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Brief technical note

A direct kinetic fluorimetric method for the enzymic determination of lactate in plasma

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Introduction

Measurement of lactate concentration is widely used as an index of oxygen deprivation of tissues and for the diagnosis of the severity of shock states [1]. Most enzymic methods for the determination of lactate require immediate deproteinisation of blood, careful handling, and are time consuming [2].

The currently preferred enzymic methods for lactate are based on the reaction of lactate with β-NAD^+ in the presence of lactate dehydrogenase (LDH) to produce pyruvate and NADH. NADH concentration is monitored absorptiometrically [3–13] or fluorimetrically [14–17] by reaction rate [6,8–13] or end-point methods [3–5,7,14–17], and related to lactate concentration. Enzymic methods using various types of automated clinical analysers have also been devised [7–12,15,17], but these methods are not suitable for the analysis of small numbers of samples.

In this paper we describe a new direct kinetic enzymic fluorimetric method for the determination of lactate in plasma. The method is especially suitable for emergencies, pediatric specimens and in general clinical laboratories where filter fluorometers or simple fluorescence spectrophotometers are available.

Materials and methods

Apparatus

A double-beam fluorescence spectrophotometer (Model 512A, Perkin-Elmer, Norwalk, USA) with a 150 W xenon lamp was used. All measurements were performed using standard rectangular cells of 1 cm path length, made of fluorescence-free fused silica. A constant temperature of 25.0 ± 0.1°C in the sample cell was maintained by using a water bath (Model ST, Sargent, Chicago, IL, USA). All measurements were carried out with constant stirring by means of a magnetic stirrer assembled below the cell holder. The following instrumental settings were used for

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measurements. Ratio mode: high voltage. 750 V; excitation wavelength. 350 nm; excitation slit, 20 nm; emission wavelength. 455 nm; emission slit, 20 nm; sensitivity, 3 and response switch slow.

Absorption measurements were carried out with a Beckman DK-1A spectrophotometer.

Reagents

All solutions were prepared in double-distilled deionised water from reagent-grade materials. All buffers used contained disodium ethylenediaminetetraacetic acid (EDTA), 5 mmol/1.

'Tris' buffer 2.0 mol/l, pH 9.2. Dissolve 24.2 g of Tris (hydroxymethyl)aminomethane and 0.19 g of $Na_2EDTA \cdot 2\ H_2O$ in 60 ml of water, adjust the pH to 9.2 with 6 mol/l HCl and dilute to 100 ml.

β-NAD$^+$ solution, 0.025 mol/l. Dissolve 179 mg of β-NAD$^+$ (Calbiochem, San Diego, CA 92112, USA) in 10.0 ml of water and refrigerate when not in use.

Lactate dehydrogenase (LDH) solution. Dissolve appropriate amount of rabbit muscle LDH (EC 1.1.1.27) (Type XI: lyophilised salt-free powder. 935 U/mg protein, Sigma Chemical Co., St. Louis, MO 63178, USA) in cold Tris buffer, 0.02 mol/l, pH 7.2, to obtain an enzyme solution of approximately 150 U/ml. Keep in an ice-bath during measurements and refrigerate when not in use.

Lactate standard solution, 20 mmol/l (stock). Dissolve 0.1920 g of L-lithium lactate (Grade L-X, Sigma Chemical Co.) in 100 ml of water and add one drop of conc. H_2SO_4. Working standards of 1.0, 4.0 and 10.0 mmol/l were prepared by appropriate dilution of the stock solution.

Specimens

Blood samples were collected according to standard procedures [1,2] in tubes containing 10 mg of sodium fluoride per ml of blood, frozen immediately in an ice-bath and centrifuged as soon as possible to separate the plasma [10].

Procedure

Pipette into the cell, 2.00 ml of the buffer solution, 10 μl standard or plasma and 0.100 ml β-NAD$^+$. Start the stirrer and after the signal has stabilised (~ 1 min), inject 0.100 ml of LDH solution and record the fluorescence intensity change for 120 s. Empty the cell by suction and repeat the procedure for the next sample.

Calculations

Read the relative fluorescence intensity change from the recorder traces, exactly 2 min after the addition of LDH solution (ΔF_{120s}) (fixed-time method). Plot (ΔF_{120s}) vs. lactic acid concentration of standards in mmol/l, to obtain the calibration curve (Fig. 1).

Results

Optimum conditions

Reaction parameters were studied in order to find optimum conditions for the kinetic fluorimetric procedure. The best conditions were selected in order to obtain high sensitivity and to avoid high blank values and background fluorescence effects. We studied the buffers Tris, ethanolamine, glycine and hydrazine(Tris), the pH range 8.5–9.5 (9.2), buffer concentration ranging from 0.1 to 2.0 mol/l (final concentration 2.0 mol/l), β-NAD^+ concentration from 2.3×10^{-4}–4.5×10^{-3} mol/l (final concentration, 1.1×10^{-3} mol/l) and the enzyme concentration from 2 to 12 U/ml (final concentration, 6 U/ml). The selected optimum conditions are indicated in parentheses. The optimum conditions are similar to those used in other kinetic procedures [3,6].

