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Exploiting the oxidative coupling reaction of MBTH for indapamide determination

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ABSTRACT

The oxidative coupling reaction between aromatic amines and 3-methylbenzothiazolin-2-one hydrazone (MBTH) in the presence of cerium(IV) has been extensively used with quantitative analytical purposes. Nevertheless, a literature survey reveals that different wavelengths (visible range) can be used to monitor the reaction products when using MBTH and Ce(IV) as colour developing reagents.

In the present work, the oxidative coupling reaction of indapamide (an oral antihypertensive diuretic drug) with MBTH in the presence of cerium(IV) was evaluated using distinct reaction approaches and was implemented in an automated multipumping flow system. The developed methodology was applied in the spectrophotometric control of the drug in pharmaceutical formulations. The optimization of the proposed multipumping flow system was performed by using an experimental design approach, namely by exploiting a Plackett–Burman factorial design and a central cubic faces design.

This work revealed the formation of products with different reaction kinetics, chemical stabilities and absorbance spectra, depending on the sequence of addition of the reagents. By exploiting a specific sequence in the addition of reagents, the proposed automatic system allowed the rapid quantification of indapamide in pharmaceutical formulations, with a determination rate of about 25 h^{-1}, and a relative deviation under 2.1% when comparing with the reference procedure. Detection limit was approximately 1 mg L^{-1}.

1. Introduction

3-Methylbenzothiazolin-2-one hydrazone (MBTH) was first introduced as a reagent in analytical chemistry for the detection and determination of aldehydes, indoles, aromatic amines, imino-hetero-aromatic compounds, arylalkylamines, carbazoles, phenols, etc. [1,2]. In the presence of oxidizing agents it forms strongly coloured products with several pharmaceutical compounds [3–11].

A literature survey focusing on the oxidative coupling reaction of MBTH and Ce(IV) [3–11] with several compounds reveals that without justification, very different wavelengths were used to monitor the formed products, probably indicating the formation of completely different reaction products.

This work was aimed at attaining an improved knowledge of the two-stage reaction involving MBTH and Ce(IV) through the spectrophotometric determination of indapamide. As no concise information is available in literature, extensive studies through mathematical modelling were carried out focusing the evaluation of the influence of several parameters on the formed reaction products. The analysis of the obtained mathematical models revealed that reaction kinetics, products stability and spectra were markedly affected by the experimental conditions.

With an analytical purpose, the reaction was subsequently implemented in a multipumping flow system. Automation of procedures for pharmaceutical analysis has been extensively exploited since it allows both a reduction in the consumption of reagents and generated wastes and a noteworthy increase in the sampling rate. In this context, flow-based techniques have proven to be valuable tools for the development of fast, simple, versatile and reliable methods that can be readily adapted for routine analysis at relatively low cost.

Multipumping pulsed flow system [12] is a low cost and simple approach to automate analytical determinations, and an advantageous alternative to the conventional flow injection analysis (FIA) [13], sequential injection analysis (SIA) [14], and multicommuted (MCFS) [15] systems relying on to the typical laminar flow regimen. MPFS rely exclusively on the utilization of multiple very small size solenoid actuated micropumps controlled by computer [16] that allow a very simplified configuration of the flow system as well as its automated control. By taking advantage of all operational characteristics provided by these flow systems, such as a high degree of mixture, high versatility in terms of both the sequence of solutions

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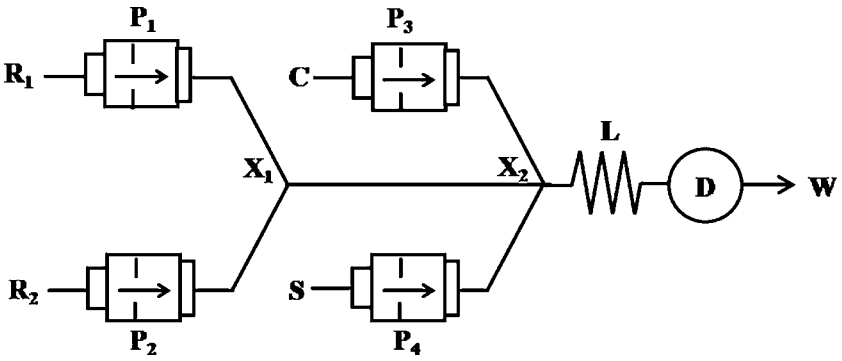

Fig. 1. Multipumping flow system. P_1–P_4: solenoid micropumps 8 μL stroke volume; X_1 and X_2, confluence points; *L*, reactor coil; *D*, detector (λ = 601 nm); *C*, carrier solution (0.1 mol L^{-1} H_2SO_4); R_1, MBTH (1.2×10^{-2} mol L^{-1} in 0.1 mol L^{-1} H_2SO_4); R_2, cerium(IV) (1.1×10^{-2} mol L^{-1} in 0.1 mol L^{-1} H_2SO_4); *S*, sample (in 5% ethanol and 0.1 mol L^{-1} H_2SO_4).

insertion and of the variability of inserted volumes, the oxidative coupling reaction was further exploited to the chemical control of indapamide formulations.

