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A spectrophotometric assay for fatty acid amide hydrolase suitable for high-throughput screening

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Abstract

Signalling via the endocannabinoids anandamide and 2-arachidonylglycerol appears to be terminated largely through the action of the enzyme fatty acid amide hydrolase (FAAH). In this report, we describe a simple spectrophotometric assay to detect FAAH activity in vitro using the ability of the enzyme to hydrolyze oleamide and measuring the resultant production of ammonia with a NADH/NAD^+-coupled enzyme reaction. This dual-enzyme assay was used to determine K_m and V_{max} values of 104 μM and 5.7 nmol/min/mg protein, respectively, for rat liver FAAH-catalyzed oleamide hydrolysis. Inhibitor potency was determined with the resultant rank order of methyl arachidonyl fluorophosphonate > phenylmethylsulphonyl fluoride > anandamide. This assay system was also adapted for use in microtiter plates and its ability to detect a known inhibitor of FAAH demonstrated, highlighting its potential for use in high-throughput screening.

Keywords: Fatty acid amide hydrolase; Assay; Spectrophotometer; Enzyme; Inhibition; Cannabinoid

1. Introduction

Although there have been therapeutic applications for the phytocannabinoid Δ^9-tetrahydrocannabinol for centuries, the discovery of the G protein-coupled CB_1 and CB_2 cannabinoid receptors [1–3] resulted in renewed interest in the clinical application of cannabinoid drugs, with potential therapeutic targets such as control of inflammation, analgesia, appetite control, treatment of glaucoma and immune system regulation [4–8]. Since the identification of the cannabinoid receptors, a number of putative endogenous ligands have been isolated, with the two major endocannabinoids being the arachidonic acid derivatives *N*-arachidonyl ethanolamide (anandamide) and 2-arachidonyl glycerol [9,10]. A key regulator of endogenous cannabinoid signalling is fatty acid amide hydrolase (FAAH, EC 3.5.1.4.), a membrane-bound enzyme responsible for catalyzing the hydrolysis of endocannabinoids as well as other fatty acid amides, including the sleep-inducing lipid *cis*-9,10-octadecenamide (oleamide) [11–14]. The in vivo effects of FAAH gene targeting [15–17] and the potentiation of endocannabinoid effects by FAAH inhibition [18–20] suggest that FAAH inhibitors may be of great therapeutic benefit. Given recent evidence for an association of problem drug use with a polymorphism which results in reduced FAAH activity [21] and a correlation between failure to implant in in vitro fertilisation embryo transfer and low lymphocyte FAAH activity [22], a rapid and facile test for FAAH activity might be a useful diagnostic tool.

Current assays for FAAH activity commonly involve the use of radiolabelled substrates and chromatographic techniques, which are expensive, laborious, not conducive to high-throughput applications and may have handling restrictions [11,23–27]. Two examples of FAAH assays that do not suffer from these drawbacks have been described. The first uses an electrode to detect ammonia generated from the hydrolysis of oleamide, but is restricted to large (10 ml) reaction volumes by the size of the electrode [28]. The second is a fluorescence-based displacement assay which detects the release of a fluorescent fatty acid analogue from fatty acid-binding protein upon its

Abbreviations: CPF, crude particulate fraction; FAAH, fatty acid amide hydrolase; GDH, glutamate dehydrogenase; MAFP, methyl arachidonyl fluorophosphonate; PMSF, phenylmethylsulphonyl fluorophosphonate

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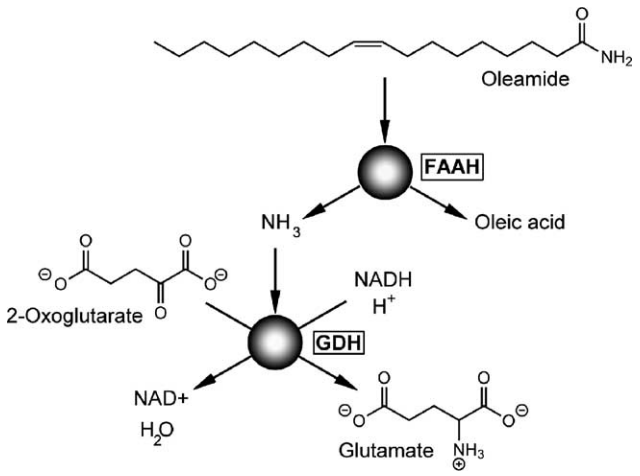

