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Short communication

Determination of arachidonic acid by on-line solid-phase extraction HPLC with UV detection for screening of cytosolic phospholipase $A_2\alpha$ inhibitors

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ABSTRACT

An on-line solid-phase extraction (SPE)–liquid chromatographic method with ultraviolet detection at 200 nm for screening of inhibitors of cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) was developed and validated. cPLA$_2\alpha$ was isolated from porcine platelets. Enzyme activity was determined by measuring the release of arachidonic acid from a phospholipid substrate using automated on-line sample clean up on a trap column followed by isocratic back-flush elution on a RP18 analytical column. While the use of a conventional RP18 column for trapping the analyte led to peak broadening only after a few runs due to pollution of the column by binding of components present in the enzyme preparation, the application of a turbulent flow column (TurboFlow Cyclone™) resulted in sharp peaks even after a plurality of injections. Interestingly, for sample introduction a turbulent flow of the mobile phase produced by high flow rates was not necessary to maintain good peak shapes. The same result could also be achieved applying low flow rates (0.5 mL/min). Several known cPLA$_2\alpha$ inhibitors were used to validate the test system.

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1. Introduction

Cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) is an esterase that selectively cleaves the *sn*-2 position of arachidonoyl-glycerophospholipids of biomembranes to generate free arachidonic acid and certain lysophospholipids [1,2]. Arachidonic acid in turn is metabolized to a variety of inflammatory mediators including prostaglandins and leukotrienes. Lysophospholipids with an alkyl ether moiety at the *sn*-1 position can be acetylated to platelet activating factor (PAF), another mediator of inflammation. Although several more phospholipases A_2 are present in the mammalian organism, the pre-eminence of cPLA$_2\alpha$ for lipid mediator generation was demonstrated especially by studies with cPLA$_2\alpha$ deficient mice. These animals, which display a reduced eicosanoid production, are resistant to disease in a variety of models of inflammation [3,4]. Therefore, cPLA$_2\alpha$ is considered as a target for the treatment of inflammatory diseases [4–9]. Meanwhile several potent inhibitors of cPLA$_2\alpha$ have been developed, which show activity in diverse animal models of inflammation after systemic or local application. However, none of these compounds is undergoing clinical development presently.

Many of the published procedures for determination of activity of isolated cPLA$_2\alpha$ and for screening of inhibitors of the enzyme are radioactive assays. Vesicles of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine ([^{14}C]PAPC) [10], covesicles of [^{14}C]PAPC or its 1-stearoyl-analogue [^{14}C]SAPC with 1,2-dioleoyl-*sn*-glycerol [11–16] and 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol [17], respectively, or mixed-micelles comprised of [^{14}C]PAPC and Triton X-100 [18–22] serve as substrates. Besides, [^{3}H]-arachidonate labeled U937 cell membranes are employed [17,19,23]. After separation from the assay mixture, the released radioactive arachidonic acid is quantified by scintillation counting. Furthermore, several fluorimetric assays have been developed for assessing activity of purified cPLA$_2\alpha$. In some of these assays phosphatidylcholines containing fluorophores are used [24,25]. Other fluorimetric substrates for the evaluation of the activity of isolated cPLA$_2\alpha$ are esters formed between a fatty acid, such as γ-linolenic acid, and 7-hydroxycoumarine [15,19,26–28]. Moreover, a spectrophotometric assay has been described for measuring the activity of cPLA$_2\alpha$ applying a thiophospholipid as substrate [29].

We have published a screening method for cPLA$_2\alpha$ inhibitors detecting the enzyme product arachidonic acid released from the natural phospholipid 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) by HPLC and UV detection at 200 nm [30]. To increase enzyme activity, 1,2-dioleoyl-*sn*-glycerol was added to the substrate in this assay. For cleaning-up of the analyte an off-line solid-phase extraction (SPE) was performed prior HPLC analysis. Now, we have optimized this test system with regard to rapidness and simpleness by application of an automated on-line SPE. The method, which does not require any sample pre-treatment, was

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validated and successfully applied for the determination of the IC_{50} values of several known cPLA$_2\alpha$ inhibitors.

