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A reagent-free method based on a photo-induced fluorimetry in a sequential injection system

Marieta L.C. Passos, M. Lúcia M.F.S. Saraiva *, João L.M. Santos, Salette Reis, Marlene Lúcio, José L.F.C. Lima

REQUIMTE, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4090-030 Porto, Portugal

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ABSTRACT

According to the current demands of environmentally friendly analytical chemistry and with a view to achieving lower reagent consumption with improved analytical performance, an automatic methodology composed of a photoreactor and fluorimetric detection (λ_{exc} = 287 nm, λ_{em} = 378 nm) was developed. To this end, a sequential injection analysis (SIA) system was developed for indomethacin determination using ultra-violet (UV) light which promotes an increase in the fluorescence of indomethacin. This increase in sensitivity makes it possible to apply this methodology to a dissolution test and to determine indomethacin in pharmaceutical formulations.

The calibration graph for indomethacin was linear between 4.10×10^{-6} and 9.00×10^{-5} mol L^{-1} and the detection limit was 1.23×10^{-6} mol L^{-1}. The method was proven to be reproducible with a R.S.D. < 5% and sampling rate of approximately 20 per hour. The potential effect of several compounds commonly used as excipients on analytical signals was studied and no interfering effect was observed. Statistical evaluation at the 95% confidence level showed good agreement between the results obtained for the pharmaceutical samples with both the SIA system and comparison batch procedures.

1. Introduction

Anastas [1] reported that green chemistry implies the use of chemical techniques and methodologies that reduce or eliminate the use or generation of feedstock, products, by-products, solvents, reagents, etc. that are hazardous to human health or the environment. This concept has evolved and different methods have been used to achieve this objective. Flow-based methodologies have been used due to their capacity for reducing reagent and solvent consumption and consequently, to reduce waste generation. In doing so, such methodologies reduce or avoid the side effects of analytical methods.

Regarding these objectives, the development of a sequential injection analysis [2] system (SIA) has some advantages when compared to the flow injection system (FIA) [3] since it readily permits a reduction in the amount of solvents and reagents consumed and waste produced while also being simpler, since it allows different methodologies to be implemented without manifold modifications.

By applying photochemistry, a greater efficiency of the method with a decreased consumption of reagents and waste generated can be obtained.

Consequently, by coupling an automatic system (such as SIA) with a photoreactor, a methodology framed in green chemistry principles can be derived. In recent years, some work has been developed based on the implementation of a photoreactor in SIA systems. In these systems, the photoreactor has a different function, such as the UV photo-oxidation of dissolved organic carbon [4], oxidation of organic phosphates for total phosphorus determination in milk [5], mineralization of the sample with sodium persulphate for total nitrogen determination [6] and photo-degradation of diclofenac for its determination in pharmaceutical formulations [7].

Therefore, by bearing in mind the advantages offered by SIA and photochemistry, a photoreactor was applied in a sequential injection analysis system for indomethacin determinations. Indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acid) is a methylated indol derivative with anti-inflammatory, analgesic and antipyretic activity, extensively used in the treatment of rheumatoid arthritis and other rheumatic diseases [8]. This determination was carried out using fluorimetric detection since combining photochemical reactions with fluorimetric detection in hydrodynamic systems has been demonstrated to be a versatile, simple and sensitive analytical strategy [7,9]. The sensitivity of the developed system was obtained since the fluorescence of the product resulting from the irradiation of indomethacin is higher than its native fluorescence. This increase in the fluores-

* Corresponding author. Tel.: +351 222078939; fax: +351 222004427.
E-mail address: lsaraiva@ff.up.pt (M.L.M.F.S. Saraiva).

