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Journal of Chromatography B, 783 (2003) 327–333

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic assay with ultraviolet spectrometric detection for the evaluation of inhibitors of secretory phospholipase A_2

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Received 15 April 2002; received in revised form 19 July 2002; accepted 5 August 2002

Abstract

A non-radioactive spectrometric assay for the evaluation of inhibitors of pancreatic group IB and non-pancreatic group IIA secretory phospholipase A_2 ($sPLA_2$) is described. Mixed-micelles consisting of 1 m*M* of a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol and 6 m*M* of sodium deoxycholate were used as substrate. The enzyme activity was determined directly without any sample clean-up by measuring the $sPLA_2$-mediated oleic acid release with reversed-phase HPLC and UV-detection at 200 nm. The known $sPLA_2$ inhibitors MJ33 and AR-C 67047MI were analyzed in this assay for their inhibitory potency. While MJ33 revealed only a very weak inhibition of group IB and IIA $sPLA_2$ at the highest test concentration (33 μ*M*), AR-C 67047MI proved to be a potent inhibitor of both enzymes with IC_{50}-values of 0.36 and 0.14 μ*M*, respectively.

Keywords: Inhibitors; Secretory phospholipase A_2

1. Introduction

The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes which all catalyze the hydrolysis of the *sn*-2 acyl bonds of glycerophospholipids to generate free fatty acids, such as arachidonic acid, and lysophospholipids. A current classification scheme divides the mammalian and non-mammalian PLA_2s into 12 groups (I–XII) with different subgroups based on their structures, enzymatic characteristics and subcellular distribution [1]. Alternatively, the PLA_2s can also be ordered into such enzymes, which utilize a catalytic histidine, and in enzymes, which have a catalytic serine. The small secretory PLA_2s ($sPLA_2$s), which require millimolar Ca^{2+} for the phospholipid cleavage, belong to the first category. In humans meanwhile nine distinct $sPLA_2$s have been identified (groups IB, IIA, IID, IIE, IIF, III, V, X and XII). The second category consists of larger enzymes that do not contain a Ca^{2+} in the active site. Members of this class are the cytosolic PLA_2s ($cPLA_2$s), the calcium-independent PLA_2s ($iPLA_2$s) and the lipoprotein-associated PLA_2s (also known as PAF-acetylhydrolases). The $cPLA_2$s comprise three enzymes, the $cPLA_2$ α (group IVA), which was first characterized

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00710-9

in 1990, and the $cPLA_2$ β and γ (group IVB and IVC) described recently.

The first mammalian PLA_2 known was the secretory enzyme from pancreas (now classified as group IB $sPLA_2$) [2]. In the late 1980s another structurally related $sPLA_2$ was isolated from platelets [3]. Because elevated levels of this non-pancreatic $sPLA_2$ (now classified as group IIA $sPLA_2$) were found in the synovial fluid of patients with rheumatoid arthritis [4,5], it was suggested that it plays the central role in initiating the inflammatory arachidonic acid cascade. Therefore, great efforts have been made to discover inhibitors of this enzyme as anti-inflammatory agents in the following time [6,7]. However, many findings obtained in the last few years favour the $cPLA_2$ α (referred to as $cPLA_2$ in this article) as the key enzyme for the intracellular liberation of arachidonic acid and the ultimate formation of the pro-inflammatory lipid mediators [7,8].

Besides various other groups, we are engaged in the development of inhibitors of the $cPLA_2$ [9]. For the discovery of such compounds we apply a cellular screening system, which measures the $cPLA_2$-mediated arachidonic acid release in intact platelets by HPLC [10]. During the development of $cPLA_2$ inhibitors, the question arises about inhibitor selectivity for $cPLA_2$ versus $sPLA_2$.

