Automated Determination of Pharmaceutically and Biologically Active Thiols by Sequential Injection Analysis: A Review

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Abstract: The present review article presents and discusses automated sequential injection (SI) based methods for the determination of pharmaceutically and biologically important thiols, namely cysteine, N-acetylcysteine, penicillamine, glutathione and captopril. The review intends to cover a range of ten years of published research on this topic (2000 – 2009). The methods are classified according to the detection systems in three categories, namely spectrophotometric, fluorimetric and electroanalytical. The determination principles of the methods are discussed and the main analytical figures of merit are tabulated.

Keywords: Thiols, review, determination, sequential injection analysis.

1. INTRODUCTION

Thiols are an important group of compounds that continue to attract the interest of analytical scientists due to their unique role in biological systems, food, pharmaceutical and aroma industries. Apart from the several review articles on the analysis of thiols [1-5] and the many research articles that appear in the literature, the analytical challenge can be even pointed out by a very recent special issue of *Journal of Chromatography B* (Elsevier) under the characteristic title "*Analysis of thiols*" (Volume 877, Issue 28, 2009).

Cysteine is a non-essential (biosynthesized in humans) α-amino acid. The side chain on cysteine is thiol, which is non-polar and thus cysteine is usually classified as a hydrophobic amino acid. The thiol side chain often participates in enzymatic reactions, serving as a nucleophile. The thiol is susceptible to oxidization to give the disulfide derivative cystine, which serves an important structural role in many proteins [6].

N-acetylcysteine is a metabolite of cysteine. It is produced within the human body. N-acetylcysteine protects the body from acetaminophen toxicity and is used in hospitals for patients with acetaminophen poisoning. It has also been shown to be effective at treating liver failure from other causes as well. Heavy metals like lead, mercury and arsenic are detoxified and removed from the body by N-acetylcysteine. It also increases the excretion of zinc and other essential minerals when taken over an extended period. It is therefore necessary to supplement zinc, copper and other trace minerals when taking N-acetylcysteine. N-acetylcysteine cleaves disulfide bonds by converting them to two sulfhydryl groups. This action results in the breakup of mucoproteins in lung mucus, reducing their chain lengths and thinning the mucus and therefore improving conditions such as bronchitis and flu [7].

Glutathione is a small protein composed of three amino acids, cysteine, glutamic acid and glycine and is produced in the human liver. It plays a key role in intermediary metabolism, immune response and health, though many of its mechanisms and much of its behavior await further medical understanding. Glutathione, in purified extracted form, is a white powder that is soluble in water and in alcohol. It is found naturally in many fruits, vegetables, and meats. However, absorption rates of glutathione from food sources in the human gastrointestinal tract are low. Glutathione is a major antioxidant highly active in human lungs and many other organ systems and tissues. It has many reported uses. It has a critical role in protecting cells from oxidative stress and maintaining the immune system. Higher blood levels of glutathione have been associated with better health in elderly people, but the exact association between glutathione and the aging process has not been determined. Among the uses that have been reported for glutathione are: i) treatment of poisoning from heavy metals, ii) treatment of idiopathic pulmonary firbosis, iii) increasing the effectiveness and reducing the toxicity of cis-platinum, a chemo drug used to treat breast cancer, iv) treating Parkinson's disease, v) lowering blood pressure in patients with diabetes etc. [8].

Captopril is an angiotensin-converting enzyme inhibitor (ACE inhibitor) used for the treatment of hypertension and some types of congestive heart failure. It was the first ACE inhibitor developed and was considered a breakthrough both because of its novel mechanism of action and also because of the revolutionary development process. Captopril is commonly marketed by Bristol-Myers Squibb under the trade name Capoten. Adverse effects of captopril include cough, angioedema, agranulocystosis, proteinuria, hyperkalemia, taste alteration, teratogenicity, postural hypotension, acute renal failure and leucopenia. Except for postural hypotension which occurs due to short and fast mode of action of captopril, most of the side effects mentioned are common for all ACE inhibitors. Among these cough is the most common

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adverse effect and is due to elevated levels of bradykinin. Hyperkalemia can occur especially if used along with other drugs which elevate potassium level in blood like potassium sparing diuretics [9].

Penicillamine is an antirheumatic drug used to treat patients with active rheumatoid arthritis. It also is classified as a metal binding (chelating) agent used for treating Wilson's disease, a genetic disease that causes excessive copper to accumulate in the body. The mechanism of action of penicillamine in rheumatoid arthritis is unknown but it may be related to reduction of collagen formation and suppression of the immune system. In patients with rheumatoid arthritis, penicillamine appears to slow the progression of the disease (specifically deformities of the joints) and improve function. For this reason it is considered a disease modifying anti-rheumatic drug (DMARD). Penicillamine binds copper, iron, mercury, lead, and cystine which then are excreted in the urine, and this mechanism is important in treating several non-rheumatic diseases including Wilson's disease. The FDA approved penicillamine in December 1970 [10].

The scope of the present study is to review recent research on the automated determination of the above-mentioned pharmaceutically and biologically active thiols using sequential injection analysis (SI). The structures of the discussed analytes are depicted in Fig. (**1**).

