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HPLC-FLUORESCENCE ASSAY FOR EVALUATION OF INHIBITORS OF FATTY ACID AMIDE HYDROLASE

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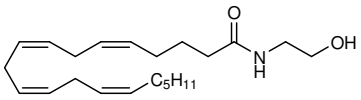
▸Introduction

Fatty acid amide hydrolase (FAAH) is the key enzyme responsible for the rapid cleavage of fatty acid amides such as the endocannabinoid anandamide (**1**). Inhibition of FAAH, which increases the anandamide levels, has been suggested as a therapeutic approach for the treatment of pain, anxiety and inflammation.
Already published assays of FAAH activity require radioactive substrates or detect cleavage products of natural anandamide or synthetic amide substrates synthesized from a fatty acid and a fluorogenic amine by HPLC-UV or fluorimetry.



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▸Experimental

Incubation procedure

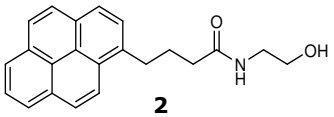
Two stock solutions were prepared consisting of Tris-HCl buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing 323 µM 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DIPAP) on the one hand and 0.2 % Triton X-100 (m/v) on the other hand. An aliquot of substrate was homogenized in each stock solution. 93 µl of the mixtures were added to 2 µl of a DMSO solution of inhibitor or 2 µl of DMSO in case of the controls. The mixtures were incubated at 37°C for 5 min. Enzyme reaction was started by adding 5 µl rat brain microsomes [10 mg/ml protein] and continued at 37°C for 45 min. Substrate concentration was 100 µM in a final incubation volume of 100 µl.
The enzyme reaction was terminated by the addition of 200 µl acetonitrile/methanol 1:1 (v/v) including the internal standard. After cooling in an ice bath for 10 min, the samples were centrifuged at 1959 x g and 4°C for 5 min. 50 µl of the obtained supernatant were injected into the HPLC system.

Analysis

The HPLC system consisted of a Bischoff 2250 HPLC pump, a Midas Cool autosampler, a Bischoff column oven and a Waters 2475 fluorescence detector. Separation was achieved on a Nucleosil 100-3 C18 (3.0 x 125 mm, 3 µm) RP column protected by a corresponding pre-column. The mobile phase consisted of acetonitrile/water/TFA (62:38:0.1; v/v/v). Separation was conducted at 20°C with a flow rate of 0.4 ml/min. Analysis time was 20 min.
The absorbance maximum of the fluorophore at 340 nm was chosen as excitation wavelength, emission was recorded at 380 nm. To cut off the high substrate signal, multiple wavelength changes during every run were required (Fig.1).

▸Results and Discussion

We have developed an assay for the evaluation of FAAH inhibitors that uses N-(2-hydroxyethyl)-4-pyren-1-ylbutanamide (**2**) as new fluorogenic substrate containing the fluorophore in the acyl part of the molecule. Enzyme activity was determined by measuring the release of 4-pyren-1-ylbutanoic acid by HPLC and fluorescence detection applying 6-pyren-1-ylhexanoic acid as internal standard. Purified microsomes from rat brain served as enzyme source. To increase substrate solubility, DIPAP or alternatively Triton X-100 was added to the assay mixture. Due to better reproducibility we chose DIPAP as additive for inhibitor screening (Fig.2).



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▸Validation

Linearity of 4-pyren-1-ylbutanoic acid was assessed in a concentration range of 0.025 nmol/ml - 2.5 nmol/ml with a correlation coefficient of R = 0.9965.
The IC_{50} of the known FAAH inhibitor URB-597 was determined with DIPAP as well as Triton X-100 as additive (Fig.2). The IC_{50}–values reported in literature range between 5 nM and 157 nM.

Fig.1

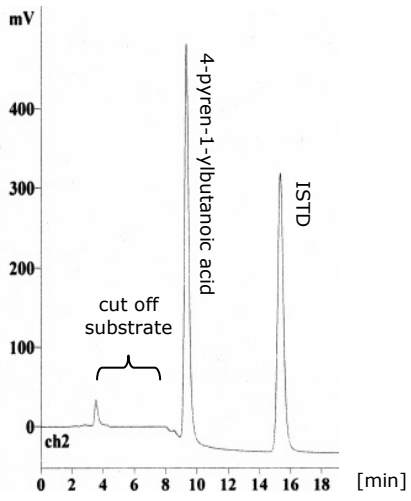

Fig.2

	IC_{50} URB-597 (nM)	
	+ DIPAP	+ Triton X-100
mean	33	121
s.d.	3.8 (13%)	39 (32%)
	(n = 5)	(n = 6)