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HPLC-fluorimetric assay of phospholipase A_2. Application to biological samples with high protein content and various reaction conditions

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ABSTRACT

Phospholipase A_2 (PLA_2) quantitation in real-time, using (7-nitro-2-1,3-benzoxadiazol-4-yl)amino-derivatives of phosphatidylcholine (NBD-PCs) as substrates, is influenced by high protein content, color or turbidity. The purpose of the study was to overcome such limitations by a complementary reversed-phase HPLC step to separate the substrates from the products of the reaction. Plasma and bronchoalveolar lavage (BAL) fluid were applied as enzymic sources, while standard porcine PLA_2 and human plasma PAF-acetylhydrolase (PAF-AH) were employed as positive controls. The method was validated with a radiometric assay and compared with the real-time fluorimetric assay. Regarding PLA_2 and PAF-AH determination, the isocratic elution systems CH_3OH–H_2O (80:20, v/v) and CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v) separated efficiently C_{12}-NBD-FA/C_{12}-NBD-PC and C_6-NBD-FA/C_6-NBD-PC, with 4.4 and 2.2 resolution, respectively. Analysis time was ~15 min/injection. The reproducibility, expressed as relative standard deviation, was ≤13% for PLA_2 and ≤16% for PAF-AH, respectively. The assay was linear for PLA_2 activities extending from 1 pmol up to at least 250 nmol FA/h/mL sample, similar to the radiometric assay. It was appropriate for samples with high protein content, where the real-time fluorimetric assay was insufficient. The HPLC method was also evaluated under elevated temperatures, various pH values and Ca^{2+} concentrations.

1. Introduction

Phospholipases A_2 (PLA_2s) (EC 3.1.1.4) are a family of highly regulated enzymes that catalyze the hydrolysis of the sn-2 ester bond of phospholipids, yielding free fatty acids and lyso-phospholipids [1]. Different PLA_2 isotypes participate in phospholipid degradation, remodeling, and in the formation of potent lipid mediators, such as eicosanoids and platelet-activating factor (PAF) [2–6]. The impact of PLA_2 has been demonstrated in sepsis, multiple injury, rheumatoid arthritis and in acute respiratory distress syndrome (ARDS) [7–13]. Therefore, its determination could give important information on the status of the patient, or even on the response to a therapeutic approach.

According to their biochemical properties and compartmentalization, PLA_2s are classified into secreted ($sPLA_2$), cytosolic ($cPLA_2$), Ca^{2+}-independent ($iPLA_2$) types and platelet-activating factor acetylhydrolases (PAF-AH) [14]. Interestingly, each subtype exhibits different specificities for the chain-length at the sn-2 acyl-chain of glycerophospholipids. These particularities may pose limitations to the assays and perplex the interpretation of the results. From the various assays for PLA_2 determination (for review, see Ref. [15]), radiometric ones are widely used due to their high sensitivity and accuracy [16,17]. However, they impose radiochemical hazards and require expensive and laborious steps for the purification of the liberated fatty acids. Fluoro-immunoassays have been developed [18,19], but these might measure even the pro-enzyme and not only the active forms of PLA_2 [20]. Sensitive assays using various fluorescent derivatives as substrates have been applied to the determination of purified PLA_2 preparations, but most of them have not addressed the issue of different specificities and requirements of the individual PLA_2 isoforms [21–25]. The combination of these methods with lipid extraction and separation of the derivatized reaction products by HPLC [26] are often necessary to avoid limitations related to intrinsic properties of the biological samples [27].

Fluorescent NBD-PC derivatives have been utilized for the determination of authentic PLA_2 and lipoprotein lipase preparations [28]. Based on this work we have previously reported a real-time fluorimetric assay for total PLA_2 activity, with preference to long acyl chains, and PAF-AH, with preference to acetyl-groups and short, oxidatively fragmented acyl chains at the sn-2

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position. The 1-palmitoyl-2-{12[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (C_{12}-NBD-PC) and 1-palmitoyl-2-{6[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (C_6-NBD-PC) were used as substrates, respectively [29]. The liberation of NBD-hexanoic or NBD-dodecanoic acid (NBD-FAs) was measured from the fluorescence increase of the reaction mixture following PLA_2 activation. The sensitivity of the method relied on the strong quenching of the substrates fluorescence for concentrations greater than the critical micellar concentration (CMC). Limitations were related to perturbation of the micellar structures of the substrates, caused by high protein content, especially albumin that non-specifically binds phospholipids. This resulted in a non-specific increase of the background fluorescence and led to reduced sensitivity. Moreover, intrinsic properties of certain samples, such as color, turbidity, low pH, high temperature, Ca^{2+} ions, and the presence of fluorescent PLA_2 inhibitors, may interfere with the NBD-FAs fluorescence [30].

The aim of the present study was to overcome such limitations by combining the real-time fluorimetric assay with an HPLC step for the separation of the intact fluorescent substrates from their relevant fatty acid products. The method was evaluated under high protein concentrations, various incubation temperatures, pH values, Ca^{2+} concentrations and in the presence of fluorescent PLA_2 inhibitors. Standard porcine $sPLA_2$ type IIA was used as reference. Furthermore, the method was applied to biological samples, such as bronchoalveolar lavage fluid (BAL) and plasma from patients with pulmonary disorders. It was validated by the radiometric assay and compared with the real-time fluorimetric one.