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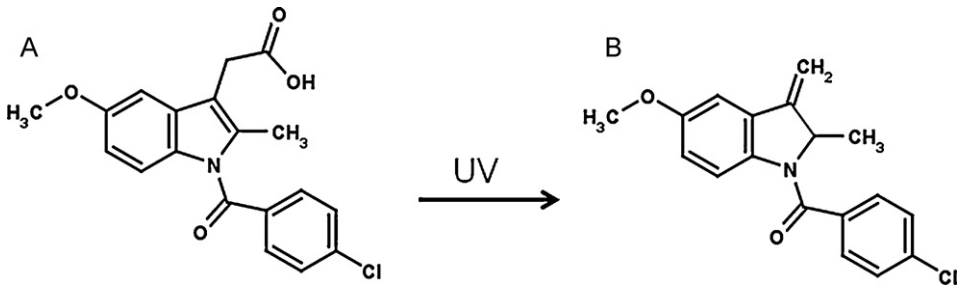

Fig. 1. Structure of indomethacin (A) and probable product (B) resulted to the irradiation of indomethacin with UV light. The structure B is unstable and can originate other different compounds.

cence intensity may be possible due to the molecular structural changes in the C3 position (Fig. 1), as it was referred before by Weedon and Wong [10].

This sensitivity in conjunction with the linear concentration range allowed this system to be used not only for determining indomethacin but also for implementing the dissolution tests. These tests are employed both for the quality control of drug dosage forms and for development of new dosage forms. Coupling these tests with flow methodologies is particularly important when low sample consumption is required, since a minimum disturbance to the volume of the dissolution medium is introduced, particularly when a high sampling frequency is necessary to achieve high resolution of the dissolution processes [11].

Determination of indomethacin using sequential injection analysis has been reported using an alkaline hydrolysis [12,13] or the tris(2,2′-bipyridyl)ruthenium (III) formed by the oxidation of tris(2,2′-bipyridyl)ruthenium (II) with Ce(IV)ammonium sulphate in diluted sulphuric acid [14]. However, no sequential injection system has ever been used in conjunction with photochemistry in the automation of indomethacin determinations reducing reagent consumption and/or avoiding the use of some toxic solvents.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical reagent grade, high purity water (milli Q) with a specific conductivity <0.1 μS cm^{-1}. All chemicals were of analytical reagent grade.

The 0.1 mol L^{-1} phosphate buffer solution was prepared by mixing adequate volumes of 0.2 mol L^{-1} solutions of NaH_2PO_4 (Fluka) and of Na_2HPO_4 (Fluka) to obtain pH value of 7.2. This buffer solution was used to prepare the indomethacin and the pharmaceutical formulations solutions.Indomethacin working standard solutions were obtained by appropriately dilution, with buffer solution, of the 1×10^{-4} mol L^{-1} stock solution of indomethacin. 100 mL of this stock solution was prepared by dissolving 3.58 mg of the solid (Sigma) in 500 μL of methanol and by dilution in the same buffer solution. The indomethacin solutions were daily prepared and they were protected from the light.

Sample solutions were prepared by dissolving the required amount of powdered preparations in methanol (it was used the same quantity of methanol that it was used to prepare the indomethacin solutions) and buffer solution. For each preparation, 20 capsules were weighed and a mean content of a single capsule was estimated in order to prepare a solution with an approximately known amount of indomethacin.

2.2. Apparatus

The SIA system (Fig. 2) consisted of a Gilson Minipuls 3 (VilliersleBel, France) peristaltic pump, equipped with a 0.90 mm i.d.

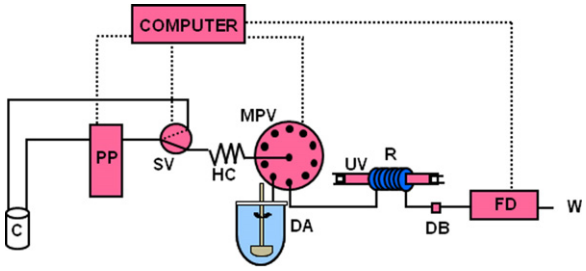

Fig. 2. SIA manifold for indomethacin determinations. C, carrier (H_2O); PP, peristaltic pump; SV, solenoid valve; HC, holding coil (2 m length/0.8 mm i.d.); MPV, multiposition selection valve; DA, dissolution apparatus; R, reactor (90 cm, around the ultra-violet lamp); DB, de-bubbler; UV, ultra-violet lamp; FD, fluorimetric detector (λ_{exc} = 287 nm, λ_{em} = 378 nm); W, waste.

