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Ethyl-propiolate as a novel and promising analytical reagent for the derivatization of thiols: Study of the reaction under flow conditions

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ABSTRACT

The present investigation demonstrates the potentials of ethyl-propiolate (EP), a novel derivatizing reagent for thiols. To the best of our knowledge this is the first systematic study of EP in analytical chemistry. The reaction was investigated under flow conditions using sequential injection (SI) analysis and UV detection at 285 nm. The reaction kinetics was affected by parameters such as the pH, the concentration of EP and the temperature and was thoroughly examined exploiting stopped-flow experiments. Cysteine (CYS) and captopril (CAP) were selected as model thiolic compounds in terms of chemical structures. Finally, the applicability of EP as a derivatization reagent for analytical purposes was demonstrated by the development and validation of a novel automated assay for the determination of CAP in pharmaceuticals.

1. Introduction

Thiols play an important role in the biological systems since they are related to a large number of biological phenomena. For instance, one of the naturally occurring thiols with simple structure, CYS, is a significant element in cellular metabolism process and protein structure [1]. On the other hand, thiol-containing drugs (e.g. CAP, tiopronine, penicillamine, etc.) are widely used for the treatment of a broad range of diseases. On this basis, there is an interest in developing analytical methodologies capable to determine both naturally occurring thiols and thiol-containing drugs in a variety of sample matrixes.

Derivatization is one of the most widely applied sample pretreatment protocols in analytical chemistry. The targets of derivatization reactions vary from improving the detectability of the analytes by tagging suitable chromo- or fluorophores, to modifying their chemical properties and increase their compatibility to the selected analytical techniques (e.g. improve the volatility for GC analysis, of fragmentation pattern for MS detection) [2]. In the case of separation techniques such as HPLC, derivatization can be carried out either pre-column (in batch or automated modes) or post-column. In capillary electrophoresis, derivatization is usually performed in batch, in-capillary or post-capillary modes. An ideal analytical derivatization reagent should fulfil most of the following demands: (i) rapid reaction under mild conditions, (ii) stability of the derivatives under the analysis conditions, (iii) minimum side-reactions, (iv) low cost and commercial availability, (v) high sensitivity and (vi) compatibility to modern analytical techniques.

The majority of the naturally occurring thiols such as CYS and its degradation product cysteamine, homocysteine, N-acetyl cysteine, reduced glutathione, the tripeptide glutathione or some drugs (CAP, penicillamine and 2-mercaptoethane sulfonate (MESNA)) lack of chromo- or fluorophore groups in their molecule. Consequently, their detectability is low and therefore their determination at low concentrations in complex matrixes may be problematic. A typical and widely accepted way to overcome this analytical problem is the derivatization of this group of compounds prior to the final measurement using suitable reagents.

The reagents used for the derivatization of thiols typically include tagging labels introducing UV–vis, fluorescent or electrochemical properties to the analytes. The analytical derivatization of thiols has previously been reviewed in detail [3–5]. Table 1 summarizes information about the most commonly used derivatizing reagents including data on the detection mode, the reaction conditions and the cost of the reagents. Fluorescent reagents such as monobromobimane (MBB) and 4-fluoro-7-sulfobenzofurazan (SBD-F) are not attractive due to the requirement of elevated temperatures and long reaction times [6,7]. Ellman's, 4,4′-dithiodipyridine (DTDP) and Thioglo®-3 (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-

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Table 1
Most popular derivatization reagents for thiolic compounds.

Reagent	Analyte	Detection mode	Reaction time (min)	Reaction conditions	Cost	Ref.
Monobromobimane (MBB)	Monothiols, polythiols	FL[a]	30	45 °C	110 €/25 mg[b]	[6]
7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)	Glutathione, cysteine, n-acetyl-cysteine, mercaptoacetic acid, γ-glutamylcysteine	FL	60	60 °C	60.5 €/5 mg[c]	[7]
Ellman's reagent	Total thiols	Vis	10	RT[d]	16.3 €/g[b]	[8]
4,4′-Dithiodipyridine (DTDP)	Mercaptans	UV	5	RT	29.7 €/g[b]	[9]
Methyl 9-maleinimido-8-methoxy-6,7-benzocumarin-3-carboxylate, (Thioglo®-5)	WR-1065	FL	10	RT	397 €/mg[b]	[10]
o-Phthaldialdehyde (OPA)	Organic thiols	FL	2	RT	47.3 €/250 mg[b]	[11]
Ethyl-propiolate	Cysteine glutathione homocysteine mercaptoethanol coenzyme A	UV	0.5	RT	36.8 €/5 g[b]	[12]

[a] Fluorescence.
[b] According to Sigma–Aldrich reagents' catalogue (2009).
[c] According to Fluka reagents' catalogue (2009).
[d] Room temperature.

3-oxo-3H-naphtho[2,1-b]pyran) react faster but the first two suffer from undesirable side-reactions while the high cost of Thioglo® – 3 limits its applicability to routine analyses [8–10]. o-Phthaldialdehyde (OPA) that is a common derivatizing reagent for primary amines, can also be used for the indirect fluorimetric or spectrophotometric determination of thiols [11]. Although OPA offer fast and sensitive assays, the main disadvantage of this reagent is the instability of the derivatives. Based on the previously-mentioned drawbacks of the already existing reagents for the derivatization of thiols and the biological significance of this group of compounds there is a continuous interest in proposing new alternative analytical approaches.

