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Sequential injection system for phospholipase A_2 activity evaluation: Studies on liposomes using an environment-sensitive fluorescent probe

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ABSTRACT

This work reports the development of an automatic methodology based on the use of 1-anilinonaphthalene-8-sulfonate (ANS) as an interfacial fluorescent probe for detecting the hydrophobic environment shift around the probe, caused by the hydrolytic action of PLA_2 on the liposomes. The implementation of this reaction in a sequential injection analysis (SIA) system along with the use of the mixing chambers permitted the evaluation of PLA_2 activity and assessment of the inhibitory effect of the non-steroidal anti-inflammatory drugs (NSAIDs) on PLA_2 activity.

Several studies were performed with the aim of establishing the appropriate flow system configuration: the liposome substrate; PLA_2 and ANS optimum concentrations and incubation times before and after the enzyme addition. Based on these studies, the optimum reaction conditions were selected. It was shown that PLA_2 is effectively inhibited by the NSAIDs tested (meloxicam, tolmetin and ibuprofen) and by the α-lipoic acid, used as a positive control.

Results obtained from the flow system are in agreement with those provided by the comparison batch procedures. The proposed methodology is in fact more efficient and rapid than the comparison batch experiments, enabling the exact timing of fluidic manipulations and precise control of the reaction conditions.

1. Introduction

Phospholipase A_2 (PLA_2) hydrolyses the membrane phospholipids [1–3], and plays a prominent role in physiological processes which include the biosynthesis of mediators involved in pro-inflammatory response [1]. Therefore, investigating inhibitors of the PLA_2 enzymatic process is of great interest in the therapy of inflammatory diseases [2]. Among the potential inhibitors of PLA_2 activity are the non-steroidal anti-inflammatory drugs (NSAIDs) which represent a wide group of molecules employed in the treatment of fever, pain and inflammation [3].

Various batch methods have been established for screening inhibitors of PLA_2 activity [4–17]. The most commonly used phospholipase assays are the radioactive TLC and the pH-stat assays [4,18]. The TLC assay is probably the most accurate, sensitive assay available. However it carries several disadvantages of being discontinuous, tedious and expensive. In other hand the pH-stat assay is often employed, however it lacks sensitivity. Spectrophotometric methods can also be used, but the sensitivity is also reduced and determined by the spectrophotometer used to carry out the experimental measurement and there is also an important drawback of using substrates that are not commercially available [16]. In view to this, despite the many assay choices available, the search continues for a convenient, generally applicable assay that is both sensitive and continuous. Of the methods that allow continuous monitoring of PLA_2 activity, the most sensitive are those that use fluorescence [11]. However to the best of our knowledge, studies on the potential inhibition of PLA_2 activity by NSAIDs using 1-anilinonaphthalene-8-sulfonate (ANS), a fluorescent probe, have not been explored.

It has been well established that although PLA_2 is water soluble, it has a catalytic preference for aggregated phospholipid substrates such as micelles, monolayers or liposomes and functions best at the water–phospholipid interface [19,20]. Accordingly, in the proposed work, unilamellar liposomes (LUV) of egg-yolk phosphatidylcholine (EPC) were used as PLA_2 enzyme substrate given that these model systems seem to mimic the interfacial character of the biomembranes [21].

Both the lipid hydrolysis by the PLA_2 enzymatic process and the potential inhibition of this process by NSAIDs are monitored with the ANS probe, because apart from its interfacial location in the lipid membranes, this probe has additional advantages as a sensitive indicator of conformational changes in the membranes [22,23]. Indeed, ANS has high fluorescence intensity when bound to lipid membranes. However when the PLA_2 enzyme binds to the membrane interface, it hydrolyses the *sn*-2 ester bond of the membrane phospholipids to hydrophilic hydroxyl acidic groups. This will in

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turn change the environment around the ANS probe, leading to a decrease in fluorescence intensity. Therefore, if the studied compounds are capable of inhibiting PLA_2 activity, the environment around the ANS probe will remain hydrophobic and the fluorescence of the probe will either not decrease or alternatively, undergo a smaller increase.