Gilson PVC pumping tube and a 10-port selection valve (Valco, Vici C25-3180EMH, Houston, USA).

In order to guarantee reproducibility in the aspirated and propelled volumes, especially when dealing with reduced volumes, the starting position of the peristaltic pump at the beginning of each cycle was controlled. For that an NResearch 161 T031 solenoid valve (W. Caldwell, NJ, USA) (SV1, Fig. 2) and a device placed on the peristaltic pump head were also introduced in the system [15]. The solenoid valve, placed between the pump and the holding coil was activated, enabling the solutions to flow through the holding coil. At the end of the cycle, the peristaltic pump returned to the initial position. During this time, the carrier solution flowed in closed circuit by inactivation of the solenoid valve.

Irradiation of the solutions with UV light was performed using a 15W Philips TUV 15W/G15T8 low pressure mercury lamp at 253.7 nm. The photochemical reactor was implemented by coiling a PTFE tubing (90 cm and 0.8 mm i.d.) around the lamp, which was subsequently placed inside a protecting chamber. The lamp was turned on 10 min prior to measurements.

As detection system, a Jasco (Model FP2020 Plus, Japan) fluorescence detector equipped with a flow-through cell of 16 μL was used. The wavelength of excitation was 287 nm and the emission was 378 nm.

In the system, before the detector, it was placed a de-bubbler 006BT from Omnifit (Cambridge, England), to avoid that any bubble, formed in the reactor, due to the high temperature inside the protecting chamber, could come in the fluorimeter.

Dissolution studies were carried out by coupling the developed SIA system to an Erweka DT (Heusenstamm, Germany) dissolution apparatus. Sample solutions were aspirated at runtime through an inline filter.

All connections, including the reactor (90 cm) and the holding coil (2 m that was serpentine-shaped in configuration), were made with 0.8 mm i.d. PTFE tubing.

The system was controlled by a homemade programme written in QuickBasic language and implemented in a microcomputer

Table 1
SIA analytical cycle used in the determination of indomethacin in pharmaceutical formulations samples.

Step	Position	Volume (μL)	Time (s)	Flow rate (mL min^{-1})	Direction	Event
1	1	50	3	1	Aspiration	Sample
2	2	533	32	1	Propulsion	Propulsion to the reactor
3	2		25	0	Stopped	Stopped flow in the reactor
4	2	1083	65	1	Propulsion	Propulsion to the detector
5	2	1933	58	2	Propulsion	Cleaning the detector

equipped with an interface card (Advantech Corp., PCL 711B, San Jose, CA) and the analytical signals were recorded on a Kipp & Zonen BD 111 (Delft, The Netherlands) strip chart recorder.

2.3. Sequential injection procedure

The developed analytical cycle (Table 1) began with the aspiration of 50 μL of sample to the holding coil, and it was sent by flow reversal, to the reaction coil (that was placed around the ultra-violet lamp), where the flow was stopped for 25 s (step 3). Thereafter, the fluids were sent to the detector in two steps (steps 4 and 5), the first one at 1 mL min^{-1} and the second one at 2 mL min^{-1}. This last period of propulsion (step 5) was only used to clean the flow cell after the measurement of the signal.

So, in each analytical cycle, one peak was obtained and the increases of signals are proportional to indomethacin levels in the sample.

2.4. Reference methods

To assess the accuracy of the results obtained by the developed SIA system, the samples were also analyzed by the reference procedures recommended by British Pharmacopoeia [16] (for capsules) and USP 28 [17] (for extended-release capsules).

