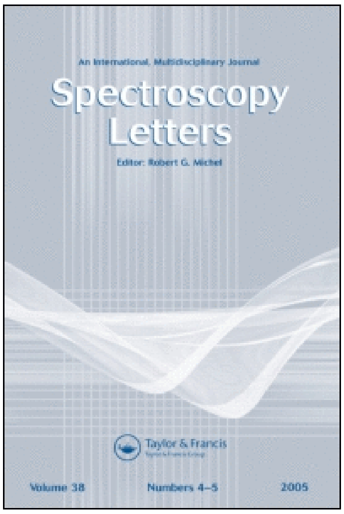

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Indirect Sequential Injection Enzymatic Determination of Allopurinol in Pharmaceuticals Based on Xanthine Oxidase Inhibition

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To cite this Article Silvestre, Cristina I. C. , Segundo, Marcela A. , Saraiva, M. Lúcia M. F. S. and Lima, José L. F. C.(2009) 'Indirect Sequential Injection Enzymatic Determination of Allopurinol in Pharmaceuticals Based on Xanthine Oxidase Inhibition', Spectroscopy Letters, 42: 6, 341 — 350

To link to this Article: DOI: 10.1080/00387010903185538

URL: http://dx.doi.org/10.1080/00387010903185538

Spectroscopy Letters, 42:341–350, 2009
Copyright © Taylor & Francis Group, LLC
ISSN: 0038-7010 print/1532-2289 online
DOI: 10.1080/00387010903185538

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ABSTRACT Two automatic sequential injection analysis methodologies were developed for the enzymatic determination of allopurinol in pharmaceutical formulations. Both methodologies were based on the inhibition by allopurinol of the reaction catalysed by xanthine oxidase, assessed by spectrophotometric detection of the reaction product (uric acid) at 295 nm. One methodology was established after a single readout at a fixed time while the other was based on the decrease of the rate of formation of uric acid upon monitoring the absorbance change. The analytical performance of both methodologies was validated, providing low environmental impact tools for assessment of allopurinol in drugs.

KEYWORDS allopurinol, automation, enzymatic determination, Green Analytical Chemistry, sequential injection analysis, xanthine oxidase inhibitor

INTRODUCTION

Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) is widely used in the management of chronic gout and hyperuricaemia, due to its capacity to inhibit the enzyme xanthine oxidase (XO). This metal flavoprotein is the last enzyme in the pathway for the degradation of purine derivatives, where it catalyzes the oxidation of hypoxanthine and xanthine to uric acid.[1] Furthermore, the increasing interest in pharmaceutical compounds that may interfere on the *in vivo* oxidant status has led to studies for putative effects of allopurinol upon organ reperfusion injuries or upon disorders where endogenous antioxidant mechanisms are compromised, such as coronary heart disease,[2,3] diabetes,[4] and cirrhosis.[5]

In this context, a variety of methods based on titrimetry,[6] spectrophotometry,[7–9] fluorimetry,[10] electrochemistry,[11–14] electrochemiluminescence,[15] and phosphorescence[16] are available for determination of allopurinol in pharmaceutical formulations. Methods for multi-analyte determination (including allopurinol) based on capillary electrophoresis[17] or HPLC with UV detection[18] have also been described. However, the majority of the methods described until

An invited paper submitted to a special issue on Green Spectroscopy and Analytical Techniques, organized by Professor Miguel de la Guardia, of the Department of Chemistry, University of Valencia, Spain, and Professor Arabinda Kumar Das, of the Department of Chemistry, University of Burdwan, West Bengal, India.

Received 30 September 2008; accepted 24 November 2008.

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now are not suitable for rapid quantification of allopurinol and some of them may raise occupational health issues because they require toxic reagents[10] or they rely on the dropping-mercury electrode.[11,12] Furthermore, they are not suitable for routine analysis, where fast and not labor-intensive protocols are mandatory. These requirements can be fulfilled by automated methods resorting to flow injection analysis (FIA).[19] In fact, FIA systems for determination of allopurinol have been described,[12–15] but some of them were not applied to real samples.[13,15]

FIA has evolved to sequential injection analysis (SIA),[20,21] a computer-controlled approach where small amounts (5 to 500 μL) of sample and reagents are stacked in a holding coil by sequential aspiration. After flow reversal, the solutions are mixed by dispersion of the adjacent segments before reaching the detection system. This type of flow system is suitable for enzymatic-based analysis, performed either in a homogeneous (solution)[22,23] or heterogeneous (immobilized)[24,25] media. Furthermore, as computer control of the manifold is inherent to SIA, kinetic protocols based on flow stop measurements can be easily implemented.[26–28] Another attractive aspect SIA methodologies is the consumption of low amounts of reagents and the consequent generation of a low volume of effluents, making this technique suitable for the implementation of Green Analytical Chemistry.[29]

Therefore, the aim of this work was to develop an automatic enzymatically based methodology for determination of allopurinol using sequential injection analysis. The methodology was based on the inhibition by allopurinol of the reaction catalyzed by xanthine oxidase, monitored in this case by spectrophotometric detection of uric acid formed at 295 nm. Two approaches, one based on a single readout at a fixed-time after reaction and another based on kinetic measurements, were developed and critically compared regarding their analytical performance and application to pharmaceutical formulations.

MATERIALS AND METHODS

Reagents and Solutions

All solutions were prepared with water from a Milli-Q system (resistivity >18 MΩ cm) and chemicals of analytical reagent grade quality with no further purification. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The buffer/carrier solution was prepared by dissolving 8.9 g of $Na_2HPO_4 \cdot 2H_2O$ in 100 mL of EDTA 1 mM. After that, the volume was made up to 1 L with water and pH was adjusted to 7.8 by dropwise addition of 2.0 M HCl solution.