2. Materials and methods

2.1. Reagents

Inorganic compounds, EDTA, Tris–HCl, TCA (trichloroacetic acid) and porcine $sPLA_2$ type IIA (EC 3.1.1.4.) were obtained Sigma Chemical Company (St Louis, MO, USA); HPLC grade organic solvents where from LabScan (Dublin, Ireland); 1-O-hecadecyl-2-[^{3}H]acetyl-sn-glycero-3-phosphocholine (7.1 Ci/mmol) was from New England Nuclear (Boston, MA); C_6-NBD-FA, C_6-NBD-PC, C_{12}-NBD-PC and C_{12}-NBD-FA were from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Real time fluorimetric assay of PLA_2 and PAF-AH

The standard incubation mixture for the determination of PLA_2 activity contained 10 mM Tris–HCl buffer solution, pH 7.4, and 2.0 mM Ca^{2+}, to which C_{12}-NBD-PC in ethanol was added at a final concentration of 5 μM, without sonication, to form lipid aggregates The reaction started with the addition of 100 μL from the source of the enzyme (BAL or 0.8×10^{-3} IU of standard porcine pancreatic PLA_2 diluted with normal saline). For PAF-AHmeasurements, C_6-NBD-PC was used as substrate at 5 μM final concentration in a 10 mM Tris–HCl buffer solution, pH 7.4, containing 10 mM EDTA instead of 2 mM Ca^{2+}. The source of PAFAH was 100 μL of either BAL or human serum in 50-fold dilution in normal saline (0.9% NaCl). Following the standard experimental conditions, PLA_2 and PAF-AH activities were determined kinetically in real-time by monitoring the fluorescence increase of the reaction mixture due to the liberation of C_{12}-NBD-FA or C_6-NBD-FA, respectively [29]. The excitation and emission wavelengths were adjusted to 475 nm and 535 nm, as resulted from the excitation and emission spectra, respectively, obtained at 5- or at 10-nm-increments. The fluorescence was recorded at 20 min intervals, by a Perkin Elmer LS-3 spectrofluorimeter (Waltham Massachusetts, USA), equipped with a constant Xenon light source (150 W) and an RCA photomultiplier. Typical duration of the reaction monitoring was 1 h for commercial porcine PLA_2, and 2 h for BAL-PLA_2 and plasma PAF-AH (depending on their activity). Positive (reaction mixture with standard PLA_2) and negative controls (with boiled samples or without the source of the enzyme) were included. Certain experiments were performed in the presence of LY311727 [(3-(3-acetamide-1-benzyl-2-ethylindole-5-oxy) propane sulphonic acid)], a fluorescent specific inhibitor of type II $sPLA_2$ [31,32]. The biological samples (BAL or standard $sPLA_2$-IIA) were pre-incubated with the inhibitor for 20 min at room temperature prior to the initiation of the reaction.

Table 1
Retention times and resolution (Rs) of NBD-lipids in reversed phase HPLC fluorimetric analysis using various elution systems: (A) C_{12}-NBD-PC and C_{12}-NBD-FA. The reaction mixture contained 10 mM Tris–HCl buffer solution, pH 7.4, 2 mM Ca^{2+}, 5 μM C_{12}-NBD-PC and C_{12}-NBD-FA. (B) C_6-NBD-PC and C_6-NBD-FA. The reaction mixture contained 10 mM Tris–HCl buffer solution, pH 7.4, 10 mM EDTA, 5 μM C_6-NBD-PC and C_6-NBD-FA. Twenty microliter were injected in the HPLC and the eluted peaks were recorded. Flow rate was adjusted at 0.5 mL/min, while excitation and emission wavelengths were adjusted to 475 nm and 535 nm, respectively.

A. Elution times and resolution of C_{12}-NBD-PC and C_{12}-NBD-FA

Elution system	Retention time (min)		Rs
	C_{12}-NBD-PC	C_{12}-NBD-FA	
CH_3OH	6.4	6.5	0.1
CH_3OH–H_2O (90:10, v/v)	6.4	7.1	0.7
CH_3OH–H_2O (80:20, v/v)	6.6	11.5	4.4
CH_3OH–H_2O–$CHCl_3$ (70:20:10, v/v/v)	6.7	6.8	0.1
CH_3OH–H_2O–CH_3CN (82:9:9, v/v/v)	6.3	7.0	0.7
CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v)	6.5	45.9	36.7

B. Elution times and resolution of C_6-NBD-PC and C_6-NBD-FA

Elution system	Retention time (min)		Rs
	C_6-NBD-PC	C_6-NBD-FA	
CH_3OH	6.3	6.4	0.1
CH_3OH–H_2O (90:10, v/v)	6.7	6.7	0.0
CH_3OH–H_2O (80:20, v/v)	6.6	6.8	0.2
CH_3OH–H_2O–$CHCl_3$ (70:20:10, v/v/v)	6.4	6.6	0.2
CH_3OH–H_2O–CH_3COOH (80:20:0.2, v/v/v)	6.7	7.0	0.3
CH_3OH–H_2O–CH_3COOH (65:35:0.2, v/v/v)	7.4	8.7	1.4
CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v)	8.0	11.2	2.2

2.3. HPLC-fluorimetric assay of PLA_2 and PAF-AH

HPLC analysis was performed on a Shimadzu LC-10AD HPLC system (Kyoto, Japan), equipped with an SPD-M10A diode array detector and an LC-10RF fluorescence detector with continuous Xenon light source, and a reversed phase column LC-18 (25 cm, 4.6 ID, 5 μm particle size), Supelco (Bellefonte, PA, USA), at a flow-rate of 0.5 mL/min. The system performed in an air-conditioned room with temperature set at 25 °C.

The excitation and emission wavelengths were adjusted to 475 nm and 535 nm, respectively. For peaks integration, DAPA software, version v1.4x, was used (DAPA Chromatography Pty. Ltd. Western Australia). Various mobile phases were tested (Table 1) to find an optimum isocratic system for the separation of the respective fluorescent phospholipid substrates and products. Authentic C_6-NBD-PC, C_{12}-NBD-PC, C_6-NBD-FA and C_{12}-NBD-FA solutions in 10 mM Tris buffer, pH 7.4, were used as reference compounds. The activities of PLA_2 and PAF-AH were calculated by plotting the integrated areas of the NBD-FAs' peaks the reaction time. Standard curves of the NBD-FAs were used for conversion of the integrated areas to pmol NBD-FA. To avoid column clogging by protein and contamination by lipids, at the end of each day the system was thoroughly washed with water, then with methanol–water (80:20, v/v)

and finally with methanol, until the signal monitored by the diode-array detector recurred to base-line, and also until the pressure dropped to 75–100 bar, depending on the elution system.