Capsules were powdered, and dissolved in water and methanol. This solution was filtered and part of this filtrate was mixed with a solution of equal volumes methanol and phosphate buffer pH 7.2. The absorbance of the final solution was determined by UV spectrophotometry at 320 nm.

Extended-release capsules were powdered, dissolved in phosphoric acid and shacked for 1 h. After that it was sonicated for 15 min, diluted with acetonitrile and sonicated again for more 15 min. Finally it was diluted in acetonitrile, and a portion of this solution was centrifuged. The supernatant was filtered and then it was analyzed by HPLC. In the chromatographic system it was used a C-18 column, the eluent was a methanol–water–phosphoric acid system and the detection was by UV spectrophotometry at 240 nm.

2.5. Dissolution studies

Dissolution studies were carried out according to the USP 28 [17]. A stirring-equipped (100 rpm) apparatus was connected to the analytical system through the sample feeding line. A mixture of 1 volume of phosphate buffer pH 7.2 and 4 volumes of water at 37.0 ± 0.5 °C was used as dissolution medium. Sample aliquots were collected at pre-set time intervals through an in-line filter and analyzed without further treatment.

3. Results and discussion

Before developing the SIA system, some preliminary batch experiments were carried out to evaluate the effect of direct UV light irradiation in indomethacin solutions. A 4×10^{-5} mol L^{-1} indomethacin standard solution was irradiated for different periods of time (0, 30, 60, and 120 s) and an increase in fluorescence was observed with irradiation time (Fig. 3).

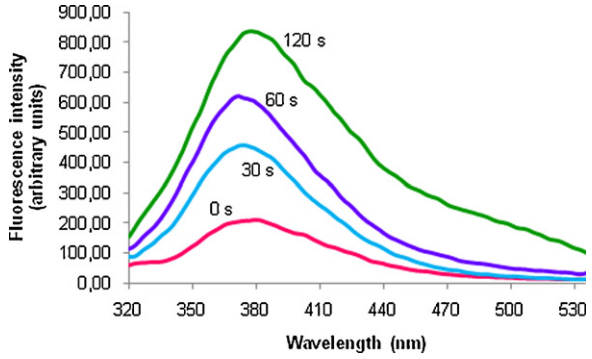

Fig. 3. Effect of irradiation time on the fluorescence intensity of indomethacin. Spectra obtained before irradiation, after 30, 60, and 120 s of irradiation of a 4×10^{-5} mol L^{-1} indomethacin standard solution.

An emission band with a maximum fluorescence of 378 nm (with an excitation of 287 nm) and with an intensity four times greater when compared to the indomethacin fluorescence band in the native form, was observed when a 4×10^{-5} mol L^{-1} solution of indomethacin was irradiated for 120 s. This occurred since the formed photoproduct has a more intense fluorescence than the native indomethacin form, possible due to the structural changes in C3 position (Fig. 1).

Since fluorescence intensity of the signals depends on sample volume and irradiation time, studies were carried out to establish the dependence of analytical signal on sample volume between 50 and 300 μL. To this end, a few calibration curves were constructed. A 21% increase in sensitivity was observed between 50 and 300 μL. However with the increase in volume, a decrease in the linear concentration range was verified. Consequently, using one or other condition it was possible to establish two different concentration ranges that could be used in different situations according to the need to use a higher sensitivity or larger concentration range. Using a volume of 50 μL, it was possible to use the system for the determination of indomethacin with concentrations between 4.10×10^{-6} mol L^{-1} and 9.00×10^{-5} mol L^{-1} while with a volume of 300 μL, the concentration range was between 3.70×10^{-6} and 2.00×10^{-5} mol L^{-1}. Determination frequency was also affected since by using 300 μL, it decreased from about 20 to 18 determinations per hour.

It was therefore decided to use a 50 μL sample volume for further studies since with a wider concentration range it was possible to apply the system for dissolution studies. With this sample volume, the sensitivity was sufficient for this application while the low consumption of dissolution medium was advantageous as it did not affect indomethacin concentration in the dissolution vessel.