Xanthine oxidase (EC 1.1.3.22) from bovine milk (ref. X-1875, 18 mg mL^{-1}, 0.6 units mg^{-1}) was also purchased from Sigma (St. Louis, MO, USA). The enzyme solution was prepared daily by dissolving 137 μL and 103 μL of commercial enzyme, for fixed-time or kinetic methods, respectively, in 5 mL of buffer solution.

Working standard solutions of allopurinol were prepared daily by rigorous dilution of 1.50 mM stock solution using buffer solution.

Xanthine solution (75 μM) was prepared by weighing it and dissolving the appropriate amount in 250 μL of NaOH 0.1 M, followed by the addition of 750 μL of water. After that, an aliquot of this solution was diluted up to 250 mL with the buffer solution. Other xanthine solutions (25 and 50 μM) were prepared in a similar way.

Apparatus

The solutions were propelled by a Gilson (Villiers-le-Bel, France) Minipuls 3 peristaltic pump, equipped with Gilson PVC pumping tubes (1.30 or 1.02 mm i.d. for fixed-time or kinetic method, respectively). This pump was connected to the central channel of an eight-port electrically actuated selection valve (C15-3118E, Valco VICI, Houston, TX).

A Thermo-Spectronic (Cambridge, U.K.) Heλios γ UV-vis spectrophotometer equipped with a thermostatic cell holder and a model 178.710 QS Hellma (Müllheim/Baden, Germany) flow-through cuvette (internal volume of 80 μL, optical path of 10 mm) was used. The wavelength was set at 295 nm. Temperature was maintained by circulating water through the cuvette holder using a thermostatic bath (I. S. Co GTR 190, Milan, Italy).

Omnifit (Cambridge, U.K.) polytetrafluoroethylene (PTFE) tubing (0.8 mm i.d.) and Gilson end-fittings and connectors were used to assemble the manifold.

A personal computer, equipped with an Advantech (Taipei, Taiwan) PCL-818 L interface card, running laboratory-made software written in QuickBasic 4.5

(Microsoft), controlled the position of the selection valve, the flow direction, the rotation speed, and the time during which the peristaltic pump was activated. Data acquisition was performed by conversion of analogue to digital signal at 4 Hz using the same software. Analytical signals were also recorded in paper using a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

For the reference procedure, the spectrophotometric measurements were carried out at 250 nm using the referred UV-VIS spectrophotometer.

Manifold and Procedure

The components of the sequential injection system were assembled as depicted in Fig. 1. The holding coil (HC) was 200 cm long, whereas the mixing coil (MC), located between the selection valve and the detector, was 150 cm long for the fixed-time approach or 60 cm long for the kinetic-based system. Other tubes connected to the selection valve were 30 cm long.

The protocol sequence for the fixed-time-based determination of allopurinol is presented in Table 1. The analytical cycle comprised the sequential aspiration of xanthine, sample, and XO into the holding coil; after flow reversal, the zones were propelled to the spectrophotometer, where a transient signal corresponding to the uric acid produced was registered (Figs. 2a–c).

For the kinetic-based approach, the protocol sequence is indicated in Table 2. The three first steps were similar to those performed in the fixed-time method (Figs. 2a, b). After that, the flow was reversed

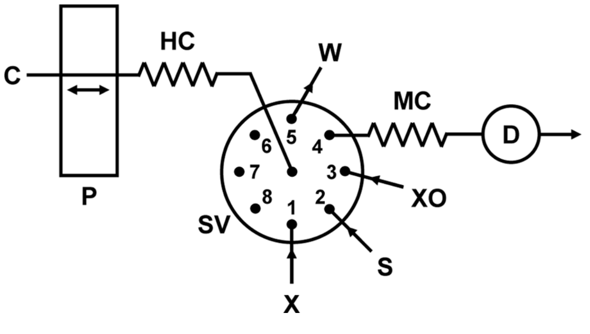

FIGURE 1 Sequential injection manifold for the enzymatic determination of allopurinol. C: carrier (buffer solution); P: peristaltic pump; HC: holding coil; SV: selection valve; XO: xanthine oxidase solution; X: xanthine solution; S: sample or allopurinol standard; MC: mixing coil; D: spectrophotometer ($\lambda = 295$ nm), W: waste.

TABLE 1 Protocol Sequence for SIA Fixed-Time Based Enzymatic Determination of Allopurinol

Valve position	Operation time (s)	Flow rate (mL min^{-1})	Volume (μL)	Description
1	20	1.2	400	Xanthine aspiration into HC
2	5	1.2	100	Sample/standard aspiration into HC
3	15	1.2	300	Xanthine oxidase aspiration into HC
4	200	0.9	3000	Propulsion of HC content toward the detector, signal acquisition

