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Enzymatic determination of total cholesterol in serum by flow injection analysis

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Abstract: Several methods are proposed for the enzymatic determination of total cholesterol in serum by flow injection analysis (FIA). The use of the merging-zones mode, with or without halting of the reacting plug in the photometric or fluorimetric detector, has allowed the development of four precise methods for the determination of this analyte; calibration curves were linear for ranges of 0.02–0.2 and 0.005–0.05 gl^{-1}. These methods have been applied to the determination of cholesterol in serum with excellent results.

Keywords: *Cholesterol; enzymatic analysis; photometry; fluorimetry; flow injection analysis; serum.*

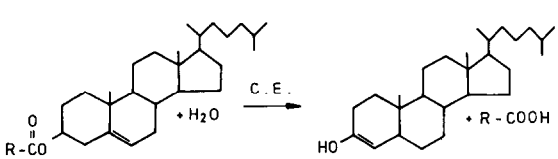
Introduction

The great catalytic potential and high selectivity of enzymes are the basic reasons for their extensive use in analytical methods, especially in the last decade [1, 2]. Although enzymatic analysis is becoming increasingly important in applications such as food and industrial analysis, the field of clinical chemistry has benefited most from the advantages associated with the use of enzymes for analytical purposes.

The association of flow injection analysis (FIA) and enzymes is an excellent marriage, as shown by their joint use [3–5].

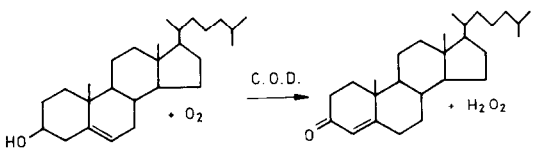
This paper reports four new optical (photometric and fluorimetric) enzymatic methods for the determination of cholesterol in serum by the merging-zones or merging-zones/stopped-flow modes. The reaction on which these methods are based consists of three steps; the first two are common to both types of detectors whereas the third step is characteristic of the detection system.

Step 1. Hydrolysis of esterified cholesterol catalysed by cholesterol esterase (C.E.):

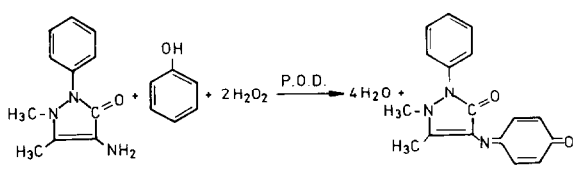


*To whom correspondence should be addressed.

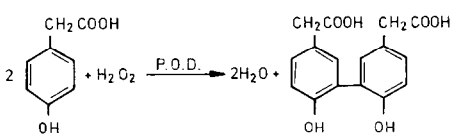
Step 2. Oxidation of cholesterol by atmospheric oxygen in the presence of cholesterol oxidase (C.O.D.):



Step 3. Indicator reaction. Hydrogen peroxide formed in the second step can react with the aid of peroxidase (P.O.D.) to form the monitored product.
(a) With 4-aminophenazone and phenol, hydrogen peroxide forms an imine derivative with maximum absorption at 500 nm.



(b) With 4-hydroxyphenylacetic acid, hydrogen peroxide forms a fluorescent dimer with a rigid structure which absorbs at 325 nm and shows maximum emission at 415 nm.



Several enzymatic–FIA methods have been proposed for the determination of cholesterol in biological fluids; all make use of amperometric detection for the determination of total cholesterol [6] through the first two steps described above. Determination of free cholesterol [7] (for which only cholesterol oxidase is necessary) is achieved by avoiding the first step of the above sequence; both free and sterified cholesterol are determined in the absence and presence, respectively, of cholesterol oxidase [8]. In either case, amperometric measurement of the hydrogen peroxide formed in the second step is performed. The lack of optical methods for the determination of cholesterol led to the development of the methods proposed in the present work.

Experimental

Apparatus

A Perkin–Elmer Lambda 1 spectrophotometer equipped with a Hellma 178.12QS flow-cell, a Perkin–Elmer LS 1LC fluorescence detector with a 4 μl flow-cell, a Tecator 5020 flow injection analyser with a home-made dual injection valve and a Tecator type II chemifold were used.