The obtained linear calibration curve depended not only on the sample volume but also on the time over which the sample was irradiated. In this way, it was necessary to study the effect of flow rate, reactor length and even stopped flow time in the reactor, since they were interfering directly in the irradiation time of the sample. Therefore, the effect of reactor length was studied using the same irradiation time. Two reactor lengths were studied of respective lengths 180 cm and 90 cm, the latter of which incorpo-

Table 2
Results obtained by the proposed SIA methodology and the comparison batch methodologies for the determination of indomethacin in pharmaceutical formulations samples and the respective relative deviation percentages (RD, %).

	Batch	Indomethacin (mg formulation^{-1})			RD (%)
		Declared	SIA	Comparison	
Indocid (capsules)	1	25	24.19 ± 0.36	23.70 ± 0.14	+2.06
	2		24.31 ± 0.34	23.76 ± 0.09	+2.31
Indocid retard (extended-release capsules)	1	75	77.51 ± 0.16	74.63 ± 0.22	+3.86
	2		74.62 ± 0.29	72.43 ± 0.22	+3.02

rated a stopped flow period to provide the same residence time as that offered by the 180 cm reactor. In this study it was observed that the sensitivity increased by 39% when the 90 cm reactor was accompanied by a stopped flow period. Therefore, having established this reactor, different stopped flow periods were evaluated up to 27 s, using 50 μL of an indomethacin standard solution of 9×10^{-5} mol L^{-1} and a flow rate of 1 mL min^{-1}. It was shown that the signals increased with time up to 25 s before remaining stable thereafter. Consequently, a 25 s stopped flow period was chosen for the sample irradiation.

As flow rate also influences the sample residence time in the reactor, flow rates between 1 and 2 mL min^{-1} were tested. Results confirmed that a flow rate of 2 mL min^{-1} led to a decrease in sensitivity as residence time in the reactor was reduced. This decrease was about 15% when compared to a flow rate of 1 mL min^{-1}. This flow rate of 1 mL min^{-1} was used as a compromise between sensitivity and determination frequency. However, to minimize the time spent to propel the sample from the reactor to the waste and through the detector, this propulsion time was divided into two steps. The first involved a flow rate of 1 mL min^{-1} and included the propulsion of the sample from the reactor to the detector. In the second step, a flow rate of 2 mL min^{-1} was used to clean the detector. In this phase this higher flow rate did not influence the signal amplitude as it took place during the propulsion of the sample to the detector and allowed a 32% increase in the determination frequency when compared to the 1 mL min^{-1} flow rate.

Having optimized all of these parameters, the increase in sensitivity caused by the UV light in the SIA system was evaluated. A pronounced increase in sensitivity (56%) was observed when the sample was subjected to UV radiation.

The developed methodology was evaluated for indomethacin concentrations up to 9×10^{-5} mol L^{-1}. The calibration curve IF = $(150.2 \pm 2.7) \times 10^2$ Conc. (mol L^{-1}) + $(1.8 \pm 1.4) \times 10^{-2}$ with 95% confidence limits for the intercept and slope was obtained. Detection and quantification limits of 1.23×10^{-6} and 4.10×10^{-6} mol L^{-1} were calculated as the concentrations corresponding to the intercept plus three and ten times $S_{y/x}$ [18], respectively. Determination frequency obtained with the developed methodology was around 20 determination h^{-1}.

3.1. Interferences

With a view to applying the developed procedure to the analysis of pharmaceutical formulations, the interfering effect of some compounds used as excipients in capsules and extended-release capsules was assessed. 4×10^{-5} mol L^{-1} indomethacin standard solutions containing increasing amounts of the excipient were analyzed by the developed flow methodology. A compound was considered as interfering if the analytical signal variation was ±3% compared to that obtained in its absence. It was verified that up to a 100 molar ratio, excipient/indomethacin compounds such as lactose, magnesium stearate, cornstarch, sucrose, hydroxypropylmethylcellulose, microcrystalline cellulose and silica did not interfere.

