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Automated Method for the Determination of 5′-Nucleotidase in Serum by Continuous Flow Analysis

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Summary: The manual method of *Persijn & Van der Slik* (J. Clin. Chem. Clin. Biochem. *6*, 441–446 (1968); *7*, 493–497 (1969); *8*, 398–402 (1970)) for the determination of serum 5′-nucleotidase has been adapted to the Auto-Analyzer II System. In the Auto-Analyzer II, the incubation temperature for the enzyme reactions is 37 °C, and the effective sample speed is 30/h, at a sample/wash ratio of 2:1. There is good agreement and correlation between the manual method and the Auto-Analyzer II method (equation of regression line: $y = x + 0.6$, correlation coefficient = 0.988; the normal range is 2.9–10.5 U/l for both methods). In routine use, within-run and between-day reproducibility are considerably smaller for the Auto-Analyzer II than for the manual method, especially when large numbers of samples are assayed.

Automatisches Verfahren zur Ermittlung der 5′-Nucleotidase-Aktivität im Serum mit kontinuierlicher Durchflußanalyse

Zusammenfassung: In diesem Aufsatz wird eine Anpassung der manuellen Arbeitsweise von *Persijn & Van der Slik* (J. Clin. Chem. Clin. Biochem. *6*, 441–446 (1968); *7*, 493–497 (1969); *8*, 398–402 (1970)) zur Ermittlung der Aktivität des Enzyms 5′-Nucleotidase im Serum an einen Auto-Analysator beschrieben.

Beim „Auto-Analyzer II" kann man eine Temperatur von 37 °C für die enzymatischen Reaktionen benutzen. Dabei ist die effektive Probenahmegeschwindigkeit 30/h, mit einem Proben/Waschflüssigkeits-Verhältnis von 2:1.

Die Übereinstimmung und die Korrelation der manuellen Arbeitsweise mit dem Auto-Analyzer II-Verfahren sind gut (Regressiongleichung $y = x + 0{,}6$; Korrelationskoeffizient 0,998; der Normalbereich ist 2,9–10,5 U/l für beide Methoden).

Bei Routineuntersuchungen sind die Reproduzierbarkeiten in der Serie und von Tag zu Tag beim Auto-Analyzer II-Verfahren bedeutend besser als bei der manuellen Arbeitsweise.

Introduction

A manual technique for the determination of serum 5′-nucleotidase[1]) has been described elsewhere (1–3). The sequence of reactions employed in this technique is as follows:

$$\text{Adenosine-5′-monophosphate} \xrightarrow{\text{5′-nucleotidase}} \text{adenosine} + \text{P}_\text{i}$$

$$\text{Adenosine} \xrightarrow{\text{adenosine deaminase}^{2})} \text{inosine} + \text{NH}_4^+$$

$$\text{NH}_4^+ \xrightarrow[\text{OH}^-]{\text{phenol/hypochlorite}} \text{indophenol blue}$$

This technique has been adapted for use on the Auto-Analyzer I (Technicon Instrument Corp., Tarrytown, N.Y. 10591) (4). The Auto-Analyzer I method required an incubation temperature of 50 °C for the enzyme reactions and had to be operated at an effective sample speed of 15/h (assay and blank determinations were run separately, each at a sample speed of 30/h). However, the demand for serum 5′-nucleotidase determinations has increased considerably; at present more than 600 specimens a week are assayed for this enzyme in

[1]) 5′-nucleotidase = 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5).
[2]) adenosine deaminase = adenosine aminohydrolase (EC 3.5.4.4)

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our laboratory. This led us to adapt the Auto-Analyzer I method for use on an Auto-Analyzer II and to optimize the procedure by modifying some of the reagents and their concentrations and by introducing a dialysis step between the enzyme reactions and the *Berthelot* reaction. Ammonium is detected by the *Berthelot* reaction as provided by Technicon for the determination of ammonium in water and sea-water (5). The enzyme reactions are carried out at 37 °C. Assays and blank determinations are run separately at a sample speed of 60/h each. Use of a double-beam colorimeter may eventually permit simultaneous running of assays and blanks at a sample speed of 40/h. The Auto-Analyzer II method described here provides a better performance at a greater speed with a greater working efficiency compared with both the manual and the Auto-Analyzer I technique.