Reagents

Standard solutions of cholesterol (Boerhinger Mannheim) were prepared by dissolution of the substance in 10% (v/v) isopropyl alcohol with surfactant (4.0% (v/v) Triton X-100

or 1.5% (v/v) Brij-35) in buffer; 0.5 M TRIS–HCl (pH 7.0) and 0.5 M phosphate (pH 8.2) were used as buffers for the photometric and fluorimetric methods, respectively.

Stock solutions of cholesterol esterase and cholesterol oxidase (Sigma Chemical Co.) were prepared by dissolution of 250 and 100 U, respectively, in 1 ml of 0.1 M phosphate buffer (pH 7.0). The stock solution of peroxidase (Boerhinger Mannheim) contained 10,000 U in 10 ml of 0.1 M phosphate buffer (pH 7.0).

Solutions were prepared by dilution of the stock solutions of the three enzymes with 10% (v/v) isopropyl alcohol and surfactant (4.0% (v/v) Triton X-100 or 1.5% (v/v) Brij-35), for the photometric and fluorimetric methods, respectively.

The reagent solution (photometric methods) comprised 0.5 mM 4-aminophenazone, 10 mM phenol and 2 mM sodium cholate in 0.5 M TRIS–HCl buffer (pH 7.0) with 10% (v/v) isopropyl alcohol and 2.0% (v/v) Triton X-100.

The reagent solution (fluorimetric methods) was 0.5 mM 4-hydroxyphenylacetic acid in 0.5 M phosphate buffer (pH 8.2) with 10% (v/v) isopropyl alcohol and 1.5% (v/v) Brij-35.

Manifolds

The merging-zones methods were conducted with the configuration in Fig. 1 without the microprocessor; the solution of the sample and the solution containing the three enzymes are simultaneously injected into the corresponding reagent stream. A single bead string reactor (SBSR) [9] favours mixing of both boluses, the different steps of the reaction taking place along the reactor R; the temperature of both solutions is maintained by a thermostat.

The methods involving merging-zones and stopped-flow incorporate a microprocessor which synchronizes the stop and start of the pump with the injection; in this case the temperature of the flow-cell requires to be kept constant by a thermostat to achieve optimum progress of the reaction. The length of reactor R in the stopped-flow method is very short so that the time that elapses between the mixing of sample and reagent and the arrival of the reacting plug at the detector is very short and the progress of the reaction can be monitored from the start.

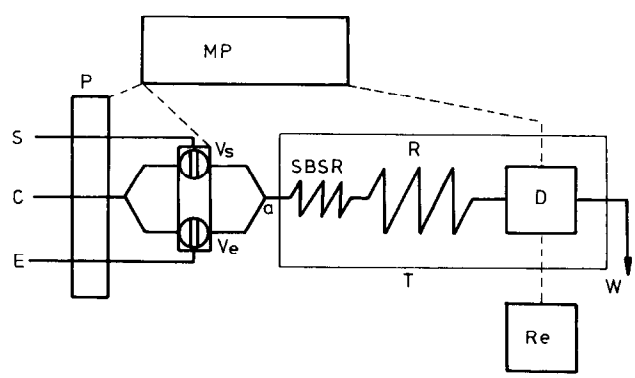


Figure 1
Configuration used for the determination of cholesterol in serum. The inclusion of a microprocessor (dashed lines) is only necessary for stopped-flow methods. P = peristaltic pump; V_s = injected sample volume; V_e = injected enzyme volume; SBSR = single bead string reactor; R = open reactor; D = detector; W = waste; Re = recorder; MP = microprocessor; C = carrier; E = enzyme solution; S = sample solution; and T = thermostat.

Proposed procedure

Fifty microlitres of serum in a 5-ml volumetric flask is diluted to 5 ml with the solution used to prepare the standard and aspirated into the sample loop with the aid of the peristaltic pump. The insertion of the contents of the sample loop simultaneously with the solution of enzymes enables mixing to occur; measurements are made of the maximum height of the FIA peak (merging-zones with photometric detection method) or of the change in the signal with time (merging-zones/stopped-flow method, kinetic mode [10] with fluorimetric detection).

Reference procedure[11]

Four millilitres of the reagent solution [2 mM 4-aminophenazone, 6 mM sodium cholate, 0.2 M KCl and 8 mM phenol with peroxidase (50 U/ml), Triton X-100 (2 ml/l), cholesterol oxidase (0.5 U/ml) and cholesterol esterase (0.5 U/ml)] are added to a test tube containing 5 μl of cholesterol standard or sample. The mixture is incubated at 37°C for 12 min, and the absorbance is then measured at 500 nm.