2. Experimental

2.1. Reagents and chemicals

Arachidonic acid, $CaCl_2$, dithiothreitol (DTT), phosphate buffered saline tablets (one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), nordihydroguaiaretic acid (NDGA), protease-inhibitor-cocktail (ingredients: 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1.5 mM pepstatin A, 1.4 mM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 4 mM bestatin, 2.0 mM leupeptin, and 0.08 mM aprotinin in DMSO), Tris, Hepes (Sigma–Aldrich, Taufkirchen, Germany); methanol HPLC-grade, acetonitrile HPLC-grade, EDTA–Na_2, KCl, NaCl (Merck, Darmstadt, Germany); dimethyl sulfoxide (DMSO), EGTA (Fluka, Taufkirchen, Germany); 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) solution (10 mg/mL ethanol), 1,2-dioleoyl-*sn*-glycerol solution (10 mg/mL acetonitrile), bromoenol lactone, RSC-3388 ((*E*)-*N*-[((2*S*,4*R*)-4-{[(1,1′-biphenyl)-2-ylmethyl](isobutyl)amino}-1-[2-(2,4-difluorobenzoyl)benzoyl]pyrrolidin-2-yl)methyl]-3-{4-[(*Z*)-(2,4-dioxothiazolidin-5-ylidene)methyl]phenyl}acrylamide) (Cayman Chemical, Ann Arbor, USA delivered via Biozol, Eching, Germany); porcine blood (slaughterhouse Schöppingen, Germany); HiTrap™ Q Sepharose Fast Flow anionic exchange columns 5 mL (total ionic capacity: 0.18–0.25 mmol Cl^-/mL) (GE Healthcare Bio-Sciences, Munich, Germany); Amicon Ultra centrifugal filter Ultracel 50K, regenerated cellulose 50,000 MWCO, Cat. No. UFC905008 (Millipore, Schwalbach, Germany); AR-C70484XX (4-[3-(4-decyloxyphenoxy)-2-oxopropoxy]benzoic acid) was synthesized according to a published procedure [15].

2.2. Isolation of cPLA$_2\alpha$ from porcine platelets

Immediately after the death of the animal, porcine blood was collected in two 250 mL polypropylene vessels, which each contained 50 mL of a solution of 0.077 M EDTA–Na_2 in 0.2% (m/v) aqueous NaCl solution. The blood was centrifuged in portions of 20 mL in 50 mL Falcon™ tubes at 195 × *g* for 15 min at room temperature and the platelet-rich supernatants were carefully separated by aspiration. The obtained platelet-rich fractions (about 180 mL) were centrifuged in portions of 30 mL at 1029 × *g* for 10 min. The pellets were resuspended in a total volume of 15 mL of a mixture of phosphate buffered saline and 3.7% aqueous EDTA–Na_2 (97:3, v/v). After centrifugation at 1029 × *g* at room temperature for 10 min, the platelets were resuspended in 20 mL of lysis-buffer (10 mM Hepes, 1 mM DTT, 2 mM EGTA, 140 mM NaCl, 27 mM KCl, pH 7.4 at 20 °C). After addition of 200 μL of protease-inhibitor-cocktail solution, the cell suspension was freezed at −20 °C. At the next day, the suspension was thawed and the cells were disrupted by sonication (Branson sonifier B15, level 4, 4× 15 s) at 0 °C and subjected to centrifugation at 48 000 × *g* at 4 °C for 1 h. Three aliquots of about 7 mL of the clear supernatant were diluted with the same volume buffer A (25 mM Tris, 1 mM EGTA, 2 mM DTT, pH 8.0 at 20 °C). Each aliquot was loaded onto a HiTrap™ Q Sepharose FF anionic exchange column (5 mL) that had been conditioned previously with 25 mL of buffer A, 25 mL of buffer B (25 mM Tris, 1 M NaCl, 1 mM EGTA, 2 mM DTT, pH 8.0 at 20 °C) and again 35 mL of buffer A at a flow rate of 5 mL/min. The column was washed with 25 mL of buffer A and then eluted at a flow rate of 5 mL/min in four steps with 15 mL aliquots of mixtures of buffer A and B containing 0.15 M, 0.30 M, 0.45 M and 0.60 M NaCl, respectively, and the four fractions were collected. The combined fractions containing the enzyme (fractions 3 obtained at a NaCl concentration of 0.45 M, total volume 45 mL) were concentrated with an Amicon Ultra centrifugal filter Ultracel 50K (4000 × *g* at 4 °C for 15 min) to a final volume of about 10 mL. The concentrate was incubated with 5 μL of a bromoenol lactone solution (1 mM in DMSO) for 10 min at room temperature to inhibit any iPLA$_2$ present in the sample. Applying the method of Bradford with bovine serum albumin (BSA) as standard, for the obtained enzyme solution a protein concentration of about 0.5 mg/mL was determined. In the enzyme assay 10 μL of this solution liberated about 0.25 nmol arachidonic acid per 100 μL in 60 min. The enzyme preparation was stored at −80 °C until used.

2.3. Incubation procedure

A mixture of 900 μL of a solution of 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) in ethanol (10 mg/mL) and 346 μL of a solution of 1,2-dioleoyl-*sn*-glycerol (DOG) in acetonitrile (10 mg/mL) was divided into aliquots of 35 μL. After removing the solvent in a stream of nitrogen, the aliqouts were stored at −80 °C until used.

