Talanta 118 (2014) 104–110

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fully automated analytical procedure for propofol determination by sequential injection technique with spectrophotometric and fluorimetric detections

Ivana Šrámková [a], Célia G. Amorim [b], Hana Sklenářová [a,*], Maria C.B.M. Montenegro [b], Burkhard Horstkotte [a], Alberto N. Araújo [b], Petr Solich [a]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 5 June 2013
Received in revised form
26 September 2013
Accepted 30 September 2013
Available online 10 October 2013

Keywords:
Propofol
Sequential Injection Analysis
Emulsions
Horseradish peroxidase
4-Aminoantipyrine

ABSTRACT

In this work, an application of an enzymatic reaction for the determination of the highly hydrophobic drug propofol in emulsion dosage form is presented. Emulsions represent a complex and therefore challenging matrix for analysis. Ethanol was used for breakage of a lipid emulsion, which enabled optical detection. A fully automated method based on Sequential Injection Analysis was developed, allowing propofol determination without the requirement of tedious sample pre-treatment. The method was based on spectrophotometric detection after the enzymatic oxidation catalysed by horseradish peroxidase and subsequent coupling with 4-aminoantipyrine leading to a coloured product with an absorbance maximum at 485 nm. This procedure was compared with a simple fluorimetric method, which was based on the direct selective fluorescence emission of propofol in ethanol at 347 nm.

Both methods provide comparable validation parameters with linear working ranges of 0.005–0.100 mg mL^{-1} and 0.004–0.243 mg mL^{-1} for the spectrophotometric and fluorimetric methods, respectively. The detection and quantitation limits achieved with the spectrophotometric method were 0.0016 and 0.0053 mg mL^{-1}, respectively. The fluorimetric method provided the detection limit of 0.0013 mg mL^{-1} and limit of quantitation of 0.0043 mg mL^{-1}. The RSD did not exceed 5% and 2% ($n=10$), correspondingly. A sample throughput of approx. 14 h^{-1} for the spectrophotometric and 68 h^{-1} for the fluorimetric detection was achieved. Both methods proved to be suitable for the determination of propofol in pharmaceutical formulation with average recovery values of 98.1 and 98.5%.

1. Introduction

Sequential Injection Analysis (SIA) is a technique which is valued for its simplicity, easy control, versatility, repeatability, easy manipulation with solutions in a closed system, and the possibility of automation of complex analytical protocols. All these features make this technique suitable for the analysis of samples with complex matrices, such as pharmaceutical formulations or food [1], where a fully automated sample pre-treatment would bring a significant benefit.

Several methods using SIA system as a tool for analysis of different pharmaceutical formulations have been suggested, measuring the drug content or evaluating various pharmaco-technological parameters stated in Pharmacopoeias such as dissolution [2] or liberation; even applications to semi-solid formulations such as ointments have been reported [3].

Emulsions are often used as pharmaceutical dosage form due to their capacity to dissolve and stabilize lipophilic compounds, while achieving a high applicability and bioavailability of the active substance. They are a heterogeneous mixtures of two immiscible liquids, one forming microdroplets within the other liquid. Emulsions can be administered either by the oral, topic, or parenteral route. If a pharmaceutically active substance possesses lipophilic properties and has to be administered intravenously, oil-in-water emulsion is generally prepared as a dosage form.

Usually, a pharmaceutical emulsion represents a complex matrix for analysis, as it contains, apart from the active substance, two different solvents, generally water and vegetable oil as main constituents and further additives such as antimicrobials, anti-oxidants and surfactants which are necessary to maintain the required stability of the active substance and the matrix over the declared time. Both, the main emulsion constituents as well as the additives can significantly affect the analysis and therefore,

* Corresponding author. Tel.: +420 495067453; fax: +420 495067164.
E-mail address: Hana.Sklenarova@faf.cuni.cz (H. Sklenářová).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.09.059

separation or extraction of the substance of interest is often required. Potential co-extraction of matrix constituents together with the active substance can require a selective reaction or detection technique. Enzymatic reactions can further be carried out to improve the selectivity or reaction rate.

Also, the oil content in the sample matrix increases the risk of analysis performance deterioration, since it can stick to hydrophobic surfaces and lead to cross over. Therefore, a more thorough and thus time consuming cleaning step is required. Additionally, emulsions are generally turbid, which disables the direct use of spectrophotometry or fluorimetry as the most commonly used detection techniques. In consequence, a tedious and time consuming sample pre-treatment process prior to analysis is needed in many analytical methods to avoid matrix effects, which can require as much as 80% of the total analysis time [4,5].

Organic solvents can be effectively used to overcome the formerly mentioned problems as their addition can lead to a homogeneous solution by breaking the surfactant micelles, which permits the use of all optical detection techniques [6].