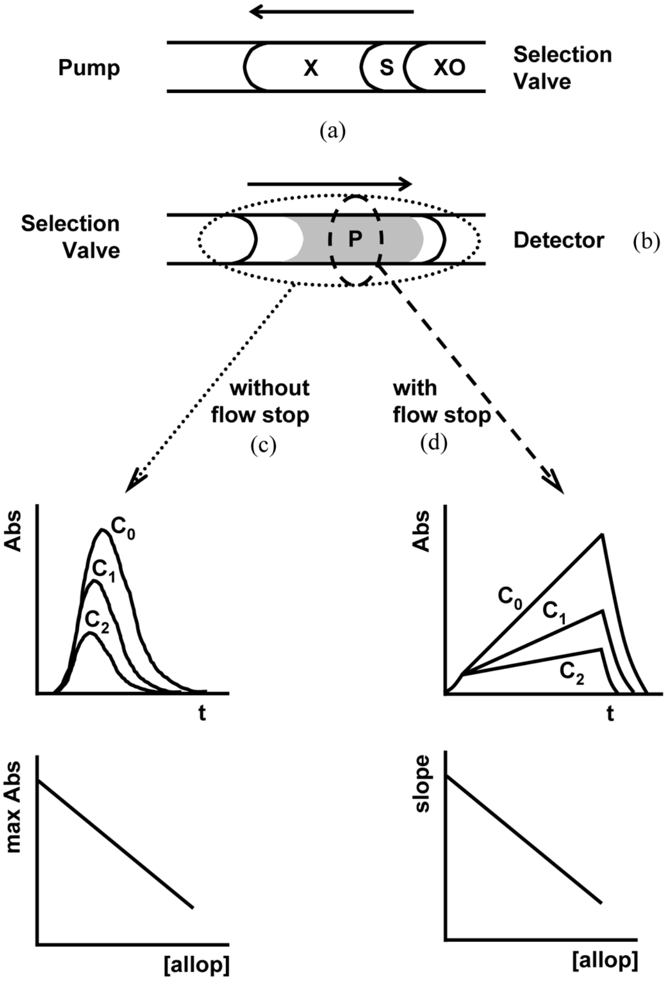

FIGURE 2 Schematic representation of sequential injection operation, comprising the aspiration of solutions into the holding coil (a) and detection of reaction product (b), with different analytical output for fixed-time (c) or kinetic (d) methodologies. X: xanthine solution; S: sample or allopurinol standard; XO: xanthine oxidase solution; P: reaction product (uric acid); C_i: allopurinol concentration; Abs: absorbance.

TABLE 2 **Protocol Sequence for SIA Kinetic Based Enzymatic Determination of Allopurinol**

Valve position	Operation time (s)	Flow rate ($mL\,min^{-1}$)	Volume (μL)	Description
1	12.81	1.9	400	Xanthine aspiration into HC
2	1.57	1.9	50	Sample/standard aspiration into HC
3	3.18	1.9	100	Xanthine oxidase aspiration into HC
4	11.5	3.8	720	Propulsion of HC content toward the detector
4	45	—	—	Stop period, signal acquisition
4	32	3.8	2000	Dispense carrier to wash flow cell

and the combined reagent + sample zone was propelled up to the spectrophotometer flow cell. The flow was then stopped and the increase of absorbance due to the production of uric acid was monitored during a preset period of time (Fig. 2d). Thereafter, the peristaltic pump was reactivated, and the flow cell was washed by the carrier solution.

Sample Treatment

Commercial tablets with a nominal content of 100 or 300 mg of allopurinol were analyzed. For this, twenty tablets were weighted and finely grounded. After that, an aliquot of 200 mg was dissolved in 250 mL of buffer solution. This solution was filtered and further diluted 60 or 200 times for determination using the fixed-time or the kinetic methodology, respectively.

Reference Method

For comparison purposes, the determination of allopurinol in pharmaceutical formulations was also performed according to the British Pharmacopoeia.[30] The methodology consisted in weighing and grounding twenty tablets. An aliquot of the powder obtained, containing about 100 mg of allopurinol, was further dissolved in 20 mL of NaOH 0.05 M with continuous stirring during 20 min. Afterward, 80 mL of HCl 0.1 M were added to this solution. After 10 min with continuous stirring, the volume of the previous solution was made up to 250 mL with HCl 0.1 M. This solution was then filtered and diluted 25 times using HCl 0.1 M before absorbance measurement at 250 nm. The concentration was calculated by direct application of Beer's Law, considering the molar absorptivity as $7.66 \times 10^3\,M^{-1}\,cm^{-1}$ and the optical path length as 1.00 cm.

RESULTS AND DISCUSSION

In the development of the fixed-time and kinetic methodologies, several studies concerning both physical and chemical parameters were performed in order to improve the performance of the systems, namely the signal amplitude, sensitivity, accuracy, repeatability, and determination rate. These features influenced the choices made during the optimization of the sequential injection systems, which was carried out using the univariate method.

Development of Fixed-Time Method

The first methodology developed was designated by fixed-time method as it was based on the maximum value of absorbance obtained from the transient signal due to uric acid formation after a pre-fixed reaction time (Fig. 2c). The initial conditions for studying this methodology were as follows: aspiration flow rate of $1.2\,mL\,min^{-1}$; propulsion flow rate of $0.69\,mL\,min^{-1}$; xanthine concentration of $50\,\mu M$; xanthine volume of $400\,\mu L$; sample/allopurinol volume of $100\,\mu L$; XO concentration of $0.148\,U\,mL^{-1}$; XO volume of $100\,\mu L$. The first studies aimed the maximization of the blank signal (absence of allopurinol) in order to enhance the sensitivity as the presence of allopurinol causes a decrease of this signal.

The first parameter studied was the length of the mixing coil, which in this case conditioned the reaction time and, consequently, the extension of uric acid formation. Three coils were tested (100, 150, and 200 cm), corresponding to reaction times of 44, 65, and 87 s, respectively. The peak height of the blank signal increased about 17% and 24% for 150 and 200 cm when compared to 100 cm coil. Therefore, further experiments were performed using the 150 cm coil as a compromise between the signal amplitude and the analysis time.