Aliquots from the enzymic reaction mixture were directly injected for HPLC analysis at specified time-intervals. Otherwise, the reaction was stopped by placing 100 μL from the reaction mixture into an Eppendorf tube containing 100 μL trichloroacetic acid (20%, w/v). The samples were collected and stored at 4 °C for a few hours before they were analyzed by an auto-sampler.

The resolution (Rs) between two peaks, a and b, was calculated by the equation:

$$\mathrm{Rs} = \frac{2(\mathrm{RT_b} - \mathrm{RT_a})}{W_a + W_b},$$

where RT is the retention time of each peak and W is the width at the baseline of the peaks.

The % reproducibility, expressed as relative standard deviation, was given by the formula:

$$\%\mathrm{RSD} = \frac{\mathrm{SD} \times 100}{\text{mean value}}$$

2.4. Radiometric determination of PLA_2

The standard assay mixture, final volume 1 mL, included 10 mM Tris–HCl buffer, pH 7.4, 2.0 mM $CaCl^{2+}$ and 5 μM dipalmitoyl-PC, 120,000 cpm (approximately 0.07 μCi in dipalmitoyl-[1-^{14}C]phosphatidylcholine), as substrate. The reaction was started with the addition of the enzyme and was stopped with 50 μL 2 N HCl and 2.2 mL of chloroform–methanol 1:1 (v/v). The mixture was thoroughly agitated and after phase separation, the chloroform-rich phase was collected and the lipids were analyzed by TLC, using chloroform–methanol–water (65:35:7, v/v/v) as solvent system. The lipids corresponding to the Rf of dipalmitoly-PC, lyso-PC and free fatty acids were scrapped off the plates separately and the radioactivity was measured with a beta counter (TriCarb 2100TR, Packard instrument company, Downers Grove, USA). Typical duration of the reaction was 5 min for commercial porcine PLA_2, and 3 h for BAL-PLA_2, depending on the activity. Positive and negative controls were applied as in the real-time assay.

2.5. Radiometric determination of PAF-AH

PAF-AH activity of the samples (50–100-fold diluted plasma or BAL) was also determined by using [^{3}H]PAF (1-O-hexadecyl-2-[^{3}H]acetyl-sn-glycero-3-phosphorylcholine) as substrate. Briefly, 100 μL of the sample were incubated with an equal volume of 20 μM PAF solution in 1 mg/mL HSA–PBS, pH 7.4 and 0.1 μCi [^{3}H]PAF per sample. The reaction was stopped after specified time intervals (usually 5–30 min), with ice-cold TCA. The radioactivity of the liberated acetyl group was measured in the 12,000 × *g* supernatant after centrifugation for 2 min. The intact substrate, which did not react, co-sedimented with the denatured HSA. For positive control, human plasma diluted 50–100-fold in normal saline was used. Negative controls were applied as in the real-time assay.

2.6. Bronchoalveolar lavage (BAL) and plasma preparation

BAL was performed by fiberoptic bronchoscopy. Patients suffering from acute respiratory distress syndrome were ventilated in Control Mechanical Ventilation Mode. During the BAL procedure the fraction of inspired oxygen (FiO_2) was set at 1.0 and the positive end-expiratory pressure (PEEP) was removed or reduced. Patients were sedated with midazolam and paralyzed with atracurium. Topical anesthetics were not used. Heart rate, arterial pressure, arterial oxygen saturation (by pulse oximetry) and mixed venous oxygen saturation were monitored throughout the procedure. The trachea was suctioned before introducing the bronchoscope through an adapter (swivel adapter), which allowed the maintenance of mechanical ventilation. The tip of the bronchoscope was then wedged in a segmental or subsegmental bronchus of the more affected area indicated by the angiogram. Six aliquots of 20 mL sterile normal saline 37 °C were infused through the working channel of the bronchoscope. The first aspirated fluid, reflecting a bronchial sample, underwent microbiological screening, while the others were collected in ice-cold tubes to avoid PAF degradation due to acetylhydrolase activity. BAL was then filtered through cell strainer filters No 7 (Becton-Dickinson-Falcon, N. Jersey, USA) and centrifuged at 500 × *g* for 15 min at 4 °C to remove mucus. Plasma was recovered after centrifugation of 10 mL of arterial blood, withdrawn just before the BAL procedure in a heparinized syringe, at 500 × *g* for 20 min.

Institutional ethics committee approved the study and written informed consent was obtained from the patients' next of kin.Statistical analysis

Each set of experiments was performed at least three independent times and each sample was measured twice. The duplicate measurements of each experiment were averaged. Results are expressed as mean ± SD of the 3 independent experiments, while comparison between groups was assessed by Student's *t*-test. The statistical significance was defined at $P < 0.05$. Regarding the comparison between the different PLA_2-determination assays, correlation and regression analysis (Statistica Software, v. 7.0) and Bland–Altman plots [33] (GraphPad software) were applied.

3. Results and discussion

This work presents an HPLC-fluorimetric assay for PLA_2 and PAF-AH determination for samples comprising components that interfere with fluorescence. It was based on the separation of NBD-PCs from their corresponding fatty acids by isocratic reversed phase HPLC.

3.1. HPLC-fluorimetric assay

The excitation and emission maxima for all the authentic NBD-derivatives were found to be 535 ± 2 nm and 475 ± 1 nm, respectively. The presence of 400 μg protein/mL of reaction mixture (bovine serum albumin, human plasma or BAL protein) did not cause any shift in the excitation and emission maxima, as previously reported [29].