The "chemistry" and properties of propiolate esters are generally well-known mainly to organic chemists [12]. Ethyl-propiolate (EP) has recently been employed in a one-pot, four-components synthesis of benzene-1,2,3,5-tetracarboxylates promoted by triphenylphosphine [13], while its halogenated derivatives were used as substrates for direct, asymmetric alkynylation of cyclic β-ketoesters using chiral phase-transfer catalysts [14]. However, the feasibility and advantages of this group of compounds for analytical purposes has been roughly investigated only very recently by Owen [15]. In this study, the author presented data on the kinetics of the reaction, structure activity correlations, stereochemistry of the derivatives and preliminary results on the reaction of EP with proteins. The aim of this work was to carry out a systematic study of this promising derivatization reaction under flow conditions. The resulting data could be useful for more extensive applications of the reagent to separation science. For this purpose, cysteine (CYS) and captopril

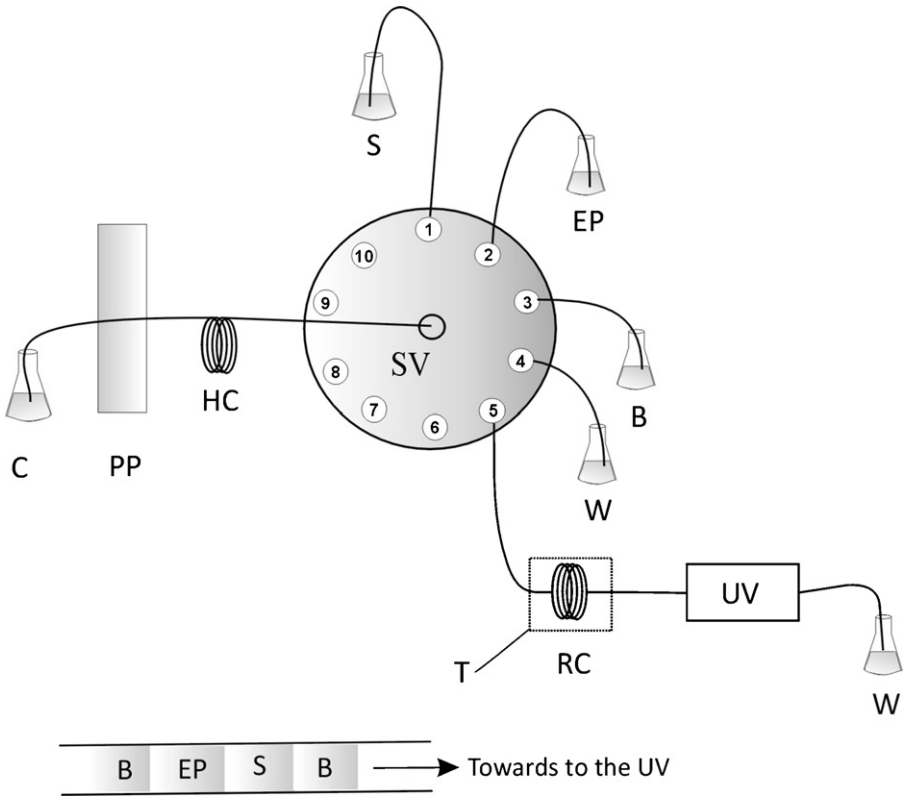

Fig. 1. SI manifold: C, carrier (water); PP, peristaltic pump; HC, holding coil (300 cm/0.5 mm i.d.); SV, selection valve; UV, detector (λ_{max} = 285 nm); S, sample; EP, ethyl-propiolate reagent; B, Britton–Robinson buffer; RC, reaction coil; T, thermostat; W, waste.

(CAP) were used as model compounds and sequential injection as the technique of choice. Finally, in order to demonstrate the applicability of the EP-based derivatization procedure a novel automated SI assay for the quality control of CAP-containing pharmaceutical formulations was developed and validated.

2. Experimental

2.1. Instrumentation

A schematic diagram of the SI manifold used is depicted in Fig. 1. It consists of a micro-electrically actuated 10-port valve (Valco, Switzerland) and a peristaltic pump (Gilson Minipuls3, Villiers-le-Bel, France) which were interfaced to the controlling PC through a multi-function I/O card (6025 E, National Instrument, Austin, TX, USA). The control of the SI system was carried out by means of a special program built-in the laboratory using the LabVIEW® 5.1.1 instrumentation software package (National Instrument). A Shimadzu SPD-10AV UV–vis detector equipped with a flow-through cell was employed to monitor the analytes' derivatives at λ = 285 nm. The flow system used 0.5 mm i.d. PTFE tubing throughout. Tygon pump tubing was used for aspirating and delivering the solutions. Data acquisition was performed through the Clarity® software (DataApex, Czech Republic).

A FIAstar 5101 thermostat (Tecator) was used to thermostate the reaction coil.

Spectra of derivatives were recorded on a Jasco V-530 UV-Vis spectophotometer equipped with 1 cm quartz cuvettes.

