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Short communication

Assessment of caspase-4 released free AFC by RP-HPLC and fluorescence detection

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ABSTRACT

A simple RP-HPLC method based on fluorescence detection was developed for the quantitation of 7-amino-4-trifluoro methylcoumarin (AFC) in cell lysates from JEG-3 choriocarcinoma cells for determination of caspase-4 activity. In contrast to the established methods of AFC detection using a fluorescence microplate reader or using a fluorescence photometer, the separation of AFC-signals from interfering fluorescence signals by a reversed phase column affords more precise quantitation of released AFC. This can be important for analyses of cell lysates with low caspase activity or experimental series with marginal differences among samples. By applying this new method, a linear dynamic range of 40 pmol/mL to 3 nmol/mL with a correlation coefficient of 0.9996 was achieved. Due to the short retention time (~7 min), the determination of AFC by RP-HPLC under isocratic conditions requires small amounts of samples (50 μL injection volume), and allows increased sample throughput. This method should be easily applied with little or no modification to other caspase assays by using the same fluorophore.

1. Introduction

Caspases are an evolutionarily conserved family of cysteine-dependent aspartate-specific proteases, with central functions in apoptotic and inflammatory signalling pathways. There is increasing evidence that caspase-activation does not necessarily lead to the execution of apoptosis, but rather plays an important role in other cellular processes, such as cell cycle regulation and differentiation [1,2].

The observation of caspase activity and the identification of caspase substrates in the absence of cell death have recently sparked a strong interest in caspase functions in cellular responses beyond apoptosis. The non-apoptotic functions of caspases suggest that they may become activated independently of the induction of an apoptotic cascade, thus leading to the cleavage of a specific subset of substrates. These substrates include members of diverse protein families such as cytokines, kinases, transcription factors and polymerases (for review see [3] and [4]).

Specific synthetic peptides with the fluorophore 7-amino-4-trifluoro methylcoumarin (AFC) at the C-terminal end are commonly used as substrates among the currently available *in vitro* assays for determination of caspase activities [5]. Substrates with this modification are available for caspase-1, 2, 3, 4, 6, 7, 8 and 9 — partially as components of different kits. After release of AFC by caspase-induced cleavage, the fluorophore can be detected at excitation/emission = 400 nm/505 nm by using a fluorescence microplate reader, or a fluorescence photometer. This convenient method has a major disadvantage: other fluorescence signals in the experimental preparation may interfere with the released AFC at the same excitation/emission wavelength, and therefore complicate the quantitative determination of AFC.

It has been shown in an earlier work of our group that leukemia inhibitory factor (LIF) stimulation of JEG-3 choriocarcinoma cells induces simultaneously invasiveness and caspase-4 expression as assessed by DNA macro-arrays and real-time PCR [6]. The aim of this study was to separate signals of free AFC, released by caspase-4, from interfering fluorescence signals by using RP-HPLC. In this article, an easy and sensitive RP-HPLC method is described for the quantitative determination of released AFC from caspase-4 substrate in cell lysates from JEG-3 choriocarcinoma cells.

2. Experimental

2.1. Materials

AFC, a proteinase inhibitor cocktail and antimycotic, antibiotic solution (AAS) was purchased from Sigma (Germany). The substrate

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Ac-Leu-Glu-Val-Asp-AFC was obtained from MP Biomedicals (USA) and Axxora (Germany). Methanol was HPLC Gradient Grade, and purchased from Roth (Germany), deionized water was generated using a Millipore Synergy UV (USA). Cell culture medium (DMEM, OPTIMEM) and Oligofectamin™ reagent for transfection were from Invitrogen (Germany) and siRNA was purchased from Ambion (UK). HEPES (Invitrogen, Germany), Glycerol, Dithiothreitol (DTT), EDTA, 1N HCl (all from Roth, Germany) were analytical grade. LIF, IL 6 and HGF were obtained from Immunotools (Germany). PBS and FCS were purchased from Biochrom (Germany). JEG-3 cells were purchased from ATCC (USA).

2.2. Standard solution and calibration standard curve

AFC standard stock solution ($c = 10\ \mu$mol/mL) was prepared in methanol, and stored in the dark at −20 °C. All further dilutions of AFC were made by diluting the AFC stock standard with 40/60 (v/v) methanol/water. Calibration working standards of AFC with concentrations from 40 pmol/mL to 3 nmol/mL were prepared. The working standards were analyzed with HPLC and a linear regression calibration equation was generated.