Scheme 1. Proposed dual-enzyme assay for fatty acid amide hydrolase activity. FAAH-catalyzed oleamide hydrolysis is measured by conversion of the resulting ammonia to L-glutamatee by L-glutamatee dehydrogenase. The reaction is followed by measuring rate of decrease in absorbance of the solution at 340 nm, a consequence of the coenzyme NADH being oxidized to NAD^+.

displacement by the free fatty acids generated by FAAH-catalyzed anandamide or oleamide hydrolysis [29]. This assay requires the isolation of fatty acid-binding protein from rat liver or recombinant sources. In addition, the assay must be calibrated to account for binding of anandamide and oleamide to this protein.

Here we describe a simple, novel spectrophotometric assay for FAAH activity based on an enzyme-coupled assay of oleamide hydrolysis. Oleamide is the primary amide of oleic acid and, therefore, its hydrolysis by FAAH yields oleic acid and ammonia. To measure the rate of ammonia release in this reaction, a dual-enzyme assay containing FAAH and L-glutamate dehydrogenase (GDH) was developed (Scheme 1). GDH catalyzes the condensation of ammonia and 2-oxoglutarate to L-glutamatee, using NADH as a coenzyme. Since NADH has a large molar extinction at 340 nm, the rate of ammonia production and, hence, oleamide hydrolysis is directly proportional to the absorbance decrease as NADH is oxidized to NAD^+. In a cuvette-based spectrophotometric assay, we demonstrate that this approach is capable of measuring the kinetic parameters of FAAH-catalyzed oleamide hydrolysis and can be used to determine the IC_{50} values of FAAH inhibitors. Adaptation of this assay for use in microtiter plates was also performed and shown to be successful in the detection of a FAAH inhibitor, making this assay suitable for use in high-throughput screening of compound libraries for novel drug candidates.

2. Materials and methods

2.1. Materials

Chemicals for chemical synthesis were obtained from Aldrich (ethanolamine, potassium hydrogen sulphate and oxalyl chloride), Rathburn Chemicals (DMF) and Fischer Scientific Ltd. (dichloromethane, ethyl acetate, hexane and magnesium sulphate). Methyl arachidonyl fluorophosphonate was obtained from Affiniti Research Products, while L-glutamatee dehydrogenase, β-NADH and 2-oxoglutarate were obtained from Calbiochem. All other chemicals were obtained from BDH Laboratory Supplies or Sigma Chemical Company.

2.2. Chemical synthesis

Endogenous FAAH substrates were prepared essentially as described by Devane et al. [9]. Oleamide was synthesized from oleic acid by conversion to its acid chloride with oxalyl chloride and subsequent reaction with ammonia solution. Similarly, anandamide was synthesized by conversion of arachidonic acid to its acid chloride and subsequent reaction with ethanolamine. Crude products were purified by flash column chromatography and analyzed by nuclear magnetic resonance spectroscopy and mass spectrometry.

2.3. Glutamate dehydrogenase assay

The GDH assay methodology described by Bergmeyer and Beutler [30] was adapted for use in spectrophotometer cuvettes. For this scaled down assay, 140 μl of solution A (71 mM 2-oxoglutarate, 4 mM ADP, 500 mM triethanolamine; pH 8.0), 14 μl of solution B (7 mM β-NADH, 121 mM $NaHCO_3$), 2 μl of GDH solution (1600 U/ml, 50% (w/v) glycerol) and 244 μl of distilled water were placed in acrylic cuvettes. Solutions were stirred and gently shaken for at least 10 min to allow equilibration, then absorbance monitored at 340 nm in a Beckman DU650 spectrophotometer until constant. Reactions were then initiated by addition of 50 μl of ammonium acetate solution (1.4 mM) to give an assay concentration of 160 μM. Solutions were mixed by stirring and absorbance automatically measured every 38 s for up to 30 min. Each assay was conducted at room temperature and consisted of four test cuvettes and two cuvettes containing a blank of distilled water in place of ammonium acetate.