For the assessment of the activity of $sPLA_2$ and for the determination of an inhibition of $sPLA_2$ by a test compound, respectively, many different assay strategies and methods can be applied [11]. The most convenient are the titrimetric, radiometric, fluorometric and the spectrophotometric assays. In the first assay type, the fatty acid obtained by the hydrolysis of phospholipids is quantified by titration with alkalihydroxide [12]. In the procedures carried out with radiolabeled substrates the release of radioactive fatty acids is determined by scintillation counting [13–16]. In the fluorescence-based assays, the $sPLA_2$ substrates contain a fluorophore, which is liberated during substrate cleavage and whose concentration can be quantified by fluorometry [17–19]. For these assays special substrates have to be employed. One type consists of phospholipids with a synthetic fluorophore in position 2, the second type of substrates are fluorogenic non-phospholipid esters such as 7-hydroxycoumarinyl-γ-linolenate. Current spectrometric assays utilize synthetic thiophospholipids, which possess a thioester linkage to the fatty acid chain instead of an oxyester linkage. Hydrolysis by $sPLA_2$ produces a free fatty acid and a thiolysophospholipid as products. The free thiol-group of the thiolysophospholipid is then available to react with a thiol-sensitive reagent to produce a chromophore [20]. Another spectrometric test system measures the fatty acids by HPLC with ultraviolet (UV) detection at 254 nm after derivatization with 9-anthryldiazomethane [21]. However, a method for assessment of $sPLA_2$ activity or inhibition, which monitors the release of fatty acids from naturally occurring, non-radioactive phospholipids by HPLC without derivatization has not been published until now. In this report we describe such an assay, which simply measures the oleic acid liberated from 1-palmitoyl-2-oleoylglycerophospholipids by $sPLA_2$ with HPLC and UV-detection at 200 nm.

2. Experimental

2.1. Materials

2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC), 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoglycerol (POPG), sodium deoxycholate (DOC), oleic acid, phospholipase A_2 from bovine pancreas (group IB $sPLA_2$) (Sigma, Germany); Tris, $CaCl_2$ (Merck, Germany); buffy coats of human blood (Institute of Transfusion Medicine, University of Münster, and German Red Cross); 4-undecyloxybenzoic acid (Aldrich, Germany); MJ33 [22,23] (1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol, lithium salt) (Calbiochem, Germany); AR-C 67047MI [24] (dicyclohexylamine salt of (*S*)-5-(4-benzylphenylsulfanyl)-4-(7-phenylheptanoylamino)pentanoic acid) (AstraZeneca R&D, Charnwood, Leicestershire, UK).

2.2. Isolation of the group IIA $sPLA_2$ from human platelets

About 100 ml buffy coat was centrifuged in four polypropylene tubes at 2000 *g* for 2 min and the platelet-rich supernatants were carefully separated by aspiration. The obtained platelet-rich fractions were combined, diluted with 50 ml cold phosphate-buf-

fered saline (800 mg NaCl, 200 mg KCl, 144 mg $Na_2HPO_4.2H_2O$, 20 mg KH_2PO_4 made up to 100 ml) and centrifuged at 1000 *g* for 15 min. The supernatant was discharged and the sedimented platelets were stored at −20 °C.

The frozen platelets were thawed, suspended in distilled water (10 ml) and homogenized by ultrasonication. To the homogenate, ice-cold 0.36 N H_2SO_4–2 *M* NaCl was added with stirring until a pH of 1.6 was reached. After standing at 4 °C overnight the mixture was centrifuged at 1000 *g* for 15 min. The supernatant was dialyzed overnight against 10 m*M* sodium acetate buffer (pH 4.4). The precipitate formed during dialysis was separated by centrifugation at 4000 *g* for 40 min. The clear supernatant containing the enzyme was concentrated by ultrafiltration in 3-ml Microsep tubes (Pall, Germany) with a cut-off of 10 000 kDa (90 min at 3300 *g*) to a final volume of about 1 ml. In the enzyme assay, 20 μl of this enzyme solution liberated about 5 nmol oleic acid per 100 μl in 30 min. The enzyme preparation was stored at −20 °C until used.

