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Monosegmented flow-analysis of serum cholesterol

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Abstract

A monosegmented flow system is designed for enzymatic spectrophotometric determination of cholesterol in blood serum. The sample (4.5 μl), enzymatic reagent (150 μl) and an air plug (100 μl) are simultaneously inserted into a carrier stream buffered to pH 7.4 (potassium dihydrogenphosphate). In order to avoid the step of air removal, a relocating detector was used. This system handles about 42 samples per hour, yielding precise results (R.S.D. usually $< 3.0\%$). Sensitivity is 46 mAU l/mmol (mAU stands for milliabsorbance units), being the method linear up to 10.3 mmol/l cholesterol. Accuracy was assessed by running 30 samples already analysed by a conventional procedure: no statistical difference between methods was found at the 95% confidence level.

Keywords: Monosegmented flow analysis; Slow enzymatic reactions; Serum total cholesterol

1. Introduction

Automation of analytical procedures by continuous flow analysis has been based on segmented flow analysis (SFA) and flow injection analysis (FIA) techniques [1]. The former approach consists in the insertion into a tubular reactor of sample volumes between two air plugs thus forming a stream regularly segmented and ensuring sample individuality [2]. However, this intense flow segmentation results in a high compressibility of the liquid stream and in consequence flow irreproducibility, therefore requiring measurements under chemical equilibrium. Regarding FIA approach, insertion of the sample into a non-segmented continuous flow stream, in which a reaction or dilution occurs, is carried out. Unlike SFA procedures, flow compression is absent so, measurements can be performed within shorter time intervals without requiring chemical equilibrium. Moreover, the lack of flow segmentation and shorter tubing lengths contribute to more simple and robust manifolds, including a lower consumption of samples and reagents [1].

Despite the above-mentioned advantages of FIA systems, these have not been widely used in clinical chemistry laboratories as it happened with SFA during the 1970s and 1980s [3]. Kinetic features of measurements generally allow the automation of only one or two specific parameters being thus rather deficient considering the screening basis of clinical analyses. Nevertheless, using FIA concept, automation of some parameters that are not analysed by automatic devices could be easily accomplished for procedures with simple pre-treatment steps as sample dilution or fast development colour reactions [3].

When considering slow kinetic reactions, the insertion of samples or reagents into the carrier stream involves an axial dispersion that increases with the residence time and originates low sensitive measurements or a high consumption of reagents. The applicability of FIA manifolds to slow kinetic determinations was assessed by using the stopped-flow mode [4], very low flow-rates [5], single bead string reactors [6] and zone trapping [7]. The former two operation modes caused a significant decrease of the sampling rate whereas the latter one originated an increase of the flow resistance and therefore required expensive propelling devices. In order to compensate for these hindrances, monosegmentation [8] based on sample insertion between two air plugs of a non-segmented carrier stream was proposed. The low amount of air bubbles did not significantly change the flow

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reproducibility and facilitated a considerable decrease of sample and reagent volumes per determination, when compared to SFA. Albeit this, the interference in the analytical signal derived from the passage of the air plugs through the detector flow cell imposes their removal by means of special devices [8–10]. Alternatively, relocation of the detector in monosegmented flows has been proposed [11]. It consists in the insertion of a single air plug to reduce the axial dispersion of the sample in the opposite direction of the flow, and relocation of the detector right after the measurement to avoid the passage of the air plug through the detector. The possibility of performing clinical determinations by using a monosegmented flow system that comprised a relocating detector assisted by an injection/commutation device was shown recently by having accomplished the automation of on-line enzymatic determinations of blood serum triglycerides [12].

The determinations of serum total cholesterol are very frequent due to its close connection to cardiovascular diseases, though the conventional enzymatic procedure used [13] is rather expensive regarding reagent and time consumption. The automatic determination of serum total cholesterol by flow injection analysis combined with amperometric detection [14], enzymes immobilisation [15], stopped-flow and merging zones [16] was already proposed. Nevertheless, the accomplishment of these determinations was always dependent on samples pre-treatment for the elimination of interference's or decrease of time and reagent consumption per determination.

2. Experimental

2.1. Reagents and solutions

Solutions were prepared with deionised water (conductivity < 0.1 μS/cm) and analytical grade reagents.