For preparing the substrate solutions, an aliquot of SAPC and DOG was dissolved in a small amount of ethanol and dried again in a stream of nitrogen. Then the residue was treated with 1372 μL of Tris-buffer (50 mM Tris, 1 mM DTT, 150 mM NaCl, 1 mM $CaCl_2$, pH 8.0 at 20 °C) and sonicated in a bath sonicator at 40–50 °C. The concentration of SAPC and DOG in the opalescent solution obtained was 227 μM and 114 μM, respectively. 88 μL of this solution were added to 2 μL of a DMSO solution of a test compound or, in case of the controls, to 2 μL of DMSO. The mixtures were pre-incubated at 37 °C for 10 min. Then the enzyme reaction was started by adding 10 μL of the enzyme preparation. The final reaction mixtures (100 μL) contained 200 μM of SAPC substrate and 100 μM of DOG. The incubations were performed at 37 °C for 60 min. In case of the kinetic tests the incubation time was variable (5–75 min). The enzyme reaction was terminated by the addition of 200 μL of a mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA–Na_2 solution (16:15:1, v/v/v), which contained nordihydroguaiaretic acid (NDGA) as oxygen scavenger (0.6 μg/200 μL). The obtained samples were cooled in an ice bath, diluted with 500 μL of a mixture of water and Tris-buffer (50 mM Tris, 1 mM DTT, 150 mM NaCl, 1 mM $CaCl_2$, pH 8.0 at 20 °C) (4:1, v/v) and then directly subjected to HPLC analysis. Control incubations in the absence of the enzyme and in the absence of substrate, respectively, were carried out in parallel and used to calculate the specific hydrolysis.

2.4. On-line solid-phase extraction (SPE) and HPLC analysis

The on-line SPE–HPLC/UV system consisted of a Waters (Milford, USA) HPLC pump 515 (pump A) coupled via a Waters autosampler model 717 plus (equipped with a 2 mL sample loop and a 2.5 mL injection syringe) to a ten-port two-position automated switching valve from Rheodyne (Waters, EV700-102-WA) and a Waters HPLC pump 515 (pump B) coupled to the switching valve directly. A TurboFlow Cyclone™ column 1.0 mm × 50 mm (Thermo Scientific, Dreieich, Germany, Cat. No. 952434) was used as trap column and a Nucleosil 100 C18 column (3 mm inside diameter × 125 mm, particle size 3 μm) (Macherey & Nagel, Düren, Germany) protected by a C18 guard column (3 mm inside diameter × 4 mm) (Phenomenex, Aschaffenburg, Germany) was applied as analytical column. Trap column and analytical column were connected to the switching valve as described recently [31]. In valve position 1 the sample (400 μL) was injected into the system and

loaded to the trap column with the mobile phase delivered by pump A (acetonitrile/water, 20:80, v/v) at a flow rate of 0.5 mL/min. After 2 min, the valve was automatically switched to position 2 and the analyte was back-flushed off the trap column onto the analytical column with the mobile phase delivered by pump B (acetonitrile/water/phosphoric acid (85%), 77:23:0.1, v/v/v) at a flow rate of 0.4 mL/min. A Waters UV detector 486 was used for detection at 200 nm and system control was performed by Waters Millennium [32] software. The temperature of the autosampler was kept at 10 °C. The analytical column was temperature controlled at 20 °C by a jetstream plus column oven from Waters. System suitability was assessed by injection of 400 μL of a mixture of 2 μL of a DMSO solution of arachidonic acid (150 μM), 198 μL of Tris-buffer (50 mM Tris, 1 mM DTT, 150 mM NaCl, 1 mM $CaCl_2$, pH 8.0 at 20 °C), 200 μL of a mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA–Na_2 solution (16:15:1, v/v/v), which contained NDGA (0.6 μg/200 μL), and 400 μL of water after each 15 injections. After about 30 injections the trap column was washed with 200 μL of isopropanol.

For calculation of enzyme inhibition the peak area of enzyme product arachidonic acid obtained in presence of a test compound was compared with the mean level of the peak area of arachidonic acid determined in absence of test compounds (=control tests, $n = 3$). The IC_{50} values were calculated with the aid of Probit transformation.

2.5. Method validation

Linearity was evaluated using enzyme product (arachidonic acid) spiked matrix samples at six concentration levels. These samples were prepared by mixing 2 μL of a DMSO solution of arachidonic acid (concentration of arachidonic acid: 20 μM, 50 μM, 100 μM, 150 μM, 200 μM and 250 μM) with 198 μL of Tris-buffer (50 mM Tris, 1 mM DTT, 150 mM NaCl, 1 mM $CaCl_2$, pH 8.0 at 20 °C), 200 μL of a mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA–Na_2 solution (16:15:1, v/v/v), which contained the oxygen scavenger NDGA (0.6 μg/200 μL), and 400 μL of water. The amount of arachidonic acid present in these solutions ranged from 0.040 nmol (12.2 ng) to 0.50 nmol (152 ng) per 800 μL. A reagent blank was prepared in the same manner by adding pure DMSO instead of the arachidonic acid solutions in DMSO. 400 μL of each sample (from a total volume of 800 μL) was directly injected onto the analytical column without using the switching valve.