Indapamide, or 4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoyl-benzamide, is an antihypertensive and diuretic drug which belongs to the indolines class [17]. Several procedures for indapamide determination in pharmaceutical and biological samples have been employed. These procedures include high performance liquid chromatography [18–20], LC–MS [21,22], gas chromatography [23], chemiluminescence [24], capillary electrophoresis with amperometric [25] and UV [26] detection, colorimetry [27,28] and UV spectrophotometry [29]. Some flow methodologies have also been used in order to quantify indapamide in pharmaceutical formulations, such as, flow injection analysis with chemiluminescence [30] and spectrophotometric [31] detection.

Thus, this work aims at contributing to an improved knowledge of the oxidative coupling reaction of MBTH and Ce(IV), by establishing mathematical models focusing the relationships between several analytical parameters (chemical and physical) and the formation of different reaction products, despite using the same chemical reagents. Furthermore, a simple, low cost, and rapid flow analysis system, exploiting the multipumping concept for the spectrophotometric determination of indapamide in pharmaceutical formulations, was implemented.

2. Experimental

2.1. Apparatus

The flow system comprised four micropumps (Bio-Chem Valve Inc. Boonton, NJ, USA, ref. 090SP) of fixed displacement diaphragm type, being solenoid operated and dispensing 8 μL per stroke.

The manifold tubing was made from 0.8 mm i.d. PTFE, and the end-fittings, connectors and confluences were also made of the same material.

Automatic control of the analytical system was accomplished by means of a Intel Pentium based microcomputer and the software was developed using Microsoft Quick-Basic 4.5. A lab-made electronic interface using a CoolDrive™ power drive board (NResearch Inc., NJ, USA) was used to activate the micropumps through the LPT1 computer port.

The miniaturized optical detector involved a S2000 spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) and a World Precision Instruments F-O-LITEH halogen lamp light source. The light source was connected to the CUV-UV Cuvette Holder (Ocean Optics) equipped with a Hellma flow-cell (30 μL inner volume, 10 mm optical path) via a single optical fiber with a 600 μm core diameter (Ocean Optics, Model: QP600-2-UV-BX), while the S2000 spectrophotometer was connected to the CUV-UV Cuvette Holder through a single optical fiber with a 200 μm core diameter (Ocean Optics, Model: QP200-2-UV-BX). An ADC1000-USB External USB A/D Converter was used to interface S2000 spectrophotometer to an Intel Pentium III computer.

Data acquisition was performed using the OOIBase32™ software version 2.0.6.5 of Ocean Optics.

2.2. Samples, standards and reagents

All solutions were prepared with doubly deionized water (conductivity <0.1 μS cm^{-1}) and analytical grade chemicals were used.

A 400 mg L^{-1} indapamide stock solution was daily prepared by dissolving 40 mg of indapamide (Sigma) in 40 mL of ethanol (Panreac, 99.5%) and diluted to 100 mL with deionized water. Then a 50 mL aliquot of indapamide stock solution was transferred to a 200 mL volumetric flask and diluted to volume with deionized water. Thus, a 100 mg L^{-1} intermediate indapamide solution in 10% ethanol was obtained.

The working indapamide standards (5–50 mg L^{-1}) were prepared by appropriate dilution of the intermediate indapamide solution, using the following experimental procedure. Aliquots of intermediate indapamide solution were transferred into a series of 50 mL volumetric flaks. After that, appropriately aliquots of ethanol (Panreac, 99.5%) were added, with the aim to obtain the same concentration of ethanol (5%) in all the working indapamide standards. Finally, 2.5 mL of 2 mol L^{-1} H_2SO_4 solution was added and the volume was made up to the mark with deionized water.

A 1.2×10^{-2} mol L^{-1} MBTH solution was prepared by dissolving 258.84 mg of 3-methylbenzothiazolin-2-one hydrazone hydrochloride hydrate (Sigma–Aldrich®) in 100 mL of a 0.1 mol L^{-1} sulphuric acid solution.

A 1.1×10^{-2} mol L^{-1} Ce(IV) solution was prepared by dissolving 444.74 mg of cerium(IV) sulphate tetrahydrate (Riedel-de Haën®) in 100 mL of 0.1 mol L^{-1} H_2SO_4.

The sample solutions made with commercially available pharmaceutical formulations were prepared by weighing and powdering a representative number of tablets. Thereafter, an appropriate amount of sample was placed in a 50 mL flask and mechanically shaken with 2.5 mL ethanol (Panreac, 99.5%) and 2.5 mL of a 2 mol L^{-1} sulphuric acid for 25 min. The solution was filtered into a 50 mL volumetric flask. The contents of flask were diluted to 50 mL with deionized water. At last, the obtained solution was filtered with a syringe filter i.d. 0.20 μm (Corning®) before its insertion in the flow system.

2.3. Flow manifold

The analytical manifold (Fig. 1) comprised four solenoid micropumps (P_1 to P_4) used as solutions insertion and propelling devices.

Prior to sample insertion the simultaneous actuation of micropumps P_1 and P_2 allowed the combined insertion of MBTH and cerium(IV) solutions through X_1, by exploiting the merging zones approach. The number of solutions pulses was enough to guarantee that the resulting mixed solution filled the flow tubing placed between the confluence points X_1 and X_2 (about 20 cm). Next, the micropump P_4 was actuated and the sample solution was inserted in the flow system reaching the confluence point X_2. Finally, by actuating the micropump P_3, sulphuric acid solution was inserted into the system through X_2 allowing the rejection to waste of the exceeding volumes of the reagents solutions and establishment of the baseline.