The efficiency of the present method was based on the separation of the substrates from the relevant reaction products during the determination of PLA_2 and PAF-AH activities. HPLC was selected over other separation techniques due to its reproducibility. Regarding PLA_2 determination, from the various isocratic elution systems tested, an efficient and rapid peak separation of C_{12}-NBD-FA from C_{12}-NBD-PC was achieved with CH_3OH–H_2O (80:20, v/v) as elution system, with retention times of 6.6 and 11.5 min, respectively, and resolution: 4.4. For the separation of C_6-NBD-FA from C_6-NBD-PC an elution system of CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v) was selected giving retention times of 8.0 and 11.2 min, respectively, and resolution: 2.2 (Fig. 1A and B and Table 1). This HPLC separation is fundamental for the quality control and the determination of the active concentration of NBD-PCs that serve as substrates for PLA_2. Especially, when they are stored for a long period, during which spontaneous, non-enzymic isomerisation of (1-palmitoyl-2-acyl-NBD)PC to (1-acyl-NBD-2-palmitoyl)PC can occur. This latter substrate does not liberate NBD-FA upon hydrolysis by PLA2 [34–36], but acyl-NBD-lyso phosphatidylcholine, instead, which is eluted at shorter times than the relevant NBD-FA [37].

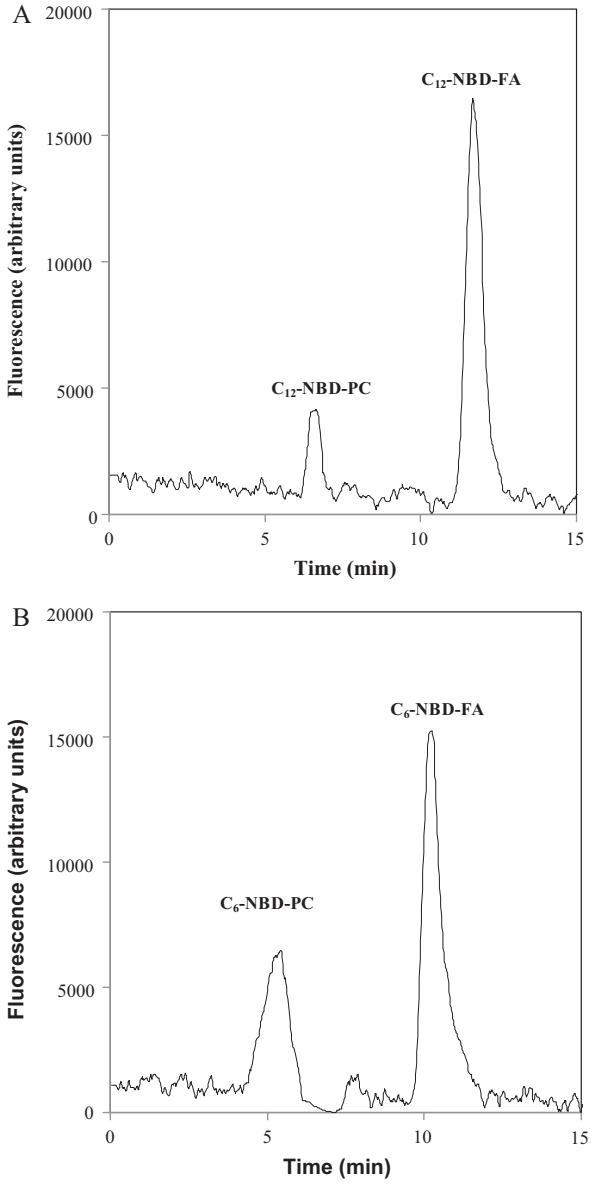

Fig. 1. Representative reversed-phase HPLC separation of reaction mixtures containing BAL fluid as source of the enzyme. (A) C_{12}-NBD-PC from C_{12}-NBD-FA, using CH_3OH–H_2O (80:20, v/v) and (B) C_6-NBD-PC from C_6-NBD-FA, using CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v), as mobile phases. The HPLC system was equipped with a fluorimetric detector with continuous Xenon light source. Excitation and emission wavelengths were set at 475 nm and 535 nm, respectively. Flow rate was adjusted to 0.5 mL/min, injection volume was 20 μL.

The HPLC separation of various NBD glycerolipid and sphingolipid analogs has been previously reported [37,38], but NBD-PCs from their corresponding NBD-FAs are analyzed for the first time.

3.2. Properties of authentic NBD-analogs and monitoring of the reaction

The response of the HPLC fluorimetric detector was linear ($r^2 = 0.997$) over a wide range of both standard C_{12}- and C_6-NBD-FA concentrations, from 1 pmol to at least 250 nmol (Fig. 2A and B).

In acidic environments, the fluorescence intensity of NBD aqueous solutions decreases due to the protonation of its amino group [39]. This can lead to an under-estimation of acidic PLA_2 activities. Under our experimental conditions, equivalent concentrations of standard solutions of C_{12}- and C_6-NBD-PC exhibited similar fluo-

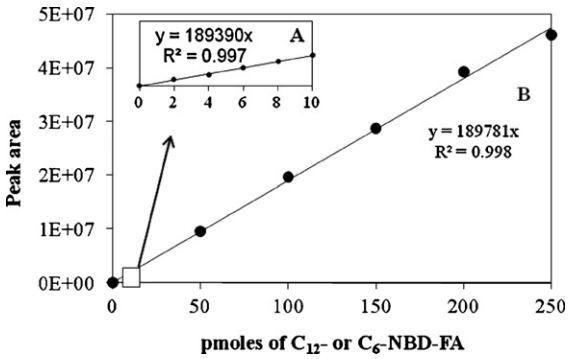

Fig. 2. Representative standard curves of authentic C_{12}- or C_6-NBD-FA subjected to reversed phase HPLC analysis at 25 °C. Mobile phases consisted of CH_3OH–H_2O (80:20, v/v) and CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v) for C_{12}-NBD-FA and C_6-NBD-FA, respectively, at a flow rate of 0.5 mL/min for both NBD-FAs. (A) Low range (0–10 pmol) and (B) high range concentrations (50–250 pmol).

rescence intensities at pH values of 5.0, 7.4, and 8.5, respectively, in the presence and absence of Ca^{2+} ions after the HPLC separation (Fig. 3A). As shown in (Fig. 3B), although C_{12}-NBD-FA fluorescence was not altered for pH values between 7.4 and 8.5 in the presence or absence of Ca^{2+} ions, at pH 5.0 the fluorescence intensity of C_{12}-NBD-FA was significantly lower than at pH 7.4 ($P < 0.05$), both in the presence and absence of Ca^{2+} ions. This is possibly due to the high lipophilicity of C_{12}-NBD-FA and in this case the PLA_2 values can be corrected by the use of external standards. In contrast, the fluorescence intensity of C_6-NBD-FA was not affected by pH changes, or by the presence of Ca^{2+} ions.