Recovery was evaluated for five different concentration levels of enzyme product-spiked matrix samples (0.040 nmol, 0.10 nmol, 0.20 nmol, 0.30 nmol and 0.40 nmol per 800 μL). The samples were prepared as described above with the exception, that 10 μL of Tris-buffer was replaced by 10 μL of the enzyme preparation. To avoid substrate cleavage by the enzyme during these experiments, the enzyme preparation was added to the mixture after the addition of the mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA–Na_2 solution. A reagent blank was prepared in the same manner by adding pure DMSO instead of the arachidonic acid solutions in DMSO. 400 μL of each sample was loaded onto the trap column and back-flushed to the analytical column using the switching valve as described above under 2.4. The recovery was calculated with the aid of the data evaluated for the determination of linearity. The obtained data were also used to calculate precision.

The variability of the assay was investigated by replicate determination of the IC_{50} values of five different $cPLA_2\alpha$ inhibitors at different days. The IC_{50} values were calculated with the aid of Probit transformation.

3. Results and discussion

3.1. Enzyme isolation and incubation procedure

In the screening assay for $cPLA_2\alpha$ inhibitors we have used so far [30], the enzyme was isolated from human platelets obtained from buffy coats by centrifugation. We now decided to use porcine platelets as enzyme source because of the lower infection risk. The isolation procedure was similar to that described for the human enzyme. Briefly, after isolation by differential centrifugation, the platelets were lysed by freezing and thawing followed by ultrasonication. Cell fragments were removed by centrifugation and $cPLA_2\alpha$ was isolated from the supernatant by anion exchange chromatography on a HiTrap™ column. The eluates with the enzyme were concentrated by ultra filtration. From 500 mL of porcine blood about 10 mL of enzyme preparation sufficient for about 1000 sample incubations were obtained. The protein concentration of the enzyme solution was about 0.5 mg/mL. In the enzyme assay, which was performed applying the same substrate composition and concentration as described for the assay with the human enzyme [30], 10 μL of the enzyme solution liberated about 0.25 nmol arachidonic acid per 100 μL in 60 min.

3.2. Incubation procedure, on-line solid-phase extraction and HPLC analysis

Since off-line solid-phase extraction (SPE) is time-consuming, we decided to apply automated on-line SPE for measuring the enzyme product arachidonic acid. With the latter method the samples can be injected onto the HPLC system without any pretreatment, resulting in a considerable reduction of the expenditure of work and an increase of the sample throughput.

We started our experiments using a conventional end-capped C18 column (LiChrospher 100 RP 18 EC 5 μ, 4 mm × 40 mm) for trapping and cleaning-up of the analyte arachidonic acid. However, with this material peak broadening occurred after only a few injections due to saturation of the column by binding of components present in the enzyme preparation, most likely proteins. In literature it has been reported that the so called turbulent flow chromatography is suitable for separating large molecules from low mass analytes [32]. The columns used in this type of chromatography consist of porous particles with sizes that generally range from 30 to 50 μm. Operating at high flow rates of the mobile phase of about 4 mL/min for 1 mm internal diameter columns leads to turbulent flows in the column preventing large biomolecules to diffuse into the pores of the stationary phase. By this means, a contamination of the column e.g. by proteins leading to a loss of column performance can be avoided. Therefore, we applied a polymeric Cyclone™ turbulent flow column as trap column alternatively.

The automated online system consisted of two isocratic HPLC pumps (a loading pump and an elution pump), and a two-position ten-port switching valve. With one of the HPLC pumps the sample provided from an autosampler was loaded onto the turbulent flow trap column. After a given time the switching valve was automatically switched into the second position, which led to disconnection of the turbulent flow column from the loading pump and its insertion between elution pump and analytical column [31]. The trapped analyte now was back flushed from the turbulent flow column onto the analytical column with analytical mobile phase delivered by the elution pump. The system is comparable to the commercially TurboFlow apparatus Aria TLX2 QuickElute System from Thermo Scientific, with the distinction that the commercial system uses two six-port valves and two gradient HPLC pumps.

In preliminary experiments it was evaluated, at which sample loading conditions the highest recoveries for the analyte could be achieved. Keeping the total loading volume constant at 2 mL and

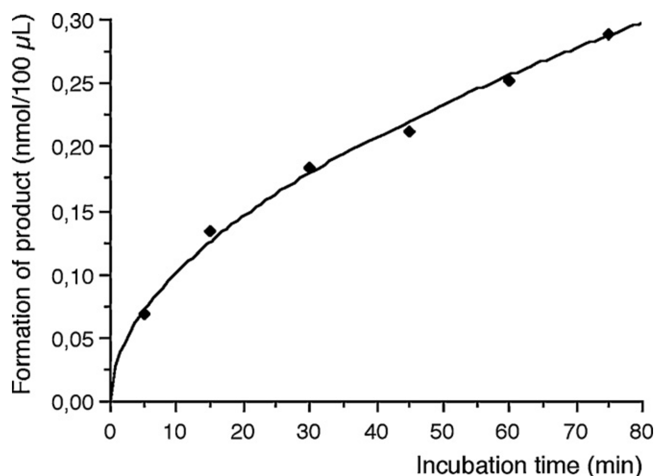


Fig. 1. Kinetics of the release of arachidonic acid from vesicles of 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (0.2 mM) and 1,2-dioleoyl-*sn*-glycerol (0.1 mM) by $cPLA_2\alpha$ isolated from porcine platelets. The reaction was conducted in a final volume of 0.1 mL of Tris-buffer buffer (Tris, DTT, NaCl, $CaCl_2$, pH 8.0 at 20 °C). Values are the means of duplicates.