2.3. Assays for group IB and group IIA sPLA$_2$

The phospholipid substrate was dissolved in $CHCl_3$ (10 mg/ml). An aliquot of this solution was thoroughly dried under a stream of nitrogen. To the residue a mixture of a solution of 10.3 m*M* sodium deoxycholate (DOC) in 0.1 *M* Tris–HCl (pH 8.5) and a solution of 6.25 m*M* $CaCl_2$ in 0.8% NaCl (58:20, v/v) was added to generate a 1.28 m*M* solution of the phospholipid. Then, 78 μl of this solution was added to 2 μl of a DMSO solution of the test compound or, in the case of the controls, to 2 μl of DMSO. The mixture was incubated for 5 min at 37 °C. Then the enzyme reaction was started by adding 20 μl of the enzyme solution. In the assay for group IB sPLA$_2$ 100 μg (in the case of POPC), 20 μg (in the case of POPE) and 5 μg (in the case of POPG) of the enzyme, respectively, were dissolved in 1 ml 0.1 *M* Tris–HCl (pH 8.5). In the assay for group IIA sPLA$_2$ the solution obtained after ultrafiltration was used. The final reaction mixture contained 1 m*M* phospholipid, 6 m*M* DOC, 1.25 m*M* $CaCl_2$. The incubation was carried out at 37 °C for 30 min. In case of the kinetic tests the incubation time was variable (0–60 min). The enzyme reaction was terminated by the addition of 400 μl of a mixture of acetonitrile, methanol and 3 m*M* aqueous EDTA–Na_2 solution (1:1:2, v/v/v), which contained 4-undecyloxybenzoic acid as internal standard (0.2 μg/400 μl). The samples obtained were cooled for 15 min in an ice bath and then stored in closed vials at −20 °C until subjection to HPLC. Control incubations in the absence of sPLA$_2$ were carried out in parallel and used to calculate the specific hydrolysis.

2.4. HPLC analysis

The HPLC system consisted of two Waters HPLC-pumps model 515, a Waters autosampler model 717 plus and a Waters UV–Vis-detector model 2487. The on-line UV absorbance spectra of oleic acid were recorded with a Waters diode-array detector model 990. Separation was achieved on a Nucleosil 100 C_{18} analytical column (125 mm×3 mm I.D., particle size 3 μm) protected with a Nucleosil 100 C_{18} guard column (20 mm×3 mm I.D., particle size 5 μm) (Macherey-Nagel, Germany). Without further purification, 100 μl of each sample was injected onto the HPLC system. The mobile phase consisted of acetonitrile–water–phosphoric acid (85%) (80:20:0.1, v/v/v). The flow-rate was 0.4 ml/min. The effluents were monitored at 200 nm. After each run the column was washed by injection of 2×2 ml of acetonitrile (in the case of the assay for group IB sPLA$_2$) or by flushing the column with 10 ml of acetonitrile using the second pump of the HPLC system (in the case of the assay for group IIA sPLA$_2$).

For the evaluation of enzyme inhibition, the mean level of oleic acid concentration obtained in the presence of a test compound (n=3) was compared with the mean level of oleic acid obtained in the absence of test compounds (=control tests, n=3). All data were analyzed by Student's *t*-test. A value of $P<0.05$ was considered significant. For the calculation of the absolute amount of the oleic acid released by the sPLA$_2$s, a calibration curve was used.

3. Results and discussion

The analysis of inhibitors of lipolytic enzymes such as PLA$_2$ is more difficult than with enzymes that operate in the aqueous phase because the