Materials and Methods

A manifold and flow system assembled for use with stock modules of the Auto-Analyzer II (see fig. 1) is employed. The most favourable sample rate and sample/wash ratio were determined on the basis of the mathematical model described by *Walker* et al. (6). For the peak value to obtain 95% of the plateau value, a sample time of about 45 s would be required. In practice, we use a sample speed of 60/h with a sample/wash ratio of 2:1.

The reagents were prepared as follows:

Buffer solution

Dissolve 4.2 g sodium diethylbarbiturate and 1.3 g $MgSO_4 \cdot 7H_2O$ (A. R. grade) in about 900 ml distilled water. Adjust the pH to 7.5 with 1 mmol/l HCl and dilute to 1000 ml with distilled water. The solution can be used for a month if stored at 4 °C.

Buffer/adenosine deaminase solution

Dilute 0.25 ml adenosine deaminase (5 mg/ml, 200 U/mg, in glycerol 50%, Boehringer Mannheim, Germany) with 1000 ml buffer solution. Stable for 2 weeks at 4 °C.

Substrate-buffer

Dissolve 0.420 g adenosine-5'-monophosphate and 0.375 g phenyl disodium orthophosphate in 300 ml of the buffer/adenosine deaminase solution. Add 0.3 ml Brij-35 (surfactant). This solution must be prepared just before use.

Complexing reagent

Dissolve 33 g sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 2H_2O$), 24 g sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 50 g sodium chloride in 950 ml distilled water. Adjust pH to 5.0 with concentrated sulfuric acid. Dilute to 1000 ml with distilled water. Before use, add 0.1 ml Brij-35 (surfactant) per 100 ml complexing reagent.

Sodium phenolate

In a liter Erlenmeyer flask, dissolve 83 g phenol in about 100 ml distilled water. Cautiously add, while cooling under tap water and in small increments with agitation, 180 ml 200 g/l sodium hydroxide solution. Dilute to 1000 ml with distilled water. When kept in an amber bottle protected from light, this solution is stable for 2 weeks at room temperature.

Sodium hypochlorite

Dilute 200 ml of 50 g/l sodium hypochlorite (Technicon product no. TO1-0114) to 1000 ml with distilled water. When kept in an amber bottle protected from light, this solution is stable for two weeks at room temperature.

Sodium nitroprusside

Dissolve 0.5 g sodium nitroprusside in 1000 ml distilled water.

Wash

Distilled water.

Standards

Dissolve 0.0869 g adenosine in 250 ml of a saturated solution of benzoic acid in distilled water. This solution contains 1.30 mmol/l