Although SIA systems are primarily applied to handling with aqueous solutions, the usage of organic solvents in automated flow systems has been reported, especially for sample pre-treatment, e.g. solid-phase extraction, [7] liquid–liquid extraction [8] or when a specific detection techniques such as atomic spectrometry are used [9]. While sticking on the hydrophobic surfaces can be desirable to coat the tubing walls with an organic film to perform extractions [10], it can also be a source of problems in analysis as it may impair the spectrometric measurement due to a different refractive index compared to aqueous solutions [11]. Therefore, analytical methods comprising an organic solvent are usually more complex than others where only aqueous solutions are used, requiring an additional/external component and a more laborious clean-up step, leading to the decrease in sample throughput and larger effluents production. Also, organic solvents can represent a limitation for implementation of specific reagents, such as enzymes, in the analysis, since they can affect their activity [12].

The application of enzymatic reaction in the analytical procedures has been studied very intensively [13]. The use of an enzyme generally provides the method with higher selectivity and offers a green alternative to inorganic catalysers which might possess toxic properties [14].

For the analysis of highly hydrophobic substances employing an enzymatic reaction, ionic liquids (IL) were suggested [15] as an alternative to organic solvents that can affect the enzyme activity. However, the cost of analysis represents a significant drawback for the combination of enzyme and IL in one analytical procedure.

In this work, the application of an enzymatic reaction for the determination of a highly hydrophobic drug propofol (2,6-diisopropylphenol) in a complex emulsion matrix in the presence of ethanol as a dissolving agent was presented.

Propofol is applied in medicine as an intravenous anaesthetic drug. Its chemical structure is not related to any other anaesthetic [16]. It is valued for its pharmacodynamic properties such as a rapid onset and offset of anaesthesia and a fast recovery of the patients without severe side effects due to a fast elimination from the human body [17]. The drug has lately increased attention after several death cases reports related to its application [18,19].

Due to the physical properties of propofol such as being an oily liquid at room temperature and an octanol: water partition coefficient of 6761:1 [20], the drug is administered in the form of an oil-in-water emulsion as a bolus in intravenous injection.

Although there are many works dealing with propofol determination in body fluids in the literature, only a limited number of papers were found referring to its determination in pharmaceutical formulation. The first method for propofol determination in bulk form was proposed in 1991 [21] and is based on the second derivative UV spectroscopy and HPLC.

Almost 10 years later, Kariem and Abounassif [22] developed a colorimetric method for propofol determination in emulsion dosage form. In this method, propofol reacted with 2,6-dichloroquinone-4-chlorimide (DCQ) and the reaction product was developed within 15 min.

Pickl et al. [23] used a more sophisticated instrumentation to determine propofol in emulsions, allowing very low detection limits. Headspace-solid phase microextraction was used as a sample pre-treatment technique prior to GC-MS analysis.

Here, the fully automated spectrophotometric method based on SIA for the determination of propofol in emulsion matrix without any sample preparation requirement but simple dilution is described and compared to a simple fluorimetric determination. The proposed method is focused on the handling of emulsion to enable the quantification of the analyte in such complex drug formulation as a matrix using SIA system.

The first method is based on an enzymatic reaction where horseradish peroxidase (HRP) was chosen as a catalyst of the enzymatic reaction applied in its determination, since it exhibits selectivity towards phenolic compounds [24]. Subsequently, the oxidation product reacts with 4-aminoantipyrine to give a coloured product, which can be spectrophotometrically measured at its absorbance maximum of 485 nm. This method was compared with respect to sensitivity, linear range, recovery and repeatability with a simple automated method with fluorimetric detection.

The development, optimization, and the achieved analytical performances and figures of merit of both methods are discussed in detail. The complexity of the first method including the problems arising from the handling of both aqueous and organic solutions in one system is described.

2. Material and methods

2.1. Reagents and solutions

Analytical grade reagents were used to prepare all solutions throughout the study. All aqueous solutions were prepared in ultra-pure water provided from a Millipore Milli-Q RG system (EMD Millipore Corporation, Billerica, MA, USA).

Peroxidase from horseradish (HRP), Type I, and propofol standard ($\geq$97%) were purchased from SAFC™ (Steinheim, Germany). Hydrogen peroxide was purchased from Fluka (Buchs, Germany). 4-Aminoantipyrine (4-AAP), sodium hydroxide and potassium phosphate were purchased from Sigma-Aldrich (Steinheim, Germany).

Propofol 1% MCT/LCT "Fresenius" i.v. emulsion (Fresenius Pharma, Graz, Austria) was used for recovery evaluation as reference material. The emulsion constituents and additives are soybean oil, purified egg lecithin, medium long chain saturated triacylglyceroles, glycerol, oleic acid, sodium hydroxide and purified water (aqua pro-injectione).

For spectrophotometric batch experiments, a propofol stock solution of approx. 1.4 mg L^{-1} was prepared by the following procedure: 60 μL of the substance was dissolved in 20 mL of ethanol 96% (V/V) and then diluted with water to 50 mL to reach a final ethanol concentration of 40% (V/V). Propofol working solutions were prepared by dilution of the stock solution with 40% (V/V) ethanol.

A 0.2 mol L^{-1} phosphate buffer was prepared from dihydrogen potassium phosphate and adjusted to pH 7.4 by addition of 0.2 mol L^{-1} sodium hydroxide solution.

Solutions of 4-AAP, H_2O_2, and HRP were prepared dissolving the adequate amounts of individual substances in buffer solution.

