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Evaluation of the UV-340 Spectrophotometric Determination for Pseudocholinesterase¹⁾ Activity (EC 3.1.1.8) in Human Serum

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Summary: A pseudocholinesterase catalytic activity assay using *p*-hydroxybenzoylcholine as substrate and measuring the decrease of NADPH at 340 nm was compared with a colorimetric method using acetylthiocholine as substrate.

The assay is simple, uses 50 µl serum and is performed at 37 °C. Precision of the UV-340 method was good except at low ranges. The catalytic activity was depressed by the anticoagulants citrate and fluoride but not by EDTA or heparin. The reference values obtained with the evaluated UV-340 method are somewhat higher than those with the colorimetric method.

As the results with both methods are comparable, the choice of procedure will depend on the local facilities.

Introduction

The determination of pseudocholinesterase catalytic activity in human serum or plasma is of value in testing liver synthesis function, and is known to be a sensitive parameter of intoxication with organophosphorus compounds (1, 2, 3).

Several colorimetric methods for the determination of serum cholinesterase catalytic concentration have been developed using iodide salts of butyryl-, benzoyl-, acetyl- or propionylcholine as substrates (4–6).

Recently, *Ashihara* and coworkers developed a method with the new substrate *p*-hydroxybenzoylcholine and with *p*-hydroxybenzoate hydroxylase as a coupled enzyme (7). Serum cholinesterase catalytic concentration is calculated by measuring the decrease of NADPH during this reaction at 340 nm. We report an evaluation of the UV-340 method. For reference we have used a colorimetric test with acetylthiocholine iodide as substrate (8).

Furthermore, we have determined reference values for serum cholinesterase activity using both methods. We also demonstrate that serum can be preserved for later determination of cholinesterase catalytic activity (9) using the UV-340 method.

Materials and Methods

Measurement of pseudocholinesterase with *p*-hydroxybenzoylcholine as substrate (UV-340 method)

Serum cholinesterase catalytic activity was determined according to a method developed by *Ashihara* et al. (7).

This method is based on the following reactions:

Protocatechuate 3,4-dioxygenase is added to decompose interfering accumulated 3,4-dihydroxybenzoic acid, which is produced during the reaction.

¹⁾ Enzymes

Pseudocholinesterase EC 3.1.1.8

p-hydroxybenzoate hydroxylase EC 1.14.13.2

protocatechuate 3,4-dioxygenase EC 1.13.11.3

Apparatus

Disposable cuvettes (Pharmaseal, cat. no. L1045M); 0.05 and 0.5 ml pipettes (Eppendorf, Hamburg, W. Germany); 2.5 ml volume pipettes; 37 °C water bath; Vitatron DCP photometer with 340 nm filter and Vitatron Digital printer (Meyvis, Bergen op Zoom, Netherlands); Spectrophotometer (Beckman 24, Mijdrecht, Netherlands).

Solutions

Solution A: 1000 U/l *p*-hydroxybenzoate hydroxylase
1000 U/l protocatechuate 3,4-dioxygenase
0.2 mmol/l NADPH
0.05 mol/l Tris-maleic acid buffer, pH 8.2

Substrate: 0.5 mmol/l hydroxybenzoylcholine iodide salt.

Reagent Blank: distilled water.

Serum samples were obtained from healthy controls and from patients with liver disease.

Procedure

1. 0.05 ml serum or Reagent Blank is pipetted into a cuvette and 2.5 ml solution A is added, mixed and preincubated at 37 °C for about five minutes.
2. Substrate solution is heated to 37 °C and 0.5 ml is added to (1).
3. After rapid mixing, the variation of the absorbance at 340 nm is measured every minute during three minutes at 37 °C.
4. Calculation (10) of serum cholinesterase catalytic concentration (U/l):

$$\frac{\Delta A_{\text{corrected}} \times 3.05 \times 10^6}{622 \times d \times 0.05} \text{ U/l} = \frac{(\dots \text{ kU/l})}{1000}$$

$\Delta A_{\text{corrected}} = \Delta A_{\text{sample}} - \Delta A_{\text{Reagent Blank}}$ (ΔA_{sample} or $\Delta A_{\text{Reagent Blank}}$ is the mean of the three variations).