A 0.5 mol/l phosphate buffer solution was prepared by adding 68.1 g potassium dihydrogenphosphate, 60 μg Triton X-100 and 2.0 g phenol to 800 ml water. The pH of the buffer solution was adjusted to 7.4 using 1.0 mol/l sodium hydroxide and the buffer volume made up to 1 l with water. This solution was used as carrier and for the reconstitution of the enzymatic reagent.

The enzymatic reagent was prepared by solubilisation of a vial of lyophilised enzymes from the CHOD-PAP Cholesterol kit (Biocon, Burbach, Germany) in 100 ml of the buffer solution. The enzymes activity in the reagent solution was 200, 300 and 1250 U/l for cholesterol oxidase, cholesterol esterase and peroxidase, respectively. This reagent solution was also composed of 0.4 mmol/l 4-aminoantipyrine. Preciset (Boehringer, Mannheim) cholesterol calibrating solutions in concentrations of 1.29, 2.59, 3.88, 5.17, 7.76 and 10.35 mmol/l were used throughout.

Precision of the results given by the proposed manifold was assessed by using laboratorial common samples randomly collected which were kept in a refrigerator prior to analysis. Two pools of commercial sera, Biocon Contronorm (Lot 302/A) and Contropath (Lot 4820) ref. 1205 and 1305, respectively, were also used.

2.2. Apparatus

The monosegmented flow system (Fig. 1) was composed of a Gilson Minipuls 3 peristaltic pump fitted with PVC tubing of the same brand for the propelling of the solutions. The reactor (R) was constructed with a PTFE tube, with 0.5 mm i.d. and 500 cm length, coiled around a 2 mm steel string and immersed in a 37°C thermostatic bath. The simultaneous insertion of air plug, sample and reagent and detector relocation was performed by a four-section injector/commutator device [17]. A Jenway 6105 spectrophotometer equipped with a Hellma 178.713QS flow cell of 8 μl optical path was used as detector. The analytical signals were displayed by a Kipp & Zonen 111BD recorder.

The flow cell was connected to the end-fittings of the mobile slide, which corresponded to the fourth insertion section of the injector/commutator device, by means of two PTFE tubes (0.5 mm i.d.) having a length of 20 cm each.

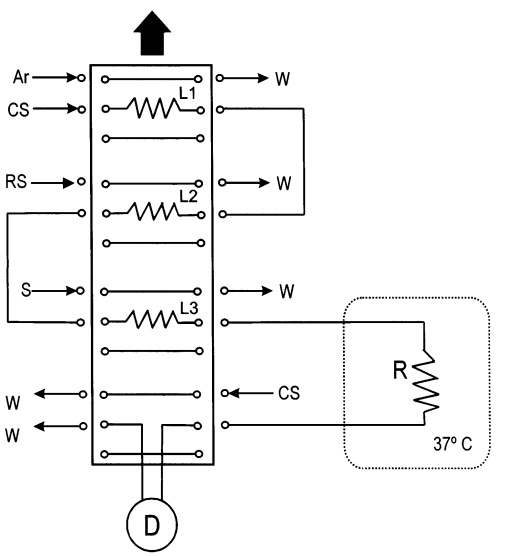

Fig. 1. Flow diagram of the monosegmented system for determination of cholesterol in blood serum. The large rectangle represents the central slide bar of the injector/commutator device in its intercalation position. Ar, air; Cs, carrier solution (sodium cholate 0.75 g/l, Triton X-100 60 μg/l and phenol 2 g/l in phosphate buffer); D, detector; L1, air loop (100 μl); L2, reagent loop (145 μl); L3, sample loop (4.5 μl); R, reaction coil (500 cm, 0.5 mm i.d.); RS, reagent solution (cholesterol esterase 300 U/l, cholesterol oxidase 200 U/l, peroxidase 1250 U/l, 4-aminoantipyrine 0.4 mmol/l in carrier solution); S, sample; W, waste. Flow rate 0.35 ml/min.

Determinations of cholesterol by the conventional procedure [13] were carried out by using a Hitachi U2000 double beam spectrophotometer.

3. Results and discussion

Hydrolysis of cholesterol ester by cholesterol esterase was the initial step of the enzymatic procedure used [13]. The free cholesterol produced was oxidised to cholest-4-en-3-one, with the concomitant production of hydrogen peroxide. In the presence of peroxidase, the latter bonded 4-aminoantipyrine with phenol, resulting in a colour product measured at 546 nm.