The hydrolysis reaction of NBD-PC analogs by PLA_2 or PAF-AH, using BAL fluid or plasma as enzyme source was monitored from 10 to at least 200 min. During this time-period, the increase in fluorescence depended upon the substrates cleavage and it was linear for both C_{12}-NBD-FA and C_6-NBD-FA ($r^2 = 0.99$) (Fig. 4). Thus, the analysis period was reduced and errors related to the evaporation of the samples were reduced.

Limitations of fluorimetric assays are related with changes in temperature, which inversely affect fluorescence. Real-time fluorimetric assays performed at 37 °C, the optimal temperature for enzymic assays, can thus result in reduced sensitivity [30]. This drawback can be inversed with the HPLC-fluorimetric assay: As shown in (Fig. 5A), a decrease in fluorescence intensity of standard concentrations of both C_{12}- and C_6-NBD-FA was observed by increasing temperature from 20 °C to 37 °C. The observed PLA_2 activity at 25 °C was significantly higher than that performed at 20 °C; however, there was not any significant difference in PLA_2 activities measured from 25 °C up to 37 °C (Fig. 5B). This could be explained by the fact that the observed fluorescence value represents the vector sum of two counteracting effects, in particular, (a) the increase of the enzymic activity with increasing temperature and (b) the decrease in fluorescence intensity caused by increasing temperature.

3.3. Applications of the HPLC fluorimetric assay

When monitoring the fluorimetric reaction in real-time, the fluorescence initially increased rapidly and this increase, which was non-specific, lasted for at least 30 min. The magnitude of the fluorescence intensity, but also the time required for the completion of this non-specific effect, depended primarily upon protein concentration. After this non-specific part, a linear component followed, the slope of which represented the cleavage of the NBD-PC by PLA_2 and the production of the relevant NBD-FA. From the slope of this line and by using internal standards we were able to quantify the enzymic activity [29]. However, when the protein content

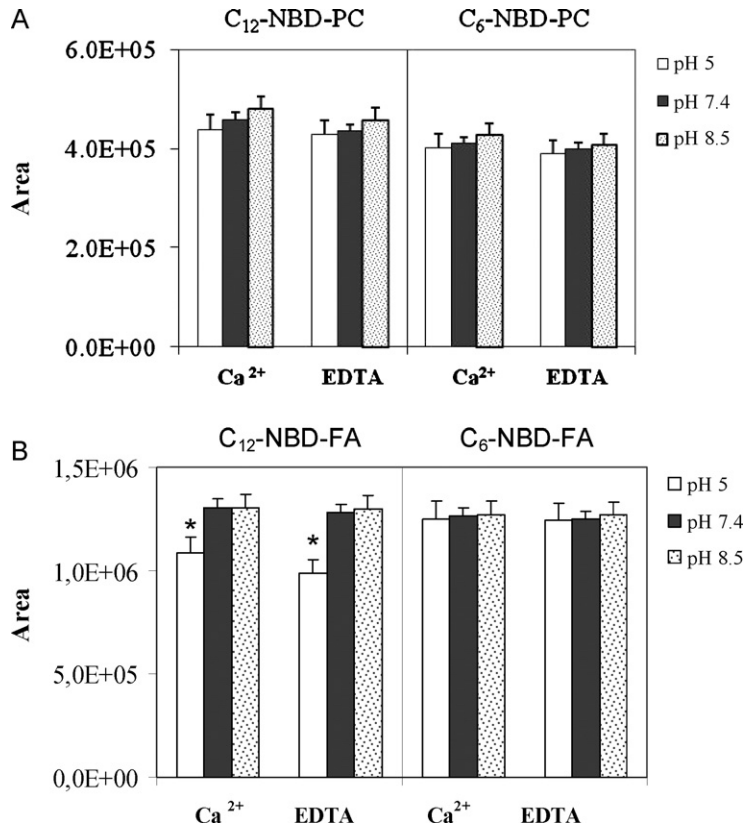

Fig. 3. Effect of pH and Ca^{2+} concentration on the fluorimetric quantitation of (A) authentic C_{12}- and C_6-NBD-PC solutions (5×10^{-3} M) and (B) C_{12}- and C_6-NBD-FA solutions (1×10^{-3} M). The analysis was performed by HPLC, under experimental conditions identical to Fig. 2. The composition of the incubation mixtures (1 mL final volume) were as follows: *pH 5.0*: 50 mM CH_3COOH–CH_3COONa, 2 mM Ca^{2+} (or 10 mM EDTA) and 10 μL of the 0.5 mM NBD-PC (or -FA) analogue. *pH 7.4*: 10 mM Tris–HCl, 2 mM Ca^{2+} (or 10 mM EDTA) and 10 μL of the 0.5 mM NBD-PC (or -FA) analogue. *pH 8.5:* 10 mM Tris–base, 2 mM Ca^{2+} (or 10 mM EDTA) and 10 μL of the 0.5 mM NBD-PC or NBD-FA analogue. The data represent the mean ± SD of at least 3 independent experiments with two measurements per sample. *Significant difference in the fluorescence in standard solutions of C_{12}-NBD-FA measured at pH 5.0, from that measured at pH 7.4 or pH 8.5.

of the samples (BAL fluid or plasma) exceeded concentrations of 250 μg protein/mL in the reaction mixture, the determination of the PLA_2 activity was not feasible due to the dramatic increase of the non-specific background fluorescence. As a result, the fluorescence corresponding to NBD-FAs was masked and the linear component representing the cleavage of the NBD-PC by PLA_2 could not be determined.

