A pulsed sequential injection analysis flow system for the fluorimetric determination of indomethacin in pharmaceutical preparations


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Abstract

This work reports the development of a pulsed flow sequential injection analysis (SIA) system based on the utilisation of pulse generating solenoid micro-pumps, which replace the conventional solutions propelling units commonly used in SIA systems. The influence on sample/reagent zones interpenetration and reaction zone formation of the reproducible pulsed flow produced by micro-pumps operation, which is characterised by a chaotic solutions movement, was comparatively evaluated with the typical laminar flow conditions of conventional SIA systems.

The enhanced sample/reagent mixing provided by the controlled pulsed flow was favourably applied in the implementation of a SIA methodology for the fluorimetric determination of indomethacin in pharmaceutical preparations upon alkaline hydrolysis in micellar medium. Linear calibration plots were obtained for indomethacin concentrations up to $10^{-5}$ mol l$^{-1}$ with a $3\sigma$ detection limit of about $1.6 \times 10^{-8}$ mol l$^{-1}$.

The obtained results complied with those furnished by the reference procedure ($RD \leq 2.3\%$) and exhibited good reproducibility (RSD < 1.2%, $n = 15$). Sampling rate was about 30 samples per hour.

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1. Introduction

The solutions propelling unit is a fundamental component of a flow system since it determines the characteristics of the flowing stream affecting solutions movement within tubing and thus sample and reagent inter-mixing. Flow techniques, such as flow injection analysis (FIA), sequential injection analysis (SIA) and multi-commutation systems, employ similar propelling units depending the selection of a particular equipment, like peristaltic pumps, syringe pumps, piston pumps, gas pressurized reservoirs, gravity-based propelling units and electro-osmotic flow-based pumps, on the manifold configuration, number of reagents involved, flow characteristics, etc. Peristaltic pumps are probably the most common choice due to its robustness and versatility [1].

However, despite evident advantages, peristaltic pumps exhibit well-known limitations that are primarily associated to indiscriminate or erratic flow oscillations (sometimes considered as a pulsating flow [2]) as a consequence of the inherent mode of operation or the loss of elasticity of the tubing. This un-controlled pulsating flow is particularly problematic when dealing with techniques that involve short pumping periods or rapid valve switching and has been minimised either by using synchronisation devices [3] or by periodical tubing replacement. Syringe and piston pumps are very reliable and consistent units capable to generate perfectly controlled pulseless flowing streams. However, they are expensive and require a periodical re-filling that affects the sampling rate [1]. The remaining types of propelling units are scarcely applied to specific situations and their utilisation is relatively restricted.

Solenoid micro-pumps are one of the latest developments in terms of propelling units [4]. They are low-cost, robust and
reliable devices characterised by producing an easily controlled reproducible pulsed flowing stream, which has been recently considered, and in opposition to the more widespread tendency, a noteworthy advantage for the implementation of analytical flow systems [2,5–8].

Unlike conventional SIA methodologies, which make use or demand smooth, consistent flowing streams, in this work a SIA system based on a pulsed flow pattern produced by two solenoid micro-pumps, was implemented. The solenoid micro-pumps responsible for solutions propelling, both in aspiration and propulsion mode, generated bursts of solution flow associated to the sudden micro-pump diaphragm actuation, which induced a chaotic movement of the solutions in all directions that contributed to a faster and more efficient mixing of the stacked sample and reagent zones [9], favouring reaction development.

The influence of solenoid pumps operation in the overall system performance, namely in terms of the hydrodynamic characteristics of the produced pulsed flow, was carried out by detailed study of zone penetration both by spectrophotometry detection (using a dye solution) and by fluorometric detection (using a fluorescent quinine solution). In a final stage the developed SIA system was used to implement a novel analytical procedure for the determination of indomethacin in pharmaceutical preparations upon alkaline hydrolysis in micellar medium and fluorometric detection of the formed products (λex = 278 nm; λem = 358 nm).

