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Exploiting Iminoquinone Free Radical Production for Thiol Based Drugs Determination in Pharmaceutical Formulations

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Um procedimento espectrofotométrico de análise em fluxo foi desenvolvido para determinação de Captopril (CPT) e *N*-acetil-*L*-cisteína (NAC) em formulações farmacêuticas baseado na reação com radical iminoquinona produzido pela oxidação de N,N-dimetil-*p*-fenilenodiamina (DMPD) em meio ácido. A ordem de adição de reagentes foi avaliada e os parâmetros analíticos foram otimizados. As faixas lineares de concentração foram de 2,5-90 e de 5,0-50 mg L^{-1} para CPT e NAC, respectivamente. Os limites de detecção (3σ) foram calculados em 0,22 e 1,8 mg L^{-1} e desvios padrão relativos (N = 10) foram inferiores que 1,0 e 2,1% para CPT e NAC, respectivamente. O procedimento foi empregado para a determinação de CPT e NAC em diversas formulações farmacêuticas e os resultados concordaram com os obtidos pelos métodos de referência para 95% de confiabilidade aplicando teste-*t* pareado.

A spectrophotometric flow analysis procedure to determine Captopril (CPT) and *N*-acetyl-*L*-cysteine (NAC) in pharmaceutical formulations was developed based on the reaction with iminoquinone radical produced from the N,N-dimethyl-*p*-phenilenediamene (DMPD) oxidation in acid medium. The reagents addition order was evaluated and analytical parameters were optimized. The linear concentrations ranges were 2.5-90 and 5.0-50 mg L^{-1} for CPT and NAC, respectively. Limits of detection (3σ) were calculated at 0.22 and 1.8 mg L^{-1} and the relative standard deviation (N = 10) were lower than 1.0 and 2.1% for CPT and NAC, respectively. The procedure was employed for CPT and NAC determination in diverse pharmaceutical formulations and by applying paired *t*-test the results were in agreement with those obtained by reference methods for 95% confidence level.

Keywords: iminoquinone radical reaction, flow injection analysis, determination of thiol drugs, Captopril, *N*-acetyl-*L*-cysteine

Introduction

The derivatization is an actual strategy to allow confident chemical information and the conversion of analyte to a colored, fluorescent or electroactive species are some of the many possibilities of this approach. In some cases spectrophotometric methods involve the reaction of the analyte with a chromogenic reagent to produce a colored species as can be exemplified by the phenothiazine dye production starting from the oxidative coupling reaction of sulfide with the iminoquinone radical which is produced by the reaction of an aromatic *p*-substituted amine, such as *N,N*-dimethyl-*p*-phenylenediamine (DMPD), with an oxidizing agent.[1,2] The reaction between sulfide and DMPD to yield methylene blue dye is presented in Scheme 1a. The same reagents (DMPD and Fe^{3+}) used for sulfide determination were employed for spectrophotometric determination of alkyl thiols[3,4] as well as to produce electroactive species from thiol aminoacids.[5] Some other bioactive species, such as synthetic and natural drugs, have thiol functional groups in their molecular structures and reactions analogous those observed in the derivatization of alkyl thiols (Scheme 1b) and thiol aminoacids (cysteine and monocysteine) with an aromatic *p*-substituted amine are expected.

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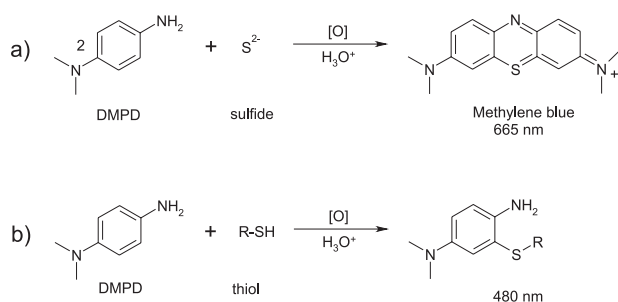
Scheme 1. Reactions of (a) sulfide and (b) thiol compound with DMPD and oxidizing agent in acidic medium.