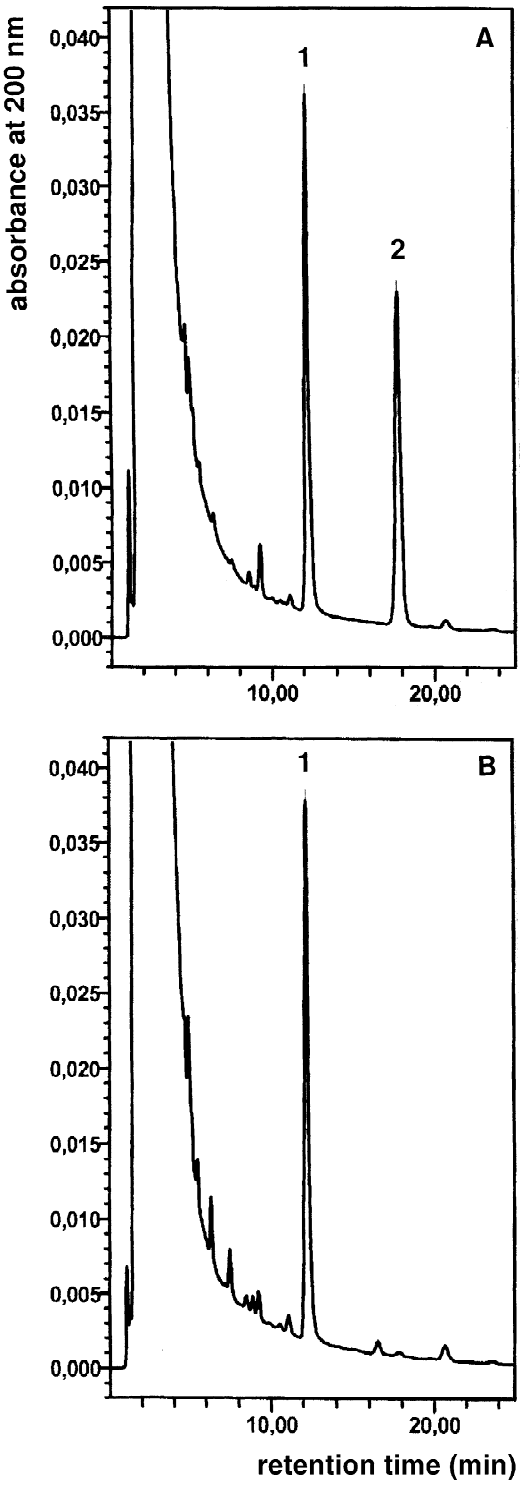

Fig. 1. (A) HPLC analysis of the oleic acid released from POPG by bovine pancreatic (group IB) $sPLA_2$. Mixed micelles of 1 m*M* POPG and 6 m*M* DOC were incubated with the enzyme for 30 min at 37 °C in the presence of 1.25 m*M* $CaCl_2$. The enzyme reaction was terminated by the addition of a mixture of acetonitrile, methanol and 3 m*M* aqueous EDTA-Na_2, which contained 4-undecyloxybenzoic acid as internal standard. (B) HPLC-analysis of a sample with inactivated bovine pancreatic (group IB) $sPLA_2$. The assay mixture containing 1 m*M* POPG, 6 m*M* DOC and 1.25 m*M* $CaCl_2$ was treated with a mixture of acetonitrile, methanol, 3 m*M* aqueous EDTA-Na_2 and internal standard prior to the addition of the enzyme, resulting in the inactivation of the enzyme immediately after its addition. Incubation of this sample was also carried out at 37 °C for 30 min. The samples were subjected to reversed-phase HPLC with UV-detection at 200 nm without further purification. Peaks: 1=internal standard (4-undecyloxybenzoic acid), 2=oleic acid.

enzymatic hydrolysis of naturally occurring, long-chain lipids and phospholipids, respectively, necessarily takes place at a lipid/water interface. Therefore, for assessment of PLA_2 inhibitors usually substrate concentrations are applied, at which aggregation of the phospholipids takes place or micelle forming compounds such as Triton X-100 or sodium deoxycholate are added, in which the substrate is intercalated. One problem of some of these assays is the fact that they are carried out with a concentration of inhibitor comparable to the concentration of the substrate used in the assay. Under such conditions, the amount of the inhibitor in the interface is quite high, and it is possible that the presence of the inhibitor causes desorption of the enzyme from the interface into the aqueous phase by altering the physical nature of the substrate thus falsely indicating enzyme inhibition. Therefore, for the development of true PLA_2 inhibitors, assays should be used in which the mol fraction of inhibitor in the interface is kept low [25].