The analytical cycle started by intercalating a unique volume of sample solution between two identical plugs of the MBTH/Ce(IV) solution. The sampling stage consisted firstly in the insertion of the MBTH/Ce(IV) solution for a pre-set number of pulses, through the simultaneous actuation of micropumps P_1 and P_2 (total volume of 16 μL per stroke), at a fixed pulse time of 1.2 s, corresponding to a pulse frequency of 50 min^{-1}, which defined the flow rate at 0.8 mL min^{-1}. Then, by inserting a pre-set number of pulses of micropump P_4, at a fixed pulse time of 0.6 s, a unique volume of sample solution was intercalated with another identical small plug of the MBTH/Ce(IV) solution. Thereafter, the reaction zone, fulfilling the second stage of the oxidative coupling reaction, was carried towards the detector through the repeated actuation of P_3 (8 μL per stroke), at a fixed pulse time of 0.6 s, corresponding to a pulse frequency of 100 min^{-1}, which defined the flow rate at 0.8 mL min^{-1}. The formed product was monitored at 601 nm.

2.4. Reference procedure

For validation of the results furnished by the developed methodology, the pharmaceutical formulations containing indapamide (tablets) were also analysed by reversed-phase liquid chromatography, according with the reference methodology [32]. The content of indapamide in the tablets was calculated from the chromatogram obtained and using the declared content of indapamide in standard solution. Each mg of indapamide in standard solution is equivalent to 1.0246 mg of the drug in tablets.

2.5. Experimental design

Different experimental strategies were adopted to optimize the values of physical and chemical parameters. For screening purposes full factorial and Plackett–Burman designs were adopted [33]. For optimization (or fine tuning) central cubic faces designs were selected [34]. The outcome of designed experiments was modelled with multivariate linear regression [35]. The structure of linear models depends on the selected experimental design. Each model structure was optimized by means of analysis of variance (ANOVA), namely by ensuring statistical significance of regression coefficients, regression and evaluating the lack-of-fit (ensured using replicates). Feedforward artificial neural networks were also used to model experiments when linear models were inadequate [36]. Three-layered networks with a non-linear hidden layer (hyperbolic tangent) and a linear output layer were selected. The training algorithm was pseudo-second-order Levenberg–Marquardt. Overfitting was avoided by an appropriate training-validation strategy (the data is split in calibration (70%) and validation (30%) subsets during network training). Models simulation was performed using data within the corresponding training range. Contour plots were produced whenever appropriate.

The experimental design tables and linear models estimation was made using Modde version 6.0 (Umetrics, Umea, Sweden). Artificial neural networks and contour plots were made using Matlab version 6.2 (Mathworks, Natick, USA).

3. Results and discussion

3.1. Influence of reagents addition sequence

Some preliminary batch assays to evaluate the reaction involved in the spectrophotometric determination of indapamide revealed that the sequence of reagents addition is very important for the reaction development. This fact become evident when was conducted an experiment in which was monitored the absorbance of formed products by two different reagent addition approaches: MBTH mixed first with Ce(IV) and then with indapamide (1st addition sequence); or, MBTH mixed with indapamide and subsequently with Ce(IV) (2nd addition sequence). For a final volume of 15 mL the reagent volumes used were: 5 mL of a 8×10^{-3} mol L^{-1} MBTH solution, 5 mL of a 1.00×10^{-2} mol L^{-1} Ce(IV) solution and 5 mL of a 25 mg L^{-1} of indapamide. The obtained results (Fig. 2) showed that if MBTH and Ce(IV) solutions were mixed prior to the addition of indapamide a blue-coloured compound was formed with maximum absorbance wavelength at 601 nm; however, when MBTH was initially mixed with indapamide and then with the oxidizing agent, a red-coloured compound was produced with maximum absorbance in the visible range at 510 nm.

In the 1st addition sequence, the results comply with others mentioned in the literature affirming that the mechanism of reaction occurs in two stages [28]. Firstly, MBTH loses two electrons and one proton due to oxidation with Ce(IV), forming an electrophilic intermediate, which is the active coupling species. In the next stage, the electrophilic intermediate and the analyte undergoes electrophilic reaction with the formation of a coloured product and the elimination of one molecule of water.

In the 2nd addition sequence, in which the products formed exhibit an absorbance maximum at 510 nm, the mechanism of reaction is most likely different, probably involving the formation of a coupled intermediate between MBTH and the analyte, that is spontaneously oxidised after the addition of Ce(IV), originating different coloured species with different formation kinetics than the ones formed in 1st addition sequence.

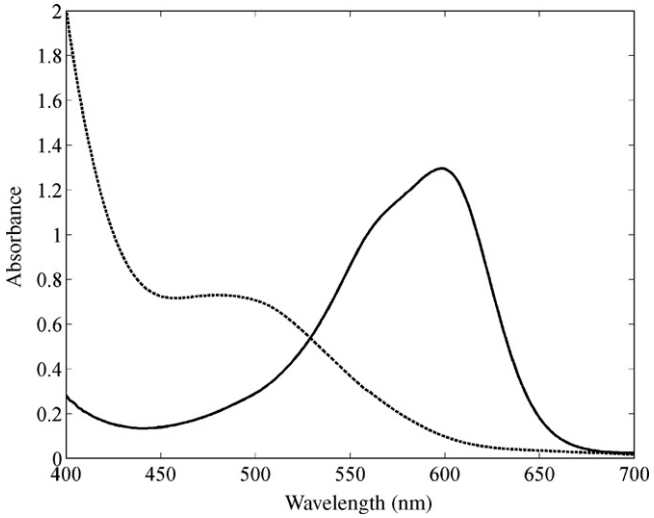

Fig. 2. Absorption spectra of indapamide (—, 1st addition sequence) and (——, 2nd addition sequence).

