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Chemiluminometric determination of captopril in a multi-pumping flow system

S. Sofia M. Rodrigues, João L.M. Santos*

Requimte, Departamento de Ciências Químicas, Laboratório de Química Analítica e Físico-Química, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, n° 164, 4099-030 Porto, Portugal

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ABSTRACT

In this work, a simple, versatile and fully automated analytical methodology for the chemiluminometric determination of captopril – an angiotensin II-converting-enzyme (ACE) inhibitor – in pharmaceutical formulations, is proposed. The developed methodology was based on the enhancement by captopril of the chemiluminescence emission of tris(2,2′-bipyridyl)ruthenium(II). In sulphuric acid medium tris(2,2′-bipyridyl)ruthenium(II) was oxidized by cerium(IV) and converted into a reactive oxidant specie $[Ru(bpy)_3]^{3+}$, which was subsequently reduced with captopril in order to yield a significant enhancement of the original chemiluminescence emission that was directly related to captopril concentration. The analytical process was implemented by resorting to an automated multi-pumping flow system (MPFS) that enabled the establishment of multiple reaction interfaces, which, in combination with the created pulsed flowing stream assured a fast and reproducible sample/reagent mixing and reaction development essential to guarantee the generation and subsequent measurement of the short-lived species involved in the chemiluminescent process. The developed system employed three solenoid micro-pumps as the only flow manifold active components. These assured the insertion, propelling and commuting of all solutions. The automatic actuation of the solenoid micro-pumps provided an easily programmed, operated and controlled analytical flow system, exhibiting high versatility, efficiency and compactness at a low cost.

Under the optimized experimental conditions, the proposed method allowed the determination of captopril for concentrations between 2×10^{-3} and 1.5×10^{-1} mmol L^{-1} ($r = 0.9996$, $n = 6$) and a sampling frequency of about 58 determinations per hour, producing 620 μL of waste per determination. The results obtained for pharmaceutical formulations were statistically comparable to those provided by the reference procedure with a relative deviation between −2.32 and 1.39%. The possible mechanism of the chemiluminescence reaction was also discussed in this work.

1. Introduction

Captopril, 1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline, whose structure is illustrated in Fig. 1, is an angiotensin II-converting-enzyme (ACE) inhibitor [1] that has been widely used to treat arterial hypertension, some types of congestive heart failure and post myocardial infarction [2,3] and to preserve the kidney function in diabetic nephropathy [4]. Approximately 50% of captopril is metabolized through the liver, being oxidized to their respective disulfides, and the remaining is excreted in the urine [5]. This compound has been also used in the treatment of cancer [6].

Despite its therapeutical usefulness, the administration of captopril for medical purposes could also originate some adverse effects, which included cough, angiodema, agranulocystosis, proteinuria, hyperkalemia, taste alteration, teratogenecity, postural hypotension, acute renal failure and leucopenia [4,7]. Thus, the monitoring of this drug is of extreme importance to assure an appropriate and adjusted pharmacotherapy.

Due to captopril pharmacological relevance, a variety of procedures have been developed and proposed for its determination in both pharmaceutical and biological samples. The most commonly used approaches involve chromatographic methods like high performance liquid chromatography [8–10] and gas chromatography [11] although voltammetry [12], amperometry [13], colorimetry [14], fluorimetry [15], spectrophotometry [16,17], atomic absorption spectrometry [18] and FT-Raman spectrometry [19] were also reported. However, some of the abovementioned methods exhibit important shortcomings that encompass laborious and time-consuming sample handling, the need for expensive instrumentation and skilled operators, complex system operation and maintenance, low analytical throughput and high consumption of reagents most of which are organic toxic ones that do

* Corresponding author. Tel.: +351 222078968/222087132; fax: +351 222078961/222004427.
E-mail address: joaolms@ff.up.pt (J.L.M. Santos).