The NSAIDs tested as potential inhibitors represent different chemical classes, namely arylpropionic acid (ibuprofen), indole (tolmetin) and oxicam derivatives (meloxicam), are monitored with the ANS probe. The naturally occurring α-lipoic acid (ALA) is also used as a positive control of the assay system since it was previously reported as a PLA_2 inhibitor [24].

With batch methodologies it is difficult to ensure reproducibility in rigorous time control within all samples. Consequently, the investigation of the effect of NSAIDs on the catalytic activity of PLA_2 would clearly benefit from a rapid, precise and exact time control of fluidic manipulations as well as reaction conditions by automated flow-through methods which are not yet available. Sequential injection analysis (SIA) was regarded as the automation resort since the exact metering of small volumetric volumes and economical characteristics inherent to this technique, are essential for the purposes of this work [25]. In this sense, the work presented here is aimed at for the first time, automating the fluorimetric evaluation of PLA_2 hydrolytic action and the anti-inflammatory effect of the drugs on its activity, using liposomes as substrate and the ANS probe for monitoring the interfacial reaction.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical reagent grade with no further purification. Water from a Milli-Q plus system with specific conductivity less than 0.1 $\mu S\,cm^{-1}$ was used throughout.

Non-steroidal anti-inflammatory drugs (NSAIDs: meloxicam, tolmetin and ibuprofen), L-α-phosphatidylcholine from egg yolk (EPC) and lyophilized powder of Bee Venom phospholipase A_2 (EC 3.1.1.4) (PLA_2) were purchased from Sigma–Aldrich. ANS was obtained from Molecular Probes. ALA was purchased from Fluka while methanol was obtained from Fisher Scientific. Tris(hydroxymethyl)aminomethane and sodium chloride were purchased from Riedel-de-Haën. Calcium chloride dihydrate was obtained from Merck.

The buffer solution was prepared with 10 mM tris(hydroxymethyl)aminomethane, 150 mM sodium chloride and 1 mM calcium chloride dihydrate and the pH was adjusted to 8.9 through addition of diluted hydrochloric acid solution. A stock solution of the ANS probe (1200 μM) was prepared in buffer containing 1% (v/v) methanol and was stable for at least 2 days at room temperature. The EPC (3000 μM) stock solution was stored in the buffer solution at 4 °C. Finally, a 5 U/mL PLA_2 solution was prepared daily in buffer after being defrosted at 25 °C and it was kept on ice during the analysis. NSAIDs solutions and the ANS and EPC working solutions were prepared daily in buffer. All solutions were protected from light exposure throughout use.

2.2. Preparation of liposomes

Liposomes were prepared by adapting the thin film hydration method as previously reported [26,27]. A known amount of EPC was dissolved in chloroform–methanol (9:1), the organic solvent was evaporated under a stream of nitrogen and the lipid film formed was then left under vacuum to remove traces of the organic solvents. Hydration of the thin lipid film was made by adding buffer. The hydrated lipid film was vortexed above the phase-transition temperature (room temperature) to yield a homogeneous white suspension of multilamellar liposomes (MLV) which were then extruded through polycarbonate filters of 100 nm pore size (Nucleopore) to form unilamellar liposomes (LUVs). EPC concentration in liposome suspensions was determined by phosphate analysis using the phosphomolybdate method [28].

2.3. Flow manifold and instrumentation

The analytical SIA system (Fig. 1) comprised a Gilson Minipuls 3 (VilliersleBel, France) peristaltic pump equipped with a 1.30 mm i.d. Gilson PVC pumping tube. This pump was connected to the central channel of a 10-port, electrically activated selection valve (Fig. 1, SV) (Valco, Vici C25-3180D, Houston, USA). Two perspex-mixing chambers (Fig. 1, MC 1 and MC 2) of ca. 350 μL volume (including a magnetic stirrer bar) connected to available inlets of the selection valve were placed on magnetic stirrers with the temperature set at 25 °C.