Captopril (CPT) and *N*-acetyl-L-cysteine (NAC) are widely used drugs and both molecules have one thiol group. CPT is used to treat hypertension, congestive heart failure, kidney problems caused by diabetes and to improve survival after a heart attack.[6] NAC is a mucolitic agent which is also used to reverse the toxicity of high doses of acetaminophen.[7] Some analytical techniques have been employed for CPT and NAC determination. Luminometric,[8] spectrophotometric,[9-11] electroanalytical[12-15] and titrimetric[16] methods have been proposed for independent determination of one among these large consumed drugs in batch mode. The application of the same pattern reaction to determine these thiol drugs was reported by exploring the reaction with 1,2-naphthoquinone-4-sulfonic acid to develop a fluorimetric method in batch mode.[17]

Obviously, NAC and CPT are not found in the same pharmaceutical formulation. However NAC and CPT are usually determined in pharmaceutical quality control laboratories and the use of the same analytical procedure to determine both analytes was considered as an important advantage for routine laboratories. In this way, the development of a generic spectrophotometric flow injection procedure for thiol based drugs determination in pharmaceutical formulations was the aim of this work. The generic approach of the proposed flow injection analysis procedure was assured once unstable iminoquinone radical reacts with the thiol drug by employing the same flow injection and chemical parameters as well as the same detection condition.

Experimental

Reagents and solutions

All chemicals used were of analytical grade and the solutions were prepared with freshly purified water (< 0.1 µS cm^{-1}). Solutions of 5.0×10^{-3} mol L^{-1} DMPD (*N,N*-dimethylphenyl-*p*-diamine, Merck) were prepared by weighting in different acidic concentration (6.25×10^{-3} –

0.1 mol L^{-1}) from H$_2$SO$_4$ (Merck) or HCl (Vetec) solutions. Acidic Fe^{3+} solutions 1.33% (m/v) (5×10^{-2} mol L^{-1}) were prepared from FeCl$_3$·6H$_2$O (Sigma) in H$_2$SO$_4$ and HCl solution.

Aqueous stock solutions (100 mg L^{-1}) of Captopril (Galena, Brazil) and *N*-acetyl-L-cysteine (Galena, Brazil) were prepared by weighting. Working reference solutions of the studied drugs were prepared from the proper dilution of CPT and NAC stock solutions immediately before the use to avoid oxidative decay. Aqueous solution of DMPD was also prepared daily.

Thiol drugs samples were purchased in local drugstores. Diverse CPT samples in tablet form (12.5 mg) were evaluated. Samples in liquid (nasal solution: 11.5 mg mL^{-1} and cough syrup: 40 mg mL^{-1}) and solid (tablet: 11.5 and 20 mg and powdered formulation 100 mg) forms were used for NAC determination.

Sample preparation

Solid samples (CPT and NAC tablets) were firstly powdered and subsequently homogenized. 50 mg L^{-1} CPT aqueous solution was prepared by dissolving an equivalent mass of 2.5 mg of the powdered formulation in water while 1.5 mg of NAC equivalent mass were weighted and dissolved in water to prepare 30 mg L^{-1} NAC solution from powdered medicines. CPT and NAC solutions were filtered and taken for 50 mL. Samples of NAC in cough syrup (20 mg mL^{-1}) and nasal solution (11.5 mg mL^{-1}) were prepared by diluting pharmaceutical formulations in water.

Flow manifold

The flow manifold employed for the determination of CPT or NAC in pharmaceutical formulations is presented in Figure 1. An eight-channel Minipuls 3 peristaltic pump (Gilson, France) fitted with Tygon tubing was employed for the propulsion of solutions. The sample was inserted in the flow path by a rotary valve Rheodyne 5020 (USA). Analytical signal was measured by employing a Femto 432 spectrophotometer (Brazil) equipped with a borosilicate flow cell with 10-mm optical path and 120 µL of useful volume. Transient signals were monitored by means of Ross 107 (Cole-Parmer, USA) monochannel x-t recorder.