Indomethacin (1-(p-chlorobenzyloxy)-5-methoxy-2-methyl-3-indolylacetic acid) is a methylated indol derivative with anti-inflammatory, analgesic and antipyretic activity comparable to that of salicylate, extensively used in the treatment of rheumatoid arthritis and other rheumatic diseases [10]. The determination of indomethacin in pharmaceutical formulations has been carried out by chromatography [11], spectrophotometric [12–14], fluorimetric [14], potentiometric [15] and voltammetric methods [16]. Most of available methodologies for indomethacin determination are either time-consuming, required specific and expensive equipment or laborious sample manipulations that affected sampling rate, or required a rigorous operator intervention that could affect precision and accuracy. The development of an automated methodology for indomethacin determination based on a SIA flow system was aimed mainly at the reduction of solutions consumption and waste generation, minimisation of operator intervention and the attainment of increased sample throughput, reproducibility and accuracy.

2. Experimental section

2.1. Reagents and solutions

All chemicals were of analytical reagent grade and solutions were prepared with deionised water (conductivity < 0.1 μS cm⁻¹).

Bromothymol blue stock solution was prepared by dissolving 0.400 g of dye in 25 ml of 96% ethanol, making the final volume up to 100 ml with 1 × 10⁻² mol l⁻¹ borax solution. Working solutions were then prepared by mixing 1 ml of the stock solution with 199 ml of 1 × 10⁻⁴ mol l⁻¹ borax solution [17]. For fluorimetric assays a 10 mg l⁻¹ quinine solution was prepared in deionised water.

Indomethacin (Sigma) working standards were prepared by dilution of the 1 × 10⁻⁴ mol l⁻¹ stock solution in a 20 mmol l⁻¹ hexadecyltrimethylammonium bromide (CTAB) solution (Fluka). A 0.1 mol l⁻¹ NaOH (Merck) solution was prepared by dissolving the required amount in deionised water.

Sample solutions (Dolovin, tablets 25 mg; Indocid, capsules 25 mg; Indocid retard, extended release capsules 75 mg) were prepared by dissolving the required amounts of powdered preparations in a 20 mmol l⁻¹ CTAB solution. For each preparation, 20 capsules or tablets were weighed and a mean content of a single capsule or tablet was estimated in order to prepare a solution with an approximately know amount of indomethacin.

2.2. Instrumentation

Spectrophotometric measurements were performed in an UV/Vis Jenway 6300 spectrophotometer equipped with an 8 or 80 μl Helma flow-cell (Mulheim/Baden, Germany).

For the fluorimetric determinations (λex = 278 nm; λem = 358 nm) a LabAlliance Fluorescence detector LC 305, equipped with an 8 μl flow-cell was used.

The analytical SIA flow system (Fig. 1) comprised a 10-port multi-position Vici Valco selection valve (MV) and two self-priming solenoid operated fixed displacement diaphragm micro-pumps (P1, P2). Depending on the evaluation studies carried out these dispersed 5 μl per pulse (090SP12-5 Bio-chem Valve Inc., Boonton, USA); 8 μl per pulse (090SP12-8 Bio-chem Valve Inc., Boonton, USA) or 25 μl per pulse (120SP12-25 Bio-chem Valve Inc., Boonton, USA). In the final manifold configuration used for indomethacin determination, the 8 μl type was used.

![Diagram of analytical pulsed flow SIA system](image-url)
The automated SIA flow system was controlled by means of
a Pentium-I based microcomputer equipped with an Ad-
vantech PCL 711B interface card. Solenoid micro-pumps
were operated by means of a CoolDrive<sup>TM</sup> power drive from
NRresearch Inc. The software was developed in QuickBA-
sic 4.5 (Microsoft) and permitted to operate the solenoid
micro-pumps and multi-position valve enabling the run-time
definition of all analytical parameters like flow rate, flow di-
rection, sample volume, reagent volume, valve position,
etc., as well as data acquisition and processing.

Sample analysis according to the reference method rec-
ommended by the U.S. Pharmacopoeia (USP 24) [18], was
carried out for delayed-release formulas in a Merck Hitachi
Lachrom Liquid Chromatograph, equipped with a L-7455
Diode Array detector, a L-7100 pump and a D-7000 interface
card, and for tablets and capsules in a UV/VIS Perkin-Elmer
Lambda 45 (λ = 318 nm) spectrophotometer.