We first developed an assay for the commercially available pancreatic $sPLA_2$ (group IB).

According to a published procedure, mixed-micelles consisting of 6 m*M* of sodium deoxycholate (DOC) and 1 m*M* of a glycerophospholipid with palmitic acid at position 1 and oleic acid at position 2 were used as substrate [21]. To study the influence of the polar part of the phospholipid substrate on the extent of enzyme inhibition, we applied phospholipids with choline (POPC), ethanolamine (POPE) and glycerol (POPG) head groups, respec-

tively. The maximal inhibitor concentration in the assay was 33 μ*M*, ensuring that the substrate concentration is much higher than that of an effective inhibitor.

The reaction mixtures were incubated in Tris-buffer in the presence of 1.25 m*M* Ca^{2+}. The enzyme reaction was terminated by adding a mixture of acetonitrile, methanol and aqueous EDTA containing 4-undecyloxybenzoic acid as internal standard. Without further purification an aliquot of the obtained solution was subjected to HPLC analysis. The oleic acid released by the enzyme was determined by reversed-phase HPLC and UV-detection at 200 nm. After each run the column was washed with acetonitrile to elute remaining compounds. Fig. 1A shows a typical HPLC-chromatogram of the oleic acid released by the pancreatic $sPLA_2$ in the absence of an inhibitor. The specifity of the chromatographic separation for oleic acid was proofed by several experiments: The recorded on-line UV-spectra of peak 2 and of an oleic acid reference were identical. After inactivation of the enzyme by a mixture of acetonitrile, methanol and aqueous EDTA, a significant peak at the retention time of oleic acid did not occur (Fig. 1B). Furthermore, the formation of the oleic acid peak could be inhibited quantitatively by 10 μ*M* of the $sPLA_2$-inhibitor AR-C 67047MI (Fig. 2).

Kinetic experiments showed that the velocity of enzyme reaction was dependent on the polar head group of the phospholipid. The specific activity of $sPLA_2$-IB was highest for POPG and lowest for POPC. To achieve about the same rate of hydrolysis for all three substrates, the amount of enzyme applied was varied. Thus, the final reaction mixture contained 20 ng/ml (POPC), 4 ng/ml (POPE) and 1 ng/ml (POPG) of $sPLA_2$-IB, respectively. Using these conditions the reaction progress curves for the hydrolysis of the phospholipids were linear up to a period of 50 min. For the evaluation of enzyme inhibitors an incubation time of 30 min was chosen. The $sPLA_2$ inhibitors MJ33 and AR-C 67047MI (Fig. 2) were evaluated under the standard assay conditions using POPC, POPE and POPG, respectively, as substrate. MJ33 has been reported to inhibit group IB pancreatic $sPLA_2$ with high specifity [22,23], while AR-C 67047MI has proved to be a potent inhibitor of both enzymes, group IB and IIA $sPLA_2$ [24]. In our assay MJ33 only produced a weak inhibition of the $sPLA_2$-IB of about 30% at the highest concentration (33 μ*M*) in all cases (Table 1). In an assay applying vesicles consisting of the non-physiological phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol, MJ33 had shown a

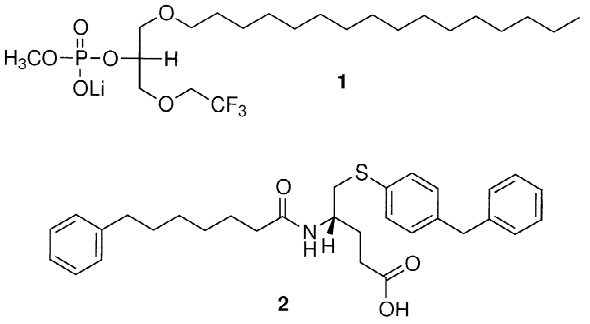

Fig. 2. Structure of the $sPLA_2$ inhibitors MJ 33 (1) and AR-C 67047MI (2).