For SIA measurements with spectrophotometric detection, propofol was dissolved in 96% (V/V) ethanol. Reagents solutions were prepared dissolving the appropriate amounts of each substance in water, and buffer was used as a carrier.

For both batch and SIA studies, H_2O_2 and 4-AAP solutions were prepared daily prior to use. The propofol stock solution was used for preparation of fresh working solutions. The HRP solution was stable for over 3 days.

For the SIA measurements with fluorimetric detection, propofol stock solution of approx. 1.4 mg L^{-1} was prepared dissolving 60 μL of the substance in ethanol 96% (V/V). The working solutions were prepared diluting the stock solution with ethanol 96% (V/V). Ethanol was also used as a carrier.

The sample solution was treated in the same way as the propofol standard solution.

All prepared solutions were stored in dark at 4 °C.

2.2. Apparatus

For batch spectrophotometric experiments, spectra were acquired using an HP diode array spectrophotometer Agilent 8453 UV–vis. All SIA experiments were performed using a commercially available FIAlab® 3500 system (FIAlab® Instrument Systems Inc., Bellevue, USA, http://www.flowinjection.com). It consists of a Cavro syringe pump equipped with a 5 mL glass syringe with a rotary three-way head valve to connect the syringe either with the solution reservoir (carrier, IN) or the tubing manifold (OUT) and an 8-port Cheminert selection valve. All connections were made using PTFE tubing of 0.75 mm i.d. The central port of the selection valve was connected to the OUT port of the syringe head valve via a holding coil (HC) of approx. 1.5 m length. Lateral ports of the selection valve were used for solution discharge to waste (port 1), aspiration of sample and reagents (ports 2–6) and propelling the reaction mixture to a detection flow cell (port 7) of 1 cm optical path length (Z-cell, PEEK). The detailed manifold configuration for spectrophotometric detection is indicated in Fig. 1.

In the system with spectrophotometric detection, the holding coil was placed into a vessel of a thermostat to maintain the temperature at 40 °C for the enzymatic reaction.

A USB 2000 spectrophotometer (Ocean Optic Inc., Dunedin, USA, http://www.oceanoptics.com) was used for signal detection. A Mikropack DH-2000 Deuterium–Tungsten Halogen lamp was used as a light source. Both instruments were coupled to the detection cell via optical fibres of 400 μm diameter (I.D.) A home-made de-bubbling device, shown in the Fig. 1, was used further. It consisted of two pieces of PMMA (3 × 2 × 1 cm). The first comprised a milled flow channel of 20 mm in length, 3 mm in width, and 1 mm in depth, which was sealed with one layer of gas permeable PTFE tape. The second piece was used to seal the cell by the help of four metal screws and had four holes for air exit. It was placed in between the selection valve and the detection flow cell, fixed by means of commercially available fittings.

For the second system, FIAlab 3500 system equipped with a Flow Through Photomultiplier based Detector (PMT-FL, FIAlab®) was used. The detector comprises a photomultiplier for data readout, a commercial fluorescence quartz flow cell with a cuvette support and fibre optic connection to the same UV light source as described above. Wavelength selectivity was achieved using optical filters. A UV 330 band pass filter with wide wavelength interval of 140 nm was used for excitation and a 295 Long Pass Filter with cut off at any wavelength under 265 nm was used for emission light filtering (Edmund Optics, Barrington, New Jersey, USA).

Control of the whole flow system as well as data acquisition and data collection evaluation was carried out using FIAlab software for Windows, version 5.9.290 (FIAlab®).

2.3. SIA – spectrophotometric procedure

The operational protocol is given in Table 1. It started with the aspiration of 600 μL of the carrier (buffer), from the reservoir at a flow rate of 50 μL s^{-1}, followed by the aspiration of H_2O_2, propofol, HRP, and 4-aminoantipyrine solutions (50 μL of each reagent) at the same flow rate. Solution mixing was improved using four flow reversals under the flow rate of 50 μL s^{-1} in the holding coil, heated to 40 °C. By this step, efficient mixing and heating were ensured and the peak shape and signal repeatability were improved.

The reaction was allowed to proceed in the holding coil (HC) for another minute to enhance the reaction product yield. In the following step, the mixture was propelled through the de-bubbling device and the detection flow cell to waste at a flow rate of 25 μL s^{-1}.