2.3. Cell cultures and preparation of cell lysates

All cultures were commenced at 10^6 JEG-3 cells/175 cm^2 flask, and maintained under standard conditions (37 °C, 5% CO_2, humidified atmosphere) in DMEM medium with L-glutamine, supplemented with 10% foetal calf serum (FCS) and 2% AAS. JEG-3 cells were trypsinized twice a week when confluence was estimated to be above 75%. For induction of different caspase-4 activities in the same cell line, different treatments were applied: 1, stimulation with different cytokines (IL-6, HGF and LIF, which is known to induce caspase-4 gene and mRNA expression), 2, caspase-4 specific siRNA induced gene silencing, 3, transfection with non-genomic siRNA, and 4, combinations of 1 and 2 or 1 and 3. Since the results of these special treatments are not the topic of this paper, they will not be explained or evaluated further herein.

Cell lysates were prepared by separating 10^6 JEG-3 cells from medium, washing with PBS (pH 7.4–8.0) and PBS/proteinase inhibitor cocktail (dilution 1/100), centrifugation, removing the medium and adding PBS/proteinase inhibitor cocktail to the pellet. Cells were lysed by successively freezing and defrosting them four times in liquid nitrogen followed by ultrasonic treatment for 1 min. Cell fragments were centrifuged at $20{,}000 \times g$ for 25 min (8 °C). The supernatant was used for determination of caspase-4 activity. Protein concentration was determined by Bradford assay.

2.4. Sample preparation procedure

Five microliters of cell lysate (protein concentration 0.2–0.8 mg/mL) were mixed with 245 μL buffer (100 mM HEPES pH 7.5, 20% (v/v) glycerol, 5 mM DTT, 0.5 mM EDTA) and 10 μL Ac-Leu-Glu-Val-Asp-AFC (20 mM in DMSO). This mixture was incubated for 2 h at 37 °C and then stopped with 10 μL 3N HCl. Samples were analyzed by HPLC. For blank values, 5 μL of deionized water was used instead of cell lysates, and treated in the same way as the samples described above.

2.5. HPLC conditions

The high-performance liquid chromatographic (HPLC) system was a Gynkotek (Dionex, Germany), which consisted of a degasser, a pump, an autosampler and a fluorescence detector. The sample injection volume was 50 μL. The excitation wavelength and emission wavelength of the fluorescence detector were set at 400 and 505 nm, respectively. The mobile phase was a mixture of methanol and water (70/30, v/v) with a flow rate of 700 μL/min. The HPLC column was a HD-SIL C18, 5 μm, 80 Å, 250 mm × 4 mm column (ORPEGEN Pharma Deutschland). An ODS Hypersil column C18, 5 μm, 10 mm × 4.6 mm was used as pre-column (MZ Analysentechnik, Deutschland). The peak area was used for quantitative calculation.

3. Results and discussion

3.1. Method development

The cleavage of AFC by caspase-4 from substrate Ac-Leu-Glu-Val-Asp-AFC is described in detail in [5]. The photometric fluorescence measurement of released AFC by a fluorescence microplate reader or fluorescence photometer seems to be an easy and fast method for the determination of caspase-4 activity in cell lysates. Commercially available kits or substrate preparations describe the quantification of AFC compared to blank value by using a standard calibration curve in a range from 1 to 5 nmol/mL (variable among different suppliers). This method generally demonstrates high blank values and sometimes questionable unexpected results. Therefore, we wondered if the measured fluorescence signal at excitation wavelength 400 nm and emission wavelength 505 nm actually reflects the released amount of 7-amino-4-trifluoro methylcoumarin.

To investigate this problem, we first analyzed samples by using the HPLC system without column. Under these conditions, the detected fluorescence signal is comparable to signals from a fluorescence photometer or microplate reader. A typical representative signal is shown in Fig. 1. The peak area (at $t = 0.37$ min) contains the released AFC and additional fluorescent components.

In a second step, using the same HPLC system but connected with a RP C18 column, the fluorescence signal from AFC in the same sample preparations was separated from other interfering signals that show the same excitation/emission wavelength. The chromatogram in Fig. 1 (insert) displays two separate peaks at 5.2 and 6.9 min. The retention time of the second peak corresponds to the retention time of free AFC as measured under the same conditions.