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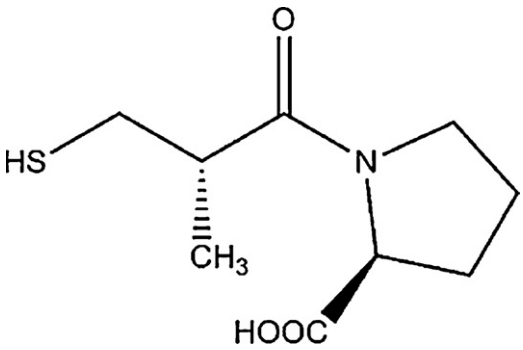

Fig. 1. Chemical structure of captopril.

not exactly meet the requirements or intents of green chemistry policies.

In recent years, chemiluminescence (CL) analysis has gained an extensive interest in the scientific community and has proved to be an important and powerful tool in different fields [20–24], mainly when it comes to pharmaceutical analysis. CL measurements have many advantages, being characterized by rapid detection with high sensitivity, wide dynamic linear range [25], no background scattering light or source instability [22] and simple and straightforwardly operated instrumentation. These characteristics make chemiluminescence detection particularly suitable for coupling with flow-based automated methodologies [26]. In this regard, chemiluminescence detection, not only in batch [27] but mostly in combination with flow injection analysis (FIA) has been reported for the determination of captopril [28–30]. Nevertheless, the limited selectivity and high reagent and sample consumption has been reported as the main shortcomings of these flow approaches.

For the determination of several compounds CL can be studied in terms of signal inhibition or enhancement. One of the most attractive CL reactions involves tris(2,2′-bipyridyl)ruthenium(II). This useful and versatile CL reagent has been applied in many analytical applications with chemiluminometric detection [31–34]. In these applications, tris(2,2′-bipyridyl)ruthenium(II) is converted in a reactive oxidant specie $[Ru(bpy)_3]^{3+}$ by chemical/photochemical/electrochemical generation, followed by reduction by a given compound to yield a light emission [35,36]. The reduction could be carried out by distinct compounds, including organic acids, amines, amino acids and pharmaceuticals such as fungicides and alkaloids [35–38].

Since the majority of the CL response is usually generated by fast reactions and the sensitivity is enhanced if measurements occur just about the maximum emission, a prompt and reproducible sample/reagents mixing ideally inside the flow cell in front of the detector would be required [39]. With this purpose, in this work an automated analytical flow systems based on multiple reaction interfaces (multi-pumping flow system – MPFS) [40] was developed for implementing the reactional scheme and carrying out the measurements. The high automation level and operationality of MPFS also enabled overcoming some of the drawbacks of FIA previously referred. In a multi-pumping flow system, multiple solenoid actuated micro-pumps act as the only active devices of the flow manifold being accountable for all stages of the analysis, such as insertion, propelling and commutation of solutions, and also for the establishment and detection of the reaction zone, providing a great flexibility and operational simplicity. Moreover, the characteristic pulsed flow generated by the actuation of the micropumps promotes a reproducible, rapid and efficient sample/reagent mixing [41] making MPFS particularly advantageous, since it promotes an immediate reaction development and thus guarantees an optimized analytical signal measurement. These characteristics diminish considerably the required sample and reagent solutions volumes and, subsequently, the production of waste amounts.

The purpose of this work was to develop a multi-pumping flow system for the selective chemiluminometric determination of captopril in pharmaceutical formulations, with simplicity, robustness, very low consumption of sample and reagents and high determination frequency. This determination was based on the high sensitizing effect of captopril on the CL of tris(2,2′-bipyridyl)ruthenium(II)–Ce(IV) system, in sulfuric acid medium. A possible mechanism of CL enhancement is discussed.

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared with water from a Milli-Q system (specific conductivity $\leq 0.1\ \mu S\ cm^{-1}$) and chemicals were of analytical reagent grade quality. Reagents were then not subject to any further purification.

Tris(2,2′-bipyridyl)dichloro-ruthenium(II)hexahydrate used in this study was purchased from Sigma (St. Louis, MO, USA). A $1.0 \times 10^{-3}\ mol\ L^{-1}$ stock solution was prepared by dissolving 74.9 mg in 100 mL of water. This stock solution was stored in a dark and fresh environment. Working standard solutions were daily prepared by suitable dilutions with deionized water.