Molar lineic absorbance of NADPH is $622 \text{ m}^2 \cdot \text{mol}^{-1}$

3.05 = total volume at the time of reaction (ml)

0.05 = volume sample (ml)

d = cell length (light path)

Measurement of serum cholinesterase with acetylthiocholine as substrate (colorimetric method)

As reference determination, serum cholinesterase catalytic concentration was determined using a colorimetric test (Boehringer Mannheim GmbH, W. Germany, cat. no. 124117) with acetylthiocholine iodide as substrate (8).

For quality control we used Precinorm U and Precipath U (Boehringer Mannheim GmbH, cat. no. 171735 and 171760 respectively) with known catalytic concentration.

Precinorm U: mean 1.02, range 0.87–1.18 kU/l

Precipath U: mean 1.13, range 1.13–1.30 kU/l

Statistical analysis

Linear regression analysis and Z-transformation of the correlation coefficient (11), the Wilcoxon two-sample test and the Wilcoxon signed rank test (12) were used. A difference was regarded significant at the $p \leq 0.05$ level.

Results

Precision

Within-run precision

The cholinesterase catalytic activity was determined 20 times with the two methods in serum from a healthy control and from two patients with high and low activity (tab. 1).

Precision with the UV-340 method was not satisfactory for very low values.

Between-run precision

In a healthy control and in a liver patient, the cholinesterase catalytic concentration was determined 20 times in each serum sample with the two methods, using different batches (tab. 2). Using different batches, precision for both methods and both sera was not fully satisfactory.

Day-to-day precision

Day-to-day precision was assessed on three consecutive working days by three different technicians in one serum sample using the two methods.

UV-340 method: mean 4.18, range 3.92–4.41 kU/l, coefficient of variation 3.3%

Colorimetric: mean 3.47, range 3.26–3.70 kU/l, coefficient of variation 3.3%

Linearity and quality control

Linearity of the method was investigated several times by diluting in saline a sample with a known high cholinesterase catalytic activity. The end volume was always kept at 200 µl and the dilutions varied from 0.5–6.00 kU/l (fig. 1).

The same dilutions were also determined using the colorimetric method. In all ranges (up to 6.00 kU/l) linearity was good.

For quality control two control sera (see: Materials and Methods) were used. The values found fell in the ranges given above.

Effect of storage at 4 °C and at –20 °C

To confirm the data that serum can be stored for longer periods and to evaluate this phenomenon for the UV-340 method, we performed nine assays after 1, 2, 3, 4, 5, 6, 8, 10 and 14 days of storage in the refrigerator (4 °C, fig. 2a) and seven assays after 1, 3, 8 and 16 weeks of storage at –20 °C (fig. 2b).

Tab. 1. Within-run precision determined 20 times in a control serum and in sera of patients with high and low pseudocholinesterase catalytic concentrations, using the two methods.

n = 20	Normal catalytic concentration			High catalytic concentration			Low catalytic concentration		
	mean (kU/l)	range (kU/l)	CV (%)	mean (kU/l)	range (kU/l)	CV (%)	mean (kU/l)	range (kU/l)	CV (%)
UV-340	2.85	2.81–2.94	1.4	5.72	5.66–5.92	1.2	0.52	0.47–0.57	6.7
Colorimetric	2.69	2.57–2.76	1.9	5.29	5.12–5.57	2.3	0.51	0.50–0.53	1.7

Tab. 2. Between-run precision of the assay of pseudocholinesterase catalytic concentration in the sera of a healthy control and a liver patient.

n = 20	Control			Patient		
	mean (kU/l)	range (kU/l)	CV (%)	mean (kU/l)	range (kU/l)	CV (%)
UV-340	4.02	3.86–4.25	2.4	1.74	1.57–1.86	5.1
Colorimetric	3.49	3.08–3.78	4.5	1.60	1.56–1.64	1.2

Fig. 1. Linearity of the pseudocholinesterase assay determined by diluting a sample with high catalytic concentration several times in saline
 a) UV-340 method
 b) colorimetric method
 Limitation was measured by increasing the volume of serum up to 200 μ l.