Procedures

The flow manifold developed for thiol drugs determination is depicted in Figure 1. After the insertion of sample aliquot in the analytical path the carrier solution directs the sample zone to mix with acidified DMPD solution in the reaction coil B$_1$. Following, the sample
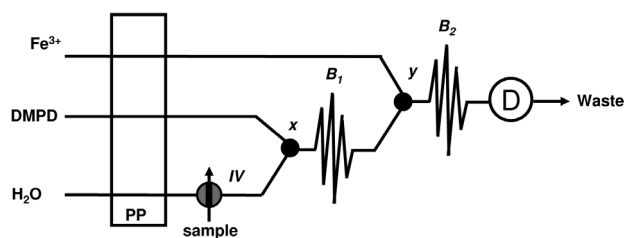
Figure 1. Flow manifold for thiol drugs determination based on the reaction with DMPD and Fe^{3+} in acid medium. ***PP*** = peristaltic pump. ***IV*** = rotary valve. ***x*** and ***y*** = confluences. ***B_1*** and ***B_2*** = reaction/mixture coils. ***D*** = detector

zone was mixed with Fe^{3+} solution in B_2 to yield a colored reaction product which was directed to the detector (D).

The initial concentration of oxidizing agent (Fe^{3+}) and DMPD were 0.05 and 0.005 mol L^{-1}, respectively. $FeCl_3$ and DMPD solutions were acidified with HCl to maintain the same acidic concentration in both solutions. The effect of HCl concentration in $FeCl_3$ and DMPD solutions was also evaluated in the concentration range from 6.25×10^{-3} to 1×10^{-1} mol L^{-1}. Notwithstanding the reagents addition order has been the first parameter evaluated by using water to direct the sample zone to the detector, the effect of HCl concentration in the carrier solution was also studied to obtain information about the main and parallel reactions.

The flow parameters were optimized by varying the carrier flow rate from 2.0 to 4.9 mL min^{-1}. The flow rates of reagents solutions were also evaluated and the flow rates of DMPD and Fe^{3+} solutions were varied from 1.1 to 3.0 mL min^{-1} and 0.8 to 3.0 mL min^{-1}, respectively. The effects of the length of mixture (B1) and reaction (B2) coils in analytical signal were evaluated by varying the length of B1 from 10 to 80 cm and B2 from 40 to 160 cm, respectively. Finally, the effect of the sampling volume (100 to 500 μL) on analytical signal was also evaluated.

The analytical characteristics of the flow injection methods for thiol drug determination were evaluated and validation tests were carried out. Volumetric reference methods[18,19] based on the thiol drugs oxidation were employed to evaluate the accuracy of the proposed method for CPT and NAC determination.

Results and Discussion

As earlier reported, the derivatization of alkyl thiols with iminoquinone radical ($DMPD^{\bullet+}$) is slow and the analytical signals were decreased (*ca.* 20%) by increasing the length of carbonic chain of alkyl thiols from 1 to 6 carbon atoms.[4] Based on this information the evaluation of chemical and flow injection parameters were accomplished for CPT (217.3 g mol^{-1}), because its carbonic chain is longer than NAC (163.2 g mol^{-1}).