To accomplish this enzymatic procedure the manifold depicted in Fig. 1 was implemented in which sera samples are introduced without any previous treatments. The manual displacement of the injector/commutator mobile slide allowed the simultaneous insertion of an air plug and a reduced volume of sample and reagent aspirated into the respective loops by means of pumping tubes mounted over the peristaltic pump. In addition the same slide displacement enabled the positioning of the detector lined up with reactor R. At the reactor, the sample dispersed into the reagent, allowing the formation of the colour product proportionally to the sample residence time in the reactor and to the total cholesterol concentration of the sample. Immediately after recording the transient analytical signal maximum, corresponding to the passage of colour product through the detector, the injector/commutator mobile slide was manually displaced to its former position in order to avoid the air plug passing through the detector and to promote the filling-up of the loops with a new air/sample/reagent set (Fig. 2).

The proposed set-up was optimised by formerly studying the chemical composition of the buffer solution used as carrier and for the reconstitution of the enzymatic reagent) and then the hydrodynamic features of the manifold.

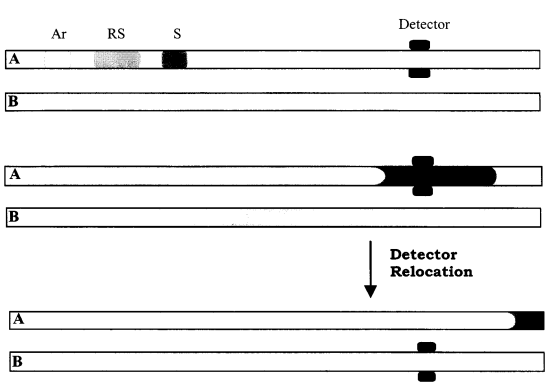

Fig. 2. Schematic representation of the stream of solutions into the reactor and detector relocation. A, reactor R with detector in line; B, lateral channel where detector is placed when mobile slide is in the loading position with new sample; Ar, air plug; RS, reagent solution plug; S, sample plug.

The effect of the buffer solution phosphate concentration was evaluated by selecting a sample insertion volume of 4.5 μl (the minimum allowed by the injector/commutator), a reagent volume of 170 μl and a carrier flow rate of 0.4 ml/min. A linear increase of the analytical signal intensity was found when the buffer solution phosphate concentration (pH 7.0) increased from 0.1 to 0.5 mol/l suggesting that rigorous control of pH was necessary to ensure maximum enzymatic activity. This increase was observed for both the calibrating solutions and the commercial sera pools. Higher phosphate concentrations produced no significant changes in the analytical signal height. The buffer solution pH was also evaluated and set to 7.4 since a wider reaction rate was attained within the range of 7.0–8.0.

Afterwards, the effect of tensioactive agents Triton X-100 and sodium cholate, on the method sensitivity was assessed. The presence of Triton X-100 seems to be rather important for the increment of cholesterol oxidase catalytic activity and free cholesterol solubility in aqueous medium [18], though higher amounts of this agent inhibit the enzymes activity and therefore, its concentration in reaction medium was studied. A pronounced decrease of the analytical signal intensity was found both for calibrating solutions and commercial sera pools when Triton X-100 concentration was over 60 μg/l. Lower concentrations originated a decrease of the analytical signals intensity attained for commercial sera pools. Sodium cholate enhances cholesterol esterase activity [13], being its optimal concentration of 0.75 g/l under the flow conditions studied.

The temperature effect on the enzymatic system performance was also evaluated and it was found that for commercial sera pools, increasing temperature from room temperature up to 38°C caused an increase in signals intensity. Higher temperatures originated a gradual loss of sensitivity.

After chemical optimisation of the carrier stream, the manifold working characteristics were also optimised. Increased flow rates were formerly evidencing a decrease of the corresponding analytical signals. For lower flow rates an additional dispersion of the sample plug in the reagent was observed. As this effect resulted in the loss of the calibration curves linearity derived from the enzymatic reagent saturation, and in a diminishing of the sampling rate due to the increase of the residence time, a flow rate of 0.35 ml/min was found to be the best compromise between net product formation and physical dispersion.

The influence of the inserted reagent volume on the analytical signals outline was equally evaluated in order to reduce the consumption of the enzymatic reagent per determination. The highest signal intensity was attained with insertion volumes of 145 μl and was kept constant

with greater volumes. This parameter was optimised after the buffer solution volume remaining between the air and reagent plugs had been reduced to that which would avoid reagent dilution.