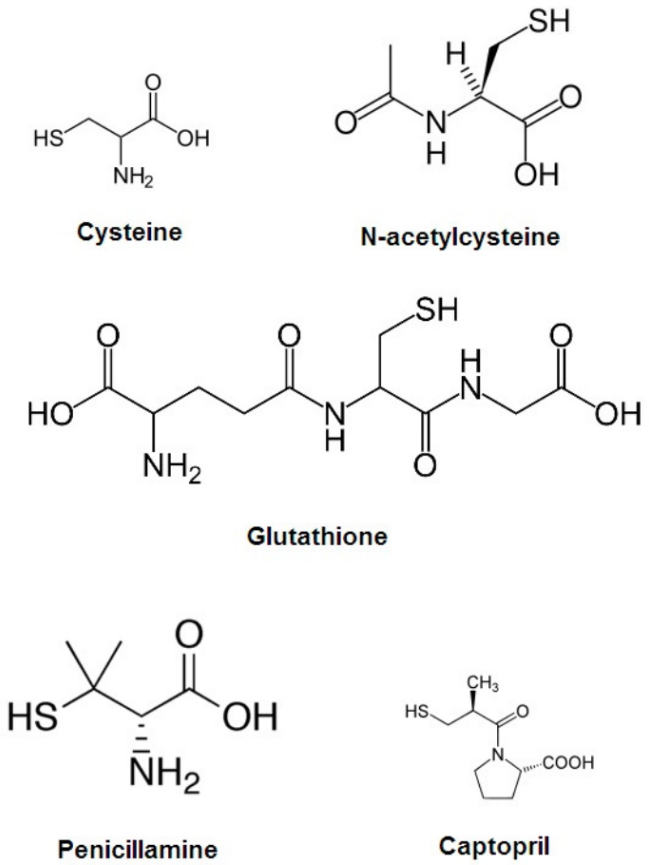

Fig. (1). Chemical structures of pharmaceutically and biologically active thiols.

2. SEQUENTIAL INJECTION ANALYSIS

Sequential-injection analysis (SI) is considered to be the second generation of flow injection techniques and was initially developed by Ruzicka and Marshall in 1990's [11,12] as an alternative sample-handling technique to the well-established FI [13-15].

Although a large number of analytical procedures have been adapted to FI, this undoubtedly useful sample-handling scheme has certain disadvantages: a) a different manifold configuration is necessary to implement each analytical method; also, in many cases of more complex solution chemistry, multi-channel manifolds (each channel providing a different reagent) are required; b) since solutions are continuously flowing through the manifold, the consumption of reagents is significant; c) optimization of the geometrical and chemical parameters for each assay is tedious and time-consuming because alterations to the configuration of the flow manifold must be made; and, d) in FIA, the sample, once introduced into the flowing carrier stream, is uni-directionally transported towards the detector and the potential of physical and chemical manipulation of the sample is limited, both spatially and temporally [16].

On the other hand, as can be seen in a typical SI setup in Fig. (**2**), the heart of a SI manifold is a multiposition selection valve. Fluids are manipulated within the manifold by means of a bi-directional pump. A holding coil is placed between the pump and the common port of the multiposition selection valve. The selection ports of the valve are reservoirs, detectors, pumps, reactors, separators, special cells, other manifolds etc. After aspiration of a discrete volume (zone) of sample into the holding coil *via* the sample line, the sample can be subjected to very complex physical and chemical pre-treatment in different ways within the SI manifold. SI offers great potential for sample handling because it is a bidirectional, stopped-flow sample-handling technique enabling the sample to be serially processed in the different modules connected to the selection valve by means of repetitive aspiration and delivery steps. The advantages of SI over FI are the following: a) SI makes use of a simpler manifold that can be employed for a larger range of analytical methods without (or minimal) alterations In its physical configuration; b) in SI, discrete volumes of sample and reagents are aspirated and their consumption is drastically reduced; c) the bidirectional and stopped-flow operation of SI provides great scope for pre-treatment of the sample. This last attribute of SI makes it ideally suited to clinical and biochemical applications for which sample pre-treatment is usually necessary prior to the actual analytical measurement. The general applications of SI in biomedical and pharmaceutical analysis have been reviewed elsewhere [17,18].

3. DETERMINATION OF THIOLS BY SI-SPECTROPHOTOMETRY

Automated spectrophotometric methods are quite popular among analytical scientists when it comes to pharmaceutical applications. Flow-through UV-Vis detectors are widely available even in low-budget laboratories, while even batch instruments can be easily converted with the aid of suitable