Table 1
Summary of regression models of chemical variables obtained for the screening and optimization stages.

Model	Design type	Design objective	Model equation (fitted using multivariate linear regression)	Analysis of variance		Q^2
				Regression	Lack-of-fit	
I	Full factorial	Screening	$A = (0.22 \pm 0.013) + (0.076 \pm 0.0071)$ [MBTH] $+ (0.11 \pm 0.015)$ [Ce] $- (0.052 \pm 0.015)$ $H_2SO_4 + (0.046 \pm 0.0075)$ [MBTH] [Ce] $+ (0.041 \pm 0.0071)$ [MBTH] [H_2SO_4] $- (0.080 \pm 0.015)$ [H_2SO_4] [Ce]	$p < 0.0001$	$p = 0.864$	0.983
II	Central cubic faces	Optimization	$A = (0.55 \pm 0.036) + (0.012 \pm 0.031)$ [MBTH] $+ (0.18 \pm 0.040)$ [Ce] $+ (0.029 \pm 0.026)$ [H_2SO_4] $+ (0.51 \pm 0.11)$ [MBTH]^2 $- (0.43 \pm 0.083)$ [Ce]^2 $- (0.17 \pm 0.057)$ [H_2SO_4]^2	$p < 0.001$	$p = 0.096$	0.911

With the aim to study the stability of the formed products through 2nd addition sequence, a study involving a programmed spectrophotometric monitoring during 120 min was performed, following the same experimental conditions as above. An absorbance spectrum (400–700 nm) was recorded at 5, 15, 30, 60, 90 and 120 min after the mixture of solutions and the obtained results are depicted in Fig. 3. The results revealed that initially, the maximum absorbance occurred at 510 nm, and that it decreases as the reaction time increases, in opposition to absorbance at 601 nm that increases at least till 60 min. This way, the red-coloured compound monitored at 510 nm is not stable, giving place to a complex with maximum absorbance at 601 nm. Moreover, at 90 min it was observed the formation of a blue precipitate, impairing spectrophotometric monitoring.

Similar stability and kinetic studies were conducted for the complexes formed by the 1st addition sequence, revealing a negligible variation in absorbance at 601 nm as reaction time increased, indicating a good complex stability. Comparing these results with the ones previously discussed for the 2nd addition sequence, it can be concluded that the formation of the complex detected at 601 nm is faster when applying 1st addition sequence approach. Additionally, no significant absorbance at 510 nm was observed.

These results justify the reason by which Youssef [28] by applying the 2nd addition sequence monitored the same reaction for indapamide determination at 601 nm. This determination methodology had to involve long waiting periods for the reaction development, because for the 2nd addition sequence the complex formation is slower than for the 1st addition sequence.

The influence on analytical signal of the waiting time before addition of the third reagent, for 1st and 2nd addition sequences, was also assessed, because it could originate some differences on the stability and formation kinetics of complexes. The analysis of the results comprising waiting times of 1, 5 and 10 min, showed that there was no observed influence of the waiting time on analytical signal before addition of the third reagent (indapamide, for the 1st addition sequence, and Ce(IV) for the 2nd addition sequence), not changing the conclusions previously stated.

One can then conclude that for indapamide determination through reaction with MBTH and Ce(IV), the 1st addition sequence approach is preferable, since it allowed a faster formation of a more stable complex, than the one formed by performing the 2nd addition sequence.

Taking into account the previous results, it was developed a multipumping flow system to implement the determination of indapamide (Fig. 1), in which the MBTH reagent was mixed with Ce(IV) solution creating a first reaction zone that was then mixed with the sample solution creating a second reaction zone.

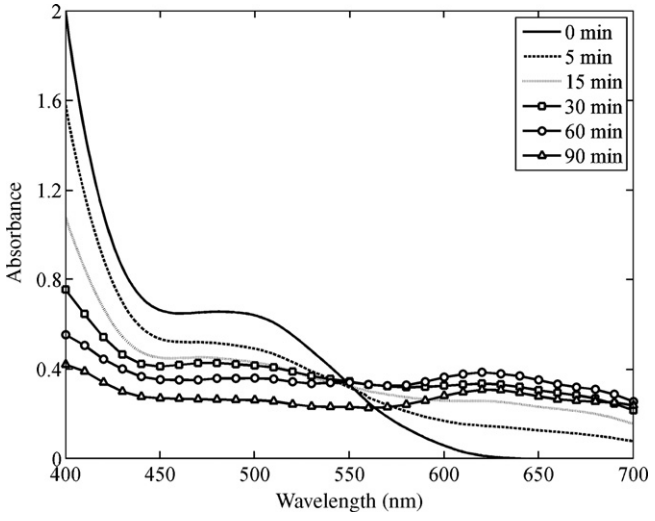

Fig. 3. Effect of development time on intensity of the coloured products of oxidative coupling reaction using the 2nd reagents addition sequence (MBTH and indapamide were mixed first and then with the Ce(IV) solution).