2.5. SIA manifold

The SIA manifold, pictured in Fig. 1, was designed in a
conventional configuration. However, the typical peristaltic
or syringe pumps were replaced by two solenoid micro-
pumps (P<sub>1</sub> and P<sub>2</sub>) that were connected through a Y-shaped
confluence point (Y). These solenoid micro-pumps were uni-
directional and for this reason they were placed in opposing
positions: P<sub>1</sub> had its inlet point attached to the confluence
point and was responsible for solutions (reagent and sam-
ple) aspiration, via the multi-position valve, into the holding
coil, while P<sub>2</sub> was attached through its outlet point and was
in charge of carrier solution insertion and reaction zone pro-
PELLING towards detection. The inclusion of the confluence
point and the fact that the solenoid pumps were independently
actuated represented an important advantage regarding con-
ventional SIA systems because the aspiration process was
separated from the propulsion process, which allowed pre-
vening any potential contamination between the aspirated
solutions and the carrier solution. Consequently, it was pos-
sible to use shorter holding coils than those typically used in
SIA systems (usually longer than 2 m) which resulted in a sig-
nificant solutions saving. Moreover, this configuration could
be exploited as a versatile dilution strategy for the analysis
of highly concentrated samples. Effectively, it is possible to
insert a given sample volume, mix it with the reagents and
then reject a selected portion of the formed reaction zone
through P<sub>1</sub> (during the aspiration stage). This way, only the
reaction zone section that remained within the holding coil
would be re-sampled (by means of P<sub>2</sub> in propulsion mode)
and sent towards detection. This approach enabled the attain-
ment of a wide range of dilution levels, without manifold
re-configuration, which would depend on the fraction of the
reaction zone that is rejected.

2.4. Analytical cycle for the determination of
indomethacin in pharmaceutical formulations

The developed flow system for indomethacin determina-
tion made use of two 8 µl per stroke solenoid micro-pumps.
The operational pulsed mode of the pumps conditioned the
strategy used for the optimization studies as distinct param-
ters like sample volume and reagent volume could be con-
trolled both in terms of number of pulses (defining a final
volume that was always a multiple of the pump stroke vol-
ume) or a time-based counting, while flow rate was controlled
in terms of pulse frequency.

The analytical cycle started when a pulse (8 µl) of NaOH
solution was inserted between two segments of sample (3
pulses–24 µl each) by means of P<sub>1</sub>, the multi-position valve
enabling the selection of the appropriate solutions. Then, by
actuation of P<sub>2</sub> and by moving the selection valve to the de-
tector’s position, the formed reaction zone was carried out to
detection where it produced an analytical signal whose mag-
nitude was proportional to the indomethacin concentration.

2.5. Reference method

Aiming at comparing the results furnished by the devel-
oped procedure, samples were as well analysed by using
the reference procedure recommended by USP 24 [18]. De-
layed release capsules were powdered, dissolved in phospho-
ric acid and acetonitrile, filtered and analysed by HPLC. In
the chromatographic system was used a C-18 column and
the eluent was a methanol–water–phosphoric acid system
[18]. Detection was by UV spectrophotometry at 240 nm.
Tablets and capsules were powdered, dissolved in methanol,
extracted with dichloromethane and the absorbance of the fi-
nal solutions was determined by UV spectrophotometry at
318 nm.

3. Results and discussion

3.1. Evaluation of the zone penetration degree within a
pulsed flowing stream

Solenoid micro-pumps actuation generates a pulsed flow-
ing stream that markedly affects the type and extension of
solutions intermixing. The successive repetition of swift di-
aphragm movements, producing a high instantaneous flow
rate, and very short resting periods, induces a chaotic move-
ment of the solutions that influence the dispersion level at-
tained [19]. In-depth dispersion studies were carried out to
evaluate this effect on a SIA manifold and to compare the
mixing features of a pulsed flow regarding the typical lami-
nar flow of conventional SIA systems. As the key operations
of the SIA systems are based on zone sequencing and mu-
tual dispersion of the zones, two fundamental parameters S<sub>f</sub>
and P [20] were assessed and used as comparative purposes
in this study.
The sample ($S_v$) and reagent ($R_v$) volumes, whether water or BTB, were identical in both assays. The extension of zones overlapping of the two assays allowed the calculation of $P$ (Fig. 2). These assays were repeated for distinct $S_v$ and $R_v$ values as well as for distinct $S_v/R_v$ ratios (Table 1).