The concentration of xanthine and XO solutions were studied simultaneously. The xanthine concentration was varied between 25 and $100\,\mu M$ while the XO concentration was tested at 0.148 and

$0.296\,\mathrm{U\,mL^{-1}}$. For both XO concentrations tested, the analytical signal obtained for xanthine concentrations of 25 and 50 μM were 60% and 83% of that obtained for 75 μM of xanthine. The 100 μM solution was not tested since precipitation occurred a few minutes after its preparation. On the other hand, for all xanthine solutions tested, an increase of 65% was observed in the analytical signal for the highest XO concentration. Therefore, the concentration values were fixed at 75 μM for xanthine and $0.296\,\mathrm{U\,mL^{-1}}$ for XO.

Next, the influence of the volume of xanthine solution was studied. The blank analytical signal increased up to 400 μL. In fact, when the carrier solution was replaced by a xanthine solution, an analytical signal similar to that of 400 μL was obtained, indicating that a volume larger than that would not enhance the signal. Fixing the volume of xanthine at 400 μL, the volume of XO solution was then varied. The blank analytical signal increased up to 300 μL, which was applied for further experiments.

Using these conditions, allopurinol standards between 10 and 750 μM were analyzed. A linear dependency between maximum peak height and allopurinol concentration was attained between 25 and 75 μM. Within this concentration range, the volume of sample/allopurinol was studied between 50 and 200 μL. For these values, calibration curves were established providing slope values of -1.85×10^{-3}, -2.27×10^{-3}, and $-1.34 \times 10^{-3}\,\mathrm{\mu M^{-1}}$ for volumes of 50, 100, and 200 μL, respectively. The maximum sensitivity was attained for 100 μL. The sensitivity was 82% lower for 50 μL because less allopurinol was present. Although more allopurinol was available in the 200 μL experiment, the distance between the other two zones (xanthine and XO) was increased (Fig. 2a), providing a lower calibration slope. Furthermore, using 100 μL volume of allopurinol, the order of solution aspiration was modified by exchanging the xanthine solution with the allopurinol solution. Results showed that, despite the increase of the absolute value of the analytical signals, this alteration of aspiration order provided a lower sensitivity (slope of $-6.50 \times 10^{-4}\,\mathrm{\mu M^{-1}}$). The increase in the absolute value of analytical signal is caused by the lower dispersion suffered by the xanthine segment in this case and the decrease in sensitivity is caused by the higher dispersion of the allopurinol solution, as the large aliquot of xanthine was placed between the XO and the allopurinol segment. Hence, the aspiration order was maintained as before.

The influence of temperature was investigated by increasing the temperature of the mixing coil and of the cell holder. For 37°C there was formation of numerous air bubbles inside the flow conduits that impaired the spectrophotometric measurement. For 23°C (room temperature) and 30°C, slope values of -2.12×10^{-3} and $-3.94 \times 10^{-3}\,\mathrm{\mu M^{-1}}$ were obtained. Therefore, the temperature chosen for further experiments was 30°C as it originated a higher sensitivity.

Finally, with the goal of increasing the determination rate, the propulsion flow rate was evaluated between 0.69 and $1.3\,\mathrm{mL\,min^{-1}}$. The sensitivity was the same up to $0.9\,\mathrm{mL\,min^{-1}}$ and it decreased for higher flow rates. Therefore, the propulsion flow rate chosen was $0.9\,\mathrm{mL\,min^{-1}}$ since it allowed a higher determination rate and it did not compromise the sensitivity. The conditions tested and the final values chosen are summarized in Table 3.

TABLE 3 **Range of Values Used in the Study of the SIA System Variables for Fixed-Time and Kinetic Methods and Chosen Conditions for its Operation**

	Fixed-time method		Kinetic method	
Parameter	Range	Chosen values	Range	Chosen values
Xanthine concentration (μM)	25–100	75	25–100	75
Xanthine volume (μL)	100–400	400	100–500	400
Xanthine oxidase concentration ($\mathrm{U\,mL^{-1}}$)	0.148; 0.296	0.296	0.074–0.592	0.222
Xanthine oxidase volume (μL)	100–400	300	—	100
Sample volume (μL)	50–200	100	—	50
Temperature (°C)	23–37	30	25–50	30
Propulsion flow rate ($\mathrm{mL\,min^{-1}}$)	0.69–1.3	0.9	—	3.8
Aspiration flow rate ($\mathrm{mL\,min^{-1}}$)	—	1.2	—	1.9
Time interval for propelling HC content to detector (s)	—	—	7–14	11.5
Time period for signal acquisition (s)	—	—	—	0–45

Development of Kinetic Method

In this case, the determination of allopurinol was based on the decrease in the rate of formation of uric acid, catalyzed by XO (Fig. 2d). The production of uric acid was followed by measuring the absorbance change at 295 nm during a pre-set time interval. The rate of reaction was determined by plotting the absorbance values versus time and by calculating the slope when the relation between them was linear ($R > 0.995$). Calibration curves were established by plotting the rate of reaction versus concentration of allopurinol.

The manifold was similar to that applied in the previous section, except for the shorter reaction coil (now 60 cm) that was not subjected to temperature control. Other operational parameters were also changed, namely the aspiration flow rate ($1.9\,\mathrm{mL\,min^{-1}}$), the propulsion flow rate ($3.8\,\mathrm{mL\,min^{-1}}$), the sample volume (50 μL), and the XO volume (100 μL).