2.2. Reagents and materials

All chemicals were of analytical-reagent grade and all the solutions were prepared in deionized water. Ethyl-propiolate (EP) and captopril were obtained from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), respectively. All other chemicals were supplied by Merck (Darmstadt, Germany). Ultra-pure quality water produced by a Milli-Q system (Millipore, Bedford, USA) was used throughout this study.

Standard stock solutions of CYS and CAP having a amount concentration of 1.0×10^{-3} M each were prepared in water and found to be stable for up to 2 days. Working standard solutions were prepared daily by appropriate dilution of the stocks in water. The standard stock EP solution (c(EP) = 0.02 M) was prepared by dissolving accurately weighed amounts of the pure reagent in 10 ml water. This solution was stable for 3 days.

The Britton–Robinson buffer consisted of a mixture of 0.04 M H_3PO_4, 0.04 M CH_3COOH and 0.04 M H_3BO_3. The pH was adjusted in the range of 5.0–12.0 by 2 M NaOH.

2.3. SI procedure for the investigation of the reaction

Equal volumes (50 μl) of buffer (B), ethyl-propiolate (EP), sample (S) and buffer (B) were sequentially aspirated in the holding coil, through ports 3, 2, 1 and 3 of the selection valve, at a flow rate of 0.6 ml min^{-1}. Then, the four zones were propelled to the detector through port 5 at a flow rate of 0.9 ml min^{-1} (see Fig. 1). The water-soluble derivative was formed as the analyte and EP zones were allowed to react in a 100 cm long reaction coil (RC). At a specific time, when the more concentrated zone passed through the detector flow cell, the peristaltic pump was stopped in order to investigate the kinetic behavior of the reaction.

When changing between standards, an additional washing step was performed to avoid carryover effects. Note that in order to avoid overpressure and/or bubble formation in the valve, the pump was stopped for 1 s between changing ports. Three replicates per standard were made in all instances, unless otherwise stated.

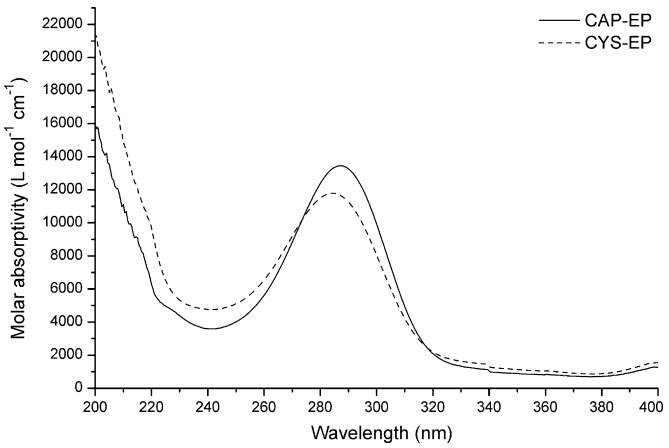

Fig. 2. UV-spectra of CYS-EP and CAP-EP derivatives (for experimental details see Section 3.1).

3. Results and discussion

Generally, propiolate esters react with compounds having thiol groups (mercaptans, sulfyhydryl compounds) under mild alkaline conditions forming thioacrylates according to the following scheme. The reaction mechanism is based on the nucleophilic attack of the thiolate ion to the α-carbon atom of the triple bond resulting in a stable alkylthioacrylate compound that absorbs in the UV region [15]. The reaction rate depends on how easy is the dissociation of the sulfylhydryl group. In order for the thiol group to act as nucleophilic reagent, it should be firstly be deprotonized which occurs when the pH of the reaction medium is greater than the pK_a of the thiol group:

$$RS + HC{\equiv}C{-}COOCH_2CH_3 \rightarrow RS{-}CH{=}CH{-}COOCH_2CH_3$$

In this study CYS and a more structurally complicated drug, captopril (CAP), were chosen as model compounds in order to investigate the reaction kinetics under flow conditions using SI configuration.

3.1. Preliminary studies

One of the objectives of the preliminary experiments was to study the absorptivity of EP derivatives in the UV region. For this reason, a spectrum of each derivative was obtained in the range of 200–400 nm using batch UV spectrophotometry using the following procedure: One milliliter each of Britton–Robinson buffer (pH = 7.0), EP reagent solution (c(EP) = 0.02 M) and standard aqueous solutions of the model thiolic compounds (CAP and CYS) were mixed in a cuvette and left to react for 5 min. Then, the cuvette was directly inserted into the spectrophotometer and the spectrum was recorded versus a reagent blank. As it can be seen in Fig. 2 a prominent UV absorbance in the range of 270–310 nm was observed due to the formation of the alkylthioacrylate derivatives. A wavelength of 285 nm was selected for further investigations. The molar absorptivities were calculated to be 11,700 and 13,400 for CYS and CAP, respectively. These values are in accordance to the finding of Owen [15].