3.2. Optimization of the MPFS

Multivariate experimental design was used to optimize the parameters of the multipumping flow system that affected the analytical results. In order to evaluate the importance of all the influencing variables on the determination, a screening experimental design method was applied. The influence of the chemical variables on the analytical signal was first evaluated. It involved the analysis of the influence on absorbance of the concentrations of MBTH, cerium(IV) ammonium sulphate and sulphuric acid solutions. This study was performed using a full factorial design with 3 central points and 24 randomised runs, comprising the following ranges: 0.01–0.001, 0.1–1 and 0.01–0.001 mol L^{-1}, for MBTH, sulphuric acid and cerium(IV) ammonium sulphate, respectively. In this screening study it was verified that maximum absorbance values were obtained for lower concentrations of sulphuric acid and higher concentrations of MBTH and Ce(IV) solutions. The obtained model was described in Table 1 (model I). According to this model, a new range for each chemical parameter was defined for optimization purposes.

Next, a central cubic faces design was applied in order to fine tune the range of concentrations of the previously variables. This type of design is often adopted for optimization purposes since it is suitable for the estimation of cubic order models. In this optimization, the concentrations ranges assessed were 0.005–0.015 mol L^{-1} for MBTH and Ce(IV), and 0.08–0.1 mol L^{-1} for sulphuric acid solutions. The results revealed that the highest analytical signal was obtained for a combination of the following concentrations: 0.012, 0.011 and 0.1 mol L^{-1} for MBTH, Ce(IV) and sulphuric acid solutions, respectively. Results are compiled in Table 1 (model II).

The physical variables of the automatic flow system affecting the analytical signal were also studied using a Plackett–Burman factorial design with 12 randomised runs and 3 central points. The studied variables and their lower and upper levels were as followed: reactor length (10–100 cm), micropumps pulse time (0.2–1.0 s) and number of inserted sample pulses (5–15). As the Plackett–Burman design only provides the tendencies of variables to optimum values,

results revealed that the absorbance values increased with pulse time and number of sample pulses. However, when approaching the higher values of sample pulses and pulse time studied in the experimental design, it was verified the appearance of double peaks with different shapes, being this fact related with the length of the reactor used, indicating that the sample volume inserted into the system was too high and was not totally reacting. According with the results, and in order to surpass the difficulty related to double peaks that make determinations unfeasible, it could be implemented a reactor with a length superior to 55 cm, but that would impair the analysis time and increase sample dispersion.

So, three studies for which were fixed a different reactor length (10, 30 and 55 cm) were conducted, and the influence on analytical signal of the pulse time and number of sample pulses were assessed in the following ranges: 0.6–1.4 s and 8–16, respectively. A full factorial design with four levels for pulse time and number of pulses was adopted for each reactor length. According to this design, a total of 48 experiments were conducted. The obtained absorbances for each reactor length were modelled with an artificial neural network (one non-linear hidden layer with three nodes and a linear output layer with one node) since a linear model was found not to be appropriate. Details regarding the calibration are available in Section 2.5. Contour plots were produced for each reactor length by simulating the calibrated artificial neural network within the two physical parameters range. It was concluded that a number of pulses higher than 13 was not adequate for the determination, as double peaks were obtained. The obtained results were treated in order to exclude the ones corresponding to double peaks, being these graphically marked as filled circles (see Fig. 4A containing the contour plot for the 55 cm-length reactor). Subsequently, for each reactor was selected the optimum combination of pulse time/number of pulses, aiming to the higher absorbance value, and comparing at the end the selected results between the three assays. That comparison revealed that the highest absorbance value was obtained using a 55 cm reactor length, 1.1 s of pulse time and 11 pulses of sample insertion.

However, it was verified that the selected values provided a determination rate of 15 h^{-1}, approximately. Aiming to increase the determination rate without impairing analytical response, all the physical variables were further optimized (with exception for reactor length already fixed at 55 cm), with the objective of obtaining a maximum value for the ratio between absorbance signal and the time needed for one determination (in this work named "period"), and by attributing the same percentage importance (50%) to both factors. The period was the difference in time for maximum absorbance values between two consecutive determinations. For each assay, five determinations were conducted, and so, the calculated period corresponded to a four values average.

Thus, the results depicted in Fig. 4B revealed that the maximum value of absorbance/period for indapamide determination were obtained when using 11 pulses of sample solution, a pulse time of 0.6 s, for a reactor with 55 cm length.

Another flow parameter of great importance was the strategy used for sample introduction in the flow system, since it can influence the degree of mixture, and hence, reaction development. Under the optimized conditions, the effect of flow sampling strategy on the absorbance signal was studied. Some assays were carried out by exploiting different sampling methods at confluence point X_2 (Fig. 1), such as merging zones, binary sampling and single sample volume, and at the same time varying the number of pulses of the inserted sample solution within 6 and 14 (corresponding to sample solution volumes between 48 and 84 μL). Two different approaches for binary sampling were evaluated, designated in this work, by binary sampling A and B. Binary sampling A and B consisted in the insertion of aliquots of sample solution with pre-mixed aliquots of MBTH and Ce(IV) solutions, that is, plugs of sample were intercalated with plugs of MBTH/Ce(IV) solution. The binary sampling approaches A and B differed in the number of sample plugs intercalated: for the type A, one pulse of sample (8 μL) was intercalated with one pulse of MBTH/Ce(IV) (16 μL) solution, while, for approach B, two pulses of sample solution (16 μL) were intercalated with one pulse of MBTH/Ce(IV) (16 μL) solution.