Omnifit polytetrafluoroethylene (PTFE) tubing (0.8 mm i.d.) and Gilson end-fittings and connectors were used to assemble the different manifold parts.

A Jasco Model FP-2020 fluorescence detector was used as detection system and the excitation and emission monitoring wavelength were set at 377 and 475 nm, respectively. Analytical signals were recorded on a strip chart recorder (Linseis, L 250 E) or acquired.

The SIA flow system was controlled by means of a microcomputer equipped with an interface card (Advantech Corp., PCL 711B, San Jose, CA). Software was developed in Quick-Basic 4.5 (Microsoft) and permitted operation of the peristaltic pump and multiposition selection valve, enabling the run-time definition of all analytical parameters to be made such as flow rate, flow direction, sample volume, reagents volume, valve positioning, etc., as well as data acquisition and processing.

2.4. Batch experiments

The hydrolytic action of PLA_2 on liposomes was monitored with the interfacial probe ANS by a fluorescence assay performed in a Perkin-Elmer LS 50B spectrofluorimeter. All data were recorded at 25 °C using a 1-cm path length quartz cuvette. Excitation and emission wavelengths were equal to those used in the SIA methodology. Initially, the reaction mixture containing buffer, EPC substrate (50 μM) and ANS (10 μM) was continuously stirred for approximately 10 min. Thereafter, PLA_2 (5 U/mL) was added to start the hydrolytic reaction.

The effect of inhibitors on PLA_2 hydrolytic action was studied by the same fluorescence assay. Two incubation procedures were tested. In the first procedure, PLA_2 (5 U/mL) was incubated with

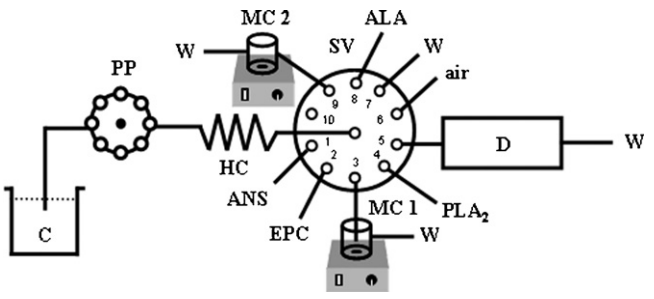

Fig. 1. The flow manifold for PLA_2 activity studies and for evaluating the inhibitory effect of NSAIDs and ALA: C, carrier, 10 mM Tris buffer pH 8.9; PP, peristaltic pump; HC, holding coil; SV, selection valve; ANS, ANS probe solution; EPC, liposomes solution or liposomes–drug incubated mixture; PLA_2, enzyme solution or enzyme–drug incubated mixture; ALA, α-lipoic acid solution; MC 1 and MC 2, mixing chambers; D, fluorescence detector ($\lambda_{excitation}$ = 377 nm; $\lambda_{emission}$ = 475 nm); W, waste.

different drug concentrations (0–8 μM) and left for 30 min after which the reaction was initiated by adding the enzyme–drug incubated mixtures to EPC stock solution (50 μM). In the second procedure, EPC stock solution (50 μM) was incubated with different drug concentrations (0–8 μM) and left for 30 min before PLA_2 (5 U/mL) stock solution was added to start the reaction.

2.5. Analytical procedure of the developed SIA-fluorimetric method

The experimental SIA-fluorimetric procedure comprises the PLA_2 activity study and assessment of NSAIDs and ALA inhibitory effect on PLA_2 activity. The resulting analytical cycle is summarized in Table 1.

For the PLA_2 activity study, the analytical cycle began with the aspiration of an air bubble (Step a). Following aspiration of the same volume of ANS probe and EPC substrate solution (Steps b and c), a second air bubble was aspirated (Step d). The aspirate sequence was then through flow reversal, propelled to mixing chamber 1 (MC 1, Fig. 1) (Step e) where the flow was stopped (Step f) and upon where the incubation of the liposomes with the ANS probe occurred. Subsequently, an aliquot of PLA_2 preceded by an air bubble (Steps g and h) was also propelled to MC 1 (Step i), where its hydrolytic action on the EPC took place during the stop period (Step j). At this point, an aliquot from MC 1 was sent to the detector (Step l), and the signal was acquired. The cycle ended by cleaning MC 1 with the carrier solution (Steps m–o).