changing the loading flow from 4 mL/min to 0.25 mL/min it was revealed that the recovery of arachidonic acid did not vary dramatically. Its absolute fluctuation range was about 15%. The highest recovery value was measured, when the sample was loaded at 0.50 mL/min for 4 min. Because a reduction of the loading time from 4 min to 2 min at this flow further increased recovery slightly, and because repeated injections of samples containing enzyme substrate and protein did not lead to a worsening of the peak shape of the analyte, the latter loading conditions were used for the validation of the method. For the analyte a carry-over effect did not arise during the automated extraction process.

A further goal within the modification of the screening assay was to reduce the amount of enzyme necessary for generation of a quantity of arachidonic acid sufficient for HPLC analysis at 200 nm. In comparison to our published $cPLA_2\alpha$ assay [30], the final dilution of the incubation volume (100 μL) was now decreased (from 1200 to 800 μL), and the volume of sample injected into the HPLC was increased (from 100 μL to 400 μL). By this way, the amount of enzyme necessary for the incubations could be reduced to about 20% of the amount necessary before.

Kinetic experiments with human $cPLA_2\alpha$ had shown that under the assay conditions used an incubation time of 60 min was suitable [30]. Because the reaction progress curve for the formation of the enzyme product arachidonic acid by the porcine enzyme had a similar shape (Fig. 1) as the curve determined with the human enzyme, in the modified assay the enzymatic reaction was carried out for 60 min too.

Fig. 2B shows a HPLC chromatogram obtained under the optimized conditions in absence of an enzyme inhibitor. Although the trap column had been reused for about 350 times before, still a sharp peak shape for the analyte arachidonic acid was observed. For comparison in Fig. 2A a chromatogram is shown, which was recorded after direct injection of a sample without on-line purification. Here, a very broad injection peak occurs and the arachidonic acid peak sits on the shoulder of this peak. With the turbulent flow column a major part of the matrix components could be separated (Fig. 2B) and almost base-line separation of the arachidonic acid could be achieved.

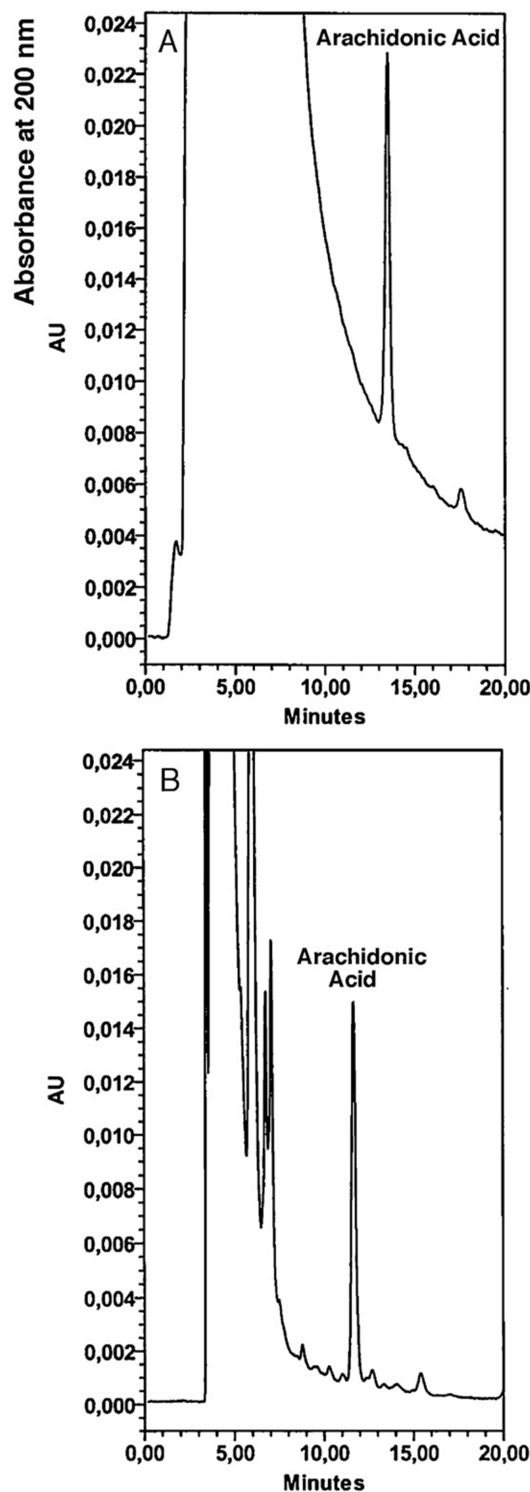
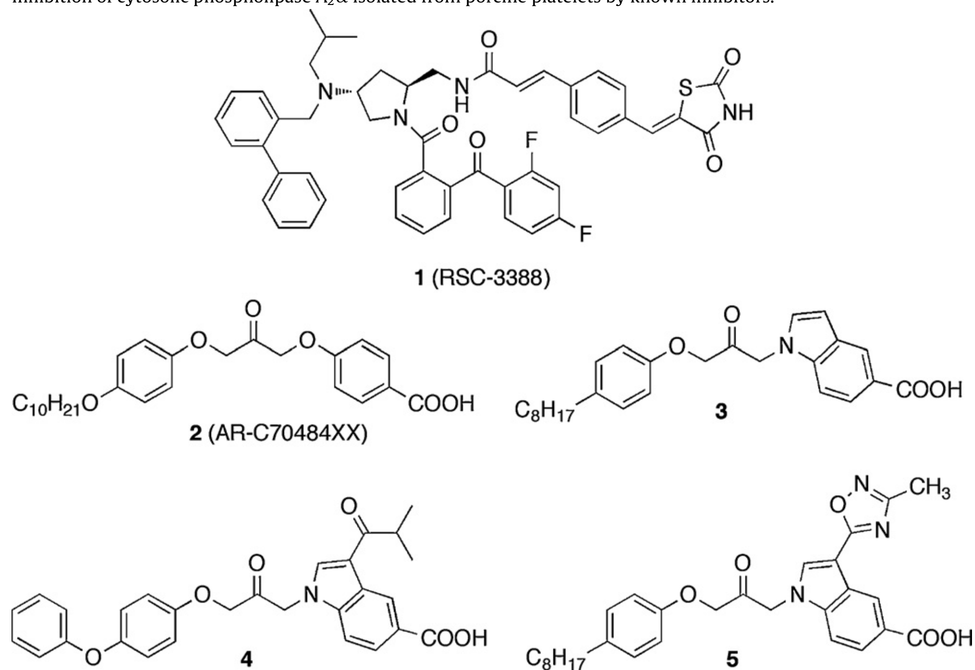