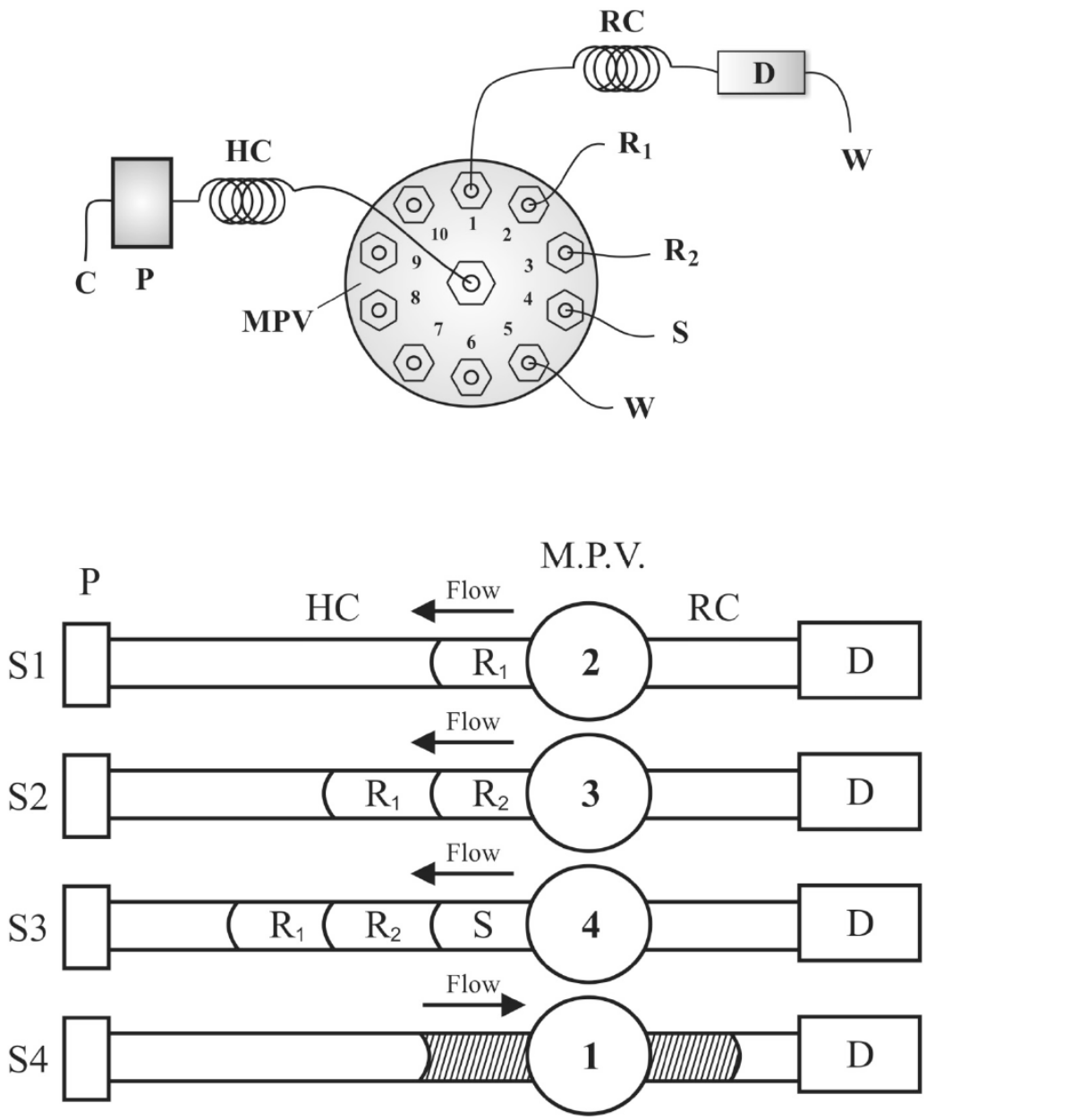

Fig. (2). Typical SI setup and SI sequence steps: P = pump; C = carrier solution; HC = holding coil; MPV = multi-position valve; RC = reaction coil; D = detector; W = waste; R_1 & R_2 = reagents; S = sample; S1-S4 = SI steps.

commercially available flow-cells. Pharmaceutically active compounds can be either detected based on their native absorbance in the UV region, or after chemical reaction / derivatization. Such methods are typically simple, easy to handle, with low operational costs and with satisfactory figures of merit for most quality control applications [19]. The main analytical figures of merit of spectrophotometric SI methods for the determination of thiols can be found in Table **1** [20-27].

Recently, our research group has started investigating the usefulness of propiolate esters as derivatizing reagents for thiols under flow conditions [20-22]. On this basis we tested three commercially available propiolate esters, namely methyl-propiolate (MP), ethyl-propiolate (EP) and butyl-propiolate (BP). Using SI as the technique of choice, we found that thiols (capropril, cysteine, glutathione and N-acetylcysteine) react under flow conditions with the esters to form UV absorbing thioacrylate derivatives. A typical reaction scheme of glutathione with EP is shown in Fig. (**3**) [21] and characteristic representative UV-spectra of BP with captopril and N-acetylcysteine are shown in Fig. (**4**) [22]. 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 in the range of 280 – 290 nm. Our research concluded that these reagents offer several important advantages; i) they are commercially available, ii) they are cost-effective (especially EP and MP) compared to other derivatization reagents for thiols, iii) they react almost instantaneously under mild conditions avoiding the use of organic solvents and iv) the free reagents does not absorb themselves.

Table 1. Overview of Methods for the Determination of Pharmaceutically and Biologically Active Thiols by Sequential Injection Analysis