For the inhibition measurements, EPC or PLA_2 solutions were pre-incubated with different drug concentrations in a similar way to the batch experiments and the EPC or PLA_2–drug mixture was aspirated instead of EPC or PLA_2 alone (Steps c or h, respectively).

Additionally, a second mixing chamber (MC 2, Fig. 1) was connected to one of the available selection valve inlets to perform the in-line incubation of the ALA and PLA_2. In this case, the analytical cycle began again with the aspiration of an air bubble, followed by the ALA, the PLA_2 solutions and a second air bubble (Steps A–D). These stacked zones were then sent to MC 2 (Step E). Therefore, the incubation of the EPC with the ANS probe in MC 1 was carried out while the incubation of the ALA with the enzyme was taking place in MC 2, since a stop period of 10 min (Step f) was used for the incubation 1. Thereafter, an aliquot of the mixture from MC 2 (Step h) (preceded by an air bubble) was propelled to MC 1 where the flow was stopped. At that point, the analytical cycle proceeded as depicted in Table 1, ending with the cleaning of MC 2 with the carrier solution (Steps M–O).

3. Results and discussion

3.1. Design of a SIA-fluorimetric method for the evaluation of the PLA_2 activity

As the methodology proposed has not yet been defined in batch experiments, we began by establishing the means by which to determine PLA_2 activity with ANS and only then did we focus on automating it. It was intended to mimic the initial conditions used in the batch experiments and to optimize the best conditions of the final SIA set-up. It was necessary to use two mixing chambers which were placed in two available inlets of the selection valve. In this way, it was possible to halt the sample and reagent mixtures and maintain them isolated from the rest of the system during the time while other steps were running.

The sequencing zones used in the batch experiments were adopted as it was necessary to guarantee the mixture of ANS with EPC before the action of PLA_2. Additionally, a monosegmented flow approach [29] was used. This implied that the sequence zones were isolated between two air bubbles before entering the mixing chambers (Table 1). In this way, it was possible to avoid the dilution of the reagents with the carrier in the holding coil and the mixing chamber. The flow was stopped after the entry of the last bubble into the mixing chamber. Air bubbles were then expelled by the waste as they entered the mixing chamber.

The 'blank' signal (considered as 100%) was obtained from the incubation of the ANS probe with the liposomes. The signal obtained after the subsequent addition of the enzyme to the incubated mixture enabled PLA_2 activity to be determined, considered as relative percentage.

The SIA set-up was optimised with the aim of establishing the EPC substrate, PLA_2 and ANS optimal concentrations as well as the incubation times before and after enzyme addition. Dispersion coefficients [30] of 8.5, 6.5 and 8.0 were obtained in MC 1 (Fig. 1) for the corresponding ANS, EPC and enzyme solutions and the corresponding working solutions were prepared taking these into account.

In the batch experiments, the incubation times before and after addition of the PLA_2 solution were 10 and 5 min, respectively. Regarding the SIA system, the first incubation period (Step f, Table 1) was reduced to 5 min since it gave the greater signal/background ratio.

For the evaluation of the incubation period after PLA_2 addition (Step j, Table 1), a 3-min period was selected as the signal/noise ratio for a 5-min incubation period was only approximately 6% higher. Additionally, the propulsion carrier flow rate towards the detector was established as 2.0 mL min^{-1} since the reaction was almost completed in the MC 1.

The EPC substrate was studied over a final concentration range of between 25 and 150 μM. An increment in the 'blank' signal along the concentration range was observed and for the chosen value of 50 μM, a greater difference between both signals was noticed.