Results and Discussion

Development of the method

A preliminary conventional kinetic study of the different steps involved (hydrolysis, oxidation and indicator reaction) was carried out to determine if the rate of each of the reactions was suitable for use in FIA; in fact even the slowest step (formation of the photometrically monitored product) was completed within 2 min. The spectra corresponding to the final product showed maximum absorption at 500 nm for photometric monitoring and maxima at 325 and 415 nm for excitation and emission, respectively, for fluorimetric monitoring.

Variables were optimized by the univariate method [12]. The study covered both chemical (type and concentration of buffer, pH and concentration of the carrier components and that of the enzymatic solution), FIA (total flow-rate, type and length of the reactors and injected volume) and physical (temperature) variables for the merging-zones, photometric and fluorimetric methods. The study included the variables characteristic of stopped-flow (delay and stop time) in the case of the merging-zones/stopped-flow methods. The optimum values found in each case are shown in Table 1.

The influence of the chemical variables is diverse. The optimum value of the analytical signal (peak height for the merging-zones methods and signal increment over a fixed interval for stopped-flow methods) for cholesterol esterase was obtained at a concentration of 0.8 U/ml. For cholesterol oxidase and peroxidase, however, there was a continuous increase in the signal with concentration; a compromise was adopted between sensitivity and analytical costs. Among the types of buffer generally used by conventional methods (PIPES, TRIS–HCl and phosphate [13–15]), the second and third are best for the photometric and fluorimetric methods, respectively; above 0.5 M the changes in their concentration are of little influence. The optimum pH in each case also appears in Table 1, as well as the reagent concentrations yielding the coloured and fluorescent products.

Temperature, a key to enzymatic activity, exerts a marked influence on the system. The value chosen, 40°C, was a compromise based on the effects of temperature on the sensitivity of the method and on the stability of the enzyme.

It should be noted that insertion of a 150-cm SBSR after the confluence point of the

Table 1
Optimum values of the variables

Variable		Photometric methods*		Fluorimetric methods*	
		mz–FIA	mz/sf–FIA	mz–FIA	mz/sf–FIA
Physical	Temperature (°C)	40	40	40	40
Instrumental	Wavelength (nm)	500	500	310 (ex) 415 (em)	310 (ex) 415 (em)
	Sensitivity	—	—	0.01	0.01
FIA	Flow-rate (ml min^{-1})	0.8	0.8	0.7	0.7
	Length open reactor (cm)	600	—	100	—
	Length SBSR (cm)	150	30	150	30
	Injected sample volume (μl)	91	91	91	91
	Injected enzyme volume (μl)	88	88	88	88
Chemical	Buffer	0.5 M TRIS–HCl	0.5 M TRIS–HCl	0.5 M phosphate	0.5 M phosphate
	pH carrier	7.0	7.0	8.2	8.2
	Cholesterol esterase (U ml^{-1})	0.8	0.8	0.8	0.8
	Cholesterol oxidase (U ml^{-1})	3.0	3.0	3.0	3.0
	Peroxidase (U ml^{-1})	100	100	100	100
	4-aminophenazone (mM)	0.5	0.5	—	—
	Phenol (mM)	10.0	10.0	—	—
	Hydroxyphenylacetic acid (mM)	—	—	5.0	5.0
	Surfactant (%)	Triton X-100 0.5	Triton X-100 0.5	Brij-35 1.5	Brij-35 1.5
	Isopropyl alcohol (%)	10	10	10	10
Times for stopped-flow method	Delay time(s)	—	34	—	17
	Stop time(s)	—	40	—	60

* mz = merging-zones; sf = stopped-flow.

enzymes and sample boluses in the non-kinetic methods (merging-zones) results in an increase in the analytical signal of about 40% compared with that produced by the use of an open reactor of the same length. The stopped-flow methods make use of a shorter SBSR (30 cm) so that the distance between the confluence point and the flow-cell is short; this results in a short residence time of the reacting plug and enables the signal to be measured at the start of the reaction. The surfactant (Triton X-100) used in the photometric methods was replaced in the fluorimetric methods by Brij-35 because of the fluorescent nature of Triton X-100.

The calibration curves for the four methods are linear for at least ten-fold ranges of concentrations, with excellent regression coefficients in all cases and sampling frequencies between 24 and 30 h^{-1}, as shown in Table 2. Because the clinical range of cholesterol concentration in serum is 1.5–2.6 gl^{-1}, the methods developed here can be used, after suitable dilution, for determination of this analyte in blood.