A $5.0 \times 10^{-4}\ mol\ L^{-1}$ ammonium cerium nitrate (Sigma, St. Louis, MO, USA) solution was daily prepared by dissolving 27.41 mg in 100 mL of a $1\ mol\ L^{-1}$ H_2SO_4 solution.

A $1.0 \times 10^{-3}\ mol\ L^{-1}$ captopril (Sigma, St. Louis, MO, USA) stock solution was prepared, on a daily basis, by dissolving 21.7 mg of captopril bulk drug in 100 mL of deionized water. Working standard solutions were prepared from dilutions of the corresponding stock solution with deionized water.

2.2. Sample preparation

Commercial tablets from different pharmaceutical laboratories with nominal contents of 25 and 50 mg of captopril were analyzed. Ten tablets were weighted and finely grounded. An accurately weighed amount of powder was dissolved in deionized water, subject to ultrasonication for 15 min and then filtered by gravity. Aliquots of filtrate was diluted with deionized water to obtain the appropriate concentration for the determination by chemiluminescence.

2.3. Apparatus

The multi-pumping flow system consisted of a set of three solenoid micro-pumps (Ref. 120SP-Bio-Chem Valve Inc., Boonton, USA), used as fluid propeller and commutation devices, and a Camspec CL-2 chemiluminescence detector (Camspec Ltd., Cambridge, UK) equipped with a three-port quartz flow cell (60 μL inner volume). The micro-pumps were of the fixed displacement diaphragm type, being solenoid operated and delivering a stroke volume of 10 μL.

The flow tubes were made of PTFE material and were of 0.8 mm internal diameter. Homemade end-fittings, connectors and acrylic confluence points were also used.

Control of the analytical system was accomplished through a PC computer equipped with a PC-LABCard model PCL-711B (Advantech, Cincinnati, OH) interface card. A CoolDrive™ power drive board (NResearch Inc., West Caldwell, USA) was used to operate the solenoid micro-pumps.

Analytical signals were also recorded on paper using a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

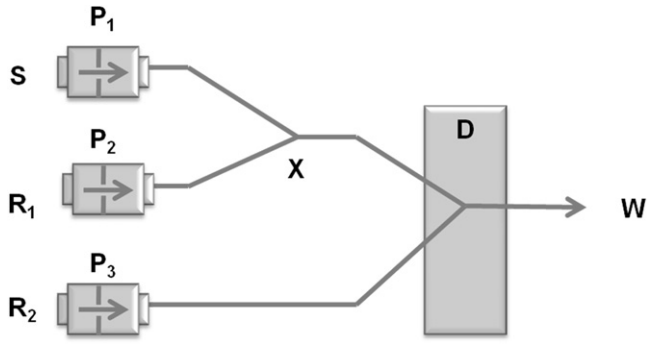

Fig. 2. Multi-pumping flow manifold diagram. P_1–P_3: solenoid micro-pumps (10 μL per stroke); X: confluence point; D: chemiluminescence detector; W: waste; S: captopril standard or sample solutions; R_1: tris(2,2′-bipyridyl)dichloro-ruthenium(II)hexahydrate solution; R_2: ammonium cerium nitrate solution.

The reference method for captopril determination was carried out by using a Jasco LC-NET II/ADC high performance liquid chromatographer furnished with a PU-2080 Plus intelligent pump, a Allsphere ODS-2 C18 column (25 cm × 4.6 mm) and a MD-2015 Plus multiwavelength detector.

2.4. Flow manifold and procedure

In Fig. 2 is represented the flow manifold exploiting de MPFS approach developed for chemiluminometric captopril determination.

It was designed with three solenoid micro-pumps (P_1, P_2 and P_3) as the only active components, used to control the flow rate, solutions volumes, sequence of insertion and transportation of the sample or the standard solutions and the reagent solutions towards the detector. The repetitive micro-pump switching on/off produced a pulsed flowing stream in which the pulse volume corresponded to the micro-pump stroke volume. The solenoid micro-pumps P_1, P_2 and P_3 were needed for individually inserting the sample or standard solutions (S), $Ru(bpy)_3^{2+}$ solution (R_1) and Ce(IV) carrier solution (R_2), respectively.