The dispersion studies also contemplated three zones reactions obtained through the insertion of a spacer solution (variable water volumes) between the reagent and the sample zones. The obtained results (Table 1) showed that zone penetration increased (higher $P$ values) when a pulsed flow was used, and that the utmost values were obtained with the lowest pulse volume, which could be explained by the fact that a 5 µl per stroke micro-pump required an increased number of pulses to propel the same volume. Furthermore, even the zone overlapping obtained with a 25 µl per stroke micro-pump was greater than the one obtained with a peristaltic pump.

A parameter that in conventional SIA systems markedly affects dispersion is the length of the reaction loop [20]. By assaying the distinct stroke volume micro-pumps (5, 8 and 25 µl) and reactors with increasing length we have inves-

![Fig. 2. Penetration of sample (S) and reagent (R) zones, using a 25 µl micro-pump and an 8 µl flow cell, with spectrophotometric detection and an 1 m reaction cell. (A) $R_v = S_{0.5}$; $P = 0.561$. (B) $R_v = S_1$; spacer = 0.25 $S_{0.5}$; $P = 0.886$.](image)

### Table 1

<table>
<thead>
<tr>
<th>Conditions of the analysis</th>
<th>SIA system with pulsed flow (5 µl)</th>
<th>SIA system with pulsed flow (8 µl)</th>
<th>SIA system with pulsed flow (25 µl)</th>
<th>SIA system with laminar flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 µl flow cell, RC 1 m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_v = R_v = 0.25 S_{0.5}$</td>
<td>140</td>
<td>0.947</td>
<td>180</td>
<td>0.936</td>
</tr>
<tr>
<td>$S_v = R_v = 2.5 S_{0.5}$</td>
<td>0.710</td>
<td>0.690</td>
<td>0.878</td>
<td>0.875</td>
</tr>
<tr>
<td>$R_v = S_{0.5}$</td>
<td>0.888</td>
<td>0.878</td>
<td>0.844</td>
<td>0.833</td>
</tr>
<tr>
<td>$R_v = S_{0.5}$; $S_v = 2.5 S_{0.5}$</td>
<td>0.654</td>
<td>0.643</td>
<td>0.569</td>
<td>0.560</td>
</tr>
<tr>
<td>$S_v = R_v = S_{0.5}$; Spacer = 0.25 $S_{0.5}$</td>
<td>0.734</td>
<td>0.690</td>
<td>0.646</td>
<td>0.666</td>
</tr>
<tr>
<td>$S_v = R_v = 0.25 S_{0.5}$; Spacer = 5.5 $S_{0.5}$</td>
<td>0.163</td>
<td>0.303</td>
<td>0.292</td>
<td>0.290</td>
</tr>
</tbody>
</table>

8 µl flow cell

| $S_v = R_v = 2.5 S_{0.5}$ | 120 | 1 | 129 | 1 | 125 | 0.950 | 122 | 0.863 |
| $S_v = R_v = 2.5 S_{0.5}$ | 0.735 | 0.690 | 0.900 | 0.666 | 0.860 |
| $R_v = S_{0.5}$; $S_v = 0.25 S_{0.5}$ | 0.974 | 0.970 | 0.800 | 0.880 | 0.823 |
| $R_v = S_{0.5}$; $S_v = 2.5 S_{0.5}$ | 0.190 | 0.680 | 0.684 | 0.684 | 0.559 |
| $S_v = R_v = S_{0.5}$; Spacer = 0.25 $S_{0.5}$ | 0.776 | 0.775 | 0.689 | 0.666 |
| $S_v = R_v = S_{0.5}$; Spacer = 2.5 $S_{0.5}$ | 0.373 | 0.333 | 0.307 | 0.258 |

$S_v$: sample volume; $R_v$: reagent volume; Spacer: water.
that the attained $P$ values. The obtained results showed that when a laminar flow was used $P$ was not significantly affected by the presence of a reaction coil. However, when operating under a pulsed flow (independently of the stroke volume used) the value of $P$ decreased as the coil length increased. This could be explained by an attenuation of the pulse effect as a consequence of the increment of the analytical pathway between the propelling unit and detector. In view of this results, when implementing a fast chemical reaction in a pulsed SIA system the reaction coil could be suppressed, which would represent an important advantage in terms of solutions saving, waste generation and sampling rate. On the other hand, when dealing with a slow reaction the reaction coil or flow strategies to increase the residence time, as is the case of a stopped flow approach, would have to be used.