The enzyme concentration was also tested until 1.250 U/mL. Regarding the decrease in fluorescence intensity, a 20.4% decrease was observed until 0.625 U/mL (Fig. 2). Between this value and 1.250 U/mL PLA_2, the decrease amounted to only 7.2%. Regarding the degree of precision associated with the measurements, values of around 2% until 0.625 U/mL and 1% for higher PLA_2 concentrations were obtained. A value of 0.625 U/mL was subsequently selected since the associated precision was still acceptable and the decrease in fluorescence intensity did not justify the 50% increase in the enzyme consumption.

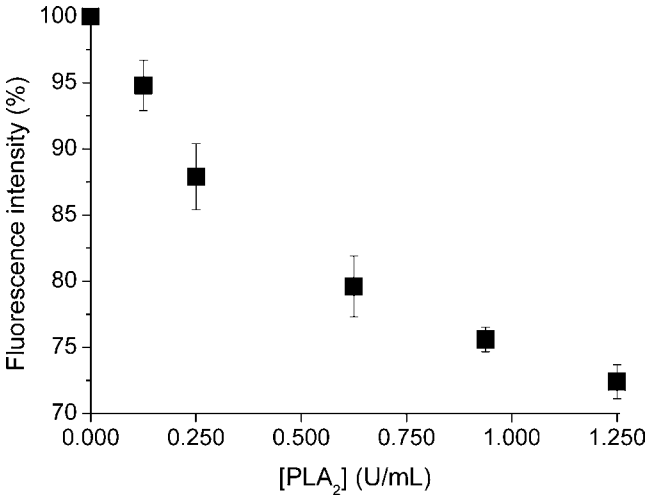

Fig. 2. Influence of PLA_2 concentration (U/mL) on the analytical signal obtained (% of fluorescence intensity).

Having established the optimum working concentrations of the lipid substrate and enzyme as 50 μM of EPC and 0.625 U/mL of PLA_2, respectively, the optimal concentration of 10 μM for the ANS probe between 5 and 15 μM tested was finally selected. It is important to note that neither the tested concentration range of ANS probe (which was always maintained in a lipid: probe ratio lower than 300:1) nor the concentration of methanol used (1%, v/v) to help with solubilising the probe, interfere with the structural environment of the liposomes [21,31–33].

3.2. Application of the SIA-fluorimetric method to test the inhibitory effect of NSAIDs and lipoic acid on PLA_2 activity

The previously optimised SIA-fluorimetric method was applied to NSAIDs representing the different chemical classes, namely arylpropionic acid (ibuprofen), indole (tolmetin) and oxicam derivatives (meloxicam) as well as to the α-lipoic acid which was used as positive control of the assay system once it has been reported as an effective PLA_2 inhibitor [24].

In this case, the analytical cycle was preceded by two different incubation procedures for the anti-inflammatory drugs and liposomes or PLA_2 as described in the batch experiments. The extension rate of those incubations remains stable for a time period greater than 10 min and on this basis, a 10-min incubation period was selected.

Activities measured with the PLA_2 or EPC and drug mixture were considered as relative percentage of the PLA_2 activity obtained without the drugs, which corresponds to 0% inhibition. Thus, the PLA_2 inhibition efficiency was expressed as the percentage inhibition of the hydrolytic action of the PLA_2 on the liposomes as a function of final inhibitor concentrations. Results obtained for the compounds tested are presented in Figs. 3 and 4.

Regarding the positive control of the ALA assay system, the results obtained point to an inhibition effect only when the incubation was performed with PLA_2. This is in agreement with the literature [24], indicating a direct interaction of the ALA inhibitor with the enzyme. Therefore, in-line incubation of the ALA and the PLA_2 enzyme was performed by, resorting to an additional mixing chamber (MC 2, Fig. 1), as detailed in the Section 2. In fact, a direct in-line coupling of the sample preparation procedure to the analytical system minimizes sample handling and thereby the risk of contam-

