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A New Method for the Determination of Nucleotidase in Serum

Automated determination of the 5′-nucleotidase by continuous flow analysis without dialysis

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A fully automated procedure is proposed for the direct determination of 5′-nucleotidase in serum, using only a simple manifold in a continuous flow technique without dialysis.

Ein automatisches Verfahren nach dem Prinzip der kontinuierlichen Durchflußanalyse für die Bestimmung von 5′-Nucleotidase im Serum wird beschrieben. Es wird ein einfaches Fließdiagram ohne Dialyse benutzt.

In previous reports (1–6) we described a new technique for the manual determination of serum 5′-nucleotidase[1]). This report presents a simple automated adaption to continuous flow analysis of the above mentioned manual procedure. We optimalised the flow scheme making use of the mathematical model described by *Walker* et al (7).

For the determination of the most favourable sample rate (sample time and sample/wash ratio) the curve characteristics "lag phase" and "experimental factor" seem to be of the greatest importance. They can be calculated from the so-called standard profile that *Walker* defined as: "The curve produced by continuously aspirating a blank to give a base-line whose absorbancy may be set at zero, then changing to continuous aspiration of a sample to produce a plateau of absorbancy E, then returning to continuous aspiration of blank until there is a return to the original base-line".

The expontial factor is defined as the time required from absorbancy y to reach 0.37 y. This factor (b) represents the slope of the exponential curve in the expression $y = E \cdot e^{-t/b}$. The value of b can be calculated by measuring the absorbancy of the "rise and fall curves" as a function of time.

The average value of b in this case is calculated to be 13.7 s. As has been pointed out by *Walker*, the attainment of 95% peaking requires a sample time of 3b. In this case the sample time therefore has to be at least 45 s. In practice we have chosen a sample speed of 30/h, with sample/wash ratio 2:1.

[1]) Enzyme: 5′-nucleotidase = 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5)
Adenosine-deaminase = adenosine aminohydrolase (EC 3.5.4.4)

Materials and Methods

Reagents

1. *Buffer solution*
   Dissolve 4.20 g sodium diethylbarbiturate and 12.6 g $MgSO_4 \cdot 7H_2O$ (A. R. grade) in about 800 ml H_2O. Adjust the pH to 7.5 with 1 mol/l HCl and dilute to 1000 ml with water. The solution can be used for a month if stored at 4° C.

2. *Buffer/adenosine deaminase solution*
   0.1 ml adenosine deaminase (400 U/ml) in glycerol 50% is diluted with 1000 ml buffer solution. Stable for 2 weeks at 4°C.

3. *Buffer substrate/adenosine deaminase solution*
   Dissolve 0.5 g adenosine-5′-monophosphate and 0.45 g phenyl disodium orthophosphate in 1000 ml buffer/adenosine deaminase.

4. *Ethylenediamine-tetraacetic acid dipotassium salt*
   Dissolve 5.6 g EDTA $\cdot 2H_2O$ in water and dilute to 50 ml.

5. *Phenol colour reagent (concentrated)*
   Dissolve 50 g phenol (A. R. grade) and 0.25 g disodium-pentacyanonitrosylferrate (A. R. grade) in water and dilute to 1000 ml. Stable at least two months if kept cool and in an amber bottle protected from light.

6. *Phenol colour reagent/EDTA solution*
   Dilute 1 volume concentrated phenol colour reagent with 4 volumes water. To 1000 ml solution is added 20 ml EDTA solution. This solution must be prepared fresh before use.

7. *Alkali-hypochlorite (concentrated)*
   Dissolve 25.0 g sodium hydroxide (A. R. grade) in 60 ml water. Add 72 ml o a commercial sodium hypochlorite solution, containing 0.5 mol/l NaClO (B.D.H., 1 mol/l in 0.1 mol/l NaOH) and dilute to 1000 ml. Care should be taken that the solution is at least 0.035 mol/l to J_2, otherwise erroneous results will be obtained. Stable at least two months if kept cool and in an amber bottle protected from light.

8. *Alkali-hypochlorite reagent*
   Dilute 1 volume concentrated alkali-hypochlorite reagent with 4 volumes water. This solution must be prepared fresh before use.