Fig. 2. HPLC chromatograms of the arachidonic acid released from 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) by porcine platelet $cPLA_2\alpha$ in the absence of an enzyme inhibitor. Sonicated vesicles consisting of 0.2 mM of SAPC and 0.1 mM of 1,2-dioleoyl-*sn*-glycerol (DOG) were incubated with the enzyme in Tris-buffer (Tris, DTT, NaCl, $CaCl_2$, pH 8.0 at 20 °C) for 60 min at 37 °C. The enzyme reaction was terminated by the addition of a mixture of acetonitrile, methanol and 0.1 mM aqueous $EDTA–Na_2$, which contained NDGA as oxygen scavenger. A C18 column protected by a C18 guard column was used as analytical column. UV detection was performed at 200 nm. (A) Direct injection of a sample (400 μL) without on-line purification. (B) On-line solid phase extraction of a sample (400 μL) with a TurboFlow Cyclone^{TM} column 1.0 mm × 50 mm. The chromatogram shown was obtained after about 350 injections of samples containing enzyme protein. The trap column was washed with 0.2 mL isopropanol after about each 30 injections.

3.3. Validation

Linearity of the detector signal was measured with matrix-spiked solutions having the same composition as the assay samples,

Table 1
Inhibition of cytosolic phospholipase $A_2\alpha$ isolated from porcine platelets by known inhibitors.



Compound	Inhibition of $cPLA_2\alpha$ IC_{50} (nM)[a]
1 (RSC-3388)	22 ± 0.23
2 (ARC-70484XX)	6.5 ± 1.1
3	24 ± 3.7
4	13 ± 0.61
5	1.0 ± 0.16

[a] Value is the mean ± standard deviation of independent experiments performed at different days; in case of **3**: $n = 5$; in all other cases: $n = 3$.

with the exception that instead of 2 μL of DMSO or DMSO solution of a test compound, 2 μL of a DMSO solution of arachidonic acid (concentration: 20–250 μM) were added per 800 μL (final concentration of arachidonic acid: 0.05–0.625 μM). Furthermore, the enzyme solution (10 μL) was replaced by assay buffer. A reagent blank was prepared in the same manner by adding pure DMSO instead of a arachidonic acid solution of DMSO. 400 μL of each mixture, consisting of a total volume of 800 μL, was directly injected onto the analytical column in duplicate (injected amount of arachidonic acid: 6.1–76 ng). Plotting the peak area of arachidonic acid versus its concentration, a non-weighted linear regression coefficient of $R^2 = 0.9985$ was obtained.