To detect the origin of these interfering signals at 5.2 min, all components of the incubation mixture (cell lysate, buffer and substrate) and cell culture medium were measured separately at the

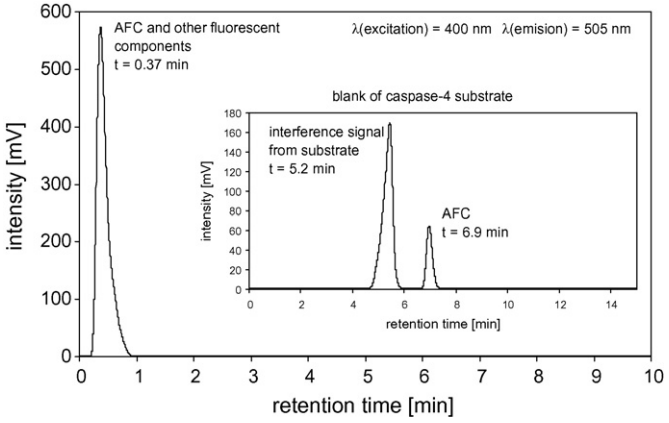

Fig. 1. The main figure shows the flow photometric fluorescence curve of a caspase-4 containing cell lysate (JEG-3 choriocarcinoma cells) incubated with the substrate Ac-Leu-Glu-Val-Asp-AFC. AFC and the interfering component produce one single signal. The insert displays an HPLC chromatogram of the same substrate, in which the peaks of the free AFC and the interfering signal are separated.

same RP HPLC conditions. The cell lysate, buffer, and free AFC do not generate the additional fluorescence signal at $t = 5.2$ min. Cell lysates spiked with AFC but without substrate do not show the signal at 5.2 min, either.

Only the caspase-4 substrate itself evokes an intense fluorescence signal at 5.2 min and a smaller one at 6.9 min for free AFC (see insert Fig. 1). Both peaks differ not only in their retention time, but they also show different fluorescence spectra, with maxima at different wavelengths (spectra not shown). Without the aid of a chromatographic step, photometric fluorescence detection at the excitation wavelength 400 nm and emission wavelength 505 nm leads to an unresolved signal overlap from both components, if the substrate peak is not separated from the AFC signal.

Minor fluorescence signals were observed for the cell culture media DMEM and OPTIMEM, but their contribution (retention time between 3 and 4 min; chromatogram not shown) is insignificant. Furthermore, this contamination can be excluded by extensive washing procedures prior to cell lysis.

The substrate-derived fluorescence signal component at 5.2 min is not constant over all experiments in the caspase-4 assay. Therefore, it is not possible to subtract this value as blank. Only the signal from the substrate at 6.9 min is constant relative to the blank value.

In Fig. 2, chromatograms show different proportions between free AFC and the signal from substrate in different samples.

Subsequently, the peak areas from flow fluorescence detection and separated AFC fluorescence signal at 6.9 min of the RP-HPLC were compared in order to evaluate the extent of these differences. The intensity of the measured free AFC signal depends strongly on the method used. Since the flow fluorescence method does not distinguish between the specific and non-specific signal, all values are higher than those obtained by the RP-HPLC method. These differences are variable (in our experiments, between 41 and 57 mV min). Also, the ratio between both values is not constant; values from RP-HPLC are between 33% and 51% of those measured by flow fluorescence (Fig. 3). Especially for samples with low caspase-4 activities, these differences can be important. Moreover, the quality of a standard curve produced by use of free AFC is questionable, because a fluorescence microplate reader or a fluorescence photometer detects the aggregate fluorescence signal of all active species in each well, and not the individual components. As long as flow fluorescence detection relies on a non-specific signal and samples contain a known competing species, additional multi-component calibrations will not be able to resolve this method's limitations.

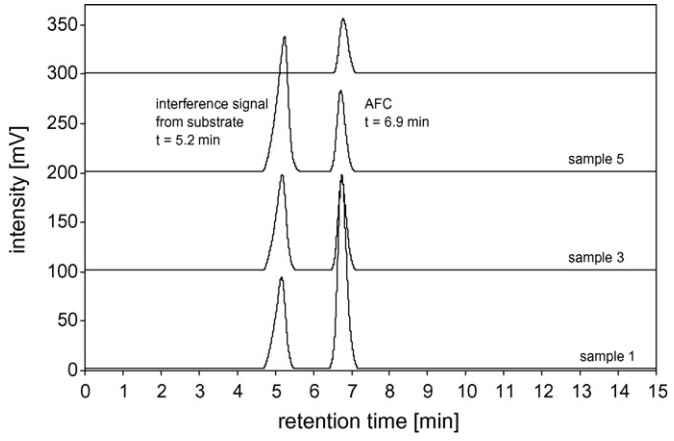

Fig. 2. Overlay of RP-HPLC chromatograms of selected JEG-3 cell samples, with different caspase-4 activity due to different treatments. Free AFC (after caspase-4 induced cleavage from the substrate) elutes after 6.9 min. A non-specific signal from the substrate itself appears after 5.2 min. The intensity of the non-specific signal does not correlate with the specific signal.