9. *Sodium chloride solution*
   Dissolve 9 g sodium chloride in 1000 ml water and add 5 drops of Brij 35 (Technicon).

10. *5'-nucleotidase stock solution* (60 U/l)
    Dissolve 3 mg 5'-nucleotidase from *Crotalus adamanteus* (B.D.H. 2 U/mg) in 100 ml buffer solution (see under 1) and add a few drops of 0.1% NaN_3 solution. This stock solution is stable for 4 weeks at 4° C. The activity of the stock-standard is checked by the manual method at 37° C (BDH assay-set). Before use dilute the stock standard with buffer solution to make working standards.

Procedure

A manifold and flow system assembled for use with stock modules of the Auto Analyzer (Technicon) as shown in figure 1 is used. Sample speed is 30/h with a sample/wash ratio 1:2.
Wave length is 625 nm. We tried to improve the sensitivity by increasing the incubation temperature. From calibration curves at serveral temperatures we decided that the most favourable temperature was about 50° C. For practical reasons an incubation temperature of 50.0° C was chosen.

Calculations

In determining serum nucleotidase activities values calculated from the calibration curve have to be diminished with the calculated blank values. The resulting activities are expressed in U/l at 37° C.

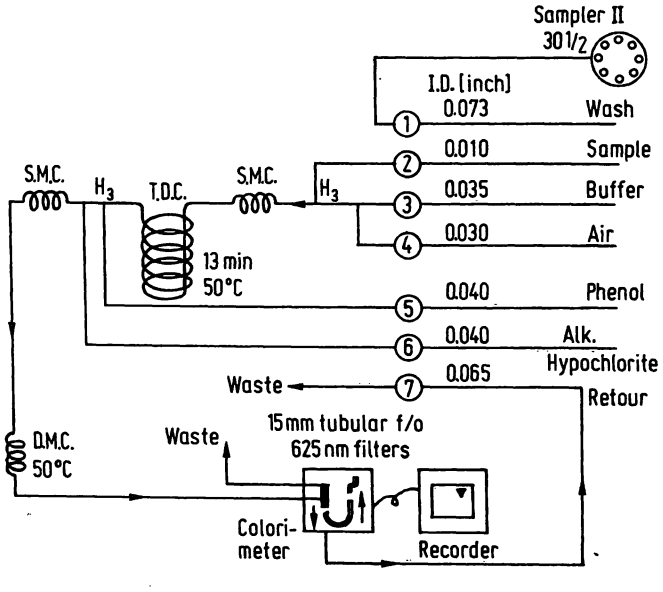

Fig. 1. Flow diagram of the automated determination of 5'-nucleotidase in serum. Tube 3 pumps buffer substrate/adenosine deaminase solution, and, in the case of the blanks buffer/adenosine deaminase solution.

Results

The automated determination of 5'-nucleotidase shows a good correlation with the manual method of *Persijn* and *van der Slik* (8). The latter is performed at an incubation temperature of 37° C. The coefficient of correlation (r), calculated from randomly collected patient serum samples (n = 40), was found to be r = 0.984 (see fig. 2).
The coefficient of variation has been shown to be 4.1% in the low range (up to 40 U/l) and about 2.5% in the higher range. As can be seen in figure 3 carry-over from the preceeding high value (69.4 U/l) is calculated to be 1.2% because the low value (7.5 U/l) increased to 0.8 U/l after the high peak. The same figure indicates that an average reading equivalent to 92% of the maximum value (steady state) is achieved with base-line and continuous trace noise levels that are quite small.