Evaluation of recovery and precision of the on-line extraction was determined by five-fold injection of 400 μL of five matrix-spiked samples prepared as described above for linearity determination (final concentration of arachidonic acid: 0.05 μM, 0.125 μM, 0.25 μM, 0.375 μM, and 0.50 μM), with the exception, that enzyme was present in the same amount (10 μL per 800 μL) as during inhibitor testing. To avoid substrate cleavage by the enzyme in these experiments, the enzyme preparation was added to the mixture after the addition of the mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA–Na_2 solution. A blank without arachidonic acid was prepared and investigated in the same manner too.

Recovery of arachidonic acid at the five control concentrations, which correspond to about 160%, 120%, 80%, 40% and 16% of the amount of arachidonic acid released in incubations with the enzyme performed in absence of an enzyme inhibitor (=controls) was 86 ± 0.6% (at 0.05 μM), 81 ± 1.8% (at 0.125 μM), 79 ± 2.8% (at 0.25 μM), 80 ± 1.5% (at 0.375 μM) and 81 ± 2.1% (at 0.50 μM) (mean ± standard deviation, $n = 5$). The mean recovery values were slightly better than those obtained by off-line solid-phase extraction on a C18 column (about 75%) [30]. The precision (relative standard deviation, RSD) at the five concentration levels was 0.7%, 2.2%, 3.5%, 1.9% and 2.6%, respectively ($n = 5$).

The method was further validated by measuring inhibition of the enzyme by the known potent $cPLA_2\alpha$ inhibitors RSC-3388 (**1**) and AR-C70484XX (**2**) from Shionogi [11] and AstraZeneca [15], respectively. Additionally, three $cPLA_2\alpha$ inhibitors with indole-5-carboxylic acid scaffold developed in our group [33–35] were evaluated. The results are shown in Table 1. The IC_{50} values determined for the five reference inhibitors lie in the same range as the corresponding values obtained with the human enzyme in our off-line HPLC/UV-assay using human $cPLA_2\alpha$ [30]. More precisely, the IC_{50}s of **4** are nearly identical in both assays, while the IC_{50}s for the remaining substances are about 1.5–2-fold lower in the on-line assay with the porcine enzyme. The rank order of potency of the inhibitors, however, does not change. The deviations of the IC_{50}s observed are not a consequence of choosing another species as enzyme source but rather a methodological outcome caused by the five-fold reduction of the amount of enzyme used in the assay. This could be seen on the basis of the inhibition values of compound **3**, which we use as a reference in each incubation series at a concentration of 33 nM. Replacement of the human $cPLA_2\alpha$ by the same amount of porcine enzyme in the off-line SPE assay did not lead to a significant change of inhibitory potency of **3**. In five independent experiments here a medium inhibition value of 41 ± 8% (mean ± standard deviation) was evaluated with the porcine enzyme, reflecting an IC_{50} value slightly above 33 nM (for comparison, IC_{50} value for **3** obtained with the human enzyme: 35 nM [33]). With the new on-line SPE method, in which a more diluted porcine enzyme solution is used, an inhibition value higher than 50% (56 ± 4%, $n = 5$) was measured

at 33 nM, resulting in an IC_{50} somewhat below this concentration (Table 1).

4. Conclusions

A validated assay for screening of $cPLA_2\alpha$ inhibitors using on-line solid-phase extraction for sample pre-treatment and reversed phase-HPLC/UV for determination of the enzyme product arachidonic acid was developed. The application of a turbulent flow trap-column (TurboFlow Cyclone^{TM}) resulted in sharp peaks even after a plurality of injections. Interestingly, for an effective separation of proteins, a turbulent flow in the column produced by a high flow rate was not necessary. The reliability of the enzyme assay was shown by the validation values and the repeated determination of the IC_{50} values of known $cPLA_2\alpha$ inhibitors.