Analyte	Method	Detection	LOD	Range	Throughput	Sample	Refs.
Captopril	Derivatization with ethylpropiolate in alkaline medium	UV-Vis at 285 nm	30 μg L^{-1}	2.0 – 40.0 mg L^{-1}	—	Pharmaceuticals	[20]
Glutathione	Derivatization with ethylpropiolate in alkaline medium	UV-Vis at 285 nm	50 μg L^{-1}	0.15 – 70.0 mg L^{-1}	100 h^{-1}	Nutrition supplements	[21]
Captopril	Derivatization with methyl (MP) and butyl (BP) propiolate in alkaline medium	UV-Vis at 285 nm	0.18 μmol L^{-1} (MP) 0.21 μmol L^{-1} (BP)	10–1800 μmol L^{-1} (MP) 10–2200 μmol L^{-1} (MP)	40 h^{-1}	Dissolution samples	[22]
Cysteine	Derivatization with methyl (MP) and butyl (BP) propiolate in alkaline medium	UV-Vis at 285 nm	0.21 μmol L^{-1} (MP) 0.30 μmol L^{-1} (BP)	10–1800 μmol L^{-1} (MP) 10–2200 μmol L^{-1} (MP)	40 h^{-1}	Nutrition supplements	[22]
N-acetylcysteine	Derivatization with methyl (MP) and butyl (BP) propiolate in alkaline medium	UV-Vis at 285 nm	0.17 μmol L^{-1} (MP) 0.23 μmol L^{-1} (BP)	10–1800 μmol L^{-1} (MP) 10–2200 μmol L^{-1} (MP)	40 h^{-1}	Pharmaceuticals	[22]
Captopril	Oxidation by Fe(III) followed by reaction of Fe(II) with 2,2'-dipyridyl-2-pyridyl hydrazone (DPPH) in acidic medium	UV-Vis at 535 nm	7 mg L^{-1}	12–1000 mg L^{-1}	60 h^{-1}	Pharmaceuticals	[23]
L-cysteine	Redox reaction of L-cysteine with Fe(III) in the presence of 1,10-phenanthroline and detection of the red complex between Fe(II) and 1,10-phenanthroline	UV-Vis at 510 nm	5 μmol L^{-1}	0 – 1000 μmol L^{-1}	15 h^{-1}	Fermentation process of *Saccharomyces cerevisiae*	[24]
Penicillamine	Complex formation with Fe(III) in acidic medium	UV-Vis at 600 nm	—	25–300 mg L^{-1}	50 h^{-1}	Pharmaceuticals	[25]
Captopril	Complex formation with Pd(II) in acidic medium	UV-Vis at 400 nm	—	0.2 – 1.4 mmol L^{-1}	—	Pharmaceuticals	[26]
Glutathione	GSSG reductase – DTNB (5,5′-dithiobis(2-nitrobenzoic acid) recycling enzymatic assay	UV-Vis at 412 nm	31 nmol L^{-1}	0 – 3.0 μmol L^{-1}	13 h^{-1}	Human blood	[27]
Captopril	Derivatization with the o-phthalaldehyde / glycine system in alkaline medium	FL at 340/455 nm	2.3 μg L^{-1}	0 – 20 mg L^{-1}	70 h^{-1}	Pharmaceuticals	[28]
N-acetylcysteine	Derivatization with the o-phthalaldehyde / glycine system in alkaline medium	FL at 340/455 nm	1.6 μg L^{-1}	0 – 15 mg L^{-1}	70 h^{-1}	Pharmaceuticals	[28]

(Table 1) contd…..

Analyte	Method	Detection	LOD	Range	Throughput	Sample	Refs.
Penicillamine	Derivatization with the o-phthalaldehyde/ glycine system in alkaline medium	FL at 320/420 nm	2.0 μg L^{-1}	0 – 15 mg L^{-1}	70 h^{-1}	Pharmaceuticals	[28]
Captopril	Derivatization with the monobromobimane in alkaline medium	FL at 390/480 nm	0.23 mg L^{-1}	0 – 100 mg L^{-1}	36 h^{-1}	Pharmaceuticals	[28]
N-acetylcysteine	Derivatization with the monobromobimane in alkaline medium	FL at 390/480 nm	0.21 mg L^{-1}	0 – 100 mg L^{-1}	36 h^{-1}	Pharmaceuticals	[28]
Penicillamine	Derivatization with the monobromobimane in alkaline medium	FL at 390/480 nm	0.81 mg L^{-1}	0 – 250 mg L^{-1}	36 h^{-1}	Pharmaceuticals	[28]
Penicillamine	Derivatization with fluorescamine in the presence of β-cyclodextrins	FL at 355/495 nm	0.1 mg L^{-1}	5 – 80 mg L^{-1}	50 h^{-1}	Pharmaceuticals	[29]
Captopril	Potentiometric titration using a Ag(I) solution as titrant	Potentiometric at a crystalline membrane $Ag(I)/Ag_2S$ electrode	—	—	5 h^{-1}	Pharmaceuticals	[26]
Captopril	Amperometric biosensors based on L-and D-amino acid oxidase for the simultaneous determination of S- and R-captopril respectively	Amperometric at 0.65 V versus a calomel electrode as reference	0.2 nmol L^{-1} (S) 15 nmol L^{-1} (R)	0.4 – 1.6 μmol L^{-1} (S) 120 – 950 nmol L^{-1} (R)	34 h^{-1}	Synthetic samples	[32]
Captopril	Amperometric biosensor based on L-amino acid oxidase for the determination of S-captopril	Amperometric at 0.65 V versus a Ag/AgCl reference electrode	16 nmol L^{-1}	0.05 – 1.5 μmol L^{-1}	80 h^{-1}	Synthetic samples	[33]
Captopril	Amperometric biosensor based on D-amino acid oxidase for the determination of R-captopril and potentiometric, enantioselective membrane electrode based on maltodextrin for the assay of S-captopril	Amperometric at 0.65 V versus a calomel electrode as reference	0.44 μmol L^{-1} (S) 14 nmol L^{-1} (R)	1 – 1000 μmol L^{-1} (S) 100 – 1000 nmol L^{-1} (R)	38 h^{-1}	Synthetic samples	[34]
Captopril	Amperometric biosensor based on D-amino acid oxidase for the determination of R-captopril	Amperometric at 0.65 V versus a Ag/AgCl reference electrode	160 nmol L^{-1}	0.2 – 1.0 μmol L^{-1}	80 h^{-1}	Synthetic samples	[35]

A well-known property of thiols is that trace metals – such as Fe(III) and Co(II) – catalyze their oxidation towards the respective disulfides. This property has turned into a useful and "popular" chemistry for the development of automated SI methods for the determination of thiols [23,24]. A typical protocol involves the following steps: i) on-line oxidation of the analyte (e.g. captopril [23] or cysteine [24]) by Fe(III) to form Fe(II), ii) on-line reaction of