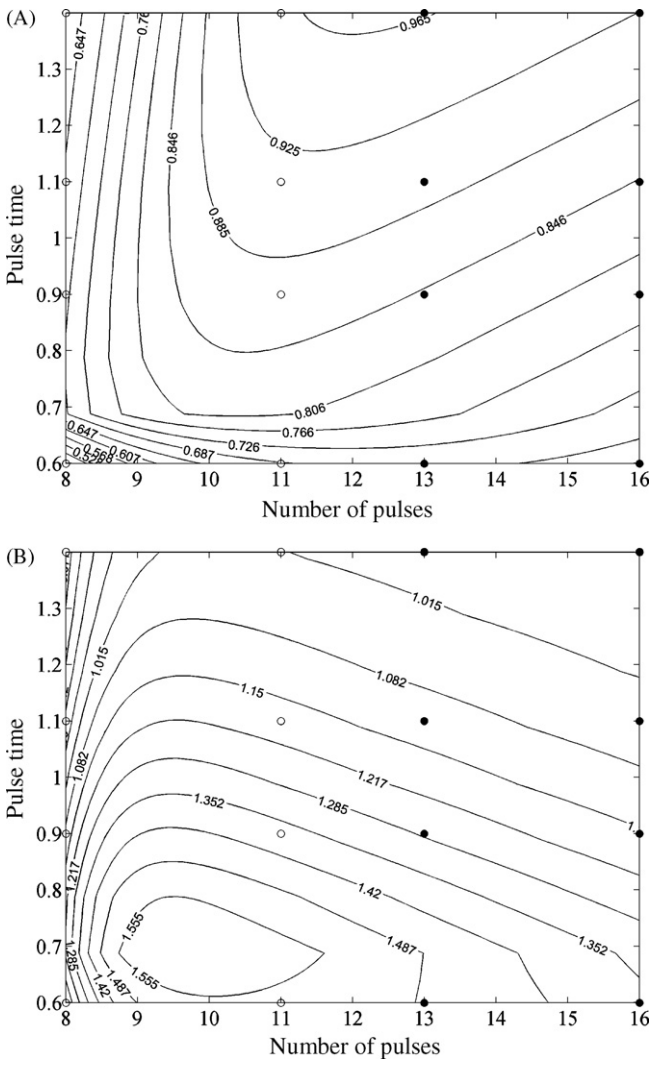

Fig. 4. (A) Effect of pulse time and number of pulses on absorbance signal using a reactor length of 55 cm (filled circles, double peak; empty circles, no double peak). (B) Effect of pulse time and number of pulses on absorbance signal per period using a reactor length of 55 cm (filled circles, double peak; empty circles, no double peak).

Performance of the merging zones process was evaluated by the simultaneous insertion of pre-mixed solution MBTH/Ce(IV) and sample solution. On the other hand, the single sample volumes approach was accomplish by inserting a unique volume of sample solution between two identical plugs of pre-mixed MBTH and Ce(IV) solution.

When programming these assays, the pulse time was modified and adjusted for all sampling strategies, in order to attain a similar flow rate throughout the system during sample insertion and transport phases. This was necessary since, in a multipumping flow system each individual solution flow rate is defined by the stroke volume and pulse time of the corresponding micropump, and when micropumps are activated simultaneously, the overall flow rate corresponds to the sum of the flow rates of all propelled solutions. This way, according to the sampling approach used, different flow rates are obtained. At the same time, the influence of the volume of sam-

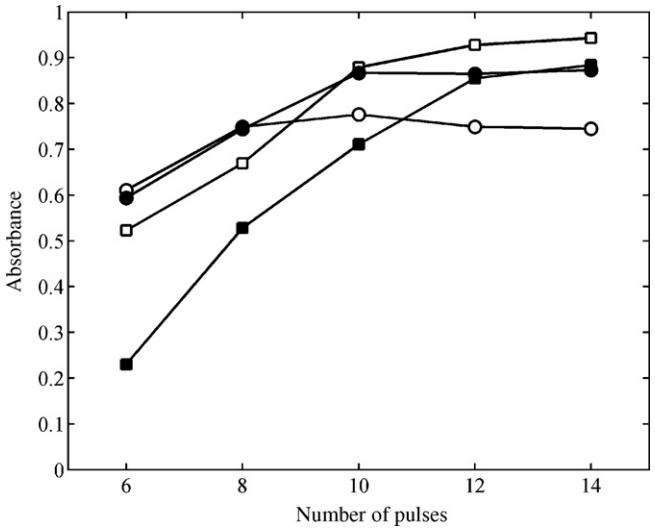

Fig. 5. Influence in the analytical signal of the number of sample pulses for distinct sampling strategies (empty squares, binary sampling A; filled squares, binary sampling B; empty circles, merging zones; filled circles, single volumes).