Spectral profiles

Spectral evaluation of the reaction products from each one of the evaluated drugs (3.4×10^{-4} mol L^{-1}) with DMPD in the presence of Fe^{3+} was performed in the visible region of electromagnetic spectrum. The spectra of the evaluated yielded colored dyes were obtained in the same acidic concentration (2.5×10^{-3} mol L^{-1}) and the profiles are presented in Figure 2. As can be observed in Figure 2, the smallest absorption peak maximum was conferred to the colored dye produced from the CPT reaction with iminoquinone free radical. The maxima absorption wavelengths related to the reaction products of $DMPD^{\bullet+}$ with CPT and NAC were 476 and 496 nm, respectively. However for the further trials the wavelength of 480 nm was selected.
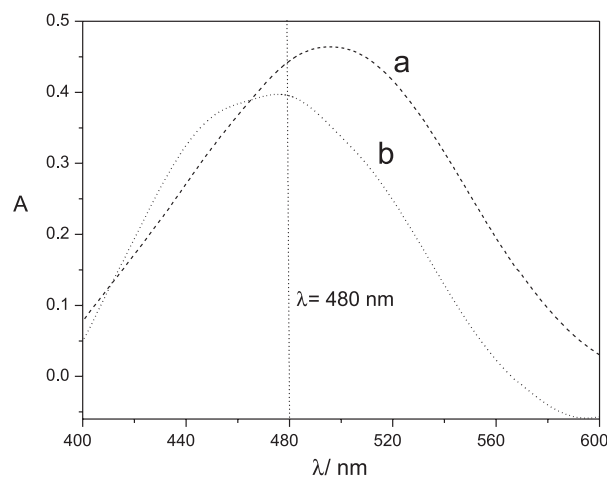

Figure 2. Spectral profiles of the reaction products from (a) NAC and (b) CPT with iminoquinone radical in acid medium

Reagents addition order

The effect of the reagents addition order on the analytical signals was evaluated in flow condition by changing the insertion point of Fe^{3+} and DMPD solutions in the flow manifold (Figure 1) and maintaining the concentration of CPT reference solution at 50 mg L^{-1}. A significant increase on the analytical signal (*ca.* 25%) was observed by mixing previously CPT and DMPD solutions before the addition of oxidizing (Fe^{3+}) solution. The sensitivity gain was explained since CPT reacts with iminoquinone free radical produced by the previous DMPD oxidation.

Evaluation of acidic concentration of reagents solutions

Hydrochloric and sulfuric acid have been used to acidify DMPD and Fe^{3+} solutions for sulfide determination by methylene blue method.[20,21] By considering that the

methylene blue method for sulfide determination involves the production of the same iminoquinone radical, the effect of the acid used (HCl or H_2SO_4) to prepare DMPD and Fe^{3+} solutions was evaluated in batch mode for CPT determination. No significant differences were observed in analytical signals (95% confidence level) by acidifying DMPD and Fe^{3+} solutions with sulfuric or hydrochloric acid. However the dispersion of analytical signals was lower for reagents solutions prepared with hydrochloric acid. Therefore, DMPD and Fe^{3+} solutions were prepared with HCl.

A significant decrease on the analytical response was observed for CPT by increasing acidic concentration of DMPD and Fe^{3+} solutions as can be observed in Figure 3. Higher sensitivity was obtained for reagents solutions prepared in 6.25×10^{-3} mol L^{-1} HCl (Figure 3). However, DMPD and Fe^{3+} solutions were prepared in 2.5×10^{-2} mol L^{-1} HCl to prevent $Fe(OH)_3$ precipitation into the analytical path.
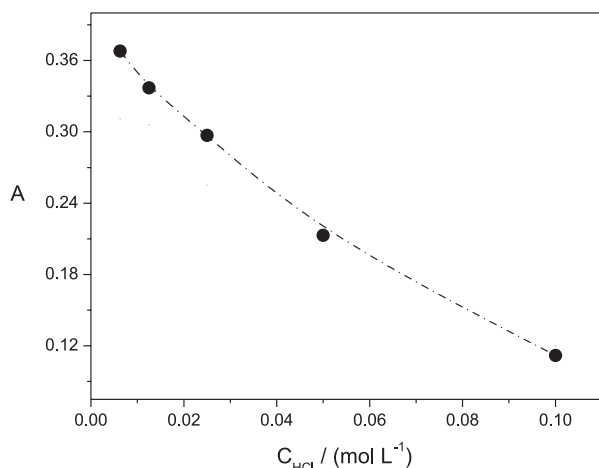

Figure 3. Effect of HCl concentration on the analytical signal for 50 mg L^{-1} CPT ($N = 3$)

Optimization of flow parameters

Flow injection parameters were optimized for the flow manifold depicted in Figure 1, by using 5×10^{-3} mol L^{-1} DMPD solution, 5×10^{-2} mol L^{-1} Fe^{3+} solution and 50 mg L^{-1} CPT.

