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Novel and sensitive method for the detection of anandamide by the use of its dansyl derivative

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Abstract

The dansyl ester of anandamide was prepared and showed intense fluorescence on silica gel thin-layer chromatography when viewed under long-wavelength ultraviolet light (detection limit, 15 fmol). A high-performance liquid chromatography method for the quantitation of anandamide was developed using a C_{18} column (250×4.6 mm) with gradient elution (1% acetic acid–methanol) and detection at 255 nm. The method was applied to the measurement of anandamide in media from cultured hepatocytes. Sample preparation involved extraction with a C_{18} cartridge, derivatization with dansyl chloride, thin-layer chromatography, and quantitation. The detection limit in hepatocyte media is 4.3 nmol at a signal-to-noise ratio of three. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Anandamide

1. Introduction

The ethanolamide derivatives of long-chain fatty acids have been known for some time [1,2]. Recently, attention has been focused on one derivative in particular, arachidonylethanolamide (anandamide, Fig. 1), since it displays pharmacological actions that suggest a role for anandamide as an endogenous cannabinoid [3,4]. This discovery has opened new avenues for better understanding how tetrahydrocannabinol (THC), the psychoactive component of Cannabis, and its synthetic analogs act to produce their varied biological responses. Moreover, it is believed that an understanding of how anandamide functions in the body will provide opportunities for the development of new drugs for the treatment of pain, inflammation, memory deficiencies, immune system related problems, etc., [3]. For these reasons research on anandamide has intensified and has become a significant area of scientific investigation.

A major impediment to progress into the biochemical and physiological role of anandamide has been the lack of convenient and inexpensive methods for the measurement of its levels in various experimental models. Thus far, an immunoassay procedure has not been reported probably due to the difficulty in generating suitable antibodies. DiMarzo [3] and Fontana et al. [5] have reviewed the assay methods developed thus far. Some of these depend primarily on the use of radiolabeled precursors such

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as arachidonic acid or ethanolamine followed by chromatographic separation. Examples of the radiolabeled precursor method for the measurement of anandamide biosynthesis in cell culture models have been reported [6–8]. A major problem with this approach has been the low conversion rate of radiolabel into anandamide resulting in sample measurements near the lower limit of detection. This may be due to dilution of the radioactivity by the endogenous pools of precursors, or it may result from a low level of biosynthetic capacity. Accurate data can only be obtained using liquid scintillation counting at the lower limit of its reliability. Mass spectrometry has been used to assay tissue levels of anandamide from a variety of sources [5,9,10], however; this method requires the use of complex and costly instrumentation not always available or readily applicable in certain experimental protocols.

Assay procedures for anandamide utilizing spectrophotometric detection have been described in response to the need for a method with a more universal applicability than techniques based on mass spectrometry [11–14]. One report by Lang et al. [11] described a procedure that uses high-performance liquid chromatography (HPLC) with UV detection of anandamide at 204 nm. These authors have applied this method to a study of anandamide amidase activity in rat brain microsomes. A potential problem with this procedure is the occurrence of interfering substances when analyzing samples from more complex sources such as intact cells. Koga et al. [12] reported on the development of a novel fluorogenic benzodioxazole derivative of anandamide and other ethanolamides that can be used with HPLC coupled to a fluorescence detector. However, no applications for this method were given and, in addition, the reagent, DBD-COCl, is not readily available. Moreover, we have found that the DBD anandamide derivative is not stable for prolonged periods (unpublished observation). A fluorescence-based method that has been successfully applied to studies of anandamide biosynthesis in microsomal preparations was reported by Sugiara et al. [13,14]. This assay involves derivatization of anandamide with 1-anthroyl cyanide followed by HPLC–fluorescence analysis. The use of this method with more complex extracts obtained from intact cells where a greater number of interfering substances could be expected was not described. Increasingly, cells in culture are being used as model systems so that any analytical procedure should be applicable to such systems.