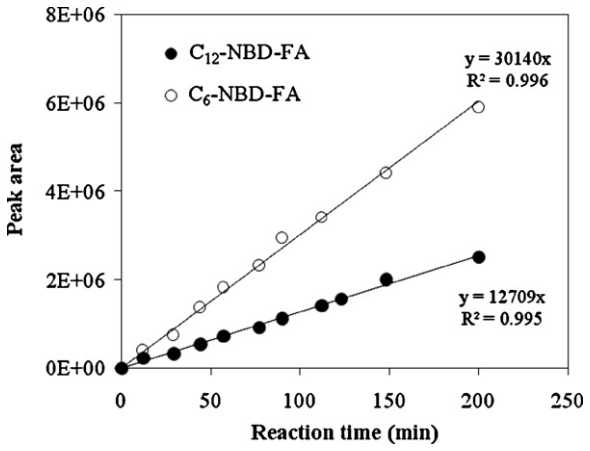

Fig. 4. PLA_2 and PAF-AH determination by the HPLC-fluorimetric assay in a BAL fluid sample using C_{12}-NBD-PC and C_6-NBD-PC as substrates, respectively. The reactions were performed at 37 °C and the buffer solution contained 10 mM Tris–HCl, 2 mM Ca^{2+}, pH 7.4, while mobile phase consisted of CH_3OH–H_2O (80:20, v/v) in the first case and 10 mM Tris–HCl, 10 mM EDTA, pH 7.4, while mobile phase consisted of CH_3OH–H_2O–CH_3COOH (60:40:0.2, v/v/v) in the second one.

With the efficient resolution for each couple of NBD-PC/NBD-FA by HPLC, the measurements could be performed with the initiation of the reaction, reducing significantly the duration of the analysis. Moreover, technical errors related to sample evaporation during reaction prolongation were avoided. With the HPLC-fluorimetric assay a wider concentration range of PLA_2 than that of the real-time one can be measured. Therefore, this assay is suitable for biological samples, given that PLA_2 levels in normal individuals are usually low, while in patients with inflammatory disorders are significantly higher [40,41]. Actually, in BAL fluid samples with high protein content, PLA_2 activity could be measured by the HPLC-fluorimetric assay, but not with the real-time one (Table 2). However, the values of these samples obtained with HPLC-fluorimetric assay are systematically lower compared to the radiometric one. The explanation of this phenomenon is not obvious. Possibly, this is related with the nature of the protein and its interaction with the different acyl-chains at the sn-2 position of C_{12}-NBD-PC and dipalmitoyl-[1-^{14}C]phosphatidylcholine, used in the fluorimetric and radiometric assays, respectively.

Table 2
PLA_2 activity determined by different methods in four samples with protein content (>250 μg/mL BAL fluid). The data are expressed as mean ± SD of three different experiments tested in duplicates.

HPLC method Activity (nmol FA/h/mL BAL)	Radiometric method Activity (nmol LysoPC/h/mL BAL)	Real-time method Activity (nmol FA/h/mL BAL)
1.32	2.03	0
1.75	2.44	0
1.06	1.4	0
0.37	0.6	0

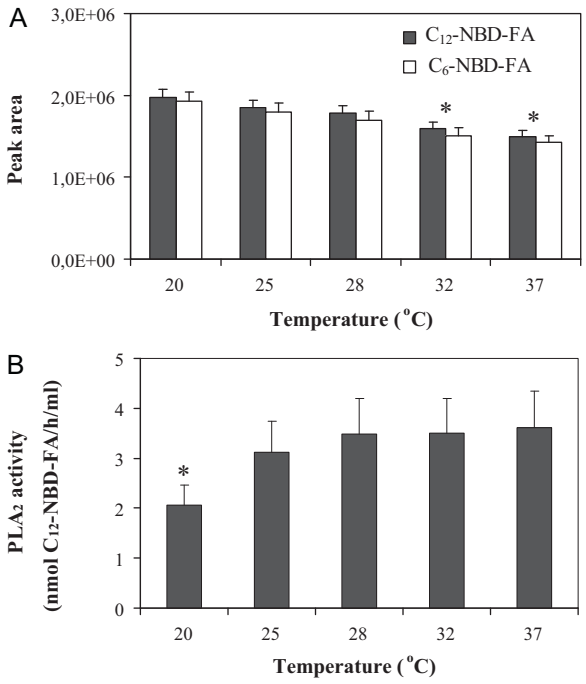

Fig. 5. (A) Effect of temperature on the fluorimetric quantitation of standard solutions of C_{12}-NBD-FA and C_6-NBD-FA subjected to HPLC analysis. The reaction mixtures and the experimental conditions of the HPLC analysis were identical to those of Fig. 4. The data represent the mean ± SD of at least 3 independent experiments with two measurements per sample. *Significant difference in the fluorescence intensities of standard C_6- or C_{12}-NBD FA solutions measured at high (32° or 37 °C) from those measured at lower temperatures (20 °C or 25 °C). (B) Effect of temperature on PLA_2 activity, using BAL fluid as source of the enzyme, measured by the HPLC-fluorimetric assay. The reaction mixtures and the experimental conditions of the HPLC analysis were identical to those of Fig. 4.The data represent the mean ± SD of at least three independent experiments with two measurements per sample. *Significant difference in the PLA_2 activity measured at 20 °C in comparison with that measured at 25 °C, 28 °C, 32 °C or 37 °C.

