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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 601–605
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Normal-phase HPLC and HPLC–MS studies of the metabolism of a cytosolic phospholipase $A_2\alpha$ inhibitor with activated ketone group by rat liver microsomes

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Received 30 May 2006; received in revised form 6 July 2006; accepted 7 July 2006
Available online 23 August 2006

Abstract

Inhibition of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) is assumed to provide a novel therapeutic approach for the treatment of many inflammatory diseases. 1-[3-(4-Octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**) is a potent inhibitor of $cPLA_2\alpha$. An important part of the pharmacophore of **2** is its activated electrophilic ketone moiety. Since it is known that activated ketones may be metabolically unstable, the metabolism of **2** by rat liver microsomes was investigated. For quantification of the metabolites normal-phase HPLC/UV on a cyano column was used, because under reversed-phase conditions with aqueous solvents **2** was partly transformed into its hydrate resulting in chromatograms with splitted peaks. Under the conditions applied about 30% of **2** were metabolized. The main metabolite was the alcohol **4** as shown by LC/MS^n.

Keywords: Metabolism; Rat liver microsomes; Activated ketone; Hydrate; Normal-phase chromatography; Cyano-phase

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 lysophospholipids [1]. 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. Thus, inhibition of $cPLA_2\alpha$ is considered as an attractive target for the design of new anti-inflammatory drugs [2,3].

Although there have been intense efforts for finding inhibitors of $cPLA_2\alpha$, no such compound has emerged to the market. The only $cPLA_2\alpha$ inhibitor reported to undergo clinical development as anti-inflammatory drug is the indole derivative efipladib from Wyeth [4]. Published compounds with high *in vitro* $cPLA_2\alpha$-inhibitory potency are thiazolidinediones of Shionogi [5] and propan-2-ones of AstraZeneca [6] such as compound **1** (Fig. 1). Recently, we have found that 1-[3-(4-octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**), which is structurally related to **1**, is also a potent inhibitor of $cPLA_2\alpha$ [7].

A common structural feature exhibited by **1** and **2** is the activated electrophilic ketone group. This moiety is an important part of the pharmacophore of the inhibitors. Its reduction leads to the inactive alcohols **3** and **4**, respectively (Fig. 1) [6,8,9]. Since it is known that activated ketones can be unstable towards keto-reduction [10], we tested the metabolic stability of **2** applying rat liver microsomes.

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC grade), isohexane (HPLC grade), phosphoric acid (85%) were obtained from Baker (Deventer, Netherlands). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium). Tetrahydrofuran (THF) (HPLC grade), diethyl ether, potassium dihydrogenphosphate,

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doi:10.1016/j.jpba.2006.07.017

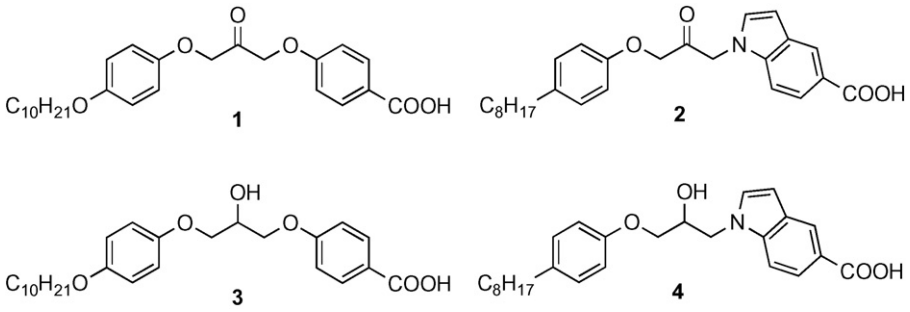

Fig. 1. Chemical structures of cPLA$_2\alpha$ inhibitors with activated ketone group (**1**, **2**) and their corresponding alcohol forms (**3**, **4**).

potassium hydrogenphosphate were acquired from VWR International (Darmstadt, Germany). Dimethylsulfoxide (DMSO) (p.a.), magnesium chloride hexahydrate (ultra) were purchased from Fluka (Buchs, Switzerland). Dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) was acquired from Roth (Karlsruhe, Germany). 2-(*p*-Toluoyl)benzoic acid was obtained from Lancaster Synthesis (Morecambe, United Kingdom). Water was purified using a Bi 18 system from Heraeus (Hanau, Germany). 1-[3-(4-Octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**) and 1-[2-hydroxy-3-(4-octylphenoxy)propyl]indole-5-carboxylic acid (**4**) were synthesized according to published procedures [7,9].