2.4. SIA – fluorimetric procedure

Fluorimetric determination of propofol was done using a very simple control programme, which started with the aspiration of 1000 μL carrier (ethanol 96%, V/V) from the reservoir at a flow rate

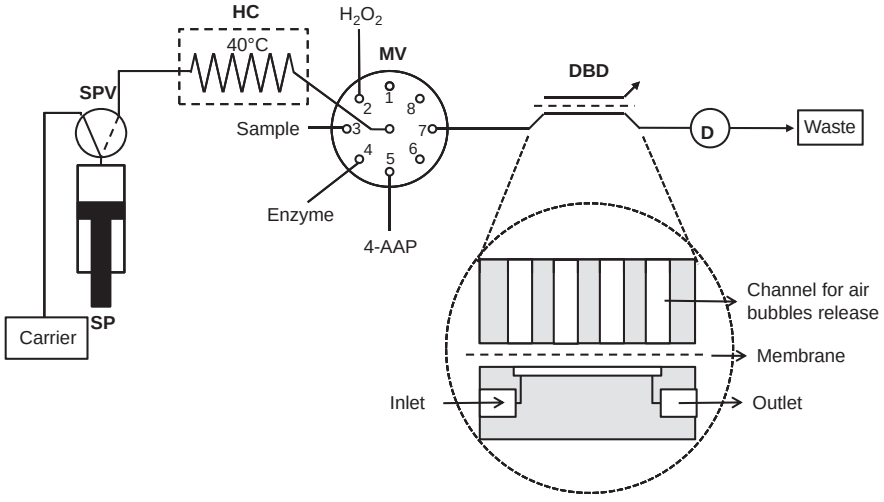

Fig. 1. Automated SIA system for determination of propofol in emulsion with spectrophotometric detection. SP: syringe pump, SPV: syringe pump valve, HC: holding coil, MV-multiposition valve, DBD: de-bubbling device, and D: detector.

Table 1
Operation protocol for spectrophotometric determination of propofol.

Step	Port	Flow rate ($\mu L\ s^{-1}$)	Operation	Description
1	–	100	Aspirate 600 μL	Syringe pump Valve in position; aspiration of the carrier
2	2	50	Aspirate 50 μL	Syringe pump Valve out position; aspiration of hydrogen peroxide
3	3	50	Aspirate 50 μL	Aspiration of sample
4	4	50	Aspirate 50 μL	Aspiration of enzyme
5	5	50	Aspirate 50 μL	Aspiration of 4-AAP
6	6	100	Aspirate 50 μL, Dispense 50 μL	Mixing, repeated 4 times
7	–			Delay 1 min
8	7	25	Empty	Propelling to the detection cell

of 80 $\mu L\ s^{-1}$, followed by 50 μL of propofol standard or sample dissolved in ethanol 96% (V/V) from the selection valve and propelling it towards the detector at a flow rate of 50 $\mu L\ s^{-1}$ while the fluorescence emission signal was registered. By the appropriate selection of excitation and emission filters, interferences of the emulsion components were significantly reduced, as described in the Results Section.

3. Results and discussion

3.1. Preliminary batch experiments

The optimum reaction conditions were investigated in batch using propofol standard solution before transferring the reaction procedure to the SIA system. This was done due to the complexity of the reaction including four components (HRP, propofol standard or sample solution, H_2O_2 and 4-AAP solutions).

The parameters to be optimized in batch were the concentrations of reagents, the temperature, and the reaction time. Once the chemical parameters were optimized in batch, hydrodynamic parameters such as solution volumes, flow rate and mixing mode had to be studied in the SIA system.

For batch, equal volumes of all reagents were used to perform these studies. All the reagents were prepared by dissolution in 0.2 mol L^{-1} phosphate buffer, adjusted to pH 7.4, which is the reported activity optimum of HRP, and as further proven by a preliminary test (data not shown). The value is also in good agreement with an earlier report [25]. The ethanol content was intended to be kept as low as possible in order not to affect the enzyme activity. Thus, propofol was at first dissolved in pure ethanol and then diluted with water to set the final ethanol concentration to 40% (V/V). This was the lowest concentration, for which two non-miscible phases were not observed. Later experiments revealed that for the analysis of a propofol emulsion, 96% ethanol was required to eliminate the matrix effects, as described in Section SIA – spectrophotometric method.

After scanning the whole spectrum range (200–700 nm), 485 nm was chosen as optimum wavelength representing maximum absorbance. It should be pointed out that after reaching the reaction's steady-state the second maximum was found at 436 nm. However, the reaction kinetics studied at this wavelength was slower and the blank value significantly higher, indicating that this absorbance corresponds to a side product, most likely, the oxidation of 4-AAP with H_2O_2.

3.1.1. Study of the enzyme concentration

HRP was prepared and used in solution to ensure a fresh portion of the catalyst for each run [14]. The concentration of the enzyme was investigated in the range of 0.16–5.10 mg mL^{-1}, with a multiplying factor of 2. Other solutions were prepared in the following concentrations: $c_{H_2O_2}$ = 18 mmol L^{-1}, $c_{4\text{-AAP}}$ = 1.36 mmol L^{-1}, and $c_{propofol}$ = 0.2 mg mL^{-1}. The reaction was performed at room temperature and the absorbance was measured after 20 min. An excess concentration of the substrate (propofol) was used to ensure that the enzyme activity was not dependent on the substrate amount but only on the enzyme concentration.

The signal increased up to 2.56 mg mL^{-1} while for higher concentration of HRP, a similar absorbance was observed, and so further experiments were performed with 2.56 mg mL^{-1} of the enzyme.

3.1.2. Study of the reaction time

After the optimum enzyme concentration was found, the influence of the reaction time was studied under the same conditions mentioned in the previous paragraph. All reagents were mixed at room temperature in a test tube. Then, an aliquot was taken and the absorbance was measured. This was repeated every 5 min over the next 45 min. The signal increased during the first 15 min and then remained stable without significant changes. So, 20 min was chosen to ensure that the reaction time is long enough to reach the steady state.