As a second step, preliminary experiments were carried out in order to confirm that the reaction between EP and the model thiolic compounds is feasible under flow conditions and can therefore be automated using SI (Fig. 1). The starting values of the chemical parameters were: c(CYS) = c(CAP) = 2.5×10^{-4} M, c(EP) = 0.02 M and

pH = 7.0. A sampling pattern of B/EP/S/B was adopted to achieve sufficient mixing and buffering of the reaction zones. On the other hand, the starting values of the instrumental variables were: $V(S) = V(EP) = V(B) = 50\ \mu l$, $q_V = 0.6\ ml\ min^{-1}$, $l(RC) = 100\ cm/0.5\ mm$ i.d., $T = 25\ ^\circ C$ and $\lambda_{max} = 285\ nm$. These experiments confirmed that the thioacrylate derivatives could be formed under flow conditions and that further investigation was necessary in order to find the most suitable conditions for analytical application of the derivatization reaction.

3.2. Investigation of the effect of the pH

The effect of the pH on the reaction kinetics was examined performing stopped-flow experiments as described in Section 2.3. The chemical parameters and values of the SI variables are those mentioned in the previous section. The most concentrated part of the reaction mixture – as indicated by the maximum peak height – was trapped in the flow cell of the UV detector by stopping the flow of the carrier stream at exactly 40 s after starting the propulsion of the zones towards the detector. The kinetic-progress of the reaction was monitored for a specified time frame of 300 s in all cases.

The experimental results confirmed that the effect of the pH on the reaction rate depended on the pK_a of the thiol group of the analyte. In the case of CYS – $pK_a = 8.3$ [15] – (Fig. 3A), for pH values in the range of 9–11, the reaction was completed in less than 40 s, that is prior to the stopped-flow time, indicating very fast kinetics. At a pH value of 8, the reaction was leveled-off at ca. 180 s, while slower kinetics were followed at lower pH values (pH = 5–7). Similar behavior was obtained for CAP where the thiol group has a higher pK_a value of 9.8 [16]. As shown in Fig. 3B, the derivatization reaction was completed in less than 40 s for pH values higher than the pK_a (pH > 10) and within 300 s at a pH = 9. Pseudo-first-order behavior was observed at more acidic pH of 5–8.

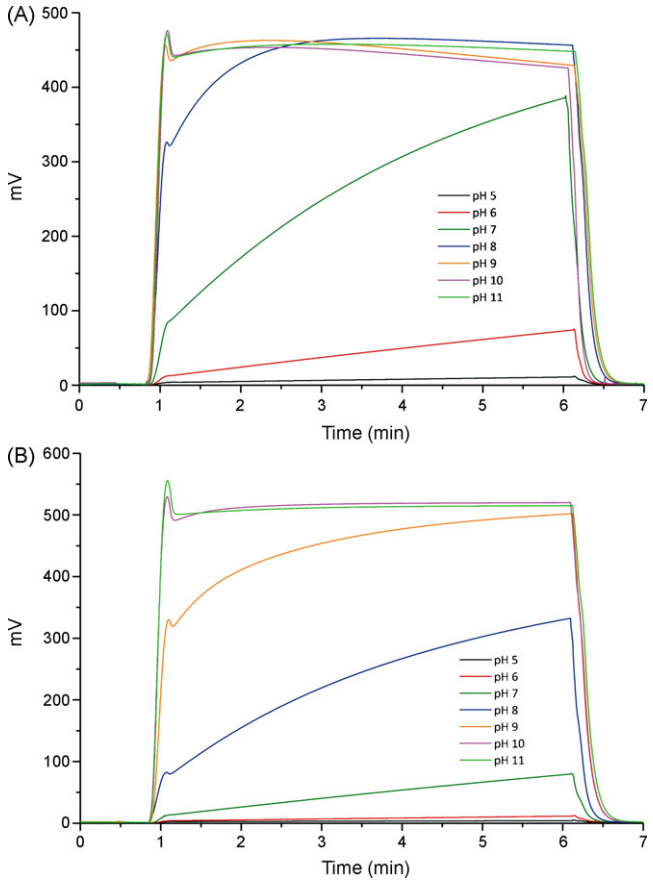

Fig. 3. Effect of the pH on the kinetics of the derivatization reaction using stopped-flow (c(EP) = 0.02 M). (A) CYS-EP and (B) CAP-EP.