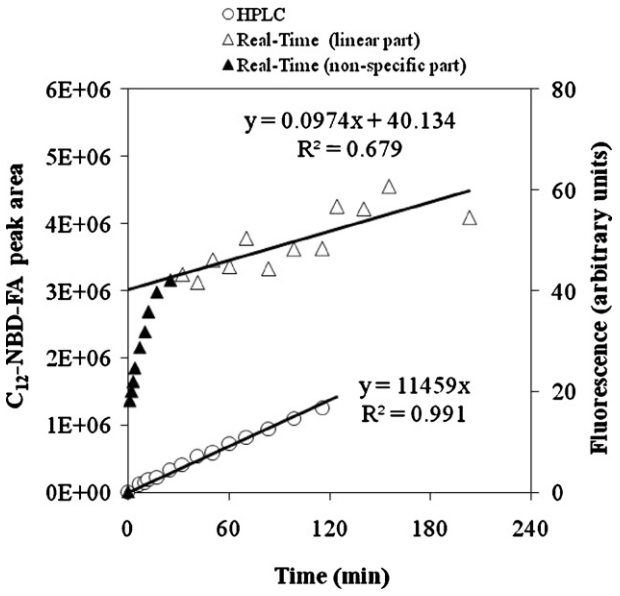

Fig. 6. Comparison between the HPLC and the real-time fluorimetric assays in a representative BAL-fluid sample with high content of suspended particles. The reaction mixtures and the experimental conditions of the HPLC analysis were identical to those of Fig. 4.

In contrast, PAF-AH levels in plasma or serum are relatively high, so in most cases the enzyme can be efficiently determined with the real-time fluorimetric assay [11], without the necessity of additional HPLC-separation step.

Another limitation is posed by turbidity of samples which causes fluorescence instability due to light scattering and gives false positive results [42]. Thus, in the real-time assay the linearity was poor (r^2: 0.679) leading to inaccurate measurements. However, it was shown that in such samples, the HPLC-fluorimetric assay corrected the linearity and the slope of the time response curve leading to precise, accurate and reproducible results (Fig. 6). Furthermore, the method could be applied even in the presence of other fluorescent organic compounds that affect the measurement of the real time method. We observed that LY311727, a specific inhibitor of secretory PLA_2-IIA, carrying indole rings, did not interfere with the fluorimetric detection of the NBD-FA product under our HPLC conditions (Table 3). In these particular BAL samples, PLA_2

Table 3
PLA_2 determination in BAL fluid by real time and HPLC fluorimetric assays in acidic pH and in the presence of a fluorescent inhibitor of $sPLA_2$. The data are expressed as mean ± SD of three different experiments tested in duplicates.

	HPLC-fluorimetric assay Activity (nmol FA/h/mL BAL)	Real-time fluorimetric assay Activity (nmol FA/h/mL BAL)
pH 5/Ca^{2+}	0.99 ± 0.2	0
pH 5/EDTA	0.47 ± 0.2	0
LY311727/Ca^{2+}	5.3 ± 0.6	2.1 ± 0.5
LY311727/EDTA	1.6 ± 0.3	0

values obtained with the HPLC-fluorimetric were systematically lower, compared to the radiometric assay. A possible explanation of this result can rely on the different specificities of each particular PLA_2 isoform, existing in the BAL sample, to the fatty acyl-chain length esterified at the sn-2 position of phosphatidylcholine. Thus, in the case of HPLC-fluorimetric assay, C12-NBD-FA is the fatty acid corresponding to C_{12}-NBD-PC, while in the radiometric one, it is palmitate.

3.4. Correlation of the methods

According to regression analysis, the results of the HPLC method presented a strong correlation with those of the reference radiometric method for both PLA_2 and PAF-AH. In particular, for the determination of PLA_2, the correlation coefficient between the HPLC-fluorimetric (H) method and the reference radiometric assay (R) was $r = 0.944$ with $P < 0.05$, while for PAF-AH activity was $r = 0.789$, and $P < 0.05$ (Fig. 7A and B). The comparison between real-time fluorimetric assay (F) and the radiometric one (R), was also statistically significant but the correlation was not as strong as the one between radiometric and HPLC method (for PLA_2 activity $r = 0.647$, with $P < 0.050$ and for PAF-AH activity $r = 0.798$ and $P < 0.050$) (Fig. 7C and D).

Furthermore, the Bland–Altman analysis clearly showed a good agreement between radiometric and HPLC-fluorimetric method. In particular, the Bland–Altman analysis for measurements obtained through the radiometric vs the HPLC-fluorimetric method, showed that the mean difference for PLA_2 activity was very low (0.49 ± 0.28) suggesting a good agreement. The 95% limits of agreement were between +1.03 and −0.06 (Fig. 8A). Moreover, the mean difference for PAF-AH activity was (2.08 ± 4.95), with 95% limits of agreement between +11.79 and −7.61 (Fig. 8B). For measurements obtained through the radiometric and the real-time fluorimetric method, the mean difference for PLA_2 activity was (0.71 ± 0.73) (Fig. 8C) and for PAF-AH it was (11.46 ± 5.77) (Fig. 8D). Finally, the results of Bland–Altman analysis show better agreement between radiometric vs HPLC-fluorimetric than between radiometric and real-time fluorimetric methods.