Linearity

Recorded curves of the relative fluorescence intensity change with time, at various lactate aqueous standards, and the corresponding calibration curves are shown in Fig. 1. The calibration curve is linear up to 10 mmol/l.

Precision and accuracy

The precision of the method was tested by analysis of two commercial control sera (Precinorm S from Boehringer Mannheim, FRG, and Validate from General Diagnostics) with target values of 3.26 and 2.30 mmol/l, respectively. The mean ±

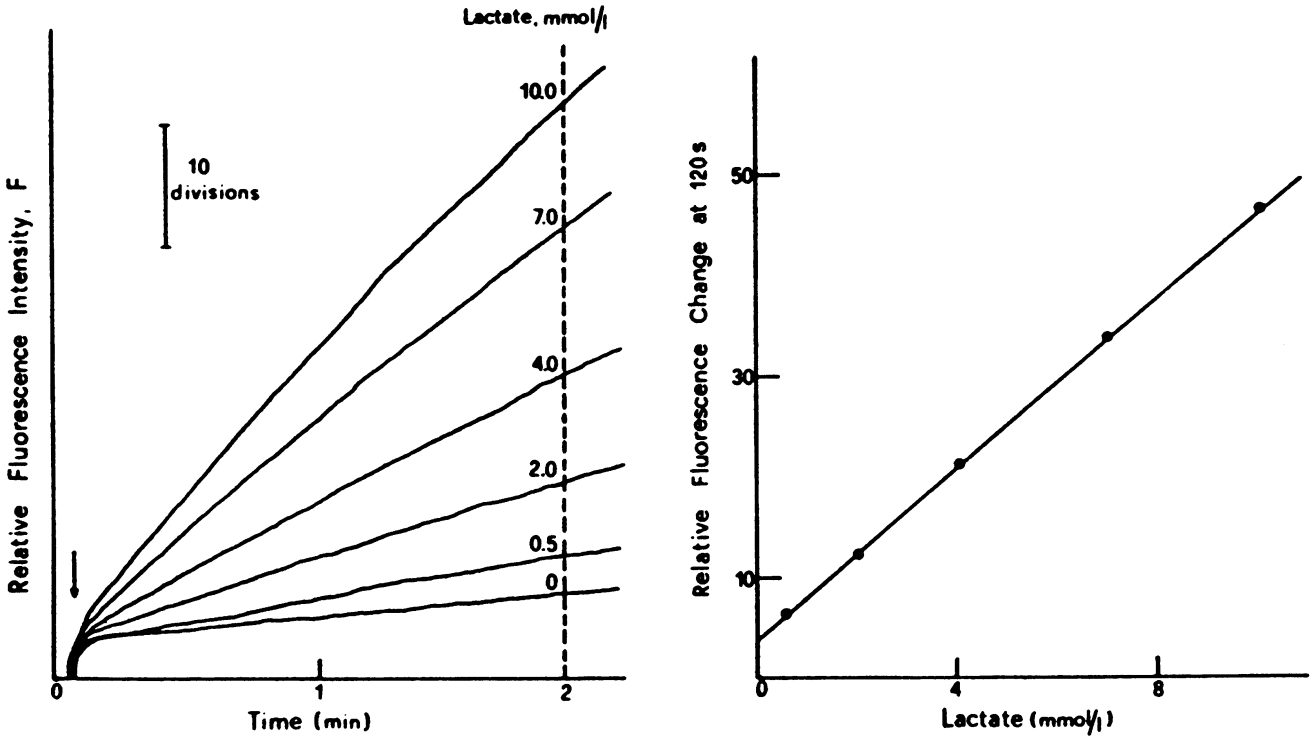

Fig. 1. Recorded curves of relative fluorescence intensity vs. time for the lactate standards and the corresponding calibration curve. Arrow indicates LDH addition.

TABLE I

ANALYTICAL RECOVERY OF LACTATE ADDED TO PLASMA SAMPLES

Lactate (mmol/l)				Recovery (%)
Initially present *	Added	Total	Found *	
2.84	2.31	5.15	5.17	101
	4.62	7.46	7.86	109
3.54	2.31	5.85	5.98	106
	4.62	8.16	8.19	101
2.47	2.31	4.78	4.67	95
	4.62	7.09	6.96	97
1.60	2.31	3.91	4.03	105
	4.62	6.22	6.39	104
1.25	2.31	3.56	3.37	92
	4.62	5.87	5.74	97
				Av.100.7

*Average of two measurements.