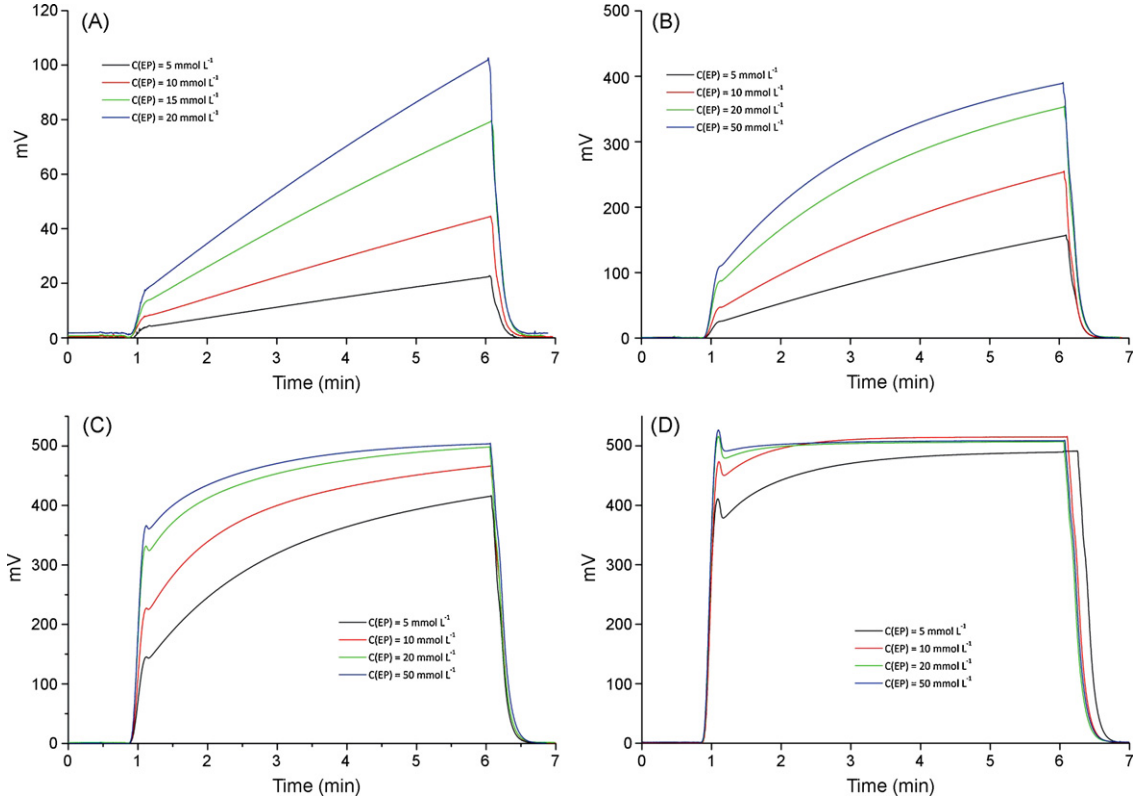

Fig. 4. Effect of the amount concentration of EP on the kinetics of the derivatization reaction using stopped-flow. (A) pH = 7, (B) pH = 8, (C) pH = 9 and (D) pH = 10.

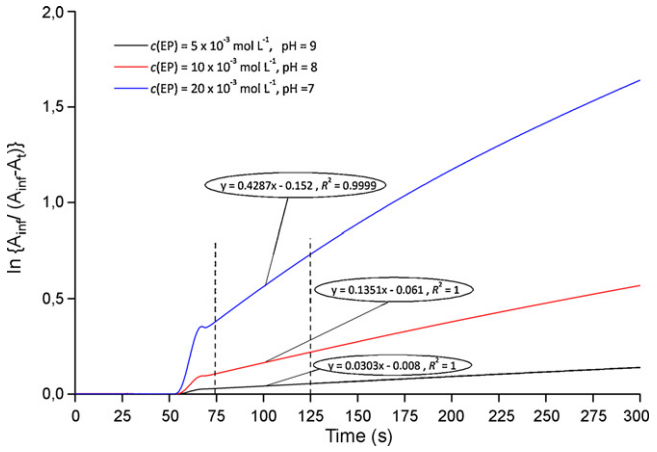

Fig. 5. Representative first-order logarithmic plots for CAP-EP.

3.3. Investigation of the effect of the EP amount concentration

The effect of the EP amount concentration on the reaction kinetics was investigated in the range of 5–50 × 10^{-3} M also performing stopped-flow experiments. Each set of EP concentrations was examined at four pH values, namely 7, 8, 9 and 10. In all cases EP was in adequate excess compared to the analytes, since the reagent-to analyte mole ratios were in the range of 20 (at c(EP) = 5 × 10^{-3} M) to 200 (at c(EP) = 0.05 M).

As expected, the concentration of EP had a significant impact on the rate of the derivatization only at pH values lower than the pK_a of the analytes. The experimental results for CAP are shown in Fig. 4A–D, while a similar pattern was obtained for CYS as well. As clearly shown in Fig. 4A, at a pH value of 7 the derivatization reaction seems to follow pseudo-first-order kinetics within the time frame of 300 s at all tested amount concentrations of the reagent. On the other hand, at a pH value of 10 which is higher than the pK_a of CAP the reaction was completed within 60 s even at low amount concentrations of the reagent (Fig. 4D). The pseudo-first-order nature of the reaction was investigated by constructing logarithmic first-order plots [17,18]. Typical data on various experimental conditions for CAP-EP are illustrated in Fig. 5. The excellent linearity in the range of 75–125 s confirmed the above-mentioned assumptions.

3.4. Investigation of the effect of temperature

The effect of the temperature on the derivatization reaction was investigated in the range of 25–60 °C by thermostating the reaction coil. The experimental results are included in Fig. 6A and B. The experiments showed a drastic effect of the temperature on the reaction only at low pH values where the reaction rate is low. For example, at pH = 7.0 there was a ca. 260 and 420% increase in the rate of the reaction for CYS and CAP, respectively. By increasing the pH the effect of the temperature was less pronounced. At pH = 10.0 there was only 12 and 18% variation of the signals at the entire range of 25–60 °C for CYS and CAP, respectively, while the respective percentages in the range of 25–40 °C were 6 and 9%. It should be noted that in all cases, the temperature had a negligible effect on the blank signals.