The utilization of higher flow rates and a shorter mixing coil are necessary to enable the observation of the reaction development. Otherwise, it would take more time for the reaction mixture to reach the detector and a plateau would be observed, corresponding to an equilibrium/end-point measurement.

As discussed before,[27,28] zone overlapping is essential to attain suitable mixing conditions in SIA. Therefore, the solutions were aspirated into the HC in the following order: 400 μL of xanthine (75 μM), 50 μL of sample/standard, and 100 μL of enzyme ($0.148\,\mathrm{U\,mL^{-1}}$). This sequence was chosen by taking into consideration that the enzyme should be the last reagent to be aspirated in order to minimize its dilution, and also that the plug of solution placed between the other two segments should be as small as possible to allow suitable overlap of the three original zones.

The study of the parameters influencing the system performance was carried out taking into consideration two aspects: first, the existence of a linear relationship between absorbance and time during the period of signal acquisition as the rate of reaction was calculated from the slope of this graph; second, the influence on the sensitivity, assessed as the rate of reaction value (using a single standard) or as the slope of the calibration curve (rate of reaction versus concentration of allopurinol, when several standards were used).

The first step in the optimization of the kinetic method was the study of the time interval during which the pump was activated before reaction monitoring. This was an important parameter in the system design, as it defined the portion of the overlapped zones (xanthine/sample/enzyme) present in the flow cell during the flow stop step. This interval was studied between 7 and 14 s, by injecting water instead of allopurinol (maximum signal). Since the highest reaction rate values were attained for 11 ($4.24 \times 10^{-3}\,\mathrm{s^{-1}}$) and 12 s ($4.04 \times 10^{-3}\,\mathrm{s^{-1}}$), the intermediate values 10.5 and 11.5 s were also tested. In this case, the highest value of reaction rate was obtained for 11.5 s ($4.60 \times 10^{-3}\,\mathrm{s^{-1}}$), which was applied in the following experiments.

Temperature increase has a drastic effect in enzyme kinetics as it increases the rate of reaction as well as deactivates the enzyme. In the proposed system, temperature studies were carried out by changing the temperature of the cell holder between 25 and 50°C. The highest rate of reaction was obtained at 30°C, and a similar value was also attained at 35°C. Above this temperature, the rate of reaction decreased, indicating possible inactivation of the enzyme.[31] Therefore, further experiments were performed at 30°C.

The volume of xanthine solution was varied between 100 and 500 μL, using two allopurinol standards (37.5 and 62.5 μM). For both concentrations, the reaction rate increased up to 400 μL ($1.91 \times 10^{-3}\,\mathrm{s^{-1}}$ and $9.27 \times 10^{-4}\,\mathrm{s^{-1}}$, respectively), remaining stable for larger volumes. Therefore, the chosen value was 400 μL.

The xanthine concentration was evaluated between 25 and 75 μM, using several allopurinol standards (10–75 μM). Comparing the results obtained for the same allopurinol standard, it was observed that the highest reaction rate was attained at 75 μM of xanthine. For instance, for the 10 μM allopurinol standard, the reaction rates were $2.54 \times 10^{-3}\,\mathrm{s^{-1}}$, $2.24 \times 10^{-3}\,\mathrm{s^{-1}}$ and $1.90 \times 10^{-3}\,\mathrm{s^{-1}}$, for xanthine concentrations of 75, 50, and 25 μM, respectively. Therefore, the xanthine concentration was fixed at 75 μM.

The XO concentration was investigated between 0.074 and $0.592\,\mathrm{U\,mL^{-1}}$, in the presence (10 and 50 μM) and absence of allopurinol. Considering the whole interval of reaction monitoring (45 s), the relation between absorbance and time was linear for the lowest concentrations of enzyme tested (0.074, 0.148, and 0.222 U mL^{-1}). For the enzyme solutions containing 0.296 and 0.592 U mL^{-1} this relation was linear in the first 30 and 15 s, respectively. For these two solutions, it was verified that the formation of a deposit in the enzyme container and poor repeatability within replicate measurements were probably due to protein settling in the reservoir flask. Considering the other solutions, the highest reaction rate values were obtained for the 0.222 U mL^{-1} solution. In fact, the sensitivity (slope of the calibration curve) was also higher for this solution: -4.81×10^{-5} s^{-1} μM^{-1} compared to -3.51×10^{-5} s^{-1} μM^{-1} obtained for the 0.148 U mL^{-1} solution. Therefore, the concentration of XO was fixed at 0.222 U mL^{-1}. The conditions tested and the final values chosen are also summarized in Table 3.

Validation of the Proposed Methodologies

The performance of the sequential injection systems for the determination of allopurinol in pharmaceutical formulations was evaluated regarding selectivity, linearity, application range, repeatability, detection limit, determination rate (Table 4), and accuracy (Table 5).

Selectivity was assessed by evaluating the interference of the excipients present in the target pharmaceutical formulations. Solutions containing 50 μM (6.8 mg L^{-1}) of allopurinol and a 100-fold excess of the potential interfering species were analyzed. The absence of interference was considered as long as the analytical signal obtained for this solution was lower than ±3% of that attained by a 50 μM of allopurinol solution. No interference was found for aerosil 200 (5 mM), lactose (5 mM), talc (680 mg L^{-1}), magnesium stearate (5 mM), starch (680 mg L^{-1}), monocrystalline cellulose (680 mg L^{-1}), povidone (680 mg L^{-1}) and sodium starch glycolate (680 mg L^{-1}). Therefore, it can be concluded that the sample matrix does not produce any significant interference in the proposed methodologies.