2.2. Preparation of rat liver microsomes

Livers of male Sprague–Dawley rats were obtained from the Institute of Pharmacology of the Medicinal Faculty of the University of Münster. Rat liver microsomes were preparated adapted to published procedures [11–13].

Six livers (95 g total) were washed with cold potassium chloride solution (1.15%, m/v) and homogenized in an Elvehjem-Potter homogenizer with an equivalent volume of cold potassium buffer (pH 7.4; 0.1 M) containing sodium EDTA (0.5 mM). The homogenate was diluted to 285 ml and centrifuged at 10,000 × *g* at 4 °C for 20 min. The supernatant was centrifuged at 100,000 × *g* at 4 °C for 60 min. The resulting microsomal pellet was resuspended in 285 ml potassium phosphate buffer (pH 7.4; 0.1 M). For removing all residual cytosolic glutathione *S*-transferases [13] the suspension was centrifuged again at 100,000 × *g* at 4 °C for 60 min. The pellet was resuspended in potassium phosphate buffer (pH 7.4; 0.1 M) (1.2 ml buffer/2 g original tissue) and stored at −80 °C. The microsomal protein concentration was determined according to the method of Bradford [14] applying bovine serum albumin (BSA) as standard and finally adjusted to 15 mg/ml.

2.3. Preparation of standards

Stock solutions containing 5 mM of **2** and **4** were prepared in DMSO. Working solutions were prepared by diluting the stock solutions with appropriate amounts of DMSO. The internal standard 2-(*p*-toluoyl)benzoic acid was dissolved in acetonitrile at a concentration of 0.2 mM and stored at 4 °C.

2.4. Incubation procedures

Microsomal incubations were carried out using 2.25 mg/ml rat liver microsomal protein in potassium phosphate buffer (pH 7.4; 0.1 M) with 3 mM $MgCl_2$ and 1.9 mM NADPH in a final volume of 498 μl. The reaction was initiated by the addition of 2 μl of the stock solution of **2** (final concentration 20 μM) and the mixtures were incubated in a shaking water bath for 30 min. The metabolisation was stopped by addition of 100 μl internal standard solution and 500 μl of ice cold acetonitrile. After addition of 1.25 ml 0.1 M phosphoric acid the sample was extracted with 3 ml diethyl ether in a rotating mixing wheel for 10 min and subsequently centrifuged at 1400 × *g* for 5 min. The organic layer was separated and the extraction step repeated. Then the combined organic layers were concentrated under a stream of nitrogen. The residue was reconstituted in 200 μl of isohexane–THF (75:25, v/v) and an aliquote (50 μl) of this solution was injected onto the HPLC column. Reference incubations without NADPH were performed in the same way. The analyte solutions were shown to be stable at 10 °C for 24 h.

The microsomal incubation samples used for HPLC/MS-analysis contained 25 μM of **2**. The incubation time was 180 min.

2.5. HPLC/UV-analysis

The HPLC system consisted of a Bischoff 2250 HPLC pump gradient system (Leonberg, Germany) with a dynamic mixing chamber from Knauer (Berlin, Germany) coupled to a Midas Cool autosampler with column oven from Spark Holland (Emmen, The Netherlands) and a UV-detector 486 from Waters (Milford, USA). System control and data processing were performed using McDAcq32 Control software. The temperature of the autosampler was kept at 10 °C. Chromatographic separation and quantitation of **2** and its metabolites was achieved by normal-phase chromatography on a Lichrospher 100-5 CN column (250 mm × 4 mm) protected by a Lichrospher 100-5 CN guard column (4 mm × 4 mm) (Merck, Darmstadt, Germany). The HPLC mobile phases consisted of isohexane–THF–TFA (92:8:0.1, v/v/v) (A) and isohexane–THF–TFA (50:50:0.1, v/v/v) (B). HPLC separation was conducted at 25 °C applying a gradient with a flow rate of 0.75 ml/min. The initial composition was 10% B. The gradient was programmed linearly to 80% B over 15 min and held for 3 min. Finally, the gradient was linearly