Fig. 2a. Influence of storage on serum cholinesterase catalytic activity for 1–14 days at 4°C in nine experiments using the UV-340 method.

Fig. 2b. Influence of storage on serum cholinesterase catalytic activity for 16 weeks at -20°C in seven experiments using the UV-340 method.

Fig. 2c. Influence of deep freezing and thawing at weekly intervals for four weeks in three experiments using the UV-340 method.

Serum of three patients suffering from liver disease were thawed and frozen again each week for four weeks (fig. 2c). In all experiments the catalytic activity was unaffected, thereby confirming earlier findings (9, 13).

Collection of blood: use of anticoagulants

To examine the effect of interfering substances on catalytic activity, different anticoagulants were tested in sera of two control persons. For collection of blood we used several vessels supplied by Omnilabo Nederland B.V. (Breda, Netherlands). The cholinesterase activity was determined using the two methods (tab. 3). Sodium fluoride and citrate should not be used as anticoagulants as they depressed cholinesterase activity as measured with both methods.

Comparison with colorimetric method

Cholinesterase catalytic concentration in samples of a normal healthy population and patients suffering from liver disease are shown in figure 3, where the correlation of the UV-340 method versus colorimetric is shown. The correlation found between the methods was excellent with a correlation coefficient of 0.963.

Reference values

The serum cholinesterase catalytic concentration was determined in the sera of 102 healthy controls (tab. 4) using the UV-340 method and the colorimetric method.

There were 47 men, aged 17–59 (median 30) and 55 women, aged 18–45 (median 25). The catalytic concentration was significantly lower in females than in males, as determined by both methods ($p <$

Fig. 3. Correlation between pseudocholinesterase catalytic concentration obtained with the UV-340 method versus the colorimetric method using acetylthiocholine as substrate.

$$y = 1.15x - 0.08; r = 0.963; p < 0.001; n = 160.$$

0.0001). This sex difference was also observed by other investigators (4, 9, 14). The catalytic activity using the UV-340 method was significantly higher ($p < 0.0001$, Wilcoxon signed rank test) than that determined by the reference method (tab. 5). Between women who were ($n = 22$) or were not ($n = 33$) using oral contraceptives there was no difference ($p = 0.14$) in cholinesterase concentration.

Tab. 3. Effect of anticoagulants on pseudocholinesterase catalytic activity in sera from two controls, using the two methods.

Anticoagulant	Control 1		Control 2	
	UV-340 (kU/l)	Colorimetric (kU/l)	UV-340 (kU/l)	Colorimetric (kU/l)
Serum without addition	5.20	4.16	3.14	2.68
Additives				
45 IU Na heparinate	5.12	4.05	3.07	2.60
3.3 mg K ₂ -EDTA	5.09	4.03	3.10	2.63
0.2 ml 0.13 mol/l citrate*)	4.08	3.21	2.84	2.43
4 mg fluoride, 2 mg K ₂ -EDTA	4.32	2.72	2.55	1.83
6.75 mg K oxalate + 6.75 mg NaF	4.41	3.05	2.35	1.83

*) The corresponding values are corrected for the dilution effect.

Tab. 4. Pseudocholinesterase catalytic concentration in serum of healthy controls using the UV-340 method and the reference method (substrate: acetylthiocholine).

n	UV-340		Colorimetric	
	males 47	females 55	males 47	females 55
Mean (kU/l)	3.71	2.91	3.22	2.56
SD (kU/l)	0.77	0.56	0.60	0.52
Range (kU/l)	1.93–5.33	1.54–3.73	2.11–4.48	1.53–3.52

Conclusions

Both methods give adequate results and are easy to perform. The choice therefore will depend on the equipment of the individual laboratory. Finally, the evaluated UV-340 method is applicable to several different types of automatic analysers.

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Tab. 5. Pseudocholinesterase catalytic concentration using the UV-340 method versus a reference method (substrate: acetylthiocholine) in 102 healthy controls.

	UV-340 method	Colorimetric
Mean (kU/l)	3.19	2.86
SD (kU/l)	0.81	0.65
Range (kU/l)	1.54–5.33	1.53–4.48

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