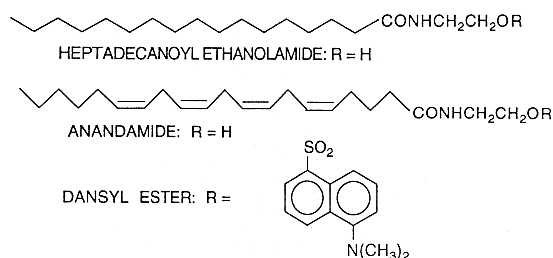


Fig. 1. The structural formulae of anandamide, heptadecanoylethanolamide and their dansyl derivatives.

Dansyl (1-*N*,*N'*-dimethylaminonapthalene-5-sulfonyl) derivatives of amino acids have been widely used as the basis for sensitive and specific methods for the quantitation of amino acids [15]. The derivatives are simple to prepare and show high detection sensitivity; 100 pmol with UV detection and can be extended into the fmol range by the use of fluorescence detectors. Although the reagent, dansyl chloride, readily reacts with primary hydroxyl groups, no reports could be found for the analysis of primary alcohols using dansylation as a reporter group. There is a publication describing a dansylation assay in which the analyte contains secondary alcohol and secondary amine groups, however, the authors did not report where derivatization occurred [16]. There is also a report on the dansylation of THC and related cannabinoids [17], however, this involves derivatization on a phenolic group. We have, therefore, examined the possibility of devising a sensitive method based on dansylation of the aliphatic alcohol group for the detection and measurement of an ethanolamide in experimental models utilizing cells in culture. The findings that we report below present both a novel application for the dansylation reaction as well as a viable method for the determination of anandamide in samples from cell culture systems.

2. Experimental

2.1. Materials

Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN, USA), heptadecanoic acid from

Sigma (St. Louis, MO, USA), 5-dimethylamino-1-naphthalenesulfonylchloride (dansyl chloride), ethanolamine, dimethylaminopyridine, 1,3-diisopropylcarbodiimide and all solvents were obtained from Aldrich. Precoated silica gel 60 thin-layer chromatography (TLC) plates (layer thickness 0.25 mm) were purchased from Merck (Darmstadt, Germany). Extraction tubes were solid-phase Supelclean LC-18 1 ml (Supelco, Bellefonte, PA, USA). Precolumn sample filters were Nylon-66 syringe filters (0.2 μm) obtained from Varian (Walnut Creek, CA, USA).

2.1.1. N-Acylethanolamines

Anandamide was synthesized by the carbodiimide method from arachidonic acid (20:4, $n-6$) and ethanolamine by using *N*,*N*-diisopropylcarbodiimide and methylene chloride as the solvent following a previously reported procedure [5]. The same conditions were used for the synthesis of heptadecanoylethanolamide, the *N*-ethanolamide derivative of heptadecanoic acid (17:0).

2.1.2. Dansyl esters

Anandamide was converted to its dansyl ester (Fig. 1) by treatment with an excess of dansyl chloride and dimethylaminopyridine in acetone in a capped vial kept at 55°C for 1 h. The reaction mixture was cooled, the acetone evaporated to dryness under a nitrogen stream. The residue was reconstituted with 100 μl of methanol and subjected to TLC on a 250-μm silica gel plate using chloroform–hexane–methanol (90:100:3, v/v) (system A). The plate was visualized by its exposure to ultraviolet light at 365 nm. The TLC zone containing the dansyl ester of anandamide was removed and eluted with methanol. Proton nuclear magnetic resonance and mass spectral analyses confirmed the structure of the isolated ester. Dansylation of heptadecanoylethanolamide and the purification and identification of the product was carried out as described above for anandamide. The purified dansyl derivatives of anandamide and heptadecanoylethanolamide were analyzed by HPLC under the conditions described below. The retention times for the dansyl anandamide and dansyl heptadecanoylethanolamide were 23.9 and 27.7 min, respectively.