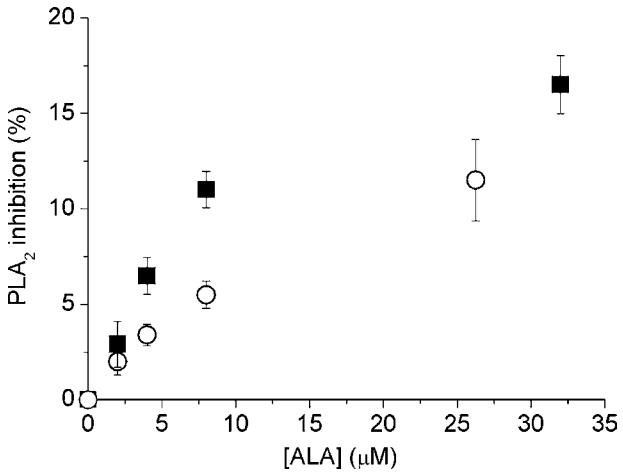

Fig. 3. Evaluation of the PLA_2 inhibition efficiency (%) exhibited by ALA according to the type of incubation: off-line (■) or in-manifold (○).

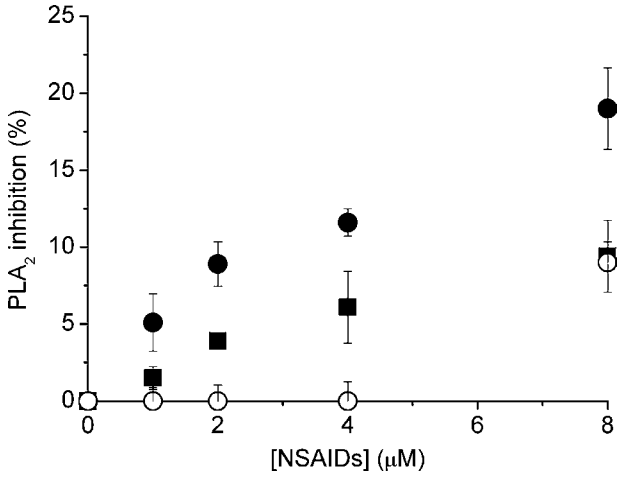

Fig. 4. Evaluation of the PLA_2 inhibition efficiency (%) by the NSAIDs: tolmetin (●); meloxicam (■); and ibuprofen (○).

Table 1
Analytical procedure for PLA_2 activity studies and for the assessment of the NSAIDs and ALA inhibition effect using the SIA-fluorescence assembly.

Step	Valve position	Operation time (s)	Flow rate (mL min^{-1})	Description
A[a]	6	1	0.8	Aspiration of an air bubble
B[a]	8	5.6	0.8	Aspiration of 75 μL of ALA
C[a]	4	11.2	0.8	Aspiration of 150 μL of PLA_2
D[a]	6	1	0.8	Aspiration of an air bubble
E[a]	9	6.8	3.0	Propulsion of the sequence to the MC 2
a	6	1	0.8	Aspiration of an air bubble
b	1	5.6	0.8	Aspiration of 75 μL of ANS
c	2	5.6	0.8	Aspiration of 75 μL of EPC (or EPC–drug incubated mixture)
d	6	1	0.8	Aspiration of an air bubble
e	3	4.5	3.0	Propulsion of the sequence to the MC 1
f	3	300 or 600[a]	–	Stop period—incubation 1
g	6	1	0.8	Aspiration of an air bubble
h	4 or 9	3.8	0.8	Aspiration of 50 μL of PLA_2 (or PLA_2–drug incubated mixture) or from the MC 2
i	3	2.5	3.0	Propulsion of the sequence to the MC 1
j	3	180	–	Stop period—incubation 2
k	3	15	0.8	Aspiration of the mixture from the MC 1
l	5	50	2.0	Propulsion of the mixture towards the detector
m	3	35	3.0	Cleaning of the MC 1 with the carrier
n	3	25	3.0	Aspiration of the cleaning carrier from the MC 1
o	7	40	3.0	Propulsion of the cleaning carrier to waste
M[a]	9	35	3.0	Cleaning of the MC 2 with the carrier
N[a]	9	25	3.0	Aspiration of the cleaning carrier from the MC 2
O[a]	7	40	3.0	Propulsion of the cleaning carrier to waste

[a] Additional steps when the in-line incubation of ALA and PLA_2 is carried out.

ination or loss of analyte. Additionally, it makes the automation of the whole process possible. Successful results were then obtained when a 10-min in-line incubation period was performed (Fig. 3).