Table 1
Inhibition of the group IB $sPLA_2$-mediated oleic acid release by the $sPLA_2$ inhibitors MJ33 and AR-C 67047MI using mixed micelles of sodium deoxycholate and 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phospholipids with choline (POPC), ethanolamine (POPE) and glycerol (POPG) headgroups, respectively

Compound	Inhibition of group IB $sPLA_2$ from bovine pancreas [IC_{50} (μ*M*)][a]		
	Phospholipid substrate:		
	POPC	POPE	POPG
MJ 33	>33[b]	>33[c]	>33[d]
AR-C 67047MI	0.32±0.04	0.34±0.06	0.36±0.07

[a] Values are means±SD (*n*=3).
[b] 24±10% inhibition at 33 μ*M* (mean±SD, *n*=3).
[c] 27±3.3% inhibition at 33 μ*M* (mean±SD, *n*=3).
[d] 28±10% inhibition at 33 μ*M* (mean±SD, *n*=3).

higher inhibitory potency. The IC_{50}-value (=inhibitor concentration, at which a 50% inhibition of the enzyme activity could be achieved) in this assay was about 2.5 μ*M* [22]. The different nature of the substrates used in both assays (phospholipid vesicles vs. phospholipid/DOC micelles) may be the reason for the differences in activity observed for MJ33. In contrast to MJ33, AR-C 67047MI inhibited the bovine pancreatic enzyme with a high potency. The IC_{50}-values obtained with the three different phospholipids used by us were nearly identical and lay in the nanomolar range (Table 1).

Next, we developed an analogous assay for group IIA $sPLA_2$. The enzyme was isolated from human platelets by acid extraction according to a method described for the isolation of the enzyme from leukocytes [26,27]. Since it has been demonstrated previously that mixed micelles of POPG and cholate are best suited for both group IB and IIA $sPLA_2$ [21,28,29], we only used this substrate system here. The concentration of the $sPLA_2$-IIA in the platelet extract was adjusted to a value, which led to the same rate of phospholipid hydrolysis as in the assay with the pancreatic enzyme. During the period of the incubation (30 min) about 5 nmol oleic acid per 100 μl were released by the enzyme. The reaction progress curve was linear for at least 60 min. As seen in Table 2, MJ33 was only a very weak inhibitor of the enzyme (13% inhibition at 33 μ*M*), while AR-C 67047MI was even more potent against this enzyme (IC_{50}=0.14 μ*M*) than against the $sPLA_2$-IB (IC_{50}=0.36 μ*M*).

In conclusion, we have developed simple non-radioactive assays for the evaluation of group IB and group IIA $sPLA_2$ inhibitors, in which natural glycerophospholipids in combination with sodium deoxycholate are applied as substrate. The enzyme product oleic acid is quantified directly without derivatization by HPLC and UV-detection at 200 nm. Since a similar substrate composition has been successfully applied in a radioactive assay for the recently discovered group IID, IIE, V and X $sPLA_2$ [16], the test system should also be valid to determine inhibitors of these enzymes.

Table 2
Inhibition of the group IIA $sPLA_2$-mediated oleic acid release by the $sPLA_2$ inhibitors MJ33 and AR-C 67047MI using mixed micelles of sodium deoxycholate and 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoglycerol (POPG)

Compound	Inhibition of group IIA $sPLA_2$ from human platelets IC_{50} (μ*M*)[a]
MJ 33	>33[b]
AR-C 67047MI	0.14±0.01

[a] Values are means±SD (n=3).
[b] 13±3.6% inhibition at 33 μ*M*.

Acknowledgements

The authors thank Dr Dave H. Robinson, AstraZeneca R&D Charnwood, Leicestershire, UK, for generously providing the $sPLA_2$ inhibitor AR-C 67047MI.

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