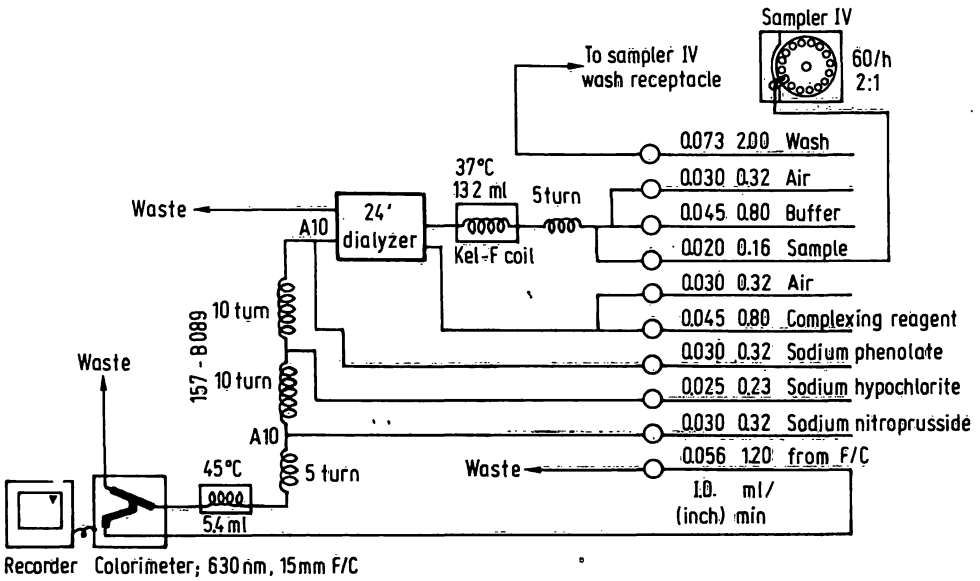

Fig. 1. Flow diagram of the automated determination of 5'-nucleotidase in serum. For blank determinations the buffer contains adenosine deaminase; for assays it contains both adenosine deaminase and substrate. As dialyzers, either 12 inch or 24 inch dialyzers can be used.

adenosine. The other adenosine standards are prepared from this standard by dilution with saturated benzoic acid.

Ammonium standards are prepared by dilution with distilled water of a stock solution of 0.7 mmol/l ammonium sulfate in distilled water, containing 1 ml/l chloroform as a preservative (5). The ammonium sulfate working standards must be prepared just before use.

Results and Discussion

The system is calibrated by sampling standard solutions of either ammonium sulfate or adenosine. Figure 2 shows a calibration curve obtained with both ammonium sulfate and adenosine standards. The points representing the adenosine standards are part of the calibration curve obtained with ammonium sulfate standards, indicating that deamination has occured with 100% efficiency. With this Auto-Analyzer II method in routine operation in our laboratory, calibration curves show no deflection from linearity when adenosine standards with concentrations up to 1.3 mmol/l (corresponding to 5′-nucleotidase activities up to 100 U/l) are used. Because of the lability of aqueous ammonium solutions, the use of adenosine standards for the calibration procedure is recommended.

By definition, the activity of one unit of serum 5′-nucleotidase leads to the formation of 1 μmol of adenosine per minute under the reaction conditions described. The activity in units per liter of serum may then be calculated as follows:

$$\frac{([\text{adenosine}]_{\text{assay}} - [\text{adenosine}]_{\text{blank}})}{\text{incubation time}},$$

with [adenosine] expressed in μmol/l and incubation time in minutes.

The incubation time is the interval between mixing of the sample with substrate-buffer and the end of dialysis. The Standard Calibration Control potentiometer of the Auto-Analyzer II colorimeter is adjusted so that continuous sampling of an adenosine standard of 1.3 mmol/l gives full-scale recorder deflection. Depending on the blank values, sera with 5′-nucleotidase activities up to 60–80 U/l can then be determined; sera with higher activity have to be diluted with distilled water before use. The effect of dilution is shown in figure 3.

The calibration procedure described above presumes an incubation temperature of 37 °C in the reaction mixture from the moment of mixing. In practice, however, the reaction proceeds at room temperature for some time before the reaction mixture enters the enzyme incubation coil at 37 °C. When the system is constructed as described here, total incubation time is about 13 minutes and the time interval between the mixing of sample and substrate-buffer and the entrance of the reaction mixture into the 37 °C heating coil is less than 1 minute. Under these conditions, the temperature effect proves to be negligible. It should be kept in mind, however, that this time interval should be as short as possible. If