The optimization process of flow injection parameters was arisen by the evaluation of the flow rate of oxidizing solution since the oxidation of aromatic amine was considered the critical reaction step. It was observed that the analytical signal was diminished by increasing the flow rate of Fe^{3+} solution. This fact was related to (*i*) the lower residence time of the sample zone in the analytical path, which decreases the oxidation rate of DMPD by Fe^{3+} and/or (*ii*) an increase of acidic concentration in the reaction coil B2. The later alternative was early confirmed (Figure 3) and

the flow rate of oxidizing solution was fixed at 1.2 mL min^{-1}. In contrast, higher analytical signals were obtained by increasing the flow rate of DMPD solution from 1.1 to 2.2 mL min^{-1}. However a significant decrease on analytical signal (> 25%) was observed for the flow rate of DMPD solution at 3.0 mL min^{-1} pointing out the requirement of Fe^{3+} excess to produce the iminoquinone radical. Therefore the flow rate of DMPD solution was set to 2.2 mL min^{-1}.

Initially water was used to carrier the sample zone to the detector and the effect of the flow rate of carrier from 2.0 to 4.9 mL min^{-1} on analytical signal was evaluated. It was observed an increase on analytical signals by increasing the flow rate of carrier. This effect was explained by the low stability of the reaction product and/or by the decrease on acidic concentration in the sample zone. It was also observed an analytical signals decrease for flow rates of carrier solution (water) lower than 3.3 mL min^{-1} and for further experiments the flow rate of carrier solution was set at 3.3 mL min^{-1}.

Notwithstanding the effects on the main and parallel reactions with NAC and CPT were appraised by increasing HCl concentration in carrier solution and maintaining the lengths of mixture and reaction coils at 50 cm (Figure 4). The acidic effect on DMPD oxidation was evaluated by inserting aliquots of thiol based drug and DMPD before the addition of oxidizing in the analytical flow path (Figure 4, curves a). In contrast, thiol drugs oxidation and/or complex formation by reaction with Fe^{3+} were highlighted by inserting aliquots of thiol drug and Fe^{3+} before DMPD addition in analytical flow path (Figure 4, curves b). For these studies CPT and NAC concentration were 50 and 25 mg L^{-1}, respectively. As can be observed (Figure 4) for CPT and NAC the highest analytical signals ($\lambda = 480$ nm) were obtained by mixing previously DMPD and thiol compound before the oxidation step and employing water to carrier sample zone to the detector. In this way, water was used throughout to carry the sample zone to the detector and the reagents addition order in advance selected was more one time confirmed. However, a decrease on analytical signal by increasing the HCl concentration in the carrier solution was observed for NAC and CPT (Figure 4, curves *a*) and it was justified by the drawback to oxidize a protonated aromatic amine.

On the other hand, some authors have reported the complex formation between thiol compounds and Fe^{3+}.[22-24] As can be observed (Figure 4, curves b) the analytical signal increases by increasing HCl concentration in the carrier solution for NAC which was explained by Fe^{3+} displacement from complex in higher acidic media. However, the same displacement effect was not confirmed for CPT since small signals variation was observed by increasing HCl
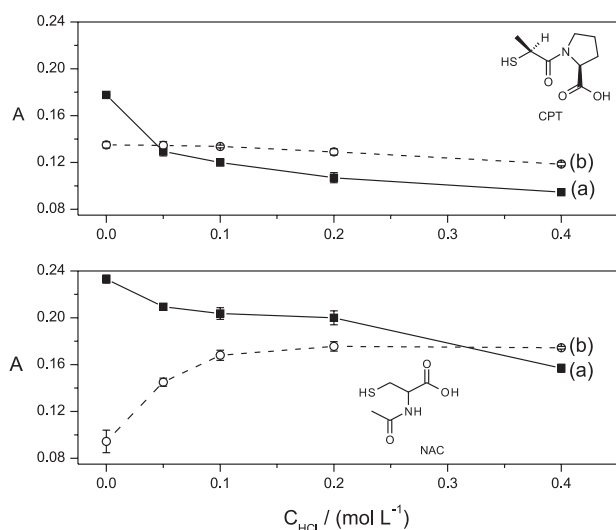
Figure 4. Effect of HCl concentration in carrier solution for different reagents addition orders. (a) Insertion of thiol and DMPD aliquots into the analytical path before Fe^{3+} solution addition. (b) Insertion of aliquots of sample and Fe^{3+} solutions, followed by the addition of DMPD solution. (N = 3)