The influence of detector flow cells with inner volumes between 8 and 80 μl was also investigated. It was verified that $P$ decreased as the cell volume increased, which could be explained by a pronounced increase in dispersion as the flow cell would act as a dilution chamber.

The evaluation of the influence of reversing successively the flow direction, when using a pulsed flow, was carried out by using a sample and reagent zone separated by a spacer solution with a 1.5 $S_{1/2}$ volume. By assaying up to 4 flow reversals the obtained results confirmed the results of Gubeli et al. [20], which demonstrated that the most significant effect on the degree of zone penetration was due to the first flow reversal. In effect, identical relationships between $P$ and $S_{1/2}$ values were obtained for the developed and for a conventional SIA system either with spectrophotometric (with DTB) or fluorimetric detection (with a quinine solution).

3.2. Optimisation of chemical and physical parameters of a pulsed flow system for fluorimetric determination of indomethacin

Indomethacin does not exhibit native fluorescence and is relatively stable at neutral pH. However, in alkaline medium indomethacin is easily hydrolysed due to the reactive OH$^-$ of the amine bond, which permits to efficiently carry out its fluorimetric determination by monitoring the formed fluorescent compound [21]. Moreover, a fluorescence enhancement is attained when an organised medium is used providing a microenvironment that favours both reaction development and fluorescence intensity of reaction products [21,22]. Aiming at implementing a pulsed flow SIA approach for fluorimetric indomethacin determination the influence of several surfactants, either neutral (Triton X), cationic (hexadecyltrimethylammonium bromide-CTAB), anionic (sodium dodecyl sulfate-SDS) or zwitterionic (hexadecylphosphocholine-HDPC), at concentrations above the critical micellar concentration (cmc), were evaluated. It was verified that with increasing concentrations of Triton X, SDS and HDPC the reaction kinetics decreased, which suggests a protective effect of the micelles on the alkaline degradation of indomethacin. On the other hand, CTAB exhibited a catalytic effect resulting in increased fluorescence intensity. A CTAB concentration of 20 mmol l$^{-1}$ was selected for the posterior experiments since solutions of higher concentration although providing improved analytical signals were of difficult manipulation due to the formation of bubbles within the flow system.

Reaction kinetics was also affected by alkali concentration which had a pronounced impact in the attained fluorescence intensity. Since one of the primarily objectives of the developed automated methodology was the enhancement of sample throughput and considering that the measurements in a continuous flow methodology are carried out in non-equilibrium conditions, the study of the hydrolysis reaction kinetics involved the assay of a wide range of NaOH concentrations from 0.05 to 1 mol l$^{-1}$. A concentration value of 0.1 mol l$^{-1}$ was considered the most appropriate, and thus selected for the posterior experiments, because above this value fluorescence intensity markedly declined probably as a consequence of the auto-absorption effect of the formed reaction products.

Following the selection of the most favourable chemical reaction conditions, the evaluation of the influence of several physical manifold parameters was also investigated. The appraisal of the most convenient propelling units dictated the selection of 8 μl rather than 5 μl per stroke micro-pump as the later, although yielding higher $P$ values, would have to be operated at a higher frequency (for the same flow rate), which affected reproducibility. Besides, considering the upper operational pulse frequency the workable flow rates range was narrower.