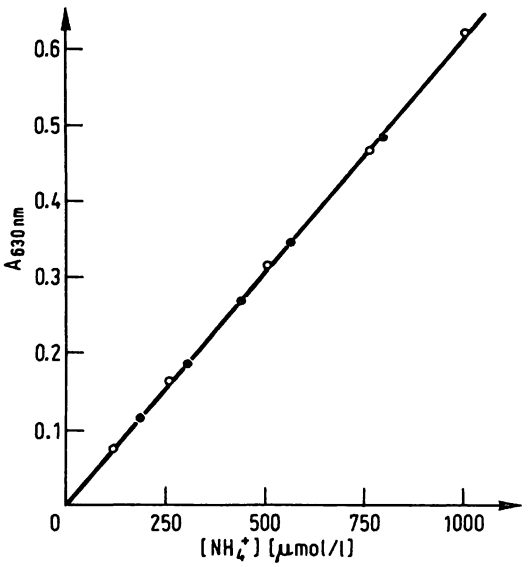

Fig. 2. Calibration curve obtained with adenosine and ammonium sulfate standards.
• = adenosine, o = ammonium sulfate.

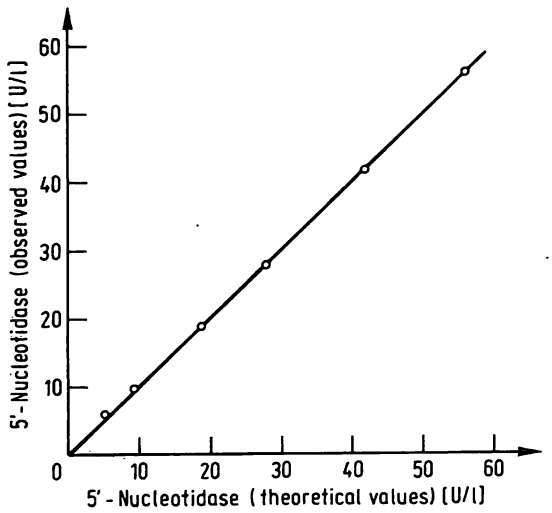

Fig. 3. Results of assays of a serum with a 5′-nucleotidase activity of 56 U/l and diluted several times with distilled water.

not, the low temperature will exert too large an effect on the final result, since we found the Q_{10} for 5′-nucleotidase to be 2.5 over a temperature range from 20 to 37 °C (data not shown). In that case, the system cannot be calibrated as described above and use must be made of patient sera and a reference method.

Figure 4 gives an indication of the within-run reproducibility and shows the effect of carry-over. Carry-over from the preceding high value (48.8 U/l) is estimated to be 0.9%, because the low value (8.0 U/l) increased to 8.4 U/l after the high peak. Figure 4 also indicates that an average reading equivalent to 93% of the maximum value is achieved with noise levels that are quite small.

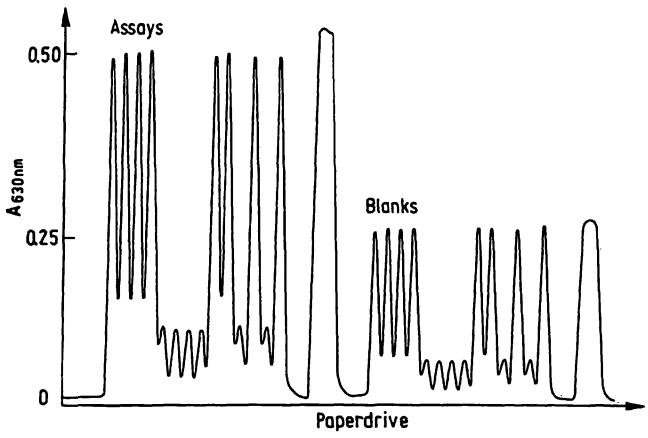

Fig. 4. Results of carry-over and steady-state experiments.

The automated procedure for the determination of 5'-nucleotidase shows good agreement and correlation with the manual method of *Persijn & Van der Slik* (3). The correlation coefficient (r), calculated from randomly collected patient serum samples (n = 120) was found to be r = 0.988; the equation of the regression line was y = x + 0.6 (see fig. 5). The normal range is 2.9–10.5 U/l for both methods (data not shown).