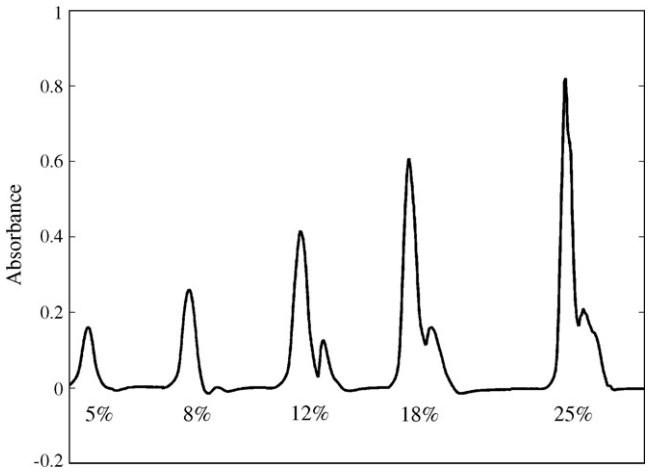

Fig. 6. Influence of ethanol concentration (%) in the analytical signal.

ple solution to be inserted into the flow system was evaluated for each sampling strategy, by analysing the effect on the analytical signal of the number of pulses of the micropump responsible for the sample insertion.

For the single sample volumes and binary sampling A approaches the obtained results revealed a more pronounced increase in the analytical signal up to approximately 10 pulses of sample solution (80 μL of sample), tending to stabilize for a higher number of pulses. However, for the merging zones approach an increase on analytical signal was verified up to 8 sample pulses (64 μL of sample), while that for binary sampling B the analytical signal increased up to 12 sample pulses (96 μL of sample).

After observing the obtained results (Fig. 5), it was concluded that both single sample volumes and binary sampling A approaches provided the highest analytical signals, and as a compromise between determination rate and sensitivity, 10 sample pulses (80 μL of sample solution) were selected for further optimizations.

Following last results, it was studied the performance of the single sample volumes and binary sampling A strategies by establishment of calibration curves with indapamide concentrations up to 50 mg L^{-1}. The results were evaluated resorting to a comparison between the slopes of calibration curves: linearity of the curves was represented by Abs = 0.0341 (±0.0005) × $C_{indapamide}$ (mg L^{-1}) + 0.24 (±0.01) and Abs = 0.0292 (±0.0004) × $C_{indapamide}$ (mg L^{-1}) + 0.20 (±0.01), for single sample volumes and binary sampling A, respectively. It was verified a sensitivity increase of about 14% when the single sample volumes approach was performed. This way, the sampling strategy applied for indapamide determinations was single sample volume.

As already mentioned in subchapter "sample, standards and reagents", since indapamide is practically insoluble in water, a stock solution of 400 mg L^{-1} was prepared in a 40% ethanol solution. The indapamide standard solutions were prepared from dilution of the stock solution with acid sulphuric solution. So, for indapamide concentrations between 5 and 50 mg L^{-1}, the ethanol concentration was between 0.5 and 5%. Nevertheless, the concentration of ethanol had a strong influence in absorbance signal, due to Schlieren effect. In order to evaluate this influence, a calibration curve with a concentration range of ethanol up to 25% was established. It was observed that the absorbance increased with the concentration of ethanol and for that reason its concentration was normalized between all standards with the objective to obtain the same influence of ethanol in analytical signal. On the other hand, it is important to mention that the utilization of ethanol concentrations higher than 5% caused intense refractive index gradients (Schlieren effect) in the sample zone, as it can be observed through the appearance of double peaks represented in Fig. 6. In order to circumvent the Schlieren effect problem, all indapamide standards were prepared in a 5% ethanol solution.

3.3. Analysis of pharmaceutical formulations

In order to apply the developed methodology to the determination of indapamide in pharmaceutical formulations, the influence of some compounds commonly used as excipients was assessed. A sample solution containing a fixed amount of indapamide (20 mg L^{-1}) and different concentrations of the excipients under evaluation were analysed by the developed method. A compound was considered as non-interfering if the analytical signal variation was ±4% compared to the analytical signal obtained in the absence of the referred compound. The results revealed that the excipients (starch, lactose, magnesium stearate, anhydrous colloidal silica, hypromellose, polyvinylpyrrolidone (povidone), stearic acid, and cellulose) on a 100-fold mass ratio regarding indapamide did not interfere.

Under all the optimal conditions previously established, a linear working response range for indapamide concentration up to 50 mg L^{-1} was obtained. The calibration curve was represented by Abs = 0.0325 (±0.0002) × $C_{indapamide}$ (mg L^{-1}) + 0.187 (±0.007) with a correlation coefficient of 0.9999. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [37] was about 1 mg L^{-1}.

The precision of the proposed methodology was evaluated through the calculation of the confidence interval of a set of 10 repeated measures for each sample. From the results in Table 2, it can be confirmed that the developed methodology presents a good repeatability, taking into account the calculated concentration ranges for a confidence level of 95%.

For accuracy assessment, the results obtained for determination of indapamide in seven commercial pharmaceutical dosage forms by the proposed flow procedure, were compared with the ones obtained through the reference procedure of the British Pharmacopoeia. The results, summarised in Table 2, revealed a good agreement between both methods, with relative deviations comprised between −1.5 and 2.1%. Additionally, this agreement was confirmed using a paired *t*-test [37], in which the *t*-value estimated (0.127) was lower than the tabulated one (2.447) illustrating the

Table 2
Comparison of analytical results obtained in the determination of indapamide in pharmaceutical formulation by the proposed and the reference method [32].