The obedience of system to the logarithmic Arrhenius' equation was evaluated by constructing the respective logarithmic plots. Linear behavior was observed in all cases. Representative experimental results are included in Fig. 6C.

3.5. Selectivity of the reaction

The selectivity of the EP reaction with thiols was investigated against similarly structured compounds such as amino-acids and

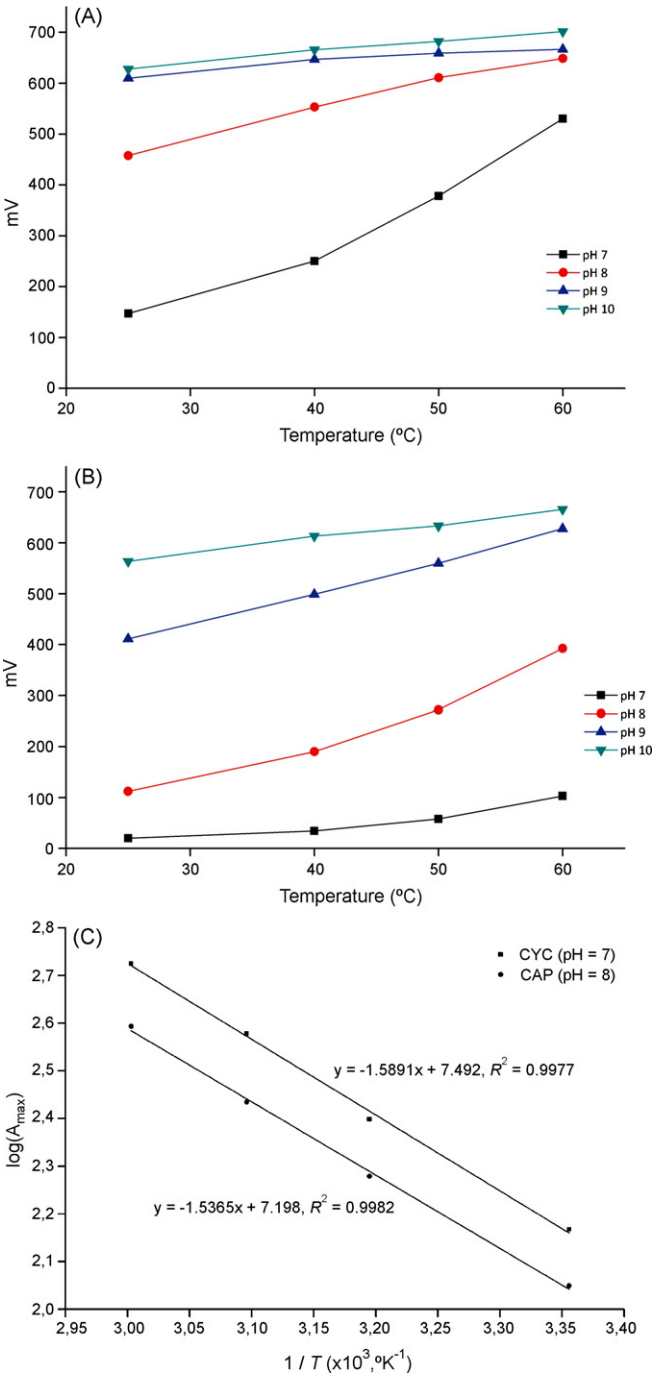

Fig. 6. Effect of the temperature on the kinetics of the derivatization reaction using stopped-flow. (A) CYS-EP, (B) CAP-EP and (C) representative logarithmic Arrhenius' equations.

disulfides. Glycine and cystine were selected as model compounds, respectively, at an amount concentration level equal to that of CAP and CYS (2.5 × 10^{-4} M). The stopped-flow approach was applied at an EP concentration of 0.02 M at pH 10. No reaction was observed indicating the selectivity of the derivatization reaction against compounds with primary amine groups and S–S bonds. The latter is an obvious advantage permitting the simultaneous determination of total thiols as well, after treatment with a suitable reducing agent.

3.6. Absorbance versus thiol concentration

In order to preliminary investigate the potential usefulness of EP as a derivatization reagent in analytical applications for the

determination of thiols, we studied the obedience of the system to the Beer's law. The linearity of the responses of CYS and CAP versus their concentrations was examined by constructing calibration curves in the range of 2.0–33.33 × 10^{-5} M. The values of the chemical and instrumental parameters were: c(EP) = 0.02 M, pH = 10.0, V(S) = V(EP) = V(B) = 50 μl, q_V = 0.6 ml min^{-1}, T = 25 °C, l(RC) = 100 cm/0.5 mm i.d, λ_{max} = 285 nm while the aspiration pattern was B/EP/S/B. The experimental data showed that in both cases the linearity was excellent over the entire concentration range. The regression coefficients were higher than 0.999 for both analytes and the percent residuals were distributed randomly around the "zero" value.

3.7. Analytical application – determination of captopril

In order to demonstrate the applicability of the new derivatization reaction, a SI spectrophotometric assay was developed and validated for the determination of CAP, an angiotensin converting enzyme (ACE) inhibitor in commercially available formulations.