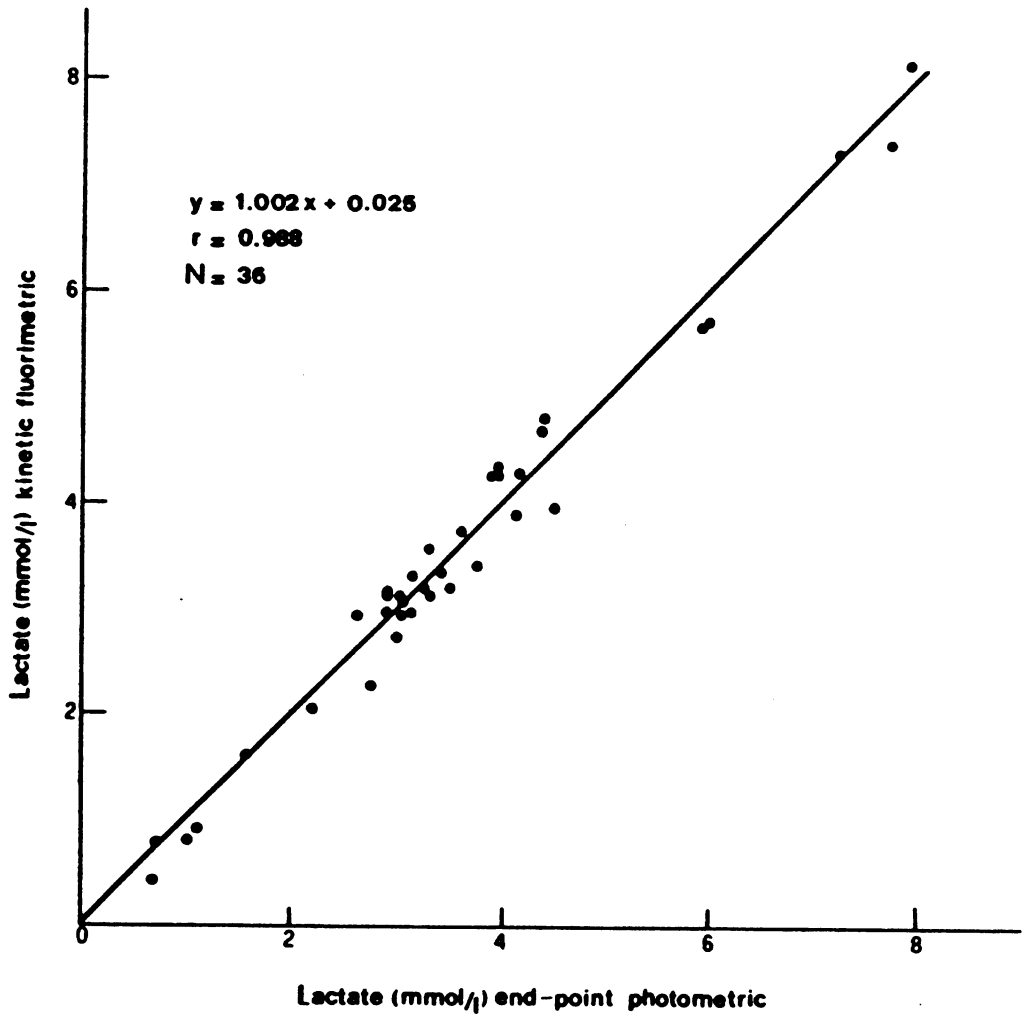

Fig. 2. Comparison of results for lactate as obtained by the manual enzymic method and the kinetic fluorimetric procedure.

standard deviation we found were 3.53 ± 0.10 ($n = 5$) and 2.11 ± 0.15 ($n = 5$), respectively. Analytical recovery of added lactate ranged from 92 to 109% (average 100.7%). Table I shows typical results of recovery studies.

Results by the proposed procedure (y) were compared with those by an established manual photometric procedure (x) [2] by analysing a series of patients' specimens by both procedures. The results are graphically presented in Fig. 2. The correlation coefficient (r) was 0.988 and the regression equation was $y = 1.002x + 0.025$ ($n = 36$).

Discussion

Most methods for the routine determination of lactate in plasma, blood or serum are photometric, end-point techniques, which require large sample volume, long incubation time and deproteinisation of the samples [1,2]. Also, these methods require addition of hydrazine in the buffer solution as a trapping reagent which is known to cause positive errors in lactate determination [18].

We attempted to devise a new method for the determination of lactate in plasma (plasma handling is convenient for this determination) [7,10] to overcome the above drawbacks of existing manual photometric end-point methods, and succeeded by using the combined merits of kinetic methodology and fluorescence technique.

Our method is simple requiring the direct addition of 10 μl of plasma into the reaction cell. No deproteinisation is required. These advantages make the method especially suitable for pediatric specimens. The measurement time is 2 min and overall analysis time less than 5 min, making the method suitable for emergencies [10,17]. Linearity covers the range 0.5–10 mmol/l lactate, and includes both normal and abnormal plasma samples [8].

In order to obtain adequate sensitivity for the measurements, a fluorometer is used to monitor the generated NADH. The method can also be applied using a low-cost filter fluorometer.

Our method is readily adaptable for automation since no pretreatment steps are required before measurements.

Using the new fixed-time kinetic procedure, short measurement times are required, thus increasing the specificity of the enzymic reaction as possible side reactions are avoided.

We conclude that the newly developed kinetic fluorimetric enzymic method for lactate in plasma is suitable for general clinical laboratories and especially for pediatric specimens and emergencies.

Acknowledgement

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