3.1.3. Study of the temperature and reaction time correction

Since the rate of enzymatic reactions is highly temperature dependent, it was necessary to choose an adequate reaction temperature. The conditions were identical as in the previous experiments: c_{HRP} = 2.56 mg mL^{-1}, $c_{H_2O_2}$ = 18 mmol L^{-1}, $c_{4\text{-AAP}}$ = 1.36 mmol L^{-1}, and $c_{propofol}$ = 0.2 mg mL^{-1}. The test tube was placed in a thermostat for temperature control. The influence of temperature was studied in the range of 25–45 °C, with 5 °C increments. A signal increase was observed up to 40 °C, while beyond that the signal decreased rapidly, which can be attributed to the thermic denaturation of the enzyme.

Regarding this observation, the reaction time was re-examined, setting the temperature to 40 °C. The results revealed that at a higher temperature, the reaction reached the steady state after 10 min. Therefore, further batch experiments were performed at 40 °C with a reaction time of 10 min.

3.1.4. Study of the 4-AAP and H_2O_2 concentration

The 4-AAP concentration was studied in the range of 6.0–11.0 mmol L^{-1} with an increment of 1 mmol L^{-1}. The highest response was obtained when a concentration of 8 mmol L^{-1} was used (Fig. 2A), so this concentration was chosen for all following experiments.

The influence of the concentration of hydrogen peroxide was tested between 16.0–20.0 mmol L^{-1}. As demonstrated in Fig. 2B, the reaction with 18 mmol L^{-1} H_2O_2 yielded the highest signals, so this concentration was adopted for next trials.

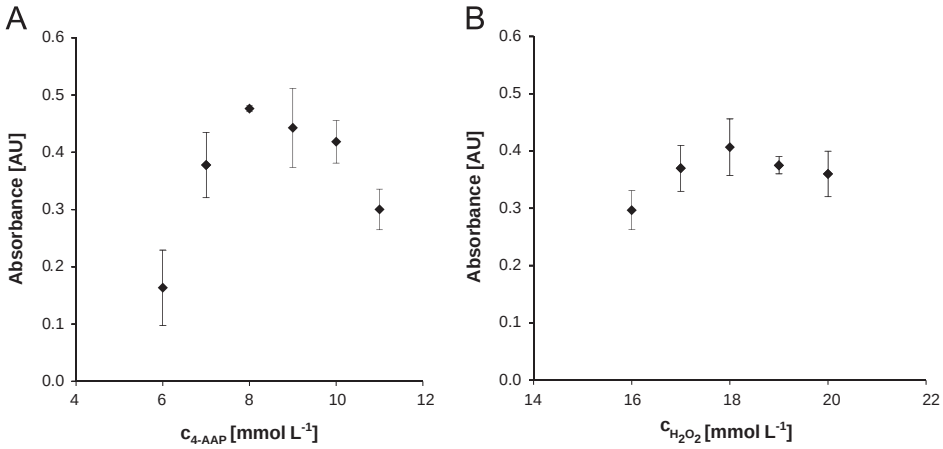

Fig. 2. Study of reaction conditions in batch; general conditions: c_{HRP}=2.56 mg mL^{-1}, $c_{propofol}$=0.2 mg mL^{-1}, T=40 °C, t=10 min (A) study of 4-AAP concentration; $c_{4\text{-}AAP}$=6.0–11.0 mmol L^{-1}, and (B) study of H_2O_2 concentration, $c_{H_2O_2}$ = 16.0–20.0 mmol L^{-1}.

3.2. SIA – spectrophotometric method

Once the reaction conditions were established, they were transferred to the SIA system for automation of propofol determination.

In the automated system, other parameters had to be optimized. The crucial role in the analysis performance had the sequence of reagents aspiration, mixing conditions, and the reaction time. Heating could not be omitted because it increases significantly the reaction rate, as confirmed by batch experiments.

A thorough penetration of, first, the sample with HRP and its substrate H_2O_2, and then the chromogenic reagent was intended. To accomplish this requirement, the following sequences of aspiration were tested: 1st H_2O_2–sample–HRP–4-AAP; 2nd H_2O_2–4-AAP–sample–HRP; 3rd H_2O_2–HRP–sample–4-AAP. The most satisfying results in terms of peak height and peak shape were obtained when the solutions were aspirated in the 1st order. This was accomplished by the sample aspiration in between the zones of H_2O_2 and HRP solutions to react first with the analyte.

Regarding the volume of the solutions, range from 25 μL to 50 μL was tested for each reagent. With the smaller one we did not obtain peaks of symmetric shape, probably due to high level of dispersion in the carrier.

Peaks of favourable shapes were obtained with the reagents´ volume of 50 μL, thus this volume was chosen for further experiments. The behaviour with higher volumes was not investigated, since large volumes caused very high consumption of reagents, especially the enzyme.