Table 1
Precision and accuracy of the method for the determination of **2** in rat liver microsomes

Sample concentration	Precision ($n=5$), R.S.D. (%)	Accuracy, R.E. (%)
0.53 μg/ml (1.25 μM)	3.9	−4.5
5.3 μg/ml (12.5 μM)	5.9	−2.8
10.5 μg/ml (25 μM)	2.5	−3.1

decreased to 10% B over 2 min and held for 7 min. The effluents were monitored at 240 nm.

2.6. Method validation

The calibration curve for linearity of **2** was constructed by spiking 2 μl of DMSO solutions with varying amounts of **2** into extraction tubes containing 2.25 mg/ml rat liver microsomal protein in potassium phosphate buffer (pH 7.4; 0.1 M) with 3 mM $MgCl_2$ in a volume of 498 μl in absence of NADPH. Seven concentration levels in the range of 1.25–25 μM (0.53–10.5 μg/ml) of **2** were taken through the extraction procedure and HPLC analysis. The peak area ratios of **2** and internal standard were plotted against the concentration of **2**. The correlation coefficient observed was $R=0.999$. The deviation of the standards from nominal concentration was less than 10%.

To determine the recovery of the assay, analogous samples spiked with 1.25, 12.5 and 25 μM (0.53, 5.3 and 10.5 μg/ml) of **2** were analyzed in the same way (each $n=5$). The concentration of the samples was determined on the basis of samples with corresponding concentrations of **2** not submitted to extraction (each $n=3$). For establishing these calibration values, aliquots of working solutions of **2** in DMSO were concentrated in a speed-vac. The residues were reconstituted in isohexane–THF (75:25, v/v), and aliquots (50 μl) of these solutions were injected onto the HPLC column. The absolute recovery was 98 ± 6.8% and the relative recovery 115 ± 4.5% (mean ± S.D.).

The precision and accuracy of the method were assayed by analyzing analogous samples ($n=5$) containing 1.25, 12.5 and 25 μM (0.53, 5.3 and 10.5 μg/ml) of **2** in the same way. The concentrations of the samples were determined on the basis of the calibration curve for **2** submitted to extraction. The results obtained are expressed as relative standard deviation (R.S.D.) and relative error (R.E.) (Table 1).

The limit of quantitation for **2** was 0.28 μg/ml (0.67 μM), based on a signal-to-noise ratio of 10:1.

2.7. HPLC/MS-analysis

The HPLC/MS system consisted of a separation module 2690 from Waters (Milford, USA) which was coupled on-line to the LCQ® ion trap mass spectrometer (Thermo-Finnigan, San Jose, USA) via an ESI-interface. The usage of an APCI-interface was found to be less suitable. The ESI voltage was set to −3 kV, the sheath gas flow was adjusted to 80 arbitrary units and the auxiliary gas flow rate was set to 0 arbitrary units. The temperature of the heated capillary was 200 °C, the tube lens offset was adjusted to 35 V and the capillary voltage set to −7.2 V. The mass spectrum was recorded in negative mode. MS^n-mode experiments were accomplished with an isolation width of *m/z* 3–5 and a normalized collision energy of 30–38%. For the simultaneous UV-detection the tunable absorbance detector 2487 from Waters (Milford, USA) was used. The detection wavelength was set to 235 nm.

A 100 μl volume of the samples was injected onto a Kromasil 100-5 C18 (60 mm × 2 mm) column (CS, Langerwehe, Germany). The mobile phase consisted of acetonitrile–ammonium formiate (20 mM) (20:80, v/v) (A) and acetonitrile–ammonium formiate (20 mM) (85:15, v/v) (B). HPLC separation was conducted at a flow rate of 0.40 ml/min. The initial composition was 45% B. The gradient was programmed linearly to 100% B over 10 min and held for 1 min. Finally, the gradient was linearly decreased to 45% B over 1 min and held for 3 min.