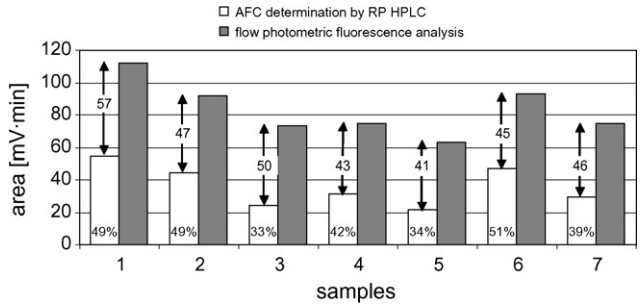

Fig. 3. Comparison of detection of caspase-4 induced production of free AFC by flow fluorescence and RP-HPLC. Representative results of various cell lysates are shown. Numbers between arrows indicate the differences in mV min. Percentages in white bars indicate the true content of free AFC (as assessed by the RP-HPLC method) as compared to the measurements obtained by flow fluorescence, which includes a non-specific signal.

3.2. The quantification of AFC by RP-HPLC

For quantification of AFC a standard calibration curve in a range from 40 pmol/mL to 3 nmol/mL was established. Under our experimental conditions, higher concentrations are detected above the dynamic linear range and, therefore, they cannot be precisely calculated (Fig. 4). Nonetheless, some suppliers recommend working in a higher concentration range (e.g. Caspase-4 Assay, MP Biomedicals, USA: 1.6–4.8 nmol/mL). Several commercially available assays do not exactly define the detection range.

The regression equation for the calibration curve has a general form $y = a_1x + a_0$, where x is the concentration of AFC standard working solutions and y is the peak area count of the fluorescence signal from AFC. In our study, the coefficients a_1 and a_0 were 0.0757 and −4.3057, and the linear regression coefficient was $r^2 = 0.9996$.

To estimate the limit of detection (LOD) and the limit of quantification (LOQ) the standards were analyzed by using the described method. LOD and LOQ were calculated from calibration data as described in Danzer [7]. In our work, the assay was determined to have a LOD of 46 pmol/mL and a LOQ of 113 pmol/mL.

For validation of the procedure cell lysate was spiked with AFC at 960 pmol/mL and had a recovery of 107% (S.D. = 0.01, $n = 3$). For determination of intra-assay precision four samples were analyzed at the beginning and the end of the measurement series of one single assay with a time interval of 10 h. This precision analysis

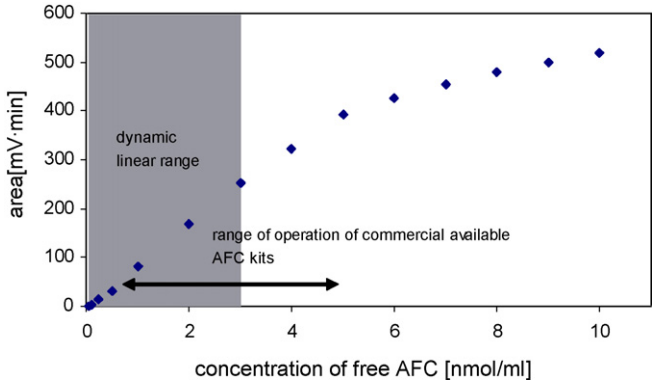

Fig. 4. Standard calibration curve for free AFC obtained by RP-HPLC method. The linear range is from 40 pmol/mL to 3 nmol/mL. Commercially available assays work in a range from 1 to 5 nmol/mL.

was repeated at four different days with a mean relative standard deviation of 1.70%. For inter-assay precision analysis 500 pmol/mL standard were compared over 7 weeks with a relative S.D. of 2.89% ($n = 6$). For this examination, each standard has been prepared independently by diluting fresh AFC.

4. Conclusion

A rapid, sensitive and robust RP-HPLC method was developed for the determination of AFC, released from substrate Ac-Leu-Glu-Val-Asp-AFC by caspase-4 in cell lysates of JEG-3 cells.

The use of the RP-HPLC method presents advantages over currently used detection methods such as detection by fluorescence microplate reading or fluorescence photometry, with respect to elimination of interfering fluorescence signals from the substrate, sample volume, and detection limits. The method has a LOD of 46 pmol/mL and a LOQ of 113 pmol/mL. The assay is linear over a range of concentration of AFC from 40 pmol/mL to 3 nmol/mL. This method should be easily transferred to other detection assays for other caspases, by using the same fluorophore with little or no modification.

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