The analytical cycle was started by establishing a baseline, which was performed by using Ce(IV) solution as carrier stream. For this, P_3 was activated in order to propel the Ce(IV) solution through the analytical path, towards the detector, at a fixed pulse time of 0.4 s, which defined the flow rate as 1.5 mL min^{-1}. Subsequently, P_3 was switched off and P_1 and P_2 pumps were actuated, simultaneously, at a fixed pulse time of 0.4 s, creating a flowing stream, at confluence point X, resulting from the merging of $Ru(bpy)_3^{2+}$ and standard or sample solutions, exploiting the merging zones flow approach [42]. Thereafter, the reagents and the sample were fully mixed inside the detector by simultaneously actuation of all three micro-pumps P_1–P_3. Taking into account the pulsed nature of the flow as well as the quick CL reactions, a rapid reaction zone homogenization was reached inside the detector flow cell and it was produced an analytical signal, which was recorded as a peak and whose magnitude was proportional to the captopril concentration. After detection, the established reaction zone was carry forward to waste.

A calibration procedure was carried out with the insertion of a set of standard solutions, to obtain the analytical curve.

2.5. Reference method

For accuracy assessment of the results obtained with the developed procedure, captopril bulk drug and captopril pharmaceutical formulations were analyzed by high performance liquid chromatography according to the British Pharmacopoeia's method [43].

3. Results and discussion

Preliminary investigations were carried out in order to assess the feasibility of this study and the possibility of exploiting the captopril sensitizing activity to develop a novel method for its determination. In this way, the CL intensities of the $Ru(bpy)_3^{2+}$–Ce(IV) reaction were recorded in the presence and absence of captopril. The experimental results showed a weak CL signal when $Ru(bpy)_3^{2+}$ was mixed with Ce(IV) solutions and a strong CL signal when captopril, $Ru(bpy)_3^{2+}$ and Ce(IV) solutions were presented in the reaction system. $Ru(bpy)_3^{2+}$ was selected as chemiluminogenic species and Ce(IV) was used as the oxidant.

3.1. Development of micro-pump flow methodology

In the development of this investigation, in order to establish the optimum conditions and improve systems performance, specifically in terms of analytical signal intensity, sensitivity, accuracy, precision, repeatability and sampling rate, various parameters were investigated using a univariate approach. These variables influenced the options for the optimization of the system.

The main parameters optimized were $Ru(bpy)_3^{2+}$, Ce(IV) and H_2SO_4 concentrations, reaction coil length, flow rate and volume of solutions, beyond of manifold configuration.

3.2. Configuration designs

Concerning the chemiluminescence reaction under study, the maximum CL emission was observed immediately after mixing the $Ru(bpy)_3^{2+}$ with the Ce(IV) solutions. Aiming at the improvement of the sensitivity, the flow system was then configured (Fig. 2). Captopril could then be inserted either into the $Ru(bpy)_3^{2+}$ or into the Ce(IV) flowing streams. Analysis of the signals obtained with both manifold configurations showed that the maximum CL intensity was achieved when the sample was previously merged with $Ru(bpy)_3^{2+}$ stream, since it improved the time for reaction development. So, the multi-pumping flow system was planned to allow merging of the sample with the $Ru(bpy)_3^{2+}$ stream through the simultaneous actuation of P_1 and P_2.

3.3. Chemical parameters

Effects of $Ru(bpy)_3^{2+}$, Ce(IV) and H_2SO_4 concentrations were investigated by using water instead of the captopril solutions.

The influence of $Ru(bpy)_3^{2+}$ concentration in the chemiluminescence intensity was investigated between 1.0×10^{-5} and 3.0×10^{-4} mol L^{-1}. It was observed that with increasing $Ru(bpy)_3^{2+}$ concentrations the intensity of the signal increased until 1.0×10^{-4} mol L^{-1}, above which the chemiluminescent signal become stable. Therefore, this value was then selected for following experiments.