3. Results and discussion

Usually, reversed-phase stationary phases are applied for analysis of drug metabolism by HPLC. Since reversed-phase chromatography of **2** with aqueous solvents resulted in splitted peaks (Fig. 2) probably due to hydrate formation of the activated keto functionality, separation of **2** and its metabolites had to be performed under water free conditions. Hence, a normal-phase HPLC method on a cyano-phase with isohexane–THF was developed.

The indole **2** was then incubated with rat liver microsomes under aerobic conditions in absence and presence of the electrogenic cofactor NADPH for 30 min according to published procedures [13]. After termination of the reaction by addition of acetonitrile and internal standard, dilute phosphoric acid was added and the resulting mixture was extracted twice with diethyl ether. The combined ether phases were concentrated under a stream of nitrogen and the residue dissolved in isohexane–THF. This solution was subjected to HPLC analysis with UV-detection at 240 nm. Representative chromatograms obtained are shown in Fig. 3.

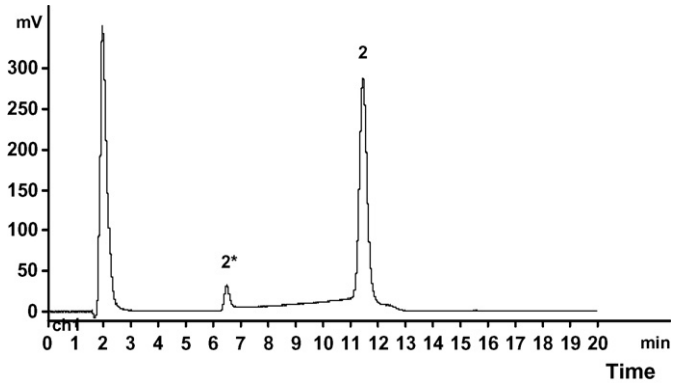

Fig. 2. HPLC chromatogram of **2** under reversed-phase conditions (column: Kromasil 100-5 C18, 250 mm × 3 mm with a guard column 10 mm × 3 mm; solvent: acetonitrile–aqueous trifluoroacetic acid (0.1%) (80:20, v/v); flow: 0.6 ml/min; UV-detection: 235 nm). 2*: front peak of $cPLA_2\alpha$ inhibitor **2**; 2: main peak of $cPLA_2\alpha$ inhibitor **2**.

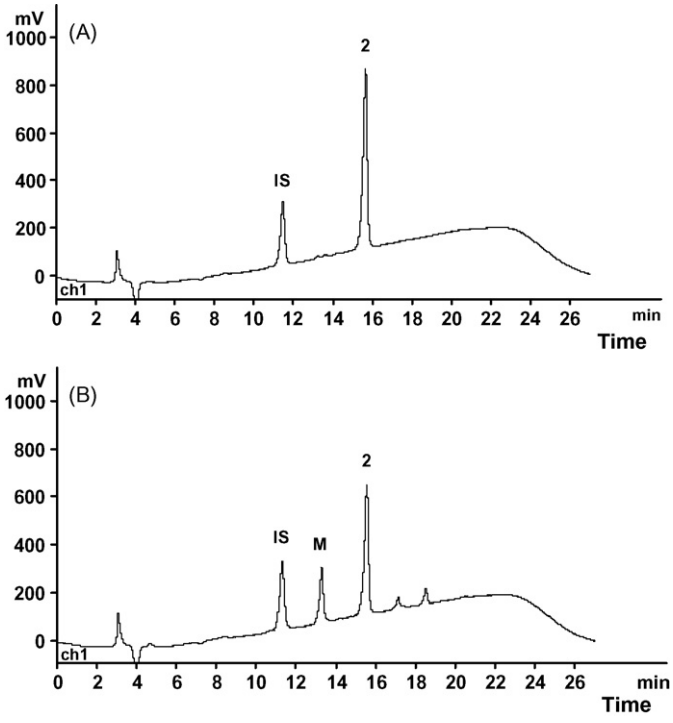

Fig. 3. Normal-phase chromatograms for **2** following incubation with rat liver microsomes in absence (A) and presence (B) of NADPH. IS: internal standard 2-(*p*-toluoyl)benzoic acid; 2: cPLA$_2\alpha$ inhibitor **2**; M: main metabolite.