2.4. Preparation of rat liver crude particulate fraction (CPF)

Freshly dissected liver from Wistar or hooded Lister rats was roughly chopped with scissors, weighed and homogenized in 10 vol of ice-cold CPF buffer (1 mM EDTA, 50 mM Tris; pH 7.4) using a Polytron homogenizer. The homogenate was centrifuged at 1000 × *g* for 5 min at 4 °C, and the supernatant layer was then centrifuged at 36,000 × *g* for 20 min at 4 °C. The resulting pellet was then suspended in 4 vol (original wet weight) of ice-cold buffer and manually re-homogenized with a glass/teflon homogenizer. Aliquots of the CPF (1 ml) were frozen at −80 °C until required.

2.5. Solubilization of CPF proteins

Equal volumes of thawed CPF preparation and solubilization buffer (1 mM EDTA, 50 mM Tris, 1% (w/v) Triton X-100; pH 7.4) were placed on ice and vortexed three times over a period of 15 min, then centrifuged at 9000 × *g* for 5 min at 4 °C. The supernatant, layer containing the solubilized protein, was removed and used in subsequent assays. The concentration of protein in the solubilized CPF preparations was determined using a minor modification of the method of Bradford [31].

2.6. FAAH assay procedure

As described above, assay solutions were made up by placing 140 μl of solution A, 14 μl of solution B, 2 μl of GDH solution, 192 μl of CPF buffer and 100 μl of solubilized rat liver CPF in acrylic cuvettes. Solutions were stirred, gently shaken for at least 10 min to allow equilibration and absorbance monitored at 340 nm in a Beckman DU650 spectrophotometer until constant. Reactions were then initiated by the addition of 2 μl of ethanolic oleamide solution. After stirring, absorbance was automatically measured every 38 s for up to 2500 s. Each assay was conducted at room temperature and consisted of five test cuvettes and one cuvette containing an ethanol blank. Reaction rates were observed to be linear for up to 500–600 s so, to determine the rate of oleamide hydrolysis, data from 0 to 494 s were analyzed. Using GraphPad Prism software, the gradient at each concentration was determined by linear regression and the blank gradient subtracted from test samples to eliminate drift. Using the Beer–Lambert equation, the absorbance gradient at each concentration was used to determine the rate of hydrolysis in nmol/min/mg protein. Rates were determined in triplicate for each oleamide concentration and the data analyzed by non-linear regression in Prism to determine K_m and V_{max} values. The mean ± S.E.M. of these data were calculated from three experiments using CPF preparations from three different animals.

2.7. Microtiter plate assays

Frozen rat liver crude particulate fraction was thawed and solubilized as described above. Oleamide was dissolved in ethanol, diluted 10-fold with buffer containing 1% Triton X-100 (w/v) and this solution was then diluted five-fold in detergent-free buffer. The solutions used in the cuvette-based assay were adapted, resulting in solution C (50 mM 2-oxoglutarate, 3 mM ADP, 690 mM triethanolamine; pH 8.0) and solution D (7.4 mM β-NADH, 125 mM $NaHCO_3$). Assay mixtures were made by combining 900 μl of solution C, 120 μl of solution D, 100 μl of GDH solution (1600 U/ml stock in 50% glycerol diluted to 284 U/ml with CPF buffer), 880 μl of solubilized rat liver crude particulate fraction and 1 ml of CPF buffer in a test tube. This solution was mixed by vortexing and 150 μl per well placed in a 96-well microtiter plate. The absorbance of the solutions was measured at 340 nm on a Dynatech MR5000 microplate reader and, when constant, 50 μl of oleamide solution or oleamide-free blank was added with a multi-tip pipette. The plate was then shaken for 10 s and the absorbance of each well was measured every 30 s for 5 min. The absorbance of each well was plotted against time and the rate of reaction (absorbance/min) determined by linear regression of the data using GraphPad Prism. The mean blank rate was subtracted from the mean rate at each oleamide concentration, measured in triplicate, and the mean ± S.E.M. rate determined using the data from three separate experiments using CPF from different animals.