The chemiluminescence intensity was also studied for Ce(IV), used as the oxidant reagent in this chemiluminescence system, between 1.0×10^{-5} and 1.0×10^{-3} mol L^{-1}. The obtained analytical signals showed that with increasing Ce(IV) concentrations the intensity of the chemiluminescent response increased until 5.0×10^{-4} mol L^{-1}. Above this concentration value the chemiluminescent intensity remained almost unchanged (Fig. 3). Consequently, taking into consideration the consumption of Ce(IV) and the sensitivity, 5.0×10^{-4} mol L^{-1} was selected as the optimum concentration of Ce(IV) for the succeeding studies.

Ce(IV) is not completely soluble in water but when it is dissolved in sulfuric acid solution becomes a stable solution. Considering that the sulfuric acid can inhibit hydrolysis of Ce(IV) and influence the oxidation ability of Ce(IV) [44], distinct H_2SO_4 solutions with increasing concentrations were assessed. The results display

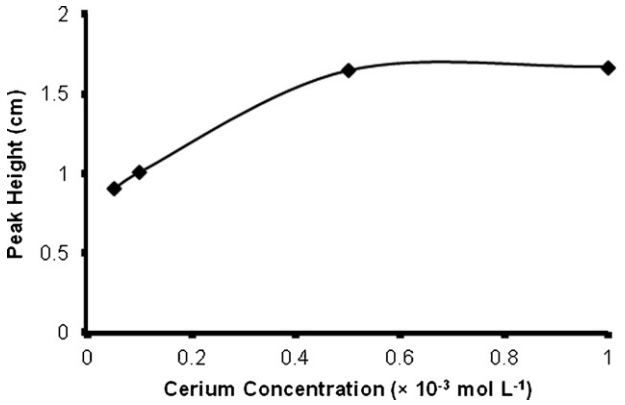

Fig. 3. Influence of cerium(IV) concentration.

that the chemiluminescent intensity achieved a maximum value for 1 mol L^{-1} H_2SO_4.

3.4. Physical parameters

By comparing chemiluminescence intensities using reaction coils, with serpentine configuration, ranging from 0 to 25 cm, it was observed that the chemiluminescence intensity gradually decreases with increasing size of the reactor, which could be probably explained by the fast kinetics of the reaction between the $Ru(bpy)_3^{2+}$ and Ce(IV) (Fig. 4). Consequently, no reactor has been selected for further experiments.

Flow rate is an important parameter responsible for establishment of the reaction time and the residence time of the reaction zone inside the flow cell conditioning the measured CL intensity. In addition, for fast CL reactions the influence of flow rate is more marked [45]. In multi-pumping flow system this is determined by the frequency of the micro-pump and the stroke volume. Influence of flow rate used to propel solutions was assessed between 1 and 3 mL min^{-1}. As the flow rate increases the magnitude of the analytical signal also increases up to 1.5 mL min^{-1} and then approaches stabilization. The best optimal flow rate was 1.5 mL min^{-1}.

Considering that in a multi-pumping flow system the stroke volume and the number of pulses of micro-pump actuation define sample and reagents volumes, the influence of reagents and sample volumes was studied. Comparing chemiluminescence intensities using equal numbers of pulses for $Ru(bpy)_3^{2+}$ and sample from 1 to 6 pulses that corresponded to a volume between 10 and 60 μL it was observed that the chemiluminescence intensity was maximum at 20 μL (2 pulses) for $Ru(bpy)_3^{2+}$ and sample. The Ce(IV) volume was studied ranging from 10 to 65 pulses. It was verified that the insertion of a Ce(IV) volume of 600 μL (60 pulses) exhibit the highest magnitude of the signal. So, for further experiments, 2 pulses of $Ru(bpy)_3^{2+}$ and sample and 60 pulses of Ce(IV) were selected.

These results revealed that volume and flow rate were fundamental parameters affecting the intensity of the analytical signal obtained with the micro-pump system.

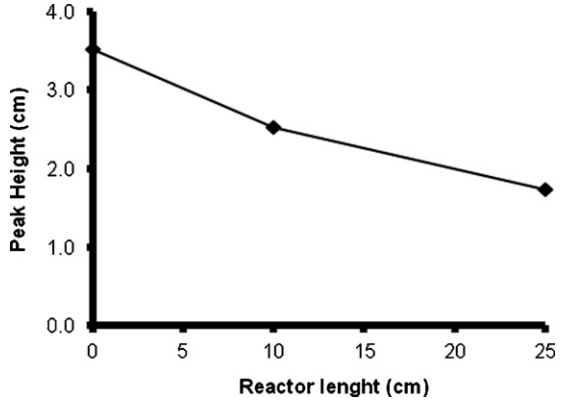

Fig. 4. Influence of the reactor length.