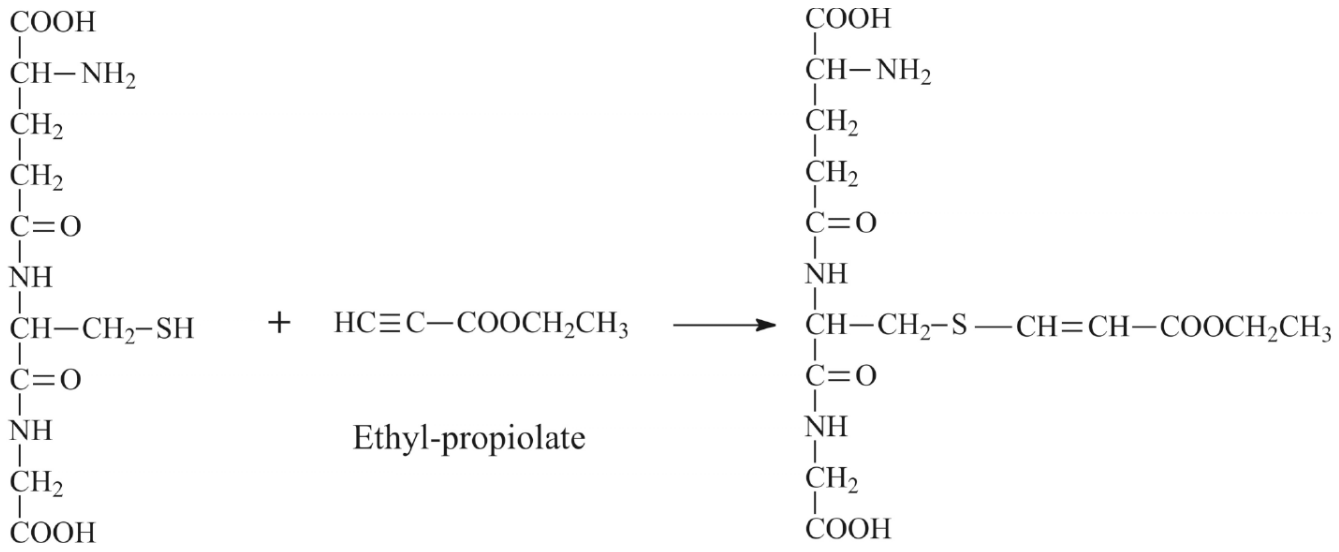

Fig. (3). Reaction of glutathione with ethyl-propiolate [21].

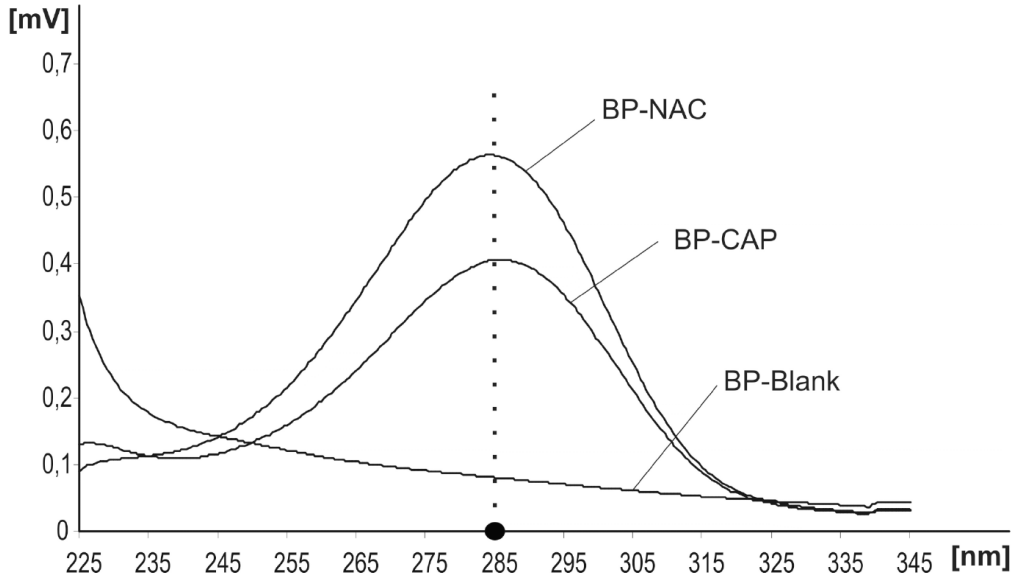

Fig. (4). UV-spectra of the derivatives of captopril (CAP) and N-acetylcysteine (NAC) with butyl-propiolate (BP) [22].

the formed Fe(II) with a suitable selective reagent (e.g. 1,10-phenanthroline [24] or 2,2-dipyridyl-2-pyridylhydrazone (DPPH) [23]) and iii) spectrophotometric detection of the formed colored complexes. Especially in [23], the authors automated the analytical procedure using both FI and SI. A critical comparison of the two modes drove – according to the authors – to the following conclusions; i) the FI method offered a two-fold increase in the sampling rate as compared to SI (120 *vs* 60 injections h^{-1} respectively), ii) the SI method was fully automated, and the instrumental parameters were easily computer-controlled, iii) in SI the determination took place in a single channel approach, while in FI a four-channel manifold was necessary, iv) the consumption of reagents was significantly less in SI compared to FI (100 μL *vs* 450 μL Fe(III) and 200 μL *vs* 450 μL DPPH per injection for SI and FI, respectively).

More simple and straightforward SI methods are based on the ability of certain thiols to form colored complexes with metal ions in acidic medium [25,26]. On this basis, penicillamine was determined by SI using its complex with Fe(III) (λ_{max} = 600 nm) [25] and captopril using its complex with Pd(II) (λ_{max} = 400 nm) [26]. It is worth to mention that according to the authors of the study, the formation of the complex between penicillamine and Fe(III) was instantaneous and faster than the oxidation reaction mentioned in the previous paragraph. The authors noticed that the due to oxidation of the analyte by Fe(III) to form the respective disulfide, the color of the complex gradually faded. Of course this behavior did not cause any problems to the accuracy and precision of the method due to the reproducible timing of the SI system.