In the case of the NSAIDs tested, they show an inhibitory effect on PLA_2 activity in a concentration-dependent manner, except for ibuprofen which only exhibits its inhibitory effect at a final concentration of 8 μM (Fig. 4). Moreover for those NSAIDs tested, it was noticed that they only exhibited their inhibition effect when the incubation was carried out with the liposomes, suggesting that they eventually change the biophysical properties of the liposomes and subsequently limit PLA_2 access to the lipid substrate. In fact for PLA_2, it was reported that both the capacity to bind to the surface of a phospholipidic aggregate and the catalytic turnover rate are highly dependent on the physical state and molecular packing of the aggregated substrate [34]. As a result, some inhibitors interact directly with the enzyme while others affect the 'interface quality' by modifying the phospholipid bilayer properties which render phospholipids inaccessible to the enzyme [24]. By comparing the capacity of the NSAIDs as enzymatic inhibitors, it is possible to conclude that tolmetin was the most effective drug, presenting an even higher inhibition efficiency than that found for the positive control (ALA). This might be explained by the previously reported membrane location of tolmetin [35]. Indeed, the predicted tolmetin superficial location positions this drug at the membrane interface, possibly interfering with enzyme adsorption to the lipid substrate with a subsequent reduction in phospholipid hydrolysis.

3.3. Validation of the results obtained through the SIA system with those provided by the batch experiments

The results obtained with the proposed methodology comply with those achieved using the batch experiments. Indeed, it was observed that for similar inhibition periods of the phospholipid hydrolysis and for the same concentrations of inhibitor compounds tested, there was a comparable PLA_2 inhibition in batch and SIA experiments (data not shown). Therefore, the batch experiments supported the fact that PLA_2 is effectively inhibited by the NSAIDs tested as well as by the ALA, through the mechanisms previously exploited. However, a significant variation in the analytical signals was reported in the batch experiments when replicates were carried out, due to ineffective time control, in contrast to the rigorous time control inherent in the flow methodology employed. Moreover, the precision of the flow procedure was estimated by calculating the relative standard deviation from 10 consecutive determinations of a 0.625 U/mL PLA_2 solution, yielding a value of 1.6%. Furthermore, a time saving advantage is seen in the developed SIA system since the batch experiments include an initial 10-min incubation period and a kinetic measurement period after PLA_2 addition to the liposome substrate [22], apart from the time spent adding the reagents. Herein, almost 720 s were required to complete an analytical cycle, taking into account the time required for each step and for proper valve port selection.

4. Conclusions

The proposed SIA methodology was successfully applied to the assessment of PLA_2 activity and evaluation of the efficiency of NSAIDs as PLA_2 inhibitors, with results comparable to those obtained by the batch procedures. Indeed, the proposed automatic flow methodology represents a noteworthy improvement in precision, efficiency and rapidity compared to batch application. Additionally, the rigorous time control throughout the analytical assays which is inherent in the proposed methodology is of utmost importance and practically impossible to ensure when analysis in series of different samples are performed in the comparison method.

The versatility offered by the SIA methodology through the use of the mixing chambers enabled favourable conditions to be created for an environmental shift to occur around the ANS probe due to the hydrolytic action of PLA_2 on phospholipids.

Finally, the SIA-fluorimetric methodology represents a convenient and economic tool for evaluating the efficiency of NSAIDs as PLA_2 inhibitors and can be extended to other interfacial PLA_2 inhibition studies. This methodology could prove to be an alternative for assessing the effect of anti-inflammatory drugs on PLA_2 activity and could therefore represent an important strategy for developing new and more potent anti-inflammatory agents in the pharmaceutical and biomedical fields.

Acknowledgments

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