The optimal temperature was ensured placing the HC into a double-wall thermostatic glass beaker connected to a thermostat with a water bath and the temperature set to 40 °C.

In the SIA system, buffer solution was used as a carrier ensuring a stable pH value (optimal for the enzyme activity, i.e. 7.4). Solutions (H_2O_2, 4-AAP, and enzyme) were prepared in water and for sample preparation 96% (V/V) ethanol was used to obtain higher solubility of propofol in real sample (emulsion). Using these conditions the emulsion matrix was completely dissolved in ethanol, which was important to achieve high recovery in case of formulation analysis.

One consequence of increasing ethanol content (in comparison to batch) and especially at increased temperature is the air bubbles formation inside the flow system. This affected the spectrophotometric measurements in the SIA system significantly. This problem was overcome by placing a simple de-bubbling device depicted in Fig. 1 right before the detection flow cell. It showed that the bubbles were effectively removed and the spectrophotometric detection was not affected by a baseline drift.

The main attractiveness of flow techniques is that a reaction steady state does not have to be reached as long as high repeatability can be achieved by careful optimization and timing. Due to the fact, that a reaction time of 10 min was required to reach steady-state in the previously described batch method, special effort was given to achieve high repeatability and sensitivity within a shorter time in the automated method.

To achieve efficient zone mixing for a homogeneous solution and to achieve a better repeatability, four flow reversals were performed at a flow rate of 50 μL s^{-1} using 50 μL zones and were followed by an additional reaction time of 5 min.

However, this mode did not bring the expected results. Double peaks and unacceptable repeatability were observed, indicating that the solutions were not yet thoroughly mixed. Increasing the number of flow reversals did not improve the results either. Using a higher flow rate of 100 μL s^{-1} for mixing, the shape of the recorded peaks improved considerably. It was found that applying the flow reversals over a period of 1 min was sufficient for complete mixing of all aspirated zones.

In the second step, the time of reaction after stopping the carrier flow was examined. It was observed that a reaction time of 5 min lowered the measurement repeatability while not increasing the method's sensitivity significantly. Based on these observations, only one additional minute of stop flow was applied. The peak heights at a given analyte concentration and repeatability achieved using the optimized conditions were within the expected limits and no carry-over between the individual measurements was observed.

3.2.1. Figures of merit of spectrophotometric method

Linearity was obtained over the range of 0.005–0.100 mg mL^{-1} for the enzymatic method with spectrophotometric detection. LOD value was calculated as three times the standard deviation of ten blank measurements divided by the calibration curve slope. LOQ value was then calculated as 3.3-fold LOD. In the spectrophotometric detection technique values of 0.0016 and 0.0053 mg mL^{-1} expressed LOD and LOQ, respectively. This method revealed good repeatability, with RSD not exceeding 5% (Table 2). Regarding the analysis time, a single run was completed within 5 min. For the spectrophotometric determination, only 50 μL of ethanol (as a sample diluent) and 50 μL of the enzyme solution (as the reagent of highest cost) were consumed. Less than 1 mL of waste was produced in a single run.

Bubble formation which aroused from mixing of organic and aqueous solutions was effectively overcome using a simple

Table 2
Summary of analytical parameters of the spectrophotometric and fluorimetric methods.

Method	Spectrophotometry	Fluorimetry
Slope (mL mg^{-1})	3.054 ± 0.161	$2.865 \times 10^6 \pm 0.308 \times 10^6$
Intercept	0.016 ± 0.004	$26.90 \times 10^3 \pm 723.9 \times 10^3$
Correlation coefficient	0.993	0.997
Linear range (mg mL^{-1})	0.005–0.100	0.004–0.243
LOD (mg mL^{-1})	0.0016	0.0013
LOQ (mg mL^{-1})	0.0053	0.0043
Repeatability (RSD%, $n=10$)	4.45 (0.075 mg mL^{-1}) 4.29 (0.125 mg mL^{-1})	1.66 (0.050 mg mL^{-1}) 0.83 (0.200 mg mL^{-1})
Recovery (%)	103.8 (0.050 mg mL^{-1}) 94.7 (0.075 mg mL^{-1}) 95.7 (0.100 mg mL^{-1})	97.5 (0.025 mg.mL^{-1}) 98.9 (0.050 mg mL^{-1}) 99.1 (0.075 mg mL^{-1})
Sample throughput (h^{-1})[a]	14	68

[a] Expressed for single injection.

membrane device. Additionally, although the sample matrix was complex, direct determination with only dilution as a sample pretreatment was possible due to the selective enzymatic reaction. To evaluate potential interferences, the effect of the sample matrix (soybean oil, purified egg lecithin, medium long chain saturated triacylglyceroles, glycerol, oleic acid, and sodium hydroxide) was tested at three different concentration levels (0.050, 0.075 and 0.100 mg mL^{-1}) using the standard addition method. Recovery values ranged from 94.7% to 103.8% with the average recovery of 98.1%. These results (Table 2) did not show any interference of the additives from the pharmaceutical matrix with the proposed method. The spectrophotometric method was found to fulfil the requirements for all tested parameters and additionally, higher selectivity of the enzymatic reaction could be expected (that is important mainly in case of real samples of biological material).