concentration in the carrier solution as well as the signals have decreased for higher acidic concentration. Thus, it was suggested that only a fraction of CPT was oxidized by Fe^{3+} in B1 and the oxidation product did not react with DMPD in B2.

The effects of mixture and reaction coils lengths on analytical signal were one by one evaluated. The effect of B1 coil length (mixture coil) on analytical response was evaluated in the range from 10 to 100 cm while the reaction coil (B2) was varied from 40 to 160 cm. Only the mixture between sample aliquot and DMPD solution occurs in B1 and better results were obtained for lower B1coil length

(10 cm). Moreover, higher analytical response for CPT was obtained for 100 cm reaction coil (B2) length besides for reaction coil length higher than 100 cm a decrease on analytical signal was observed. Therefore the residence time on reaction coil of *ca.* 5 s (total flow rate equal 6.7 mL min⁻¹) was enough to produce iminoquinone radical and to permit its reaction with CPT.

The sampling volumes were varied from 100 to 500 μL and the analytical response was linearly dependent for aliquots volumes lower than 275 μL. For sampling volume higher than 275 μL did not set off a significant difference (95% of confidence level). Therefore, sample aliquot of 275 μL was selected throughout.

Feature of the method

The linear concentration ranges from 2.5 to 90 mg L⁻¹ and from 5 to 50 mg L⁻¹ were obtained for CPT and NAC, respectively. The sensitivity for NAC (0.011 uA L mg⁻¹) was higher than for CPT determination (0.0079 uA L mg⁻¹) due to CPT has higher carbonic chains. Limits of detection (3σ) were calculated for each thiol based drugs[25] and they were 0.22 and 1.8 mg L⁻¹ for CPT and NAC, respectively. For 10 consecutive determinations, the highest relative standard deviation (2.1%) was obtained for NAC (5 mg L⁻¹), while higher precision (0.93%) was observed for CPT (50 mg L⁻¹). Analytical throughput of 120 determinations h⁻¹ was achieved for both thiol drugs. The figures of merits of the proposed flow injection procedure for CPT and NAC determination and other spectrophotometric flow methods earlier published were summarized in Table 1. The

Table 1. Characteristics of spectrophotometric methods for NAC and CPT determination in flow analysis systems

Analyte	Comments	Linear range / (mg L⁻¹)	LOD / (mg L⁻¹)	RSD / (%)	Throughput / h⁻¹	Ref.
CPT	(FIA) Kinetic method. Reaction with IO_3^-	10-60	ni	< 4.5	100	11
	(FIA) Fe^{3+} reduction by CPT. Fe^{2+} reacts with *o*-phen	2.2-170	1.1	< 0.2	60	26
	(SIA) Complex formation with Pd^{2+}	43-304	ni	< 1.3	5	27
	(FIA) Fe^{3+} reduction by CPT. Fe^{2+} reacts with DPPH	7-1000	4.0	< 0.8	120	28
	(SIA) Fe^{3+} reduction by CPT. Fe^{2+} reacts with DPPH	12-1000	7.0	< 1.2	60	28
	(FIA) Reaction with DMPD	2.5-90	0.22	< 1.0	120	Proposed method
NAC	(FIA) Zn^{2+} extracted from solid phase $[Zn_3(PO_4)_2]$ by NAC react with alizarin red	4.9-24.5	1.3	< 0.5	60	29
	(FIA) Reaction between NAC and Br_2 produced in line	19-190	9.7	< 1.2	60	30
	(FIA) Oxidation of NAC or ferroine by Ce^{4+}	1-16	0.6	< 1.4	60	31
	(FIA) Fe^{3+} reduction by NAC. Fe^{2+} reacts with *o*-phen	0.4-52	0.08	< 1.5	60	32
	(FIA) Complex formation with Pd^{2+}	6-600	1.2	< 1.4	45	33
	(FIA) Reaction with DMPD	2.5-50	1.8	< 2.1	120	Proposed method