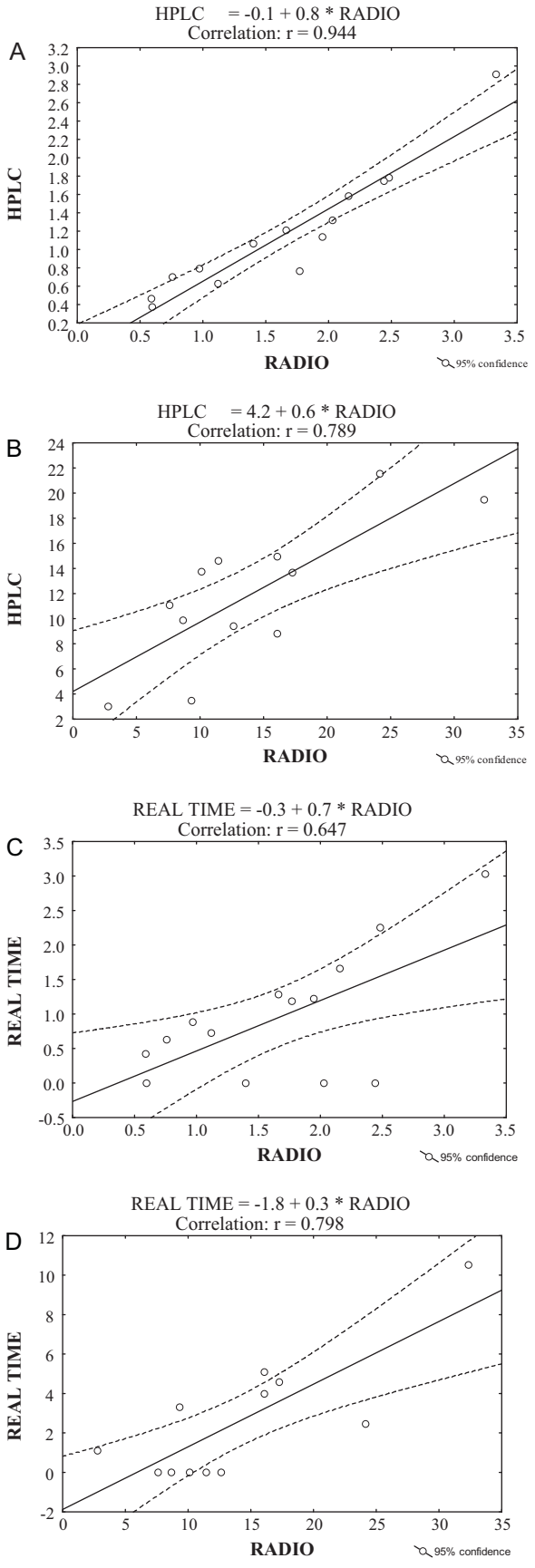

Fig. 7. Relationship between the HPLC fluorimetric and radiometric assays using regression analysis. (1) Radiometric vs HPLC-fluorimetric assays for the determination of (A) PLA_2 or (B) PAF-AH activities. (2) Radiometric vs real-time fluorimetric assays for the determination of (C) PLA_2 or (D) PAF-AH activities.

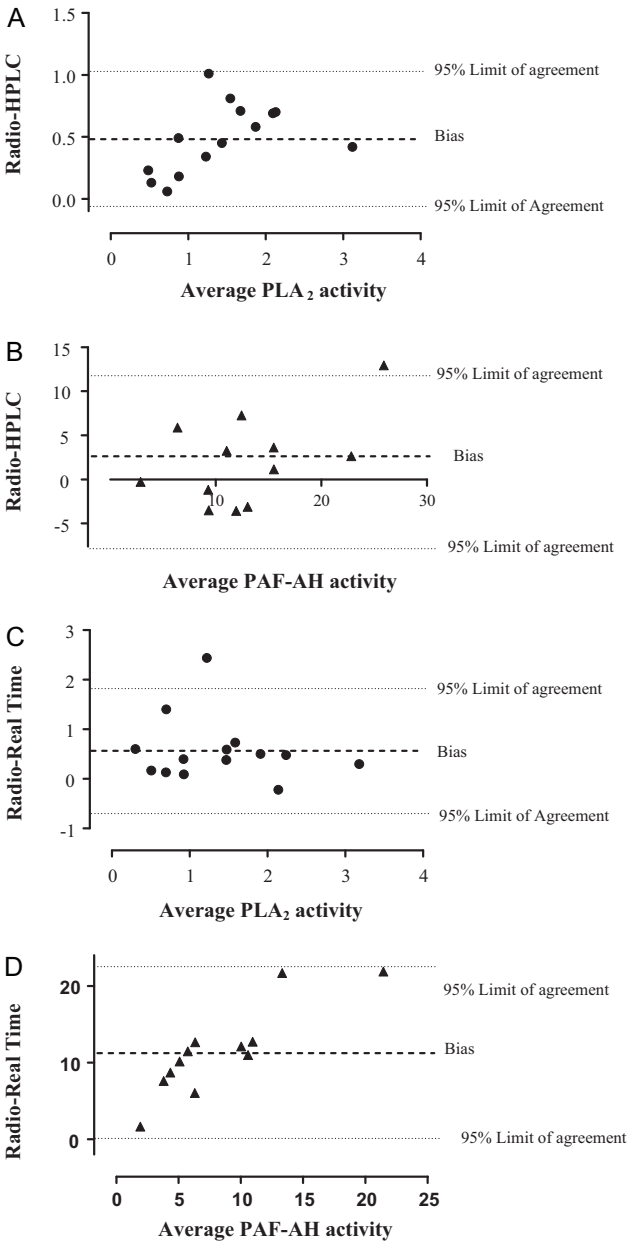

Fig. 8. Relationship between the HPLC fluorimetric and radiometric assays using the Bland–Altman analysis. (1) Radiometric vs HPLC-fluorimetric assays for the determination of (A) PLA_2 or (B) PAF-AH activities. (2) Radiometric vs real-time fluorimetric assays for the determination of (C) PLA_2 or (D) PAF-AH activities. The *X* axis represents the average of parameter values measured by the two methods, while the Ψ axis represents the absolute difference between the parameter values measured by the two methods.

4. Conclusion

The HPLC fluorimetric assay described in the present work is applicable under various experimental conditions. Due to the fact that the fluorescence intensity of all the NBD-lipids was not significantly changed in the presence of Ca^{2+} ions or EDTA that are required by several PLA_2s isoforms, the present method could prove valuable for the discrimination between different PLA_2 isoforms. The method is suitable for biological samples where PLA_2 activity cannot be measured with the real-time fluorimetric assay. Bland–Altman analysis showed that the HPLC-fluorimetric assay gave similar results with the radiometric one. Furthermore, com-

pared with the widely used radiometric methods, the HPLC assay is of low cost and friendly to the environment.

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