3.3. SIA – fluorimetric method

Since fluorimetric detection has been often used for propofol determination [27, 28], fluorimetric determination in the SIA system was carried out for comparison with the developed spectrophotometric determination. For this determination, the sample was prepared in ethanol 96% (V/V). Therefore, the possibility of reducing the organic waste production using water as a carrier was examined. However, a strong baseline drift was observed in this case which affected the detection considerably. Comparing the results obtained with standard solutions and real samples of the same concentration level (as declared by the producer of the pharmaceutical formulation), significantly different results were observed. For this reason, ethanol was used as a carrier, which improved the repeatability and analyte recovery (evaluated with real samples) significantly.

Hence, the primary radiation from 260 nm was applied and emission at $\lambda \geq 295$ nm was measured. This led to recovery values near to 100%, so the matrix effects were eliminated by the wavelength selection.

3.3.1. Figures of merit of fluorimetric method

Fluorimetry as a commonly used detection in other propofol determinations was tested in the SIA system, too. The linear range of 0.004–0.243 mg mL^{-1} for the fluorimetric method was found. LOD and LOQ values were 0.0013 and 0.0043 mg mL^{-1}, respectively. The analysis time of 1 min was needed and about 1.05 mL of waste was produced in a single run. The fluorimetric determination required approx. 1 mL of ethanol per analysis as environmentally harmless and economic organic solvent. The fluorimetric method showed excellent repeatability, with RSD less than 2%.

The recovery values in case of spiked matrix of pharmaceutical emulsion ranged from 97.5% to 99.1% with average recovery of 98.5% (Table 2). Simple fluorimetric determination revealed similar values of all tested parameters which were within the required limits. Only low selectivity of this detection decreases its application in real biological samples analysis.

3.4. Comparison of spectrophotometric and fluorimetric methods

The parameters characterizing the analytical performance of both methods were evaluated and compared in Table 2.

Linearity of both tested methods was found in similar range. The fluorimetric method showed broader range towards the higher concentration levels. Limits of detection and quantitation were comparable and the described values were more than three orders of magnitude smaller than the content of propofol in the pharmaceutical formulation declared by the producer, being 10 mg mL^{-1}.

The time of analysis per run was longer for the complex spectrophotometric method which included the enzymatic reaction. However, this did not lead to higher solvent and reagent consumption, compared to other flow methods. Low waste volume generation was proved in both methods. As for repeatability, the RSD values of the spectrophotometric method were found to be a little bit higher but did not exceed the common values found in automated determinations using different flow techniques. The difference in RSD values was caused by higher number of steps in the spectrophotometric system compared to the simple fluorimetric one.

The recovery values from the tests with real samples (pharmaceutical emulsion matrix) showed a slightly wider range in case of spectrophotometric method but the average values were found to be very similar. The data obtained from real sample measurement proved that the suggested spectrophotometric method did not exhibit the problems that are highly likely to occur when an organic solvent and/or highly hydrophobic substance is handled in a flow system, such as carry-over, Schlieren effect or the need of an external device.

Comparing both methods (spectrophotometric and fluorimetric detections), it can be seen that the more complex one (spectrophotometric) provided a sensitivity comparable to the simple fluorimetric detection. Higher selectivity is expected due to the enzymatic reaction. As the recovery measurements revealed, in both cases, the components of the studied emulsion sample did not influence the analysis.

3.5. Comparison with the formerly reported methods

Most of the analytical parameters were found to be better than the first reported method for propofol determination in pharmaceutical formulation [21]. The detection limits were almost 100 times lower; also higher recovery and better repeatability were achieved with the automated SIA methods.

In comparison with the manual spectrophotometric method reported by Kariem and Abounassif [22], we observed an about four times higher LOD. However, only one organic solvent (ethanol) was used in the SIA method, unlike in their method, where two solvents (2-propanol and dimethylsulphoxid) were necessary to perform the analytical reaction. Moreover, the analysis time is

about three-times shorter and the determination is carried out fully automatically.

Pickl [23] presented a highly sensitive method, enabling the detection of very small quantities of analyte in the sample (emulsion). This was possible due to the use of a very sensitive but very costly detection technique (mass spectrometry) which is not accessible to all laboratories. Sample pre-treatment was carried out prior to analysis (solid phase microextraction). This step, however, increased the analysis time significantly.

Other separation methods with spectrophotometric and fluorimetric detection were designed for propofol determination in biological fluids matrix. They provided low detection limits, required for such kind of samples. In [26], SPE pre-concentration step is followed by HPLC/UV detection with total analysis time of 20 min. The HPLC/fluorimetry method developed by Boulieu at al. [27] required less than 6 min for the elution of propofol, using 500 μL of the sample and producing 3.6 mL of waste in a single injection. Similarly, an HPLC method with fluorimetric detection was proposed by Cox et al. [28]. Double liquid–liquid extraction using 1.5 mL of acetonitrile–methanol mixture was performed to increase the sensitivity. The following separation step took then almost 10 min, with almost 15 mL of organic waste per analysis. In comparison to these methods, the proposed SIA procedure offers a faster, simpler, more economic and especially automated alternative for the determination of propofol.