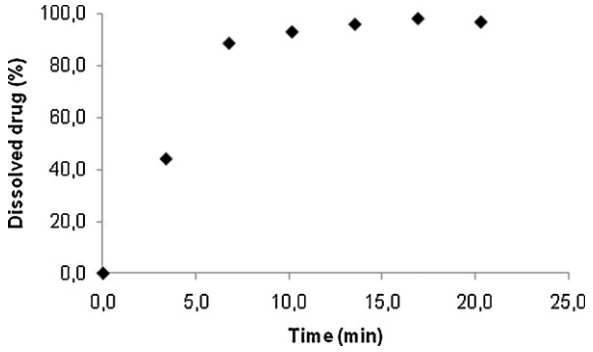

Fig. 4. Dissolved drug during the time in the dissolution studies of capsules of indomethacin.

3.2. Analysis of pharmaceutical formulations

The developed methodology was applied to the determination of indomethacin in pharmaceutical formulations such as capsules and extended-release capsules.

To evaluate the accuracy of the proposed system, the samples were analyzed (Table 2) according to the SIA and reference batch methods and the relative deviations were calculated. It was verified that these were lower than 4%, confirming the accuracy of the system.

Agreement between both methods was also evaluated using the *t*-test, carried out as a comparison of two experimental means [18]. The calculated *t* values were 1.46, 5.09, 12.22 and 6.95 for capsules (batch 1 and 2) and extended-release capsules (batch 1 and 2), respectively. The tabulated *t* value of 12.71 when compared to the calculated *t* values, showed the absence of statistical differences for those results obtained by the methodologies at the 95% confidence level.

The relative standard deviations (RSD%) of 2.50 and 2.42% were calculated from ten consecutive sample injections with concentrations of 2.22×10^{-5} and 7.80×10^{-5} mol L^{-1}. Therefore, the methodology showed a good repeatability, confirming its applicability within a distinct concentration range.

3.3. Dissolution studies

For the dissolution studies, the dissolution apparatus was connected to the system and it was verified that all of the previously optimized analytical parameters (including the aspiration flow rate) were adequate for the dissolution studies and did not require any adjustment.

The capsules' dissolution profiles resulting from periodic measurements showed an increase in indomethacin concentrations in the dissolution medium with time (Fig. 4) which approached stabilization.

It was also observed that not less than 80% of the labeled amount of indomethacin was dissolved in 20 min, being in accordance with the condition imposed by USP28 [17].

With the use of a SIA system, it was possible to carry out multiple successive determinations to establish the dissolution profile without affecting the concentration in the dissolution vessel, since the solution consumption was only 215 μL (50 μL for the determination and 165 μL to fill the valve port before the determination) and the total volume of dissolution medium was 750 mL.

4. Conclusions

Sequential injection analysis in conjunction with photochemistry was for the first time applied to the determination of indomethacin. The use of the photoreactor permitted sensitivity to be increased since the fluorescence signal increased with irradiation time. Therefore, the use of the photoreactor and fluorimetric detector allowed the linear concentration range to be extended and sensitivity to be increased, making it possible to apply the system not only to the indomethacin concentrations in pharmaceutical formulations but also to dissolution tests. The obtained results were reliable and in agreement with those obtained by the comparison methods. However, the developed methodology has some advantages when compared to the conventional methodologies. The method does not consume any reagent beyond that required for indomethacin dissolution, since the reaction is performed by the UV light. The sample frequency was higher, especially when compared to the reference methodology that USP [17] recommend for extended-release capsules which spend 60 times more time than the developed methodology. When compared with other methodologies that use SIA systems to determine indomethacin [12–14], the developed methodology reduces reagent consumption and/or avoids the use of some toxic solvents.

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