Between-day and within-run reproducibility of both the manual and the Auto-Analyzer II method were estimated under routine conditions with the use of pooled sera. 5–11 samples of pooled sera were distributed randomly among the 120–200 patient sera routinely assayed for 5'-nucleotidase each day. The results, shown in tables 1 and 2, indicate that both between-day and within-run variability are considerably smaller when the Auto-Analyzer II method is used. Data obtained with the manual method, in smaller routine work loads of maximally 80 patient sera, are also included in tables 1 and 2. These data illustrate the decline in performance of the manual method that occurs in routine use when the work load increases, and thus underline the need for automation of this method, especially when large numbers of samples have to be assayed[3]). The mean values in table 1 and 2 are not comparable, because different pool sera were used for the Auto-Analyzer II method.

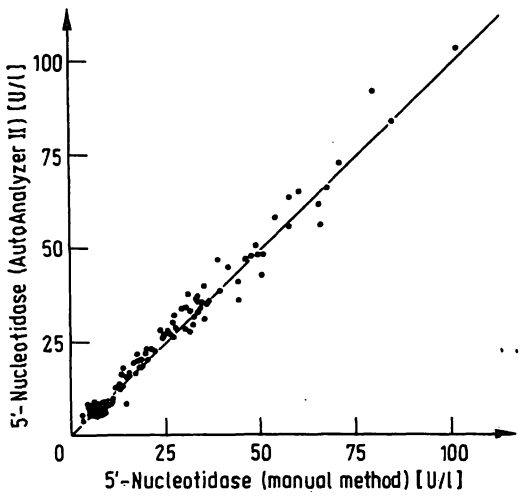

Fig. 5. 5'Nucleotidase values obtained with the Auto-Analyzer II method and the manual method of *Persijn & Van der Slik* (3). Correlation coefficient r = 0.988; equation of regression line: y = x + 0.6.

Tab. 1. Between-day variability.

Manual			Auto-Analyzer II		
n	$\bar{x}$ (U/l)	CV (%)	n	$\bar{x}$ (U/l)	CV (%)
13	63.2	6.2	9	67.1	2.2
16	30.3	9.9	54	26.4	3.9
15	6.5	18.0	59	8.6	6.7
60*	8.8*	9.8*			

* These results were obtained with a pooled serum distributed regularly among routine work loads of maximally 80 patient sera.

Tab. 2. Within-run variability*.

Manual			Auto-Analyzer II		
n	$\bar{x}$ (U/l)	CV (%)	n	$\bar{x}$ (U/l)	CV (%)
10	45.2	3.8	8	47.7	1.7
9	31.1	10.9	10	35.5	1.7
9	7.0	21.4	10	8.0	2.5
11**	8.7**	9.1**			

* For the Auto-Analyzer I method, within-run coefficients of variation of 2.8 to 4.1% have been reported for sera with 5'-nucleotidase activities in the range considered here (4).
** These results were obtained with a pooled serum distributed regularly among routine work loads of maximally 80 patient sera.

Use of a double-beam colorimeter allows the simultaneous running of assay and blank determinations at a sample speed of 40/h and a sample/wash ratio of 2:1. Comparison of the absorbance values measured manually with those obtained with the Auto-Analyzer II gave a correlation coefficient of 0.994 (n = 21, range 3.5–79.6 U/l). Here, aqueous standards cannot be used, because assay and blank signals are subtracted in the colorimeter. To the best of our knowledge, no calibration sera containing 5'-nucleotidase are available at present. Therefore, in this case calibration cannot be absolute, but requires the use of patient sera and a reference method. For this reason, we run assays and blanks separately in our laboratory.

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[3]) Compare also data of table 4 in l. c. (7), which refer to a series of 30 patients.

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