The chromatogram of the reference (Fig. 3A) displays the internal standard (IS) and the analyte **2** as main peaks. Incubation in presence of NADPH (Fig. 3B) resulted in the formation of metabolites. Peak M was the main metabolite with about 18% of the peak area ratio of **2** in the reference. The amount of parent compound **2** remaining after 30 min was 66 ± 0.84% (mean ± S.D., $n = 5$). Kinetic experiments showed that prolongation of the incubation time up to 2 h does not cause a further significant increase of metabolism of **2**.

After spiking the incubation probe with the alcohol **4**, it could be assumed that this compound was the main metabolite. LC/MS^n was employed to verify this assumption.

Since LC/MS investigations with normal-phase chromatography on a cyano-phase were not successful, a reversed-phase chromatographic method was applied using a mixture of aqueous ammonium formiate solution and acetonitrile as eluent. As expected, peak splitting of compound **2** could be observed again. The main metabolite eluted between the front peak (**2***) and the main peak of **2** (Fig. 4). The mass spectra of this metabolite and of the alcohol **4** were identical, revealing a formiate adduct at *m/z* 468 $[M + 45]^-$. After isolation and collision of that ion, a daughter ion appeared in the MS^2-spectrum at *m/z* 422 $[M - H]^-$, which could be further fragmented to the main product ions at *m/z* 378, 216 and 203 in both cases.

The MS-spectrum of the front peak **2*** contained an ion at *m/z* 484, which allowed to assign the structure as the hydrate of **2** $[M + 18 + 45]^-$. In the MS^2-spectrum it fragmented to the ketone **2** by elimination of water and formiate (*m/z* 420 $[M - H]^-$) (Fig. 5). The ketone **2** itself appeared in the MS as formiate adduct at *m/z* 466, and fragmented to *m/z* 420 $[M - H]^-$.

In conclusion, a normal-phase HPLC-method for detection of the metabolites of the cPLA$_2\alpha$ inhibitor **2** formed by rat liver microsomes was described. Under the test conditions **2** is only metabolized in part. The main metabolite is the inactive alcohol **4** as proved by MS^n-experiments. Further investigations will measure stability of **2** in rats during the carrageenan-induced rat paw edema assay, an *in vivo* test system widely applied for testing anti-inflammatory drug candidates.

