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Clinica Chimica Acta 281 (1999) 159–162

Technical note

Colorimetric micro-determination of free fatty acids in plasma using microplate readers

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Received 11 May 1998; received in revised form 16 November 1998; accepted 2 December 1998

Keywords: Free fatty acids; Plasma microplate reader; Rabbits

1. Introduction

In 1967 Laurell and Tibbling developed a colorimetric method for measurement of the total free fatty acid (FFA) content in blood plasma [1]. Between 1974 and April 1998 (Science Citation Index Expanded, ISI Citation Database), their paper was cited 411 times. Their assay has excellent working characteristics such as high sensitivity, accuracy, precision, and specificity, but has two disadvantages. First, it requires 50 μ l of plasma and this is difficult to achieve when small plasma volumes are available, particularly in small mammals or birds, and when a variety of other plasma components must also be measured. Second, it requires chloroform as the major solvent and consequently requires quartz cuvettes or multiwell plates (both of which are expensive). Chloroform is both noxious to humans and causes degradation of plastics such as may be found in spectrophotometers and from which multiwell microplates are typically made.

Our purpose is to present a modification of the Laurell and Tibbling method to address both these disadvantages and to permit a rapid assessment of numerous samples using commercial multiplate readers. First, we scaled down

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the original procedure so as to utilize only 10 μ l of plasma. Second, we replaced the measurement solvent (which included chloroform) with warm ethanol to permit rapid measurement in UV multiplate readers. Finally, we validated the modified method against the original one.

2. Methods

The same reagents and working solutions as in [1] were used with the following modifications and clarifications. The working solution of 10 ml of 0.5 M $\text{Cu}(\text{NO}_3)_2$, 10 ml of 1.0 M triethanolamine, and 3.5 ml of 1 M NaOH was diluted with double distilled water to 100 ml and 33 g of NaCl was added and the pH was adjusted to 8.1. This solution (the Cu-TEA solution) was stable for up to 1 month at room temperature when kept in the dark. The 0.4% diphenylcarbazide (DCP solution) in ethanol was stored under nitrogen and stable for at least 1 week in the refrigerator; it was discarded when clarity changed (became pink). Palmitic acid standards (Sigma) were prepared in CHM (chloroform–heptane (4:3, v/v), 2% methanol) with the 0.2 M stock solution (5.128 g in 100 ml of CHM) diluted serially to 0.2 mM working standard and are stored at room temperature in the dark.

Plasma samples ($n = 20$) were obtained from New Zealand white rabbits, which were serially bled from an ear artery after injection with ACTH to produce a range of FFA values (see [2] for the method). ACTH is a potent lipolytic hormone [3]. A 10- μ l volume of plasma was added to glass extractions tubes (10 ml) and 1 ml of CHM solution added. A 10- μ l volume of double distilled water was added to all tubes to be used as standards. Standards (0, 0.005, 0.01, 0.02 and 0.04 mM solutions of palmitic acid corresponding to 0.0, 0.5, 1.0, 2.0 and 4.0 mM FFA in plasma, respectively) were treated in the same way as plasma samples. All tubes were vortexed on a multivortexer for 2 min. Silicic acid (50 mg, activated overnight at 120°C) was then added, vortexed again for 4 min, and centrifuged for 3–5 min at 2000 g. The upper 0.8 ml subsample was transferred with a glass pipette to another tube containing 0.4 ml of Cu-TEA solution, vortexed for 5 min and centrifuged for 3 min at 2000 g. A 0.6-ml volume of the upper phase of each tube was transferred into a new tube, being careful not to transfer any of the Cu-TEA reagent into the new tube. This upper phase was then evaporated under nitrogen at 37°C in a fume hood, 0.2 ml ethanol was added, and the tubes were again placed into a 37°C water bath for 15 min. To ensure complete uptake by the ethanol, we vortexed for 5 min. To each tube we added 0.1 ml of warm (room temperature) DPC solution (the above DPC solution to which TEA was added immediately before: 0.1 ml of 1 M TEA per 10 ml of DPC). The tubes were vortexed for 15 s and 0.2 ml pipetted into the wells in the microplate (Nunc MicroWell 96U plates). Blanks (0.2 ml

ethanol) were placed into the first four wells. Absorbance was read on a Bio-Rad microplate reader (Model 3550-UV) at 490 nm after the color was allowed to develop for 15 min. Though Laurell and Tibbling used 550 nm, fixed filters were available on our microplate reader and our results were robust relative to the standard method.

Fig. 1 shows that the calibration curve at this wave length is linear over the range of standard concentrations used. We assessed the precision of the method by measuring five plasma samples from each of five rabbits (plasma concentrations ranged from 0.246 to 3.253 mM) and the coefficient of variation averaged 10.3% (range 8.9–14.2%). Finally, Fig. 2. shows that there is a 1:1 correspondence between the original method and the modified method, with a slope of essentially 1 and the regression line going through the origin. Thus, we conclude that modified method gives results identical to those of the old one and, given its simplicity of use, its requirement for small sample volume, its lack of chloroform vapor in contact with the investigator or the machinery, and its ability to measure large numbers of samples rapidly, we recommend its use.

Fig. 1. Calibration curve using the palmitic acid standards using the microdetermination method described in the paper and measured at 490 nm on a Bio-Rad microplate reader.

Fig. 2. Correspondence between the free fatty acid concentrations obtained by the Laurell and Tibbling method [1] with that obtained by the microdetermination method on the same rabbit plasma samples.

Acknowledgements

We thank the Natural Sciences and Engineering Research Council of Canada for funding.

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