For assays containing PMSF, the inhibitor was dissolved to 1 mM in ethanol and 40 μl added to the initial assay mixture with the volume of buffer reduced accordingly. After preincubation for 30 min at room temperature, plates were transferred to the plate reader and reactions initiated. The absorbance of each well was plotted against time and the rate of reaction (absorbance/min) determined by linear regression of the data using GraphPad Prism. The mean ± S.E.M. rate was calculated from the eight determinations at each assay condition and the blank rate subtracted from the observed rates of hydrolysis for oleamide and oleamide in the presence of PMSF. To test if the rate of oleamide hydrolysis in the presence of PMSF was significantly different from that of the control, data were analyzed using Student's unpaired *t*-tests with GraphPad Prism.

3. Results

3.1. Modified glutamate dehydrogenase assay

Before attempting the dual-enzyme FAAH assay, it was necessary to first demonstrate that modifying the GDH assay conditions described by Bergmeyer and Beutler [30] for use in cuvettes was successful. Using bovine L-glutamatee dehydrogenase, with ammonium acetate at 160 μM as the initiating substrate, conversion of 2-oxogltarate to L-glutamatee was observed by a decrease in absorbance of the reaction mixture at 340 nm as shown in Fig. 1. The rate of this reaction was dependent on the concentration of ammonium acetate with sensitivity down to the low micromolar range (not shown). To determine whether the ammonia generated by oleamide hydrolysis could be detected by this assay, oleamide and a FAAH source were combined in the dual-enzyme assay.

3.2. Glutamate dehydrogenase–FAAH dual assay

FAAH is highly expressed in brain and liver so, for initial attempts at the dual-enzyme assay, rat liver was

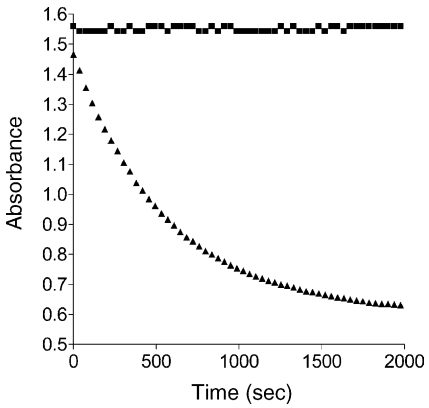

Fig. 1. Raw spectrophotometer data from a cuvette-based assay demonstrating the decrease in absorbance at 340 nm when ammonium acetate (▲, 160 μM) is converted to L-glutamatee by L-glutamatee dehydrogenase in comparison to a control assay with no ammonia source (■).

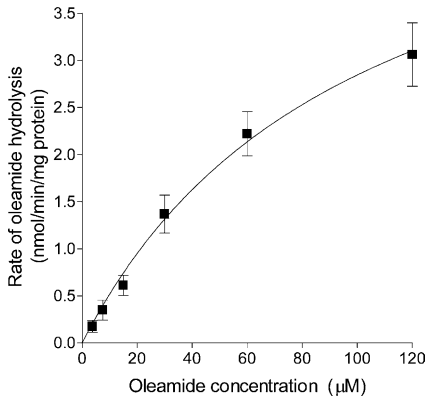

Fig. 3. The effect of oleamide concentration on FAAH-catalyzed oleamide hydrolysis in solubilized rat liver crude particulate fraction using a cuvette-based dual-enzyme assay. Data are expressed as the mean ± S.E.M. of three independent experiments.

chosen as a FAAH source. Initial attempts at the assay using intact liver microsomes, membranes or crude particulate fraction (CPF) proved unsuccessful due to absorbance readings at 340 nm being off the scale of the spectrophotometer, possibly due to light scattering by the cloudy suspension. However, on solubilization of membrane proteins using 1% (w/v) Triton X-100, acceptable absorbance values were obtained. When 160 μM oleamide was used to initiate the dual-enzyme reaction, each of the soluble preparations from the different FAAH sources exhibited a decrease in absorbance at 340 nm with respect to controls (Fig. 2). As rates of hydrolysis were very similar in each preparation (not shown), CPF was used for all subsequent assays due to its ease of preparation.