2.2. Apparatus

HPLC analyses were performed at ambient temperature on a high-performance liquid chromatograph that consisted of dual Rainin pumps and a Spectroflow 757 absorbance detector (Kratos Analytical Instruments). Separations were carried out on a Dynamax Microsorb C_{18} (80-225 C5) 5 μm particle size column (250×4.6 mm I.D.), at a flow-rate of 1 ml/min; a Rainin guard column of the same composition was used in all of the assays. The mobile phase consisted of a methanol–1% aqueous acetic acid gradient [40% methanol (0 min), 86% methanol (12 min); 90.5% methanol (19 min); 99.5% methanol (30 min); and 99.5% methanol (30–35 min)]. The quantitation of anandamide was based on the integration of peak areas at 255 nm. Data were recorded starting at 10 min following injection for a period of 25 min and normalized for 100% display. Recoveries were calculated by comparing peak areas of the isolated analyte standards to the peak area of the internal standard dansyl heptadecanoylethanolamide.

2.3. Linearity, detection limit and recovery

The stock solutions of anandamide (1 mg/ml) and heptadecanoylethanolamide (1 mg/ml), used as internal standard (I.S.), were prepared in dry acetone and stored at −20°C until analysis. Five standards of anandamide in acetone, ranging from 0.5 to 100 μg and heptadecanoylethanolamide (25 μg) were prepared by the addition of each from their respective stock solutions to 1 ml of acetone followed by 100 μl of dansyl chloride (10 mg/ml in acetone) and 100 μl of dimethylaminopyridine (10 mg/ml). The sample was vortex-mixed for 15 s, the vials capped under nitrogen and incubated at 55°C for 1 h. The reaction mixture was evaporated; the residue was dissolved in 100 μl of methanol and subjected to TLC on 250-μm silica gel plates using system A for development. The dansyl anandamide and the dansyl derivative of heptadecanoylethanolamide were spotted as markers on separate lanes of the plate. After elution, the plate was visualized by UV light at 365 nm. The TLC zones between the markers were removed and extracted with methanol (3×1 ml). The methanol solution was filtered (0.2 μm Nylon-66) and evaporated to dryness under a nitrogen stream,

the residue dissolved in 200 μl of methanol, and an aliquot of 150 μl was injected onto the HPLC column. The curves were constructed by plotting the ratios of the integrated peak areas for the analyte to the internal standard integrated peak areas at 255 nm vs. the concentration of the analyte.

2.3.1. Recovery

Extraction efficiencies of anandamide from the culture media were determined at the low, medium and high levels of the concentration range used for calibration. Absolute peak areas obtained after injection of anandamide standards (1.5, 50 and 100 μg) which underwent derivatization and TLC were compared to those obtained after injection of spiked media that underwent the entire procedure described in Fig. 4. The absolute extraction efficiency was determined as the sample/standard peak area ratio. In all cases the extraction efficiency exceeded 85%.

2.4. Preparation of the culture medium samples for standard curve or unknown and precolumn derivatization procedure

To construct calibration curves, hepatocyte culture, without anandamide, was incubated at 37°C for 6 h. The media were separated from the cells by centrifugation at 1500 *g* for 20 min. To construct a calibration curve, 25 ml of medium was spiked with known concentrations of anandamide ranging from 1.5 to 100 μg in 2 ml of medium. The mixture was vortex-mixed for 1 min and processed in duplicates

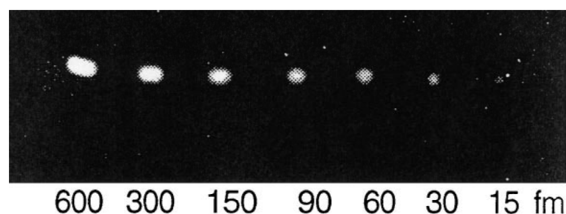