Calibration curves were established by plotting the absorbance value (single-point method) or the rate of reaction (kinetic method) versus concentration of allopurinol and typical values are given in Table 4 ($N = 15$). For the single-point method, linearity was lost for concentrations higher than 75 μM. The slope value was significantly different from zero ($|t_{\text{exp}}| = 131 > t_{\text{tab}} = 2.16$; $\alpha = 0.05$) and it did not include the zero value in its confidence interval ($\alpha = 0.05$), thus indicating proportionality. In addition, the residuals distribution exhibited a random behavior. As the method was based on the inhibition of the analytical signal, the intercept value was also significantly different from zero ($|t_{\text{exp}}| = 434 > t_{\text{tab}} = 2.16$; $\alpha = 0.05$).

TABLE 4 **Figures of Merit for the Proposed SIA Methods**

	Fixed-time method	Kinetic method
Calibration curve		
Slope[a]	-0.00237 ± 0.00016[b]	$(-6.30 \pm 0.22) \times 10^{-5}$[d]
Intercept[a]	0.426 ± 0.008[c]	$(4.48 \pm 0.04) \times 10^{-3}$[e]
Correlation coefficient	−0.9941	−0.9984
Application range (μM)	25–75	5–25
Repeatability		
Method precision (intra-assay)	<2.4%	<9.2%
Method precision (inter-assay)	<5.2%	<5.5%
Detection limit (μM)	6.2	1.3
Quantification limit (μM)	20.7	4.3
Determination rate (h^{-1})	15	34

[a] Mean ± limits of confidence ($\alpha = 0.05$, $N = 15$).

[b] In μM^{-1}.

[c] In absorbance units.

[d] In absorbance units s^{-1} μM^{-1}.

[e] In absorbance units s^{-1}.

For the kinetic method, linearity was lost for concentrations higher than 25 μM. The slope value was also significantly different from zero ($|t_{\text{exp}}| = 236 > t_{\text{tab}} = 2.16$; $\alpha = 0.05$) and it does not include the zero value in its confidence interval ($\alpha = 0.05$). The residuals distribution also exhibited a random behavior. As expected, the intercept value was also significantly different from zero ($|t_{\text{exp}}| = 1010 > t_{\text{tab}} = 2.16$; $\alpha = 0.05$).

Concerning the repeatability of the methods, the intra-assay precision was estimated by calculating the relative standard deviation from three and five consecutive injections of each sample analyzed ($N = 7$), for the fixed-time and kinetic methods,

TABLE 5 Results (Mean Value ± Standard Deviation, in mg) Obtained by Fixed-Time Method (SIA_{tfix}), Kinetic Method (SIA_{kin}) and Reference Method, and Values of Relative Deviation (RD; %) Between Each Proposed Method and the Reference Method

Sample	Reference method	SIA_{tfix}	% RD (SIA_{tfix})	SIA_{kin}	% RD (SIA_{kin})
Ratiopharm 300 mg	298.9 ± 0.0	299.1 ± 7.2	+0.1	294.2 ± 9.0	−1.6
Zyloric 300 mg	302.5 ± 0.2	301.7 ± 5.6	−0.3	297.6 ± 27.5	−1.6
Uriprim 300 mg	297.9 ± 0.3	307.5 ± 2.4	+3.2	280.1 ± 19.1	−6.0
Zurim 300 mg	292.0 ± 0.3	301.1 ± 6.4	+3.1	272.6 ± 6.9	−6.6
Zurim 100 mg	97.3 ± 0.1	93.6 ± 0.8	−3.8	107.1 ± 9.8	+10.1
Zyloric 100 mg	97.3 ± 0.1	101.2 ± 0.8	+4.0	88.8 ± 1.1	−8.7
Uriprim 100 mg	101.4 ± 0.1	99.9 ± 1.9	−1.5	104.9 ± 3.5	+3.6

respectively. Values ranging from 0.8% and 2.4%, and from 1.2% and 9.2% were obtained for the fixed-time and kinetic methods, respectively. The inter-assay precision was calculated for two samples, analyzed at different days. RSD values lower than 5.2% and 5.5% were attained for the fixed-time and kinetic methods, respectively.

In order to evaluate the accuracy of the proposed systems, samples of pharmaceutical formulations were analyzed ($N = 7$). The results were compared with those obtained by the reference procedure (Table 5). The results obtained with the proposed methodologies were in a good agreement with the reference method (relative deviations, RD < 4% for fixed-time method and RD < 10% for kinetic based method). Clearly, the fixed-time method provided more accurate results and the repeatability was also better, considering the values obtained for the samples analyzed.

For comparison purposes, paired *t*-test was performed and $|t|$ values of 1.21 and 1.50 were obtained when the fixed-time or the kinetic method was compared to the reference batch method. The comparison between those values with the tabulated $t_{(P=0.05,\ d.f.=6)} = 2.45$ indicated no statistically significant difference for the mean concentrations obtained by each proposed method and the reference method.

The determination rate was calculated by adding the time taken by each step of the protocol sequence. Therefore, the time required for a complete analytical cycle was 240 and 106 s, corresponding to 15 and 34 determinations per hour for the fixed-time and kinetic methods, respectively.