The statistical study performed to test the reproducibility of the methods with 11 different samples in each case (0.1 g l^{-1} of analyte), prepared under identical conditions and injected in triplicate, yielded results which show the precision of the proposed methods; the relative standard deviation was less than 2.1% in all cases for a confidence level of 95% (Table 2).

The detection limit of the proposed methods, calculated as three times the standard deviation of the blank divided by the slope of the calibration curve, is also shown in Table 2.

The study of the potential interferents was based on those commonly considered in the literature [10, 11, 13] for the determination of this analyte: glucose, urea, bilirubin, haemoglobin, uric and ascorbic acids in foreign species: analyte ratios from 100-fold that existing in human serum to 1:1, in the case of haemoglobin the ratio used was that normally found in haemolysed serum. The results showed that: glucose, urea and creatinine are tolerated in a 100:1 ratio; bilirubin and haemoglobin do not cause interference in a 10:1 ratio; there is no interference with uric and ascorbic acids in a 1:1 ratio, except in the fluorimetric method with merging-zones, in which ascorbic acid causes a slight disturbance at this level.

Application of the proposed methods to the determination of cholesterol in serum

Two of the proposed methods (photometric merging-zones and fluorimetric merging-zones/stopped-flow) were selected to test their validity for application to the determination of cholesterol in serum. Samples of human serum corresponding to both healthy and sick individuals were used. The analyte concentration in each sample was previously determined by the conventional reference method after applying the two selected methods to each sample, with a prior 1:100 dilution, and also performing two additions of standard to determine the recovery of cholesterol. Table 3 lists the results of this study. Agreement between these results and those obtained by the conventional methods is excellent. The recovery of analyte is also quite good.

Thus it can be concluded that the proposed methods are suitable for application in clinical laboratories and are better than the amperometric FIA methods [7–9], both in determination limit and in reproducibility. Unlike the present study, none of the previous studies on other methods included an investigation of interferents normally present in biological fluids, a comparison with conventional methods, or determination of the recovery of analyte, which in the present work corroborate the merits of the proposed methods. Interference caused by the presence of ascorbic acid in the merging-

Table 2
Features of the methods*

Detection	FIA mode	Equation of calibration curve	Regression coefficient	Linear range ($g\ l^{-1}$)	Detection limit ($g\ l^{-1}$)	RSD (%) ($n = 33$)	Sampling frequency (h^{-1})
Photometric	mz	$A = 1.643x - 0.001$	0.999	0.01–0.10	0.0013	1.28	24
Photometric	mz–sf	$\Delta A = 0.764x - 0.199$	0.997	0.01–0.20	0.0006	2.08	30
Fluorimetric	mz	$\%I_f = 1.137x - 0.038$	0.991	0.02–0.20	0.0020	1.45	30
Fluorimetric	mz–sf	$\Delta I_f = 4.509x - 0.001$	0.996	0.005–0.05	0.0004	1.66	26

*RSD = relative standard deviation; mz = merging-zones; sf = stopped-flow; x = cholesterol concentration, $g\ l^{-1}$; A = absorbance; I_f = fluorescence intensity; sampling frequency in h^{-1}.

Table 3
Determination of cholesterol in serum*

Sample No.	Concentration ($g\ l^{-1}$) Conventional method	Concentration ($g\ l^{-1}$) Photometric method			Concentration ($g\ l^{-1}$) Fluorimetric method		
		Found ($g\ l^{-1}$)	Recovery (%)†		Found ($g\ l^{-1}$)	Recovery (%)‡	
			First addition	Second addition		First addition	Second addition
1	1.79	1.77	100	101	1.78	106	108
2	1.88	1.88	99	108	1.86	100	96
3	1.90	1.89	98	100	1.88	104	101
4	1.96	1.91	100	103	1.93	97	97
5	2.06	2.04	106	101	2.05	99	104

*Dilution: 1:100.
†First and second additions: 0.02 and 0.06 $g\ l^{-1}$, respectively.
‡First and second additions: 0.015 and 0.03 $g\ l^{-1}$, respectively.

zones fluorimetric method is not a serious problem for the application of the method to the determination of the analyte in serum since ascorbic acid is rapidly oxidised by atmospheric oxygen to form products that do not interfere with the method.

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