colour reagent resulted in a linear standard curve (Fig. 1).

Storage of samples

Samples of plasma and their corresponding CHM extracts were stored at −20 °C in order to determine whether losses occur at low temperatures. Replicate samples were analysed at the times given in Table 1. There was a gradual increase in FFA concentrations in both sets of samples. The rate of increase was higher in plasma than in CHM extract.

Influence of phospholipids

The influence of phospholipids was evaluated by adding known amounts of lecithin to plasma samples and proceeding with the usual analyses. Table 2 shows that lecithin in concentrations varying from 10,02–35,05 mg% had very little effect on the final concentration of FFA.

TABLE 1 Increase in FFA concentration (% of initial) and CHM extracts following freezing at −20 °C

	24 h	48 h	216 h	384 h
Plasma.....	+5,5	+19,4	+33,5	n.d.**
CHM extract.....	+4,4	+16,8	+18,0	+28,7

** n.d.=not done

TABLE 2 Effect of added lecithin on the concentration of FFA

Plasma FFA before Lecithin mM (A)	Measured plasma FFA after Lecithin mM (B)	Difference in FFA. mM (B-A)	Lecithin added mM
0,265	0,280	+0,015	0,107
0,265	0,258	−0,007	0,223
0,265	0,249	−0,016	0,350
0,752	0,764	+0,012	0,110
0,752	0,733	−0,019	0,222
0,752	0,735	−0,017	0,350
1,167	1,153	−0,014	0,100
1,167	1,188	+0,021	0,219
1,167	1,171	+0,004	0,350

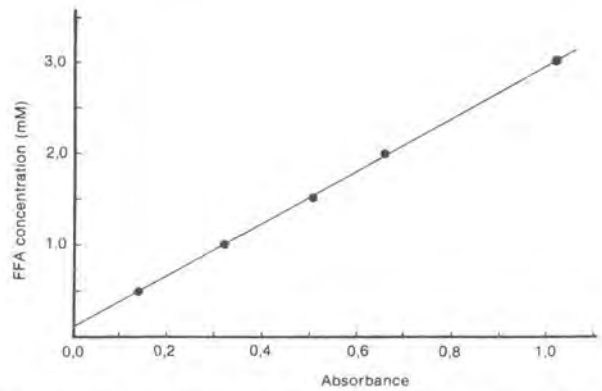

FIG. 1 Standard curve for normal concentrations of FFA 0,125–3,000 mM read at 500 nm

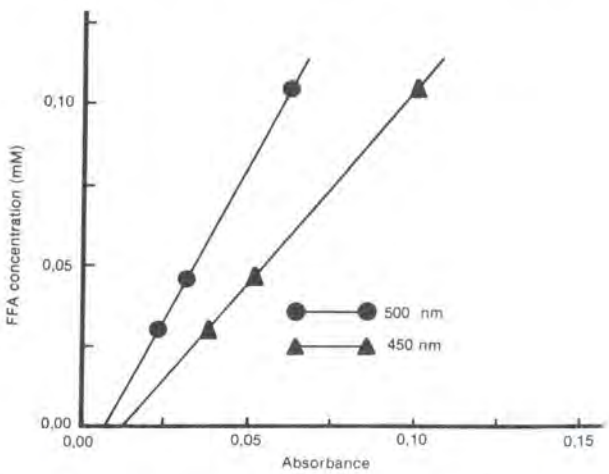

FIG. 2 Standard curve for low concentrations of FFA 0,030–0,100 mM

* Model 202, Labotec (Pty) Ltd, RSA
† Radiochemical Centre, Amersham, England

TABLE 3 Normal FFA levels of Merino wethers in mM, determined on 7 consecutive days

B66	B67	B68	B70	B75	B77	B82	B83	B85	B86
0,147	0,269	0,321	0,648	0,210	0,568	0,499	0,789	0,484	0,838
0,320	0,810	0,682	1,069	0,565	0,927	0,535	0,474	0,583	0,727
0,168	0,213	0,205	0,504	0,189	0,502	0,481	0,732	0,479	0,714
0,225	1,036	0,777	0,981	0,306	0,415	0,504	0,900	0,544	0,896
0,245	0,535	0,684	0,634	0,148	0,432	0,829	0,795	0,503	0,580
0,293	0,261	0,343	0,585	0,208	0,668	0,784	1,197	0,838	0,903
0,271	0,360	0,323	0,683	0,281	0,839	0,397	0,466	0,353	0,597

Overall mean: 0,547 mM
Range: 0,147–1,197 mM

Comparison of reproducibility

The reproducibility of the present method was compared with that of other techniques by computing the standard error of the mean ($S\bar{x} = \frac{Sx}{n}$, where Sx represents the standard deviation) of a number of triplicate determinations performed during the course of our studies. The results clearly indicate that the standard error ($S\bar{x} = 0,006\ 5$) of the present method differs by as much as an order of magnitude from the standard errors obtained by us when using other established methods.