ni = not informed.

analytical characteristics of the proposed method for CPT determination were better than those obtained in previous works while the analytical characteristics of the proposed method for NAC were similar (Table 1).

Application

The proposed flow procedures were employed for thiol drugs determination in pharmaceutical formulations. Solids samples of CPT (tablets) were selected to evaluate the proposed flow methods and NAC samples in solid (tablets and powdered formulation) and liquid (syrup and solution) form were used. The CPT and NAC amounts as well as the related standard deviations (N = 3) were summarized in Table 2. The results obtained from the proposed FIA methods for CPT and NAC were compared with reference methods[18,19] and no significant differences were found for CPT and NAC by applying paired *t*-test (95% confidence level).

Table 2. Determination of CPT and NAC in pharmaceutical formulations (N = 3).

Drug	Sample	Thiol amount / mg			Error / (%)
		Declared value	Proposed method	Reference method	
CPT	1	12.5	13.56 ± 0.08	12.1 ± 0.2	+12
	2	12.5	11.36 ± 0.04	11.93 ± 0.09	−4.8
	3	12.5	12.5 ± 0.2	11.7 ± 0.1	+6.8
	4	12.5	11.8 ± 0.1	11.8 ± 0.2	0
	5	12.5	12.69 ± 0.03	11.67 ± 0.08	+8.7
	6	12.5	11.07 ± 0.04	11.25 ± 0.01	−1.6
NAC	1	11.5	10.4 ± 0.3	10.2 ± 0.1	+2.0
	2	11.5	10.6 ± 0.3	10.0 ± 0.1	+6.0
	3[a]	11.5	11.72 ± 0.04	12.3 ± 0.2	−4.7
	4	20	17.3 ± 0.3	18.0 ± 0.2	−3.9
	5[a]	40	34.4 ± 0.8	37.8 ± 0.6	−9.0
	6	100	108 ± 1	117 ± 6	−7.7

[a]Liquid samples (cough syrup and nasal solution).

Conclusions

A flow injection procedure for thiol based drugs determination was developed. The procedure was based on the same pattern reaction and the analytical performance was evaluated as an alternative for the official methods to check the amount of captopril and *N*-acetyl-*L*-cysteine in pharmaceutical formulations. The pattern reaction for thiol drugs derivatization was not the same involved in the methylene blue method for sulfide determination, since

the coupling reaction do not occur for CPT and NAC. The proposed procedure was developed in a conventional flow injection system to highlight its versatility and high analytical throughput. The results obtained by the proposed flow procedure were compared with those obtained by reference methods for CPT and NAC determination by applying paired *t*-test and no significant differences were observed for 95% confidence level.

The better reagents addition order to produce a colored dye from the reaction of iminoquinone free radical (DMPD$^{\bullet+}$) with thiol drugs was established and the effects related to the acidic concentration of carrier and reagents solutions were discussed. By considering the reaction involved between iminoquinone radical and the evaluated thiol molecules, the proposed flow procedure can be applyied for some thiol bioactive species determination, such as penicillamine, cysteine, glutathione, thiopronine and cysteamine.

The generic attributes of the proposed procedures were enlighten and they were successfully applied for CPT and NAC determinations in pharmaceutical formulations (tablets, powdered, syrup and nasal solution) with a single chromogenic reagent (DMPD) and in the same wavelength (480 nm).

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