3.3. Oleamide concentration–response assays

To validate this approach, it was necessary to demonstrate that the measured enzyme kinetics were comparable with those described in the literature. As is apparent from Fig. 2, the absorbance decrease due to utilization of NADH in the dual-enzyme assay is linear for approximately 600 s. To determine accurately the rate of absorbance decrease at 340 nm and, hence, the rate of oleamide hydrolysis by FAAH, data from 0 to 494 s were analyzed as described above in Section 2. Oleamide hydrolysis by FAAH was concentration-dependent with calculated apparent K_m and V_{max} values of 104 ± 13 μM and 5.7 ± 0.4 nmol/min/mg protein, respectively (Fig. 3).

3.4. FAAH inhibitor studies

To determine whether the dual-enzyme was sensitive to known inhibitors of FAAH activity, the ability of FAAH to hydrolyze 100 μM of oleamide was examined in the presence of three different inhibitors (Fig. 4). Phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor, methyl arachidonyl fluorophosphonate (MAFP), a potent and irreversible inhibitor of FAAH [32], and anandamide, itself a substrate of FAAH, all inhibited oleamide

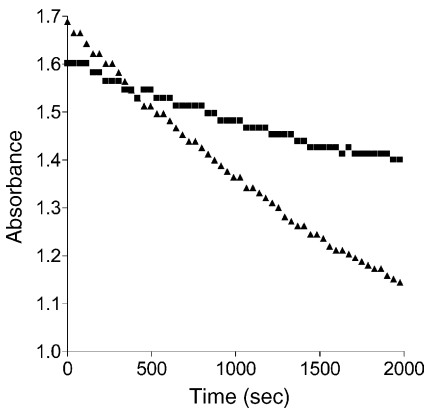

Fig. 2. Raw spectrophotometer data from a cuvette-based dual-enzyme assay for fatty acid amide hydrolase activity using rat liver CPF. Data demonstrate the difference between the change in absorbance at 340 nm due to the hydrolysis of oleamide (▲, 160 μM) or blank solution (■) when incubated with solubilized rat liver crude particulate fraction in the presence of glutamate dehydrogenase assay components.

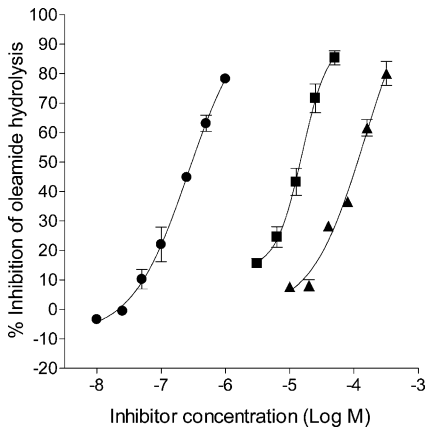

Fig. 4. The effect of methyl arachidonyl fluorophosphonate (●), phenylmethylsulphonyl fluoride (■) and anandamide (▲) on the FAAH-catalyzed hydrolysis of 100 μM oleamide in solubilized rat liver CPF using a FAAH–GDH dual-enzyme assay. Data are expressed as the mean ± S.E.M. of three independent experiments.

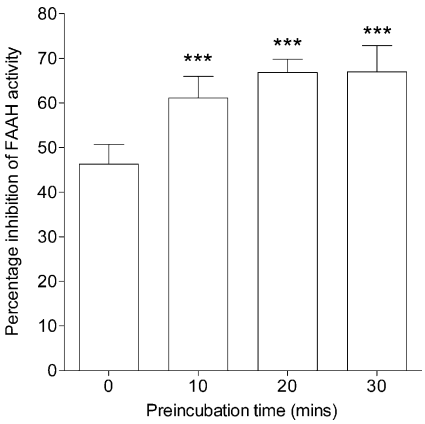

Fig. 5. The effect of phenylmethylsulphonyl fluoride (10 μM) preincubation on inhibition of the FAAH-catalyzed hydrolysis of 100 μM oleamide using a FAAH–GDH dual-enzyme assay. Data are expressed as the mean ± S.E.M. of three independent experiments and were analyzed by one-way ANOVA using GraphPad Prism software ($^{***}P < 0.001$).