In the optimised manifold, an air (100 μl), sample (4.5 μl) and reagent (145 μl) set was simultaneously inserted into the buffer solution stream (0.5 mol/l phosphate, 7.4 pH) that flowed at a 0.35 ml/min rate, by displacement of the injector/commutator mobile slide. This set was then led through a coiled tubular path (500 cm length, 0.5 mm i.d), that was kept immersed in a 37°C thermostatic bath, towards the detector for a residence time of 210 s. After attainment of the highest transient signal, the injector/commutator was once more displaced to allow the relocation of the detector to a side channel, thus avoiding the air passage through the detector and restoring the signal to the baseline. At the same time, the loops were filled up with a new air/sample/reagent set for about 25 s. MCFA technique combined with a long reactor allowed the new reaction set to be inserted before the previous reached the detector. Hence, the sampling rate was solely limited by the time interval required for the air plug to pass along the sample and reagent loops towards the reactor (42 s), and thus a maximum sampling rate of 42 samples per hour was achieved (Fig. 3).

3.1. Application to real sample analysis

Accuracy of the proposed method was assessed by determining the total cholesterol in 30 serum samples (3.0–6.5 mmol/l) and comparing the results accomplished with those provided by the conventional procedure [13]. This was carried out by adding a 15 μl sample to 1500 μl of enzymatic reagent followed by a 10 min incubation period. Afterwards, the mixture absorbency was measured at 546 nm. The samples of cholesterol concentration was obtained by graphic interpolation in a calibration curve which was composed of the same calibrating solutions used for the proposed method.

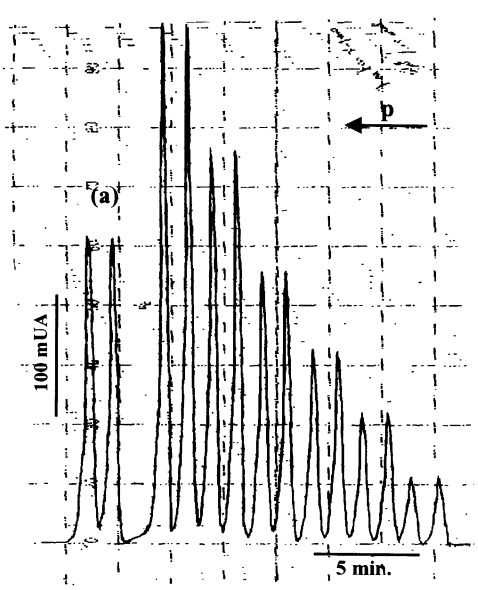

Fig. 3. Typical recorder output relating to determination of cholesterol in blood serum. S, cholesterol standard solutions 1.29, 2.59, 3.88, 5.17, 7.76 and 10.35 mmol/l; (a) sample.

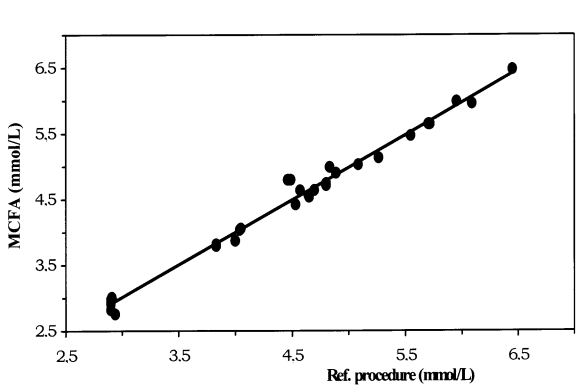

Fig. 4. Correlation between cholesterol determined by the reference and the proposed methods.

The relationship: C_{MCFA} (mmol/l) = 0.989($\pm$0.045) $\times$ C_{Ref} (mmol/l) + 0.042($\pm$0.205); r = 0.993 (95% confidence level), where C_{MCFA} expresses the result obtained by the proposed methodology and C_{Ref} that of the conventional methodology, was obtained (Fig. 4).

The precision was also evaluated by estimating the variation coefficient for 12 repeated injections of two sera mixtures in concentrations of 3.7 and 5.9 mmol/l which are a variation coefficient of 3.0 and 2.5%, respectively.

4. Conclusions

MCFA is a simple alternative to the automation of slow kinetic reaction procedures as it allows to increase the residence time without sample dispersion and prejudice of the sampling rate.

When compared with conventional procedure, the proposed methodology presented reduced consumption of sample and reagent, higher sample throughput and gives results of similar quality.

Relocation of the detector avoids the air plug passage through the detector as this is displaced from the analytical channel to another side channel without flow interference.

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