CONCLUSIONS

The proposed sequential injection system allowed fast and reliable determination of allopurinol, and it was shown to be useful for quality control purposes in pharmaceutical formulations. Furthermore, this work also accounts for the adequacy of SIA to enzymatic analysis, allowing the easy implementation of time-based approaches by taking advantage of its inherent computer control. It should also be emphasized that the methodologies proposed here follow Green Analytical Chemistry principles by not using toxic reagents as they rely on enzymatic analysis with direct spectroscopic detection of the reaction product, without the need of further derivatization. In the SIA systems proposed here, enzymes were applied in solution. In a straightforward view, the utilization of immobilized enzymes may present a greener alternative when compared to the utilization of enzymes in solution. However, from our previous experience with immobilized enzymes, the reactors have a short life (about one week, generally). Furthermore, several reagents are required for immobilization, most of them not being environmentally friendly. Considering that a typical enzymatic reactor lasts about one week, that the quantity of enzyme immobilized may vary from 200 to 1000 μL, and that the SIA systems require about 100–140 μL per day of commercial enzyme, the total enzyme consumption would be about 700 μL per week, which is comparable to the consumption of immobilized enzyme.

Compared to the reference method described by the British Pharmacopoeia,[30] the proposed SIA systems are faster, with analysis time of 2 to 4 min, which is at least 10 times less than the time taken by the batch procedure. Furthermore, the SIA systems are also a greener alternative to the reference procedure. Besides the "green" advantages inherent to automation (the utilization of less materials, namely those that are disposable or that must be washed or cleaned, for instance), the SIA procedure produces about 3 mL of effluent per assay while the reference method originates 300 mL. For both methods, sample dissolution is required, which produces about 250–270 mL of effluent, which may be reduced by taking a smaller aliquot of drug sample. Concerning reagent consumption, the SIA procedures require micro-amounts of the reagents involved (μL of μM solutions). In fact, SIA procedures consume 400 μL of 75 μM xanthine solution and 300 or 100 μL of 0.3 U/mL xanthine oxidase solution per assay while the reference procedure requires 20 mL of NaOH 0.05 M and 250 mL of HCl 0.1 M. As a possible disadvantage, the linear response ranges of the SIA methods are narrower than that obtained by the reference method. However, as the concentration expected in drug samples is generally known, it is not difficult to apply a dilution factor that matches the determination range.

Compared to previously described FIA methods, the proposed systems are a more environmentally friendly alternative to the FIA system proposed by Cataldi et al.,[12] which used a mercury electrode. Despite the better sensitivity and lower detection limit, the FIA system proposed by Chi et al.[15] was not applied to any sample. In contrast, both methods proposed here were successfully applied to real samples, with results statistically comparable to the British Pharmacopoeia procedure. Compared to the electrochemical FIA procedure proposed by Zen et al.,[14] based on the inhibition of oxidation of ascorbate, a lower determination frequency and a narrower working range were attained by the SIA systems. Nevertheless, the strategy proposed here, based on the *in vivo* action of allopurinol, provides information not only about the quantity of this compound but also about its activity, a value-added asset for quality control.

Considering the system proposed by Martin and Rechnitz,[13] which is also based on the catalytic activity of xanthine oxidase (but using allopurinol as substrate), the lifetime of the biosensor described is limited to a few days, with decreasing sensitivity along consecutive working days. This limitation was overcome in the present work by the utilization of enzymes in solution. This strategy, combined to the low reagent consumption associated to SIA, makes the proposed methodologies affordable when compared to FIA, where the reagents are consumed continuously.

Finally, comparing the two methods proposed here, better results were obtained by the fixed-time approach regarding precision and accuracy. On the other hand, the kinetic-based method presented a higher determination rate, offering a suitable tool for screening analysis, where a high throughput is more relevant than an excellent precision.

ACKNOWLEDGMENT

Cristina I. C. Silvestre thanks Fundação para a Ciência e Tecnologia and FSE (III Quadro Comunitário de Apoio) for the Ph.D. grant (BD/31107/2006).