4. DETERMINATION OF THIOLS BY SI-FLUORIMETRY

Fluorimetry is a very useful analytical technique due to its increased sensitivity and selectivity compared to simple UV-Vis spectrophotometry. The advantageous properties of fluorimetry have popularized it among analytical scientists with special emphasis to bioanalytical applications. Direct analysis or coupled to separation techniques, fluorimetry can be based on either the measurement of the endogenous fluorescence of an analyte, or on the formation of a suitable derivative. Although there are numerous published methods on the fluorimetric determination of thiols after derivatization, as can be seen in Table **1** the SI applications are rather limited [28, 29].

In a recent MSc Thesis carried out by our research group we explored the development of automated fluorimetric methods for the determination of pharmaceutically active thiols (captopril, N-acetylcysteine and penicillamine) using two different chemistries [28]: i) the first approach was based on the well-known reaction of o-phthalaldehyde (OPA) with primary amines in alkaline medium to form highly fluorescent derivatives. This derivatization reaction proceeds only in the presence of a suitable nucleophilic reagent and thiols can certainly play this role. The studied reactions are shown schematically in Fig. (**5**). Glycine was proved to be the most suitable primary amine compound in terms of sensitivity. As can be seen in Table **1** the developed SI methods were fast enabling a sampling rate of 70 h^{-1} and quite sensitive with the limits of detection being in the ppb-range for all thiols; ii) the second method involved the investigation of the reaction of the selected thiols with monobromobimane (MBB) under SI conditions. Although MBB is a well-known reagent for thiols [2], up to now it was restricted to applications involving pre-column derivatization prior to separation with liquid chromatography and capillary electrophoresis. Despite of its advantages, the high cost of the reagent was exclusionary for on-line FI-based applications. However, taking advantage of the ability of SI to handle reduced volumes of reagents and samples in a discontinuous mode, we reported the first applications of MBB in flow systems. A typical reaction scheme between MBB and penicillamine is shown in Fig. (**6**). Comparing the MBB and OPA SI methods based on the figures of merit of Table **1**, it was concluded that OPA offers significantly higher sensitivity. On the other hand, the MBB assay is simpler and more selective for thiols.

Penicillamine (Fig. **1**) apart from the thiol moiety contains a primary amine group as well. Suliman and co-workers took advantage of this property in order to develop a SI-FL method for penicillamine using fluorescamine in alkaline medium as derivatizing reagent [29]. Besides the analytical part of this study, two interesting aspects are: i) the use of β-cyclodextrin in order to enhance the sensitivity of the procedure and ii) the application of molecular modeling calculations in an attempt to understand the mechanism of the enhancement.

5. DETERMINATION OF THIOLS BY SI-ELECTROCHEMISTRY

Electrochemical methods have their solid place in modern analytical chemistry in diverse areas, including process analysis, environmental protection, agriculture analysis and food control, or some areas of clinical analysis [30]. Especially when it comes to flow systems, electrochemical detectors have gained significant popularity as they offer some advantageous features such as cost effectiveness, ease of construction, increased robustness compared to static measurements, and miniaturization potentials [31].

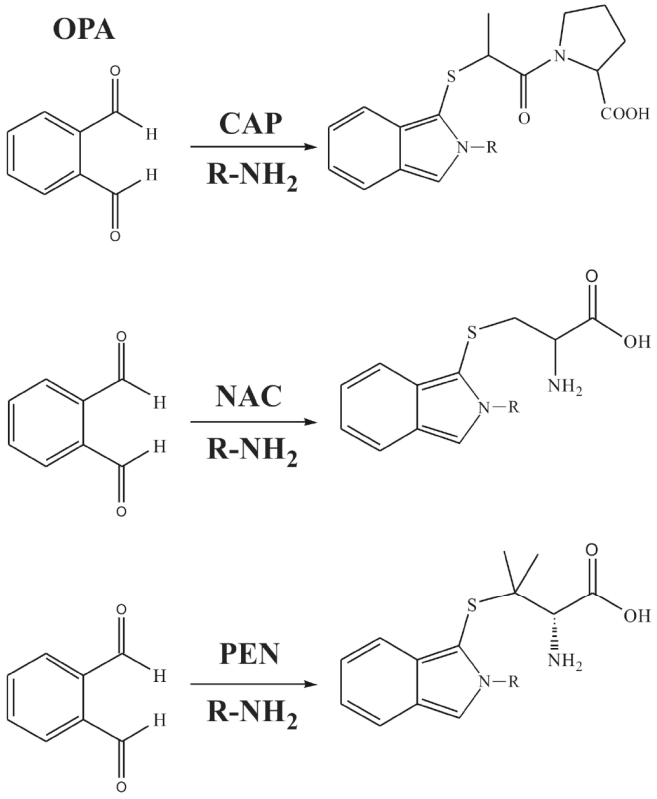

Fig. (5). Reaction of o-phthalaldehyde (OPA) with captopril (CAP), N-acetylcysteine (NAC) and penicillamine (PEN) in the presence of a primary amino compound [28].