Sample	Dosage (mg)/formulation	Amount found (mg)[a]		R.D. (%)[b]
		MPFS Methodology	Reference method	
Generis 2.5	2.5	2.76 ± 0.09	2.75 ± 0.18	0.2
Alter 2.5	2.5	2.56 ± 0.08	2.58 ± 0.12	−0.8
Fluidema 2.5	2.5	2.58 ± 0.08	2.59 ± 0.05	−0.3
GP 2.5	2.5	2.51 ± 0.06	2.54 ± 0.04	−1.5
Fludex LP 1.5	1.5	1.50 ± 0.03	1.46 ± 0.06	2.1
Tandix 1.5	1.5	1.52 ± 0.05	1.51 ± 0.05	0.7
Ind Generis 1.5	1.5	1.51 ± 0.04	1.49 ± 0.04	0.8

[a] Mean ± $t0.05$ (Student's t-test) × (S.D./$\sqrt{n}$).
[b] Relative deviation of the development method regarding the reference procedure.

Table 3
Analytical figures of merit of the proposed multipumping flow system (MPFS) and batch methodology [28].

Parameter	MPFS	Batch
Linear dynamic range (mg L^{-1})	Up to 50	1.2–9.6
Equation of linear calibration	$A = 0.0325 \times C_{indapamide} + 0.187$	$A = 0.1179 \times C_{indapamide} + 0.0086$
Detection limit (mg L^{-1})	1.00	0.14
Sampling rate (h^{-1})	25	Not mentioned
MBTH (mg)/determination	0.207	6.00
Ce(IV) (mg)/determination	0.356	10.00
H_2SO_4 (mg)/determination	12.55	49.04

absence of any statistical differences for a confidence level of 95% ($n = 7$).

The time required to complete an analytical cycle was approximately 147 s and consequently the determination rate equated to about 25 determinations per hour. For that reason, the developed automatic flow methodology represents a significant improvement in determination rate when compared to the reference method [32], in which, almost 12 min were required to complete a chromatographic run (determination rate of about 5 h^{-1}), meaning an improvement of about 80%.

3.4. Comparison of the proposed MPFS with other methodologies

Additionally, the MPFS and chromatographic methodologies were compared in terms of production of residues per determination. The HPLC procedure produced approximately 14.4 mL of residues per determination (containing: SDS 4.21 mg, glacial acetic acid 2.62 mg, triethylamine 100.91 mg, butan-2-ol 224.62 mg and acetonitrile 3.39 g), while the MPFS only produced 1.44 mL per determination (containing: MBTH 0.207 mg, Ce(IV) 0.356 mg and sulphuric acid 12.55 mg). This means that the proposed MPFS allowed a significant reduction in the production of residues in the order of 90%.

The developed MPFS was also compared with another spectrophotometric methodology carried out in batch for indapamide determination based on the same reaction [28]. In Table 3 are compiled some analytical figures of merit of both methodologies and also the reagents consumption per determination. According to Table 3, the obtained linear working range with MPFS was increased to up 50 mg L^{-1}. In batch methodology the determination rate is not mentioned, but since it involves waiting periods for reaction development (15 min total waiting time, according with authors), it can be predicted that the determination rate is significantly lower than that obtained by the proposed MPFS. Moreover, the comparison between the two methods demonstrated that the proposed MPFS allowed a significant reduction in the consumption of reagents in the order of 97, 96 and 74%, for MBTH, Ce(IV) and sulphuric acid, respectively. A lesser wastefulness of reagents was accomplished due to sulphuric acid solution that was the only reagent in carrier solution, allowing the very low consumption of MBTH or Ce(IV).

4. Conclusions

This work contributed to an improved knowledge of the oxidative coupling reaction involving the reagents MBTH and Ce(IV), often used in the pharmaceutical chemical monitoring.

The results obtained in this work showed that the oxidative coupling reaction involving MBTH and Ce(IV) can led to distinct reaction products depending on the experimental conditions, namely on the sequence of reagents addition. This assumption was confirmed when this reaction was applied in the spectrophotometric determination of indapamide by resorting to an automated flow-based system. Effectively, different reaction products with different kinetics, stabilities and absorbance spectra were obtained simply by varying the sequence of reagents insertion. If the reagents addition sequence involves mixing first MBTH and indapamide, and only then Ce(IV), a product with fast formation kinetics, relatively unstable and with a maximum absorbance wavelength at 510 nm is formed. But, by mixing first MBTH and Ce(IV), adding latter the indapamide solution, then the formed product is much more stable, with maximum absorbance wavelength at 601 nm.

This variability can be exploited as an advantageous versatile feature whenever the presence of given specie interfere with the determination, a situation that could be overcome by using a specific addition sequence that favoured the formation of a product with either distinct kinetics or a different maximum absorbance wavelength. For example, when applying this methodology for the determination of a substance in several matrices, and if interfering specie absorbing at one of the monitored wavelengths are formed, then the same reaction can still be used by exploiting one specific addition sequence of reagents, avoiding the detection at the wavelength where the interference is noticed.

The automation of a reactional scheme involving MBTH and Ce(IV) by means of a flow-based analytical system requires immediately high versatility in terms of solutions manipulation in order to enable the exploitation of these possibilities. In this regard, the multipumping flow system implemented for indapamide determination allowed the individual insertion, commutation and propelling of all solutions in a pre-determined and programmed sequence, making very easy the implementation of the two different addition sequences of reagents by using only one analytical

system. At the same time, it generated a pulsed flowing stream with improved mixing conditions that facilitate reaction development.

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