References

[1] Y. Kita, T. Ohto, N. Uozumi, T. Shimizu, Biochim. Biophys. Acta 1761 (2006) 1317.
[2] M. Ghosh, D.E. Tucker, S.A. Burchett, C.C. Leslie, Prog. Lipid Res. 45 (2006) 487.
[3] A. Sapirstein, J.V. Bonventre, Biochim. Biophys. Acta 1488 (2000) 139.
[4] J. Bonventre, Trends Immunol. 25 (2004) 116.
[5] S. Connolly, D.H. Robinson, Expert Opin. Ther. Patent. 5 (1995) 673.
[6] J.D. Clark, S. Tam, Expert Opin. Ther. Patent. 14 (2004) 937.
[7] M. Lehr, Anti-Inflamm. Anti-Allergy Agents Med. Chem. 5 (2006) 149.
[8] V. Magrioti, G. Kokotos, Expert Opin. Ther. Patent. 20 (2010) 1.
[9] E.A. Dennis, J. Cao, Y.H. Hsu, V. Magrioti, G. Kokotos, Chem. Rev. 111 (2011) 6130.
[10] E. Amandi-Burgermeister, U. Tibes, B.M. Kaiser, W.G. Friebe, W.V. Scheuer, Eur. J. Pharmacol. 326 (1997) 237.
[11] K. Seno, T. Okuno, K. Nishi, Y. Murakami, F. Watanabe, T. Matsuura, M. Wada, Y. Fujii, M. Yamada, T. Ogawa, T. Okada, H. Hashizume, M. Kii, S. Hara, S. Hagishita, S. Nakamoto, K. Yamada, Y. Chikazawa, M. Ueno, I. Teshirogi, T. Ono, M. Ohtani, J. Med. Chem. 43 (2000) 1041.
[12] J. Varghese, R.E. Rydel, E.G. Thorsett, WO98/25893 (1998).
[13] J. Varghese, R.E. Rydel, M.S. Dappen, E.G. Thorsett, WO00/27824 (2000).
[14] I.P. Street, H.K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N.M. Tremblay, Z. Huang, P.K. Weech, M.H. Gelb, Biochemistry 32 (1993) 5935.
[15] S. Connolly, C. Bennion, S. Botterell, P.J. Croshaw, C. Hallam, K. Hardy, P. Hartopp, C.G. Jackson, S.J. King, L. Lawrence, A. Mete, D. Murray, D.H. Robinson, G.M. Smith, L. Stein, I. Walters, E. Wells, W.J. Withnall, J. Med. Chem. 45 (2002) 1348.
[16] F. Märki, W. Breitenstein, E. Beriger, R. Bernasconi, G. Caravatti, J.E. Francis, R. Paioni, H.U. Wehrli, R. Wiederkehr, Agents Actions 38 (1993) 202.
[17] J.R. Burke, L.B. Davern, P.L. Stanley, K.R. Gregor, J. Banville, R. Remillard, J.W. Russell, P.J. Brassil, M.R. Witmer, G. Johnson, J.A. Tredup, K.M. Tramposch, J. Pharmacol. Exp. Ther. 298 (2001) 376.
[18] R. Lucas, A. Ubeda, M. Paya, M. Alves, E. del Olmo, J.L. Lopez, A. San Feliciano, Bioorg. Med. Chem. Lett. 10 (2000) 285.
[19] F. Ghomashchi, R. Loo, J. Balsinde, F. Bartoli, R. Apitz-Castro, J.D. Clark, E.A. Dennis, M.H. Gelb, Biochim. Biophys. Acta 1420 (1999) 45.
[20] G. Kokotos, S. Kotsovolou, D.A. Six, V. Constantinou-Kokotou, C.C. Beltzner, E.A. Dennis, J. Med. Chem. 45 (2002) 2891.
[21] W.C. Hope, T. Chen, D.W. Morgan, Agents Actions 39 (1993) C39.
[22] K. Conde-Frieboes, L.J. Reynolds, Y.C. Lio, M.R. Hale, H.H. Wasserman, E.A. Dennis, J. Am. Chem. Soc. 118 (1996) 5519.
[23] J. Banville, P. Serge, G. Yonghua, B. Neelakantan, US 6,492,550 (2002).
[24] T. Bayburt, B.Z. Yu, I. Street, F. Ghomashchi, F. Laliberte, H. Perrier, Z. Wang, R. Homan, M.K. Jain, M.H. Gelb, Anal. Biochem. 232 (1995) 7.
[25] H.S. Hendrickson, E.K. Hendrickson, I.D. Johnson, S.A. Farber, Anal. Biochem. 276 (1999) 27.
[26] Z. Huang, F. Laliberte, N.M. Tremblay, P.K. Weech, I.P. Street, Anal. Biochem. 222 (1994) 110.
[27] J.S. Seehra, Y. Xiang, J. Bemis, J. McKew, N. Kaila, L. Chen, WO 99/43672 (1999).
[28] J.C. McKew, M.A. Foley, P. Thakker, M.L. Behnke, F.E. Lovering, F.W. Sum, S. Tam, K. Wu, M.W.H. Shen, W. Zhang, M. Gonzalez, S. Liu, A. Mahadevan, H. Sard, S.P. Khor, J.D. Clark, J. Med. Chem. 49 (2006) 135.
[29] L.J. Reynolds, L.L. Hughes, L. Yu, E.A. Dennis, Anal. Biochem. 217 (1994) 25.
[30] M. Schmitt, M. Lehr, J. Pharm. Biomed. Anal. 35 (2004) 135.
[31] J. Fabian, J. Chromatogr. B 875 (2008) 557.
[32] P.J. Rudewicz, Bioanalysis 3 (2011) 1663.
[33] J. Ludwig, S. Bovens, C. Brauch, A. Schulze Elfringhoff, M. Lehr, J. Med. Chem. 49 (2006) 2611.
[34] A. Drews, S. Bovens, K. Roebrock, C. Sunderkötter, D. Reinhardt, M. Schäfers, A. van der Velde, A. Schulze Elfringhoff, J. Fabian, M. Lehr, J. Med. Chem. 53 (2010) 5165.
[35] S. Bovens, A. Schulze Elfringhoff, M. Kaptur, D. Reinhardt, M. Schäfers, M. Lehr, J. Med. Chem. 53 (2010) 8298.