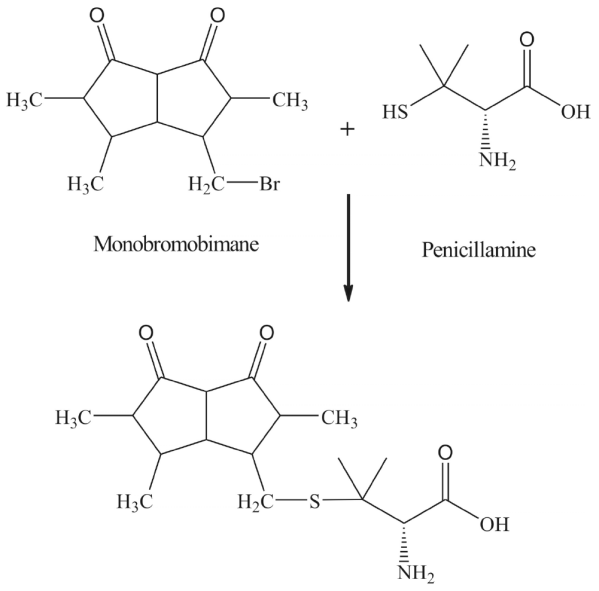

Fig. (6). Reaction of monobromobimane with penicillamine [28].

A SI method with potentiometric detection for the determination of captopril was based on the use of a $Ag(I)/Ag_2S$ ion selective electrode [26]. The method took advantage of the property of the thiol group present in the captopril molecule to promote the quantitative reaction with Ag(I) cation in acid media, resulting in the formation of a precipitate under stoichiometric conditions. Besides the advantages offered by automation by SI, the proposed method is characterized by two important features: i) there is no need for filtration of the samples prior to injection, minimizing this way the pretreatment steps and ii) the standards of the analyte at various concentrations are generated on-line.

Finally, a series of interesting articles was published by the group of Stefan, van Staden and Aboul-Enein using SI for the enantiomeric analysis of captopril using SI [32-35]. S-captopril was determined by a SI amperometric biosensor using a carbon paste electrode modified with L-amino acid oxidase [33]. The LOD was 16 nmol L^{-1} and the sampling rate 80 h^{-1}. In an analogous manner, R-captopril was determined selectively by a SI amperometric biosensor using a carbon paste electrode modified with D-amino acid oxidase [35]. The sampling rate was the same as above, but the LOD was 10 times higher. The simultaneous determination of the two enantiomeric forms of captopril was reported by combining the two previous biosensors in a single SI setup [32]. Analysis of enantiomeric mixtures of the analyte at different ratios confirmed the suitability of the automated scheme, while the method was adequately selective against D- and L-proline and polyvinylpyrolidone. In a fourth paper, the same authors reported the use of a potentiometric enantioselective membrane electrode based on maltodextrin for the assay of S-captopril, while R-captopril was determined amperometrically with the same protocol as mentioned above [34]. The potentiometric electrode design was similar to the amperometric biosensor one, by replacing the enzyme with maltodextrin solution in citrate buffer (1:1 molar ratio).

6. CONCLUSIONS

Since 2000, SI has proven to be a useful analytical platform for the development of methods for the determination of thiols in various substrates. Pharmaceutically and biologically active thiols such as captopril, cysteine, N-acetylcysteine, glutathione and penicillamine have been determined efficiently by spectrophotometry, fluorimetry and electroanalytical-based detection systems. In terms of new trends, propiolate esters seem to be quite promising derivatizing reagents for thiols offering significant advantages. However, further research is needed in order to expand SI methods for thiols to more complicated and challenging matrixes such as biological material and food samples. A viable suggestion would be the coupling of SI to liquid chromatography towards the development of fully automated pre-column derivatization schemes that could combine the advantages of SI to the separation power of chromatography.

REFERENCES

[1] Kuśmierek, K.; Chwatko, G.; Głowacki, R.; Bald, E. Determination of endogenous thiols and thiol drugs in urine by HPLC with ultraviolet detection. *J. Chromatogr. B*, **2009**, *877*, 3300.

[2] Toyooka, T. Recent advances in separation and detection methods for thiol compounds in biological samples. *J. Chromatogr. B*, **2009**, *877*, 3318.

[3] Pandey, S.K.; Kim, H.K. A review of methods for the determination of reduced sulfur compounds (RSCS) in air. *Environ. Sci. Technol.*, **2009**, *43*, 3020.

[4] Supalkova, V.; Huska, D.; Diopan, V.; Hanustiak, P.; Zitka, O.; Stejskal, K.; Baloun, J.; Pikula, J.; Havel, L.; Zehnalek, J.; Adam, V.; Trnkova, L.; Beklova, M.; Kizek, R. Electroanalysis of plant thiols. *Sensors*, **2007**, *7*, 932.

[5] White, P.C.; Lawrence, N.S.; Davis, J.; Compton, R.G. Electrochemical determination of thiols: A perspective. *Electroanalysis*, **2002**, *14*, 89.

[6] Wikipedia, the free encyclopedia: Cysteine, URL: http://en.wikipedia.org/wiki/Cysteine (accessed May 25, **2010**).

[7] Advanced Health, URL: www.advance-health.com/nacetylcysteine.html (accessed May 28, **2010**).

[8] Healthline, URL: http://www.healthline.com/galecontent/glutathione (accessed May 25, **2010**).

[9] Wikipedia, the free encyclopedia: Captopril, URL: http://en.wikipedia.org/wiki/Captopril (accessed May 25, **2010**).

[10] MedicineNet, URL: www.medicinenet.com/penicillamine/article.html (accessed May 25, **2010**).

[11] Ruzicka, J.; Marshall, G.D. Sequential injection: A new concept for chemical sensors, process analysis and laboratory assays. *Anal. Chim. Acta*, **1990**, *237*, 329.

[12] Lenehan, C.E.; Barnett, N.W.; Lewis, S.W. Sequential injection analysis. *Analyst*, **2002**, *127*, 997.