REFERENCES

1. Borges, F.; Fernandes, E.; Roleira, F. Progress towards the discovery of xanthine oxidase inhibitors. *Curr. Med. Chem.* **2002**, *9*(2), 195–217.
2. Reyes, A. J.; Leary, W. P. Allopurinol or oxypurinol in heart failure therapy – a promising new development or end of story? *Cardiovasc. Drugs Ther.* **2005**, *19*(5), 311–313.
3. Zhao, L.; Roche, B. A.; Wessale, J. L.; Kijtawornrat, A.; Lolly, J. L.; Shemanski, D.; Hamlin, R. L. Chronic xanthine oxidase inhibition following myocardial infarction in rabbits: Effects of early versus delayed treatment. *Life Sci.* **2008**, *82*(9–10), 495–502.
4. Inkster, M. E.; Cotter, M. A.; Cameron, N. E. Treatment with the xanthine oxidase inhibitor, allopurinol, improves nerve and vascular function in diabetic rats. *Eur. J. Pharmacol.* **2007**, *561*(1–3), 63–71.
5. Spahr, L.; Bresson-Hadni, S.; Amann, P.; Kern, I.; Golaz, O.; Frossard, J. L.; Hadengue, A. Allopurinol, oxidative stress and intestinal permeability in patients with cirrhosis: An open-label pilot study. *Liver Int.* **2007**, *27*(1), 54–60.
6. Hassib, S. T.; Safwat, H. M.; Elbagry, R. I. Spectrophotometric determination of some antiinflammatory agents using N-bromosuccinimide. *Analyst* **1986**, *111*(1), 45–48.
7. Korany, M. A.; Bedair, M. M.; Elsayed, M. A.; Fahmy, O. T. Colorimetric determination of pharmaceutical thiocompounds and allopurinol using mercurochrome. *Anal. Lett.* **1989**, *22*(8), 1909–1925.
8. Shoukrallah, I.; Sakla, A.; Wintersteiger, R. Determination of allopurinol and flucytosine in tablets by differential UV spectroscopy. *Anal. Lett.* **1990**, *23*(5), 843–861.
9. Abdelhay, M. H.; Barary, M. H.; Elsayed, M. A.; Hassan, E. M. Use of derivative spectrophotometry for the invitro determination of allopurinol and uric-acid mixtures. *Anal. Lett.* **1991**, *24*(9), 1517–1530.
10. Ehaid, M. Y.; Habeeb, A. A.; Ayad, M.; Taha, E. A. Quantitative-determination of allopurinol by fluorescence labeling using 5-Dimethylamino Naphthalene-1-Sulphonyl chloride. *Anal. Lett. B—Clin. Biochem. Anal.* **1984**, *17*(9), 759–770.
11. Chatten, L. G.; Boyce, M.; Moskalyk, R. E.; Pons, B. S.; Madan, D. K. Determination of allopurinol in tablets by differential-pulse polarography. *Analyst* **1981**, *106*(1260), 365–368.
12. Cataldi, T. R. I.; Palmisano, F.; Zambonin, P. G. Flow-injection with anodic polarographic detection for the determination of allopurinol in pharmaceutical formulations. *Analyst* **1989**, *114*(11), 1449–1452.
13. Martin, G. B.; Rechnitz, G. A. Electrochemical determination of allopurinol based on its interaction with xanthine-oxidase. *Anal. Chim. Acta* **1990**, *237*(1), 91–98.
14. Zen, J. M.; Chen, P. Y.; Kumar, A. S. Flow injection analysis of allopurinol by enzymeless approach at glassy carbon electrodes. *Electroanalysis* **2002**, *14*(10), 645–649.
15. Chi, Y. W.; Xie, J. L.; Chen, G. N. Electrochemiluminescent behavior of allopurinol in the presence of Ru(bpy)(3)(2+). *Talanta* **2006**, *68*(5), 1544–1549.
16. Perez-Ruiz, T.; Martinez-Lozano, C.; Tomas, V.; Martin, J. Determination of allopurinol by micelle-stabilised room-temperature phosphorescence in real samples. *J. Pharm. Biomed. Anal.* **2003**, *32*(2), 225–231.

17. Perez-Ruiz, T.; Martinez-Lozano, C.; Tomas, V.; Galera, R. Development of a capillary electrophoresis method for the determination of allopurinol and its active metabolite oxypurinol. *J. Chromatogr. B* **2003**, *798*(2), 303–308.
18. Shostak, D. Liquid-chromatographic determination of allopurinol in tablets—collaborative study. *J. Assoc. Offic. Anal. Chem.* **1984**, *67*(6), 1121–1122.
19. Ruzicka, J.; Hansen, E. H. *Flow Injection Analysis;* 2nd ed.; John Wiley & Sons: New York, 1988.
20. Ruzicka, J.; Marshall, G. D. Sequential injection—A new concept for chemical sensors, process analysis and laboratory assays. *Anal. Chim. Acta* **1990**, *237*(2), 329–343.
21. Pimenta, A. M.; Montenegro, M.; Araujo, A. N.; Calatayud, J. M. Application of sequential injection analysis to pharmaceutical analysis. *J. Pharm. Biomed. Anal.* **2006**, *40*(1), 16–34.
22. Pascoa, R.; Vidigal, S.; Toth, I. V.; Rangel, A. O. S. S. Sequential injection system for the enzymatic determination of ethanol in wine. *J. Agric. Food Chem.* **2006**, *54*(1), 19–23.
23. Economou, A.; Panoutsou, P.; Themelis, D. G. Enzymatic chemiluminescent assay of glucose by sequential-injection analysis with soluble enzyme and on-line sample dilution. *Anal. Chim. Acta* **2006**, *572*(1), 140–147.
24. Segundo, M. A.; Rangel, A. O. S. S. Sequential injection flow system with improved sample throughput: Determination of glycerol and ethanol in wines. *Anal. Chim. Acta* **2002**, *458*(1), 131–138.
25. Stefan-van Staden, R. I.; Bokretsion, R. G.; van Staden, J. F.; Aboul-Enein, H. Y. Simultaneous detection of creatine and creatinine using a sequential injection analysis/biosensor system. *Prep. Biochem. Biotechnol.* **2006**, *36*(4), 287–296.
26. Hansen, E. H. Exploiting kinetic-based flow-injection methods for quantitative chemical-assays. *Anal. Chim. Acta* **1992**, *261*(1–2), 125–136.
27. Oliveira, H. M.; Segundo, M. A.; Lima, J. L. F. C.; Grassi, V.; Zagatto, E. A. G. Kinetic enzymatic determination of glycerol in wine and beer using a sequential injection system with spectrophotometric detection. *J. Agric. Food Chem.* **2006**, *54*(12), 4136–4140.
28. Segundo, M. A.; Rangel, A. O. S. S. Kinetic determination of L(-)malic acid in wines using sequential injection analysis. *Anal. Chim. Acta* **2003**, *499*(1–2), 99–106.
29. Armenta, S.; Garrigues, S.; de la Guardia, M. Green analytical chemistry. *Trac-Trends Anal. Chem.* **2008**, *27*(6), 497–511.
30. British Pharmacopoeia Commission, *British Pharmacopoeia*. The Stationery Office—MHRA: London, 2005; Vol. III.
31. Bailey, J. E.; Ollis, D. F. *Biochemical Engineering Fundamentals;* 2nd ed.; McGraw-Hill: New York, 1986.