One minor limitation associated with the utilisation of solenoid micro-pumps for solutions aspirations and/or propelling was that the sample or reagent volumes introduced in the analytical system were always multiple of the micro-pump stroke volume. Accordingly, the evaluation of the influence of sample volume, a very important parameter in a flow methodology, was carried out by using sample volumes between 16 and 64 μl (corresponding to 2–8 sample pulses). Although higher sample volumes could have been used, assuring a sensitivity improvement, it was verified that the upper limit of the linear concentrations range was significantly abridged by using sample volumes higher than 64 μl, which could be probably explained by a reagent shortage in the element of fluid situated at the peak maximum. A proportional reagent increase could have solved this problem, but the concomitant analytical signal increment would result in a pronounced increase in the sample and reagent consumption. Accordingly, it was decided to use a 48 μl sample volume. The evaluation of the influence of NaOH volume was carried in the 8–32 μl range by inserting 1–4 NaOH pulses. It was observed that a reagent volume higher than 8 μl had no influence on reaction development acting simply as a dilution factor. On the other hand, and taking into account the dissimilarity of viscosity between the sample and the NaOH solutions, it was decided to take advantage of the pulsed nature of the flowing stream by creating two reaction zones, which would
3.3. Analysis of indomethacin pharmaceutical formulations

After system optimisation linear calibration plots for indomethacin concentrations up to $10^{-3}$ mol $\text{l}^{-1}$ were obtained. The determination detection limit, estimated as three standard deviation, was $1.6 \times 10^{-8}$ mol $\text{l}^{-1}$.

By multiple analysis of indomethacin solutions of two distinct concentrations ($4 \times 10^{-7}$ and $4 \times 10^{-8}$ mol $\text{l}^{-1}$) the developed methodology exhibited a good reproducibility with relative standard deviation (RSD%) lower than 1.2% ($n=15$), confirming its applicability in distinct concentrations range.

The results obtained with the proposed flow procedure were comparatively evaluated with those furnished by the reference methodology recommended by USP 24 for the analysis of capsules and tablets. No significant disparity was obtained between both methods with relative standard deviations, expressed in percentage, (RSD%) lower than 2.3% (Table 2). This resemblance was confirmed by a paired $t$-student test, which, for a 95% confidence level, showed no significant statistical differences between the results furnished by both methods ($t$ calculated = 0.626; $t$ tabulated = 4.302). Sampling rate was about 30 samples per hour.

The analytical flow system was robust and stable, and no baseline drift was observed. Micro-pumps operational features remained unchanged over an extended utilisation period, which confirmed their long-term lifetime even considering that they were actuated at pulse frequencies as high as 150 min$^{-1}$. The consumption of reagents per determination was about 32 μg of NaOH and 35 μg of CTAB.

The proposed flow system exhibited operational characteristics similar to those of a conventional SIA system, even considering that the features and the mode of operation of the propelling unit consisting on two solenoid micro-pumps were considerably different than that of more conventional peristaltic or syringe pumps. Moreover, it showed evidence of a great robustness, versatility, simplicity and easy of operation. However, in what could be considered a decisive diverging aspect, the utilisation of solenoid micro-pumps as propelling units generated a pulsed flow that revealed, both on the zone penetration studies and in the analysis of pharmacu...
tical preparations, an enhanced sample/reactant intermixing aptitude that resulted in a significant increment in reaction zone homogenization, particularly in the case of low stroke volume micro-pumps.

The pulsed nature of the flowing stream could assume a great importance in the management of viscous solutions. Usually, solutions of high viscosity disturb the general behaviour of a flow system because they markedly restrain solutions mixing originating pronounced concentrations gradients. This problem is commonly surpassed by resorting to complementary strategies to improve sample dispersion, as is the case of the utilisation of dilution chambers or flow reversal, which normally compromised the sample throughput. In this sense, the developed pulsed SIA system could represent a valuable alternative for all situations requiring enhanced mixing conditions.

The developed analytical methodology applied in the determination of indomethacin in pharmaceutical preparations could be considered an advantageous alternative to other already available proposals [11–16] including the recommended method of USP 24 [18], because it employs a flow manifold with a very simple configuration, it enables fast sample determinations without the need for extensive sample manipulations and it provides a wide working analytical range with good reproducibility and high sampling rates. Moreover, reagent consumption was significantly reduced as multiple pulses were not needed to propel the reaction zone to the detector, and less solution was required for flushing.

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