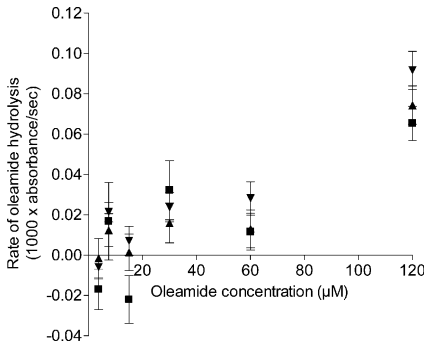

Fig. 6. The effect of oleamide concentration on FAAH-catalyzed oleamide hydrolysis in solubilized rat liver crude particulate fraction using a microtiter plate dual-enzyme assay. Absorbance was measured at 340 nm and data are expressed as the mean ± S.E.M. of three determinations of rate using CPF preparations from three different animals.

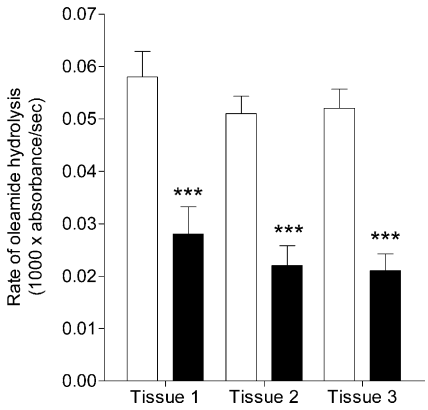

Fig. 7. Detection of FAAH inhibition using a microtiter plate dual-enzyme assay. FAAH-catalyzed hydrolysis of 100 μM oleamide was measured in the absence (white bars) or presence (grey bars) of 10 μM PMSF by measurement of absorbance at 340 nm. The data, from the solubilized liver crude particulate fraction of three different rats, represents the mean ± S.E.M. of eight determinations of rate at each condition and were analyzed by un-paired *t*-tests using GraphPad Prism software ($^{***}P < 0.0001$).

hydrolysis in a concentration-dependent manner with calculated IC_{50} values of 17 ± 3 μM, 264 ± 49 nM and 171 ± 40 μM, respectively. Since the IC_{50} for PMSF was higher than expected, 10 μM PMSF was incubated for various times with FAAH and the effect on the hydrolysis of 100 μM oleamide measured. Preincubation of the enzyme with PMSF significantly increased the extent of inhibition ($P < 0.0001$, Fig. 5) when compared to immediate exposure to the inhibitor, suggesting that this may have resulted in the high IC_{50} values obtained with this assay. No significant differences in extent of inhibition were observed between 10, 20 and 30 min preincubation.

Finally, to confirm a direct inhibition of FAAH, further assays were performed with GDH alone in the presence of FAAH inhibitors. Using 100 μM ammonium acetate as the substrate, inclusion of PMSF (100 μM), MAFP (1 μM) or anandamide (320 μM) in the assay failed to alter the GDH reaction rate (99 ± 1; 97 ± 1 and 101 ± 2% of control rates, respectively).

3.5. Adaptation of assay for microtiter plates

To avoid issues with precipitation of oleamide in establishing a microtiter plate assay, ethanolic oleamide was diluted ten times with CPF buffer containing 1% (w/v) Triton X-100 and a further five times with detergent-free buffer before addition to the assay solution. Scaling down the assay resulted in a failure to detect the hydrolysis of low concentrations of oleamide (Fig. 6). However, at 120 μM, the highest concentration tested, hydrolysis was clearly detected.

The ability of PMSF to inhibit FAAH activity in the microtiter plate assay was assessed using 100 μM oleamide as substrate and preincubation at room temperature for 30 min with 10 μM PMSF (Fig. 7). In this assay, PMSF inhibited CPF-derived FAAH activity by 56 ± 2% in comparison to controls. This compares to an inhibition of 34 ± 2% using 10 μM PMSF in the cuvette-based dual-enzyme assay, in the absence of a preincubation. These results clearly demonstrate that this assay has potential for use as a high-throughput screen of compound libraries for FAAH inhibitors (Scheme 1).