[13] Ruzicka, J.; Hansen E. H. *Flow Injection Analysis*; Wiley and Sons: New York, **1981**.

[14] Hansen, E.H. The impact of flow injection on modern chemical analysis: Has it fulfilled our expectations? And where are we going? *Talanta*, **2004**, *64*, 1076.

[15] Smith, J.P.; Hinson-Smith, V. Flow injection analysis: Quietly pushing ahead, *Anal. Chem*., **2002**, *74*, 385A.

[16] Economou, A. Sequential-injection analysis (SIA): A useful tool for on-line sample-handling and pre-treatment. *TrAC - Trends Anal. Chem.*, **2005**, *24*, 416.

[17] Economou, A.; Tzanavaras, P.D.; Themelis, D.G. Sequential-injection analysis: A useful tool for clinical and biochemical analysis. *Curr. Pharm. Anal.*, **2007**, *3*, 249.

[18] Solich, P.; Polášek, M.; Klimundová, J.; Ruzicka, J. Sequential injection technique applied to pharmaceutical analysis. *TrAC - Trends Anal. Chem.*, **2004**, *23*, 116.

[19] Tzanavaras, P.D.; Themelis, D.G. Review of recent applications of flow injection spectrophotometry to pharmaceutical analysis. *Anal. Chim. Acta*, **2007**, *588*, 1.

[20] Zacharis, C.K.; Tzanavaras, P.D.; Themelis, D.G. Ethyl-propiolate as a novel and promising analytical reagent for the derivatization of thiols: Study of the reaction under flow conditions. *J. Pharm. Biomed. Anal.*, **2009**, *50*, 384.

[21] Zacharis, C.K.; Tzanavaras, P.D. High throughput automated determination of glutathione based on the formation of a UV-absorbing thioacrylate derivative. *Comb. Chem. High Through. Screen.*, **2010**, *13*, 461.

[22] Tsiomlektsis, A. *Alkyl-esters of Propiolic Acid as Derivatizing Reagents for Thiols: Study of the Reactions Under Flow Conditions and Development of Sequential Injection Methods for the Determination of Pharmaceutically Active Thiols*. MSc Thesis, Aristotelian University of Thessaloniki, Greece, **2010**.

[23] Tzanavaras, P.D.; Themelis, D.G.; Economou, A.; Theodoridis, G. Flow and sequential injection manifolds for the spectrophotometric determination of captopril based on its oxidation by Fe(III). *Microchim. Acta*, **2003**, *142*, 55.

[24] Lee, S.H.; Sohn, O.J.; Yim, Y.S.; Han, K.A.; Hyung, G.W.; Chough, S.H.; Rhee, J.I. Sequential injection analysis system for on-line monitoring of L-cysteine concentration in biological processes. *Talanta*, **2005**, *68*, 187.

[25] O. Suliman, F.E.; Al-Lawati, H.A.J.; Al-Kindy, S.M.Z.; Nour, I.E.M.; Salama, S.B. A sequential injection spectrophotometric method for the determination of penicillamine in pharmaceutical products by complexation with iron(III) in acidic media. *Talanta*, **2003**, *61*, 221.

[26] Pimenta, A.M.; Araújo, A.N.; Montenegro, M.C.B.S.M. Sequential injection analysis of captopril based on colorimetric and potentiometric detection. *Anal. Chim. Acta*, **2001**, *438*, 31.

[27] Araujo, A.R.T.S.; Saraiva, M.L.M.F.S.; Lima, J.L.F.C. Determination of total and oxidized glutathione in human whole blood with a sequential injection analysis system. *Talanta*, **2008**, *74*, 1511.

[28] Karakosta, T. *Development of Fluorimetric Sequential Injection Methods for the Determination of Pharmaceutically Active Thiols Using o-phthalaldehyde and monobromobimane as Derivatizing Reagents*. MSc Thesis, Aristotelian University of Thessaloniki, Greece, **2009**.

[29] Suliman, F.E.; Al-Lawati, Z.H.; Al-Kindy, S.M.Z. A spectrofluorimetric sequential injection method for the determination of penicillamine using fluorescamine in the presence of β-cyclodextrins. *J. Fluoresc.*, **2008**, *18*, 1131.

[30] Trojanowicz, M.; Szewczynska, M.; Wcislo, M. Electroanalytical flow measurements - Recent advances. *Electroanalysis*, **2003**, *15*, 347.

[31] Pérez-Olmos, R.; Soto, J.C.; Zárate, N.; Araújo, A.N.; Montenegro, M.C.B.S.M. Sequential injection analysis using electrochemical detection: A review. *Anal. Chim. Acta*, **2005**, *554*, 1.

[32] Stefan, R.-I.; Van Staden, J.F.; Aboul-Enein, H.Y. Simultaneous detection of S and R captopril using sequential injection analysis. *Talanta*, **2000**, *51*, 969.

[33] Stefan, R.-I.; Van Staden, J.F.; Aboul-Enein, H.Y. On-line assay of S-captopril using an amperometric biosensor/sequential injection system. *Anal. Chim. Acta*, **2000**, *411*, 51.

[34] Stefan, R.-I.; Van Staden, J.F.; Aboul-Enein, H.Y. Amperometric biosensors/sequential injection analysis system for simultaneous determination of S- and R-captopril. *Biosens. Bioelectron.*, **2000**, *15*, 1.

[35] Stefan, R.-I.; Van Staden, J.F.; Aboul-Enein, H.Y. On-line monitoring of R-captopril using an amperometric biosensor/sequential injection analysis system. *Instrum. Sci. Technol.*, **2002**, *30*, 243.

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