In our hands the reproducibility of Dole's (1956) titrimetric method ($S\bar{x} = 0,028$) was similar to that of Falholt *et al.* (1973), but not as good as that obtained by the method of Novak (1965) ($S\bar{x} = 0,020$). On the other hand, Duncombe's (1964) technique ($S\bar{x} = 0,049$) proved rather erratic and, despite much effort, we were unable to obtain meaningful results with either the Lappin (1971) or the Mikac-Dević *et al.* (1973) method.

Normal FFA levels

The present method was used to determine the normal FFA levels on samples taken from 10 Merino wethers each morning for 7 consecutive days. The results shown in Table 3 indicate that the levels fall into 2 distinct categories. Wethers B70, B83 and B86 exhibited FFA levels (mean 0,748 mM, range 0,474–1,197 mM) which were consistently higher than those obtained from the rest of the animals (mean 0,460 mM, range 0,147–1,036 mM). The reason for these higher levels is not entirely clear but they seem to be associated with a somewhat abnormal nervousness on the part of the 3 animals. The calculated overall mean was 0,547 mM and the range was 0,147–1,197 mM.

Discussion

As a result of our investigation a highly sensitive and reproducible method permitting the estimation of free fatty acids with an optimum range of 0,030–3,000 mM and a standard error of 0,006 5 has been developed. This range is considerably wider than the next best range (0,125–2,000 mM) obtainable with the methods of Novak (1965) and Laurell & Tibbling (1967), and, in addition, it has 2 distinct advantages; firstly, it obviates the repetition of the estimations when plasma samples containing >2,000 mM of FFA have to be analysed, and, secondly, it permits the determination of low levels of FFA which may be encountered in tissues other than blood. The reproducibility of most other FFA methods leaves much to be desired. In our hands only the method of Falholt *et al.* (1973) and our modification of Novak's (1965) technique gave the fairly satisfactory reproducibilities of 0,028 and 0,020 respectively. Nevertheless, even these differed by an order of magnitude from that (0,006 5) obtained by the present method.

From our past experience with several colorimetric methods for the determination of FFA, we have come to the conclusion that all suffer from one or more of the following drawbacks: incomplete extraction of FFA, interference by phospholipids, lack of sensitivity, danger of contamination due to the two-phase system, and, most serious of all, extremely poor reproducibility.

It is now generally accepted that the chloroform-methanol mixture of Folch, Lees & Sloane-Stanley (1954) is the most efficient yet devised for the extraction of lipids from various tissues. Blankenhorn & Ahrens (1955) found heptane as efficient as chloroform and it had the added advantage of carrying the lipids into an upper phase. Laurell & Tibbling (1967), in an extensive study of various extraction procedures, came to the conclusion that a chloroform-heptane mixture containing 2–5% methanol was by far the most efficient for the complete extraction of FFA from plasma. This was later confirmed by both Regouw, Cornelissen, Helder, Spijkers & Weeber (1971) and Falholt *et al.* (1973) and was finally adopted by us for the present procedure. The reason why so few workers have actually used the chloroform-heptane-methanol extraction procedure is not entirely clear. However, it could be connected to the fact that, as shown by Laurell & Tibbling (1967), alcohols, under certain conditions, tend to induce abnormally high blank values which subsequently interfere with the colorimetric evaluation of the FFA.

The problem of the interference by phospholipids was resolved by Itaya & Ui (1965) who made use of phosphate buffer to eliminate these metabolites. The efficacy of the treatment was confirmed by Fukui, *et al.* (1972), Falholt *et al.* (1973) as well as by us in the present method. Other attempts to exclude phospholipids using either silicic acid (Laurell & Tibbling, 1967) or sodium chloride (Mikac-Dević *et al.*, 1973) have proved far less successful. Silicic acid was shown by Regouw *et al.* (1971) to coabsorb FFA in addition to the phospholipids while sodium chloride, in our hands as well as those of Falholt *et al.* (1973) gave rise to irreproducible results.

Our main standard curve read at 500 nm, consistent with the curves of Iwayama (1959), Duncombe (1964), Regouw *et al.* (1971), Falholt *et al.* (1973) and to a lesser degree of Laurell & Tibbling (1967) and Lappin (1971) did not pass through the origin. This would have precluded the estimations of FFA levels below 0,125 mM. However, by adopting a second standard curve read at 450 nm, we were able to detect levels as low as 0,030 mM with ease.

All FFA methods including the present one suffer from 2 inherent and unavoidable weaknesses. Firstly, in order to dissolve FFA, highly volatile organic solvents such as heptane and chloroform have to be used and this often leads to pipetting errors. Secondly, the fact that a 2-phase system is a prerequisite for the formation of the copper or cobalt salts is also responsible for further inaccuracies. Despite these intrinsic disadvantages, we have succeeded in developing a highly reproducible, sensitive and accurate method by adopting an efficient extraction procedure linked to a most sensitive colorimetric technique.

The present method was used by us to determine the normal FFA levels of Merino wethers fed lucerne hay. The values (mean 0,547, range 0,147–1,197 mM) found by us are higher than the baseline values (0,24–0,36 mM) obtained by Reid & Hinks (1962) for Border Leicester × Merino wethers given a mixture of lucerne hay chaff and oat grain, but somewhat lower than those (0,600–1,000 mM) of Annison (1962) for Merino wethers on a mixture of lucerne chaff and maize grain.

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