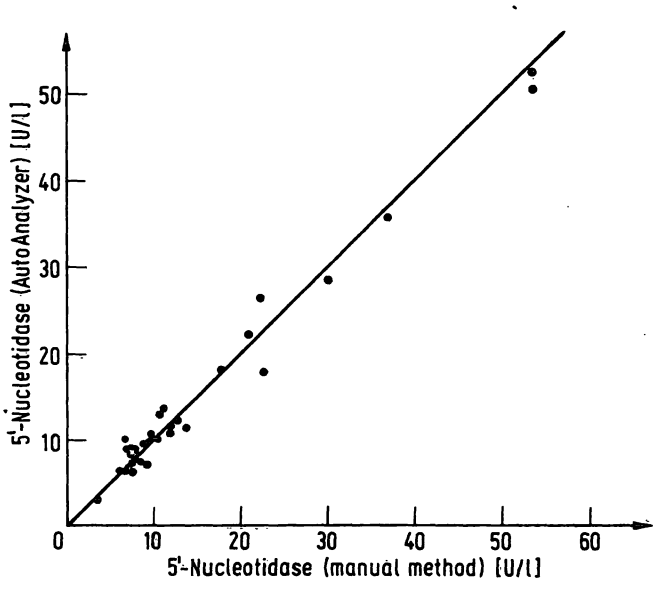

Fig. 2. Comparison of 5'-nucleotidase-values (U/l) obtained by the automated method and the manual method of *Persijn* and *van der Slik* (8).

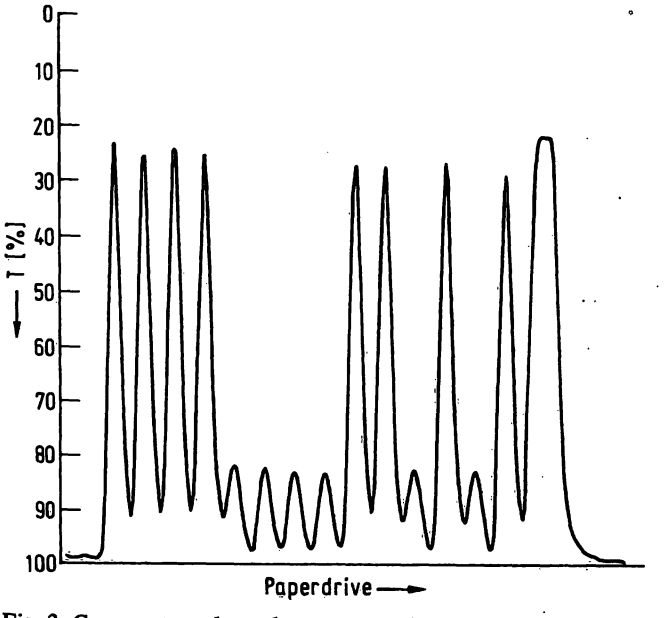

Fig. 3. Carry-over and steady-state experiments.

Tab. 1. Coefficients of variation (C. V.) for different series of serum.

Range U/l serum	C. V. %	number of samples
0–20	4.1%	15
20–40	4.5%	25
40–60	2.8%	20
60–80	2.1%	13

Comments

1. The measured 5′-nucleotidase activity remains constant for a substrate concentration range of 1.0 to 6 mmol/l, which is in a good agreement with the manual method (see l. c. 5). In this respect it should be noted that there exists a linear relationship between the recorder scale units and the 5′-nucleotidase activity, expressed as U/l at 37°C.
2. The interference by bone alkaline phosphatase in this 5′-nucleotidase assay is suppressed by the addition of phenylphosphate to the incubation medium as described under reagent 3 (see l. c. 4). Phenylphosphate does not interfere with the colorimetric determination of adenosine and can be omitted from the blanks.
3. *Activity of adenosine deaminase*
   0.2 ml adenosine stock solution is added to a tube containing 1.0 ml buffer/adenosine deaminase. After stoppering and standing at 37°C for 2 minutes, the reagents for ammonia are added as described in the standard manual procedure (8). The absorbancy is read at 625 nm against a blank which contains water instead of adenosine solution and should not be below 1.10.
4. As can be seen from table 2 a deviation from the linear relationship between optical density and amount of protein becomes apparent if the quantity of protein is about 95 g/l. The deviation from linearity is small; it amounts to only about 10%.
5. The addition of 1000 mg/l hemoglobin serum was found to have no significant effect on the results of the 5′-nucleotidase assay. In this experiment aliquots of cell-free haemolysate of four times washed erythrocytes were used.

Tab. 2. Influence of protein on 5′-nucleotidase determination in serum

protein concentration	5′-nucleotidase activity
65 g/l	16,4 U/l
75	16.6 U/l
85	15.8 U/l
95	14.6 U/l
125	12.0 U/l

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