In comparison with formerly published methods, this work resulted in lower detection limits. SIA is a technique based on flow where the steady state is not reached, unlike in batch methods. Other methods might have reached lower detection limits than the proposed method; however, the SIA method offers other advantages (low solvent consumption, feasibility, automation, high sample throughput, and easy manipulation with solutions).

4. Conclusion

The use of a fully automated system with spectrophotometric detection for the determination of a highly hydrophobic substance in a complex matrix, and the application of the selectivity of an enzymatic reaction to eliminate sample matrix effects, was demonstrated. The obtained results were described and compared with an automated method with fluorimetric detection in terms of analytical performance.

Both methods and the used analyser system stand out by simplicity, rapidness, and high sensitivity, and were proven to be applicable to the determination of the lipophilic analyte propofol in emulsion taking advantage of both organic solvent and an enzymatic reaction.

Acknowledgements

The authors greatly acknowledge the financial support of the Charles University in Prague (Project SVV265002). The publication is co-financed by the European Social Fund and the State budget of the Czech Republic, TEAB, Project no. CZ.1.07/2.3.00/20.0235.

References

[1] R. Pérez-Olmos, J.C. Soto, N. Zarate, A.N. Araujo, J.L.F.C. Lima, M.L.M.F.S. Saraiva, Food Chem. 90 (2005) 471–490.
[2] Z. Legnerová, H. Sklenářová, P. Solich, Talanta 58 (2002) 1151–1155.
[3] P. Solich, H. Sklenářová, J. Huclová, D. Šatínský, U. Schaefer, Anal. Chim. Acta 499 (2003) 9–16.
[4] H. Kataoka, Trends Anal. Chem. 22 (2003) 232–244.
[5] G. Vas, K. Vékey, J. Mass Spectrom. 39 (2004) 233–254.
[6] M. Almgren, S. Swarup, J.E. Lofroth, J. Phys. Chem. 89 (1985) 4621–4626.
[7] Z. Legnerová, D. Šatínský, P. Solich, Anal. Chim. Acta 497 (2003) 165–174.
[8] C.C. Acebal, H. Sklenářová, J. Škrlíková, I. Šrámková, V. Andruch, I.S. Balogh, P. Solich, Talanta 96 (2012) 107–112.
[9] C. Mitani, A.N. Anthemidis, Anal. Chim. Acta 771 (2013) 50–55.
[10] A. Cladera, M. Miro, J.M. Estela, V. Cerda, Anal. Chim. Acta 421 (2000) 155–166.
[11] B. Horstkotte, M. Alexovič, F. Maya, C.M. Duarte, V. Andruch, V. Cerdà, Talanta 99 (2012) 349–356.
[12] K. Ryu, J.S. Dordick, Biochemistry 31 (1992) 2588–2598.
[13] M. Trojanowicz, Flow Injection Analysis: Instrumentation and Applications, World Scientific Publishing, Singapore (2002) 2002; 162–194.
[14] C.I.C. Silvestre, P.C.A.G. Pinto, M.A. Segundo, M.L.M.F.S. Saraíva, J.L.F.C. Lima, Anal. Chim. Acta 689 (2011) 160–177.
[15] U. Kragl, M. Eckstein, N. Kaftzik, Curr. Opin. Biotechnol 13 (2002) 565–571.
[16] M.S. Langley, R.C. Heel, Drugs 35 (1988) 334–372.
[17] C. Vanlersberghe, F. Camu, Mod. Anesthetics 182 (2008) 227–252.
[18] J. Holzki, C. Aring, A. Gillor, Pediatr. Anesthesia 14 (2004) 265–270.
[19] E.F. Kranioti, A. Mavroforou, P. Mylonakis, M. Michalodimitrakis, Forensic Sci. Int. 167 (2007) 56–58.
[20] ⟨http://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?id=12155⟩.
[21] L.C. Bailey, K.T. Tang, B.A. Rogozinski, J. Pharm. Biomed. Anal. 9 (1991) 501–506.
[22] E.A.G. Kariem, M.A. Abounassif, Anal. Lett. 33 (2000) 2515–2531.
[23] K.E. Pickl, V. Adamek, R. Gorges, F.M. Sinner, J. Pharm. Biomed. Anal. 55 (2011) 1231–1236.
[24] Y.C. Lai, S.C. Lin, Process Biochem. 40 (2005) 1167–1174.
[25] P.C.A.G. Pinto, M.L.M.F.S. Saraiva, J.L.F.C. Lima, Talanta 77 (2008) 479–483.
[26] D. Teshima, H. Nagahama, K. Makino, Y. Kataoka, R. Oishi, J. Clin. Pharm. Ther. 26 (2001) 381–385.
[27] X. Cussonneau, E. De Smet, K. Lantsoght, J.P. Salvi, M. Bolon-Larger, R. Boulieu, J. Pharm. Biomed. Anal. 44 (2007) 680–682.
[28] J. Yarbrough, R. Harvey, S. Cox, J. Chromatogr. Sci. 50 (2012) 162–166.