Since the molecule of CAP does not possess a strongly UV-absorbing or fluorescent moiety, various derivatization reactions have been described to increase the detectability of the analyte. Typical reagents include 2-chloro-1-methylquinolinium tetrafluoroborate (λ_{max} = 355 nm) [19], 2,4-dibromoacetophenone (λ_{max} = 263 nm) [20], Pd(II) (λ_{max} = 380 nm) [21], o-phthalaldehyde/isoleucine (λ_{max} = 355 nm) [22], monobromobimane (λ_{ex} = 400 nm/λ_{em} = 480 nm) [23,24], ThioGlo®3 (λ_{ex} = 365 nm/λ_{em} = 445 nm) [25], 3-bromomethyl-propyphenazone (λ_{max} = 254 nm) [26], 5,5′-dithio-(bis-2-nitrobenzoic acid) (Ellman's reagent, λ_{max} = 412 nm) [27], o-phthalaldehyde/glycine (λ_{ex} = 345 nm/λ_{em} = 455 nm) [24], N-(1-pyrenyl)maleimide (λ_{ex} = 340 nm/λ_{em} = 389 nm) [28], 1,1′-[ethenylidenebis(sulfonyl)]bisbenzene (λ_{max} = 254 nm) [29], 1-benzyl-2-chloropyridinium bromide (λ_{max} = 312–314 nm) [30,31], p-bromophenacyl bromide (λ_{max} = 260 nm) [32]. Some of the reported disadvantages of the above-mentioned reagents/methods include: non-automated batch pre-column procedures [19–27], high purchase costs [23,24,27] or no commercial availability [29], long reaction times [20,24–26], time-consuming procedures for the removal of the excess of the reagent [20,28,31].

On the other hand, automated methods of analysis and especially flow-based approaches such as flow (FI) and sequential injection (SI) offer rapidity, cost-effectiveness, high-throughput and enhanced precision and accuracy due to the strictly controlled experimental conditions. Previously reported FI and SI methods for the determination of CAP are based on UV–vis [33–38], chemiluminescence [39–43] and electroanalytical [44–50] detection.

3.7.1. Study of SI variables

The effect of the SI main variables such as the mixing order, the flow rate of the carrier, the reaction coil length and the injection volumes of sample, EP and buffer were studied by using the univariate approach. Based on the investigation of the reaction carried out in the previous sections, a pH value of 10 and an EP amount concentration of 20 mM were selected. The temperature of the reaction coil was set at 25 °C. According to the findings described in Section 3.6, the experiments were carried out at c(CAP) = 1.0 × 10^{-4} M.

The SI variables, the studied range and the selected value of each variable studied are shown in Table 2. The order of mixing of the reagents and the sample was examined at three combinations: (i) B/EP/S, (ii) B/S/EP and (iii) EP/B/S. The first aspiration pattern produced the highest signals and it was therefore adopted for all subsequent experiments.

Among the studied SI variables, it is worthy to commend on the influence of the length of the reaction coil on the sensitivity of the assay. The stopped-flow experiments described in Sections 3.2–3.4 indicated fast reaction kinetics at pH > 10 even at room temperature. These results were verified since the highest signals were obtained in the absence of a reaction coil. The sample injection volume had also a profound effect on the sensitivity of the determination. The signals increased almost linearly in the range of 25–100 μl and remained practically unaffected up to 150 μl. An injection volume of 100 μl was therefore adopted for further investigations.

Table 2
Study of the SI variables for the determination of CAP in pharmaceutical formulations.

SI variable	Studied range	Selected value
$L_{(RC)}$ (cm)/(0.5 mm i.d.)	0–100	0
V(S) (μl)	25–150	100
V(EP) (μl)	25–100	50
V(Buf) (μl)	25–100	50
q_v (ml min^{-1})	0.45–1.2	0.9

3.7.2. Method validation

Under the selected chemical and SI variables, the proposed method for the determination of CAP was found to be linear in the range 2.0–40.0 mg l^{-1}, obeying the regression equation:

$$A = (21.45 \pm 0.05) \times \gamma(\text{Cap}) + (3.52 \pm 1.08)$$

where A is the absorbance in mV units (peak height) and γ(Cap) is the mass concentration of the analyte. In order to validate the determination range, a mass concentration of 20.0 mg l^{-1} was selected as the 100% level. The range was therefore evaluated between 10.0 and 30.0 mg l^{-1} (50–150% of the target concentration) using six calibration points according to the ICH guidelines [51]. This range is suitable for both assay and content uniformity quality control applications. The validity of the calibration curve was proved by both the value of the regression coefficient (r^2 = 0.9999) and the range of the residuals (−1.0 to +1.4%). Additionally, the plot of the residuals indicated random distribution around the "zero" line.

The repeatability was excellent, as indicated by the value of the relative standard deviation, s_r, of 8 replicates of a CAP standard (20 mg l^{-1}) that was 0.34%. The reproducibility was evaluated by recording calibration curves in the range of 10.0–30.0 mg l^{-1} CAP for a time period of 6 consecutive days. The relative standard deviation, s_r, of the slopes was 2.8%, confirming the robustness of the derivatization reaction.