4. Discussion

We report here a novel, simple and inexpensive spectrophotometric assay for the study of fatty acid amide hydrolase activity. As well as removing the need for radiochemicals and chromatography, this assay has the potential for adaptation for the high-throughput screening of compound libraries for FAAH inhibitors. The cuvette-based spectrophotometer assay was able to measure oleamide hydrolysis to allow determination of K_m and V_{max} values of 104 ± 13 μM and 5.7 ± 0.4 nmol/min/mg pro-

tein, respectively for rat liver extracts. These figures are broadly in agreement with previously reported values. For example, hydrolysis of oleamide by rat liver membranes and solubilized fractions showed K_m values of 5 and 31 μM, respectively [28,33]. Thumser et al. [29] using a fluorescence displacement assay observed K_m values ranging from 2 to 14 μM and V_{max} values of 0.3–6 nmol/min/mg protein for oleamide hydrolysis by rabbit brain and mouse neuroblastoma cell preparations. It is likely that the relatively high K_m values reported here using the dual enzyme assay result from conducting the assay at room temperature and near neutral pH, compared to other studies conducted at 37 °C and at the enzyme's pH optimum of 9–10 [34–36].

When the effect of known FAAH inhibitors was examined using this dual-enzyme assay, the order of potency was determined to be MAFP > PMSF > anandamide, with IC_{50} values of 264 ± 49 nM, 17 ± 3 and 171 ± 40 μM, respectively. The values for inhibition with MAFP and PMSF, however, are higher than those reported in the literature. For example, MAFP has been reported to exhibit an IC_{50} of 0.082 and 0.1 nM against FAAH-mediated oleamide hydrolysis in mouse liver and brain membranes, respectively [37]. Again, it is likely that the assay conditions employed affected these results. In the inhibition studies, inhibitors were preincubated with FAAH for approximately 30 s before initiation of the assay with oleamide. However, as demonstrated in Fig. 5, when a single concentration of PMSF was preincubated with the enzyme for 10, 20 or 30 min, inhibition of oleamide hydrolysis was significantly greater than that observed without preincubation. Both MAFP and PMSF inhibit FAAH by covalent modification and, hence, the preincubation step would enable this reaction to occur prior to initiation of the hydrolysis assay, leading to a left-shift in the inhibitor potency curves.

Adaptation of the cuvette-based spectrophotometer assay for use in microtiter plates was attempted with some success. In the format employed here, it was not possible to ascertain rates of hydrolysis at lower concentrations of oleamide (Fig. 6), although higher concentrations were clearly measurable. This effect was not apparent in the cuvette assay and may be due to the formation of micelles by Triton X-100, as the detergent concentration was above its critical micelle concentration of 0.2–0.3 mM [38] in both assay systems. Light scattering from micelles may have affected absorbance readings and, while not deleterious to the cuvette assay, may have caused background noise in the microtiter plate assay. This would be exacerbated by two factors. Firstly, the path length in the plate-based assay was much shorter than in the cuvette, resulting in greatly reduced absorbance readings. Fluctuations caused by the presence of micelles would, therefore, be much more pronounced. Secondly, as the plates were read from the bottom, the absorbance of the entire assay mixture would be measured. In the cuvette assay, absorbance was measured from the side, reducing any scattering effects caused by the presence of micelles. This phenomenon had less influence on the larger absorbance values obtained with high oleamide concentrations, thereby allowing more accurate detection. It is likely that, with further development of assay conditions, this problem can be overcome. Despite this problem, however, the adaptation of this assay for use in microtiter plates was shown to be successful for the detection of FAAH inhibitors. When the hydrolysis of 100 μM oleamide was examined in the absence or presence of 10 μM PMSF, there was a very significant difference ($P < 0.0001$) in the apparent reaction rate. This convincingly demonstrates that this plate-based assay, in a matter of minutes, has the ability to detect the inhibition of FAAH-catalyzed oleamide hydrolysis.

In conclusion, we have developed a novel, spectrophotometric dual-enzyme assay for the detection of fatty acid amide hydrolase activity. This assay removes the need for radiochemical substrates or chromatography and is sufficiently sensitive to enable kinetic measurements and inhibition studies. Although only able to utilize oleamide as its substrate, this assay is comparable to previously reported techniques with the benefit that it is quick and inexpensive. Its greatest strength, particularly with regard to the current therapeutic interest in endocannabinoids signalling, is its potential for use in high throughput applications. The adaptation of this assay for use in multiwell plates will enable the rapid screening of chemical libraries for FAAH inhibitors.

Acknowledgements

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