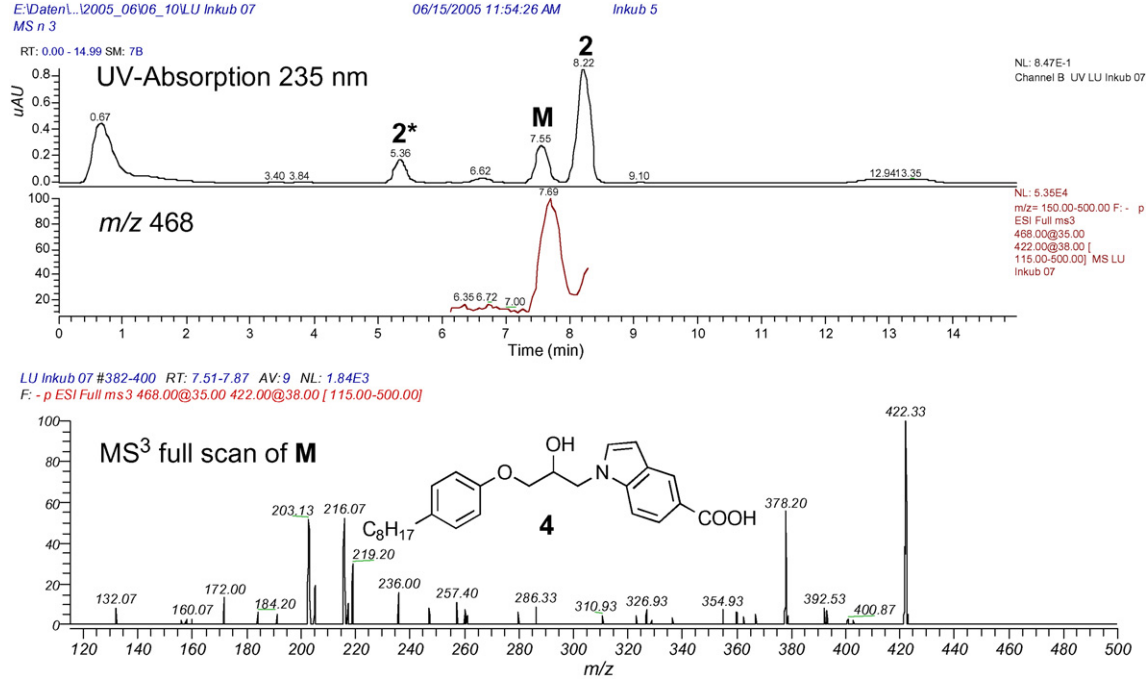

Fig. 4. UV-chromatogram, ion-chromatogram (*m/z* 468), and MS^3 full scan of the main metabolite (M) of rat liver microsome incubation of the cPLA$_2\alpha$ inhibitor **2**.

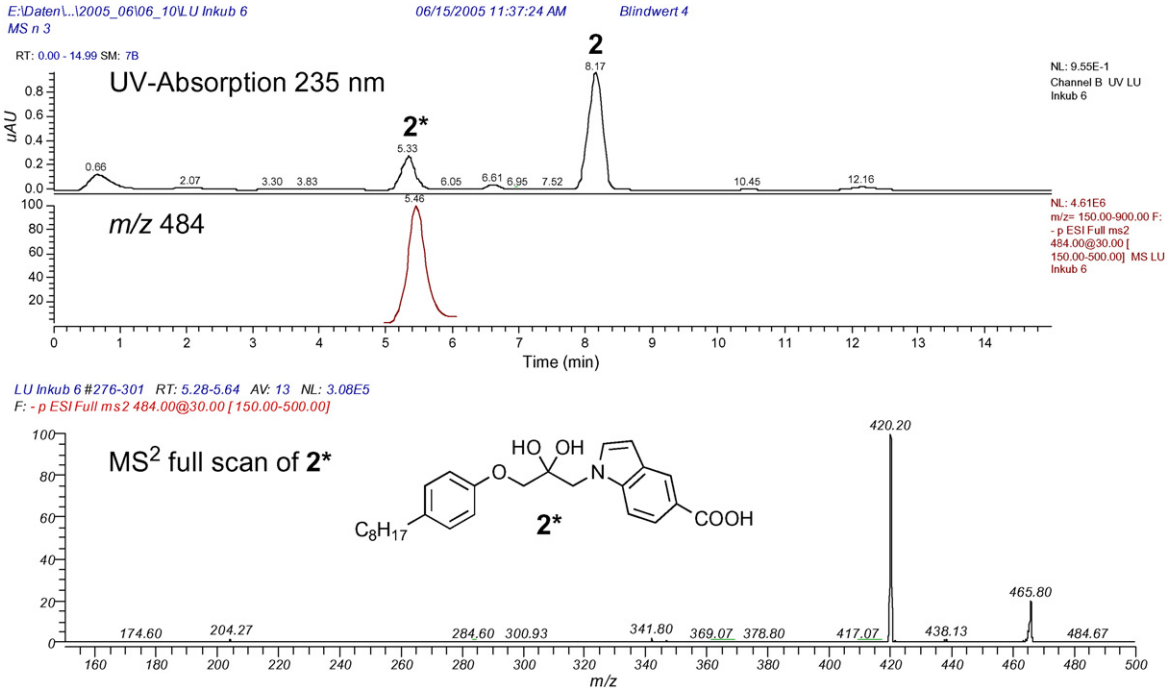

Fig. 5. UV-chromatogram, ion-chromatogram (*m/z* 484) of the cPLA$_2\alpha$ inhibitor **2**, and MS^2 full scan of **2*** (front peak of **2**).

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