The limits of detection (c_L) and quantitation (c_Q) of the proposed assay were calculated according to the IUPAC recommendations, as 3.3 × s_b/m and 10 × s_b/m, respectively, where s_b is the standard deviation of the blank measurements (n = 8), and m is the slope of the calibration graph [52]. On this basis, the c_L and c_Q of the proposed method were calculated to be 30 and 90 μg l^{-1} CAP, respectively.

The selectivity of the SI method was evaluated against common pharmaceutical excipients (colloidal silicon dioxide, pregelatinized starch, talc, lactose monohydrate, magnesium stearate, citric acid, titanium dioxide, gelatin and sodium saccharin). The placebo mixture was prepared by mixing 1 g of each excipient in a mortar until a homogenous fine powder was obtained. An accurately weighed amount of the resulting placebo was dispersed in water, ultrasonicated for 30 min, centrifuged at 3000 rpm for 15 min and filtered through 0.45 μm syringe filters. The resulting stock solution had a nominal placebo concentration of 10 mg ml^{-1}. Standard CAP solutions at the 20 mg l^{-1} level were spiked with elevated placebo concentrations in the range of 200–1000 mg l^{-1}, corresponding to mass ratios from 10 to 50:1. The obtained recoveries were in the range of 98.8–101.3% verifying the specificity of the assay even at high excipients/analyte ratios.

The accuracy of the proposed method was validated by analyzing nine synthetic samples – containing 1000 mg l^{-1} of placebo – spiked

Table 3
Determination of CAP in pharmaceutical formulations using SI.

Sample	Recovery (%)	
	Capoten® (25 mg/tab)	Dosturel® (50 mg/tab)
Assay		
S1	98.6	99.8
S2	97.4	101.2
S3	102.1	102.3
Content uniformity		
S1	96.4	99.6
S2	95.2	105.7
S3	105.3	101.9
S4	105.2	99.0
S5	99.4	97.9
S6	100.3	101.1
S7	97.3	98.7
S8	96.4	99.5
S9	99.7	100.8
S10	102.7	104.1

with different amounts of captopril in the range of 10.0–30.0 mg l^{-1} (50–150%). Synthetic samples pretreatment included ultrasonication for 15 min and filtration through 0.45 μm filter prior to SI analysis. The percent recoveries were satisfactory in all cases, ranging between 98.0 and 101.8%.

We examined the ruggedness of the assay against deliberate variations of the flow rate towards the detector (±0.1 ml min^{-1}), the pH (±0.5), the amount concentration of the derivatizing reagent (±10%) and the injection volumes of the sample, reagent and buffer (±5% in all cases). All experiments were carried out using synthetic samples containing 20.0 mg l^{-1} CAP and 1000 mg l^{-1} placebo. In all cases the variation of the above-mentioned variables caused a relative error of less than ±5% compared to the optimal conditions, verifying the satisfactory ruggedness of the proposed automated analytical procedure.

3.7.3. Analysis of real samples

For assay analysis, the content of 20 CAP-containing tablets (Capoten® tabs, 25 mg/tab and Dosturel® tabs, 50 mg/tab) was mixed and homogenized in a mortar. Accurately weighed amounts were dispersed in water and ultrasonicated for 15 min. A portion of the resulting solution was filtered through 0.45-μm disposable syringe filters, diluted and analyzed according to the optimal SI procedure. For content uniformity analysis, 10 CAP-containing tablets of each product were individually dissolved in 100 ml of water under shaking and ultrasonication. Aliquots of the resulting solutions were filtered through 0.45-μm disposable syringe filters diluted appropriately and finally analyzed using the proposed SI protocol.

The experimental results from the application of the validated SI method are tabulated in Table 3. The percent recoveries were, in all cases, within the limits established by the US Pharmacopoeia [53], ranging between 97.4 and 102.3% for assay and between 95.2 and 105.7% for content uniformity tests.

4. Conclusions

The present study reports data from the investigation of the behavior of ethyl-propiolate, a new derivatizing reagent for thiolic compounds, under flow conditions using sequential injection analysis. Using cysteine and captopril as model compounds, the experimental work that was carried out clearly demonstrated the feasibility and advantages of this new reagent for analytical applications: (i) the reaction is simple, (ii) it proceeds in aqueous solutions, (iii) it reacts rapidly and selectively with thiols under mild conditions, (iv) the reaction rate can be controlled effectively by adjusting the pH, (v) it offers adequate sensitivity at the μg l^{-1} level using simple UV instrumentation, (vi) the blank signals due to the native absorbance of the reagent are negligible and is therefore suitable for post-column applications, (vii) it is commercially available at a low cost compared to many derivatizing reagents for thiols and (viii) the reaction can be readily carried out under flow conditions.

On going research is carried out in order to demonstrate the applicability of this reagent to the determination of thiols in biological materials. On this basis, research is focused on coupling to HPLC *via* automated post- and pre-column derivatization protocols. Matters currently under investigation include stability and optimization of the separation of the derivatives under pre-column derivatization-HPLC conditions, effect of commonly HPLC solvents on the reaction and stability of the derivatives in the post-column mode and optimization of sample pretreatment protocols for maximum sensitivity and selectivity. Parallel experimental work is orientated towards the exploration of the application of the reagent to capillary electrophoresis and especially to in-capillary derivatization based assays.

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