Table 1
Results obtained in the evaluation of the interfering effect of excipients by using a 1×10^{-2} mmol L^{-1} captopril solution on the developed methodology.

Interference	Tolerance limit
Lactose	10
Stearic acid	40
Magnesium stearate	65
Starch, microcrystalline cellulose, anhydrous colloidal silica, castor oil	100[a]

Data refer to the concentration ratio (expressed in mmol L^{-1}) between the interfering specie and the analyte.
[a] Maximum tested concentration ratio.

3.5. Study of the possible mechanism of the system

The proposed reaction mechanism is probably analogous to that described previously for determinations that involved $Ru(bpy)_3^{2+}$–Ce(IV) system [32,46,47]. Thus, the captopril determination is based on the monitoring of the light emitted in reduction of $Ru(bpy)_3^{3+}$ to the excited state by the oxidizing species generated during the reaction investigated [36]. In these CL reaction, Ce(IV) in sulfuric acid oxidize $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ and captopril to an active intermediate. The active species generated can react with $Ru(bpy)_3^{3+}$ promoting the production of excited state $[Ru(bpy)_3^{2+}]^*$ that emits light as shown below:

$$Ru(bpy)_3^{2+} + Ce^{4+} \rightarrow Ru(bpy)_3^{3+} + Ce^{3+} \quad (1)$$

$$Cap + Ce^{4+} \rightarrow Cap^{\bullet +} + Ce^{3+} \quad (2)$$

$$Cap^{\bullet +} \rightarrow Cap^{\bullet} + H^+ \quad (3)$$

$$Ru(bpy)_3^{3+} + Cap^{\bullet} + H_2O \rightarrow [Ru(bpy)_3^{2+}]^* + \text{otherproducts} \quad (4)$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow [Ru(bpy)_3^{2+}] + h\nu \quad (5)$$

3.6. Interferences study

In order to assess the selectivity of the developed methodology to the determination of captopril in pharmaceutical formulations, the influence of several species commonly used as excipients was evaluated. Samples containing a fixed amount of captopril (1×10^{-2} mmol L^{-1}) and increasing concentrations of the excipient under evaluation were analyzed by the developed methodology. A compound was considered as non-interfering if the analytical signal variation was ±3% regarding the one obtained in its absence. The obtained results (Table 1) revealed that under the used reaction parameters, no significant interfering effect for the majority of the tested compounds was found. These results confirmed that the

Table 2
Parameters evaluated during the optimization of the multi-pumping flow system performance and values selected for its operation.

Parameter	Evaluated range	Selected values
$Ru(bpy)_3^{2+}$ volume (μL)	10–60	20
Ce(IV) volume (μL)	10–650	600
Sample volume (μL)	10–60	20
Reactor length (cm)	0–25	0
Flow rate (mL min^{-1})	1–3	1.5
$Ru(bpy)_3^{2+}$ concentration (mol L^{-1})	1.0×10^{-5}–3.0×10^{-4}	1.0×10^{-4}
Ce(IV) concentration (mol L^{-1})	1.0×10^{-5}–1.0×10^{-3}	5.0×10^{-4}
H_2SO_4 concentration (mol L^{-1})	1.5×10^{-1}–1	1

Table 3
Comparison of analytical results obtained in the determination of captopril in pharmaceutical formulations, by the developed methodology and the reference procedure.

Pharmaceutical formulation	Declared dosage (mg/formulation)	Amount found (mg/formulation)		R.D. (%)[b]
		Developed methodology[a]	Reference methodology[a]	
Captopril Generis®	25	24.90 ± 0.37	24.56 ± 2.70	1.39
Captopril Mepha®	50	49.75 ± 0.47	50.67 ± 0.64	−1.81
Capoten®	25	24.20 ± 0.36	24.48 ± 2.24	−1.15
Captopril GP®	25	24.63 ± 0.28	24.95 ± 2.62	−1.31
Captopril ratiopharm®	50	50.60 ± 0.56	51.80 ± 4.16	−2.32

[a] Mean ± $t_{0.05}$ (Student's t test) × SD/$\sqrt{n}$.
[b] Relative deviation of the developed methodology with respect to the reference procedure.

proposed methodology can be applied in the analysis of captopril in pharmaceutical formulations.

3.7. Application to pharmaceutical formulations

After system optimization, the feasibility and accuracy of the proposed flow procedure was demonstrated by applying it to the determination of captopril in commercially available pharmaceutical formulations using the previously referred optimized experimental conditions exhibited in Table 2.

A linear working concentration range was obtained between 2×10^{-3} and 1.5×10^{-1} mmol L^{-1} of captopril. The analytical curve was represented by the equation: $h = 34.1\ (\pm 0.6) \times C + 0.13\ (\pm 0.04)$, in which h was the height of the recorded peak (expressed in cm) and C was the captopril concentration (expressed in mmol L^{-1}), with a correlation coefficient (r) of 0.9996 ($n = 6$). The detection limit, calculated from the equation of the calibration curve according to Skoog et al. [48] using the following expression $LOD = 3\sigma/m$, where σ is the standard deviation of 20 measurements of the blank and m is the slope of the analytical curve, was estimated at 1×10^{-3} mmol L^{-1}, whose result demonstrate the sensitiveness for the proposed procedure.

In order to evaluate the applicability and the accuracy of the proposed methodology, this was applied to the determination of captopril in five commercially available pharmaceutical formulations. The obtained results, summarized in Table 3, were in agreement with those furnished by using the reference method, revealing a relative deviation between −2.32 and 1.39%. In addition, the repeatability was satisfactory, with a relative standard deviation lower than 0.6% ($n = 3$). A paired Student's t-test [49] was also performed on the data obtained by both methods and confirmed that, for a confidence level of 95% ($n = 5$), there were no significant statistical differences between the results obtained by both procedures: the estimated t-value (1.773) was lower than the tabulated t-value (2.776).

The developed flow system revealed stability, no baseline drift was verified and robustness. The sampling rate was about 58 determinations per hour. The consumption of reagents per determination was about 1.5 μg of $Ru(bpy)_3^{2+}$ and 164 μg of Ce(IV), which generated 620 μL of waste *per* determination.

4. Conclusions

The proposed chemiluminometric methodology, based on the CL enhancing effect of captopril on $Ru(bpy)_3^{2+}$–Ce^{4+} system in the medium of sulphuric acid, allowed fast and reliable determination of captopril in pharmaceutical formulations, with good system stability, low cost and no sample pre-treatment. Additionally, the obtained results confirmed that this developed flow approach exhibits high sensitivity, accuracy, reproducibility, robustness, a wide working concentration range and was selective due to the limited influence of some compounds commonly used as excipients. The relative standard deviation could be considered very satisfactory, which demonstrated a good repeatability, and when compared to the reference procedure, the relative deviations were inferior to 2.32%. The accuracy of the developed methodology regarding to the reference procedure was confirmed by paired t-test for pharmaceutical formulations.

The employment of solenoid micro-pumps, as the only active components, responsible for the insertion, transport and mixing of all solutions, beyond guaranteeing a high operational simplicity, also generated a pulsed flowing stream that provided a fast and very efficient homogenization between sample and reagents, reducing sample dispersion and facilitating reaction development. Such advantages are crucial to promote low solutions consumptions and, subsequently, the minimization of waste generation, resulting in an environment friendly strategy and a reduction of the costs of the analysis. Moreover, this procedure demonstrates high automation level. The versatile operational characteristics of MPFS anticipate that the developed flow system can be easily applied in the analysis of other compounds without noteworthy physical changes in the manifold. Furthermore, due to the versatility of the proposed methodology, it can be also applied to more complex samples, such as biological fluids, although it would be eventually necessary the carrying out of an in-line sample pre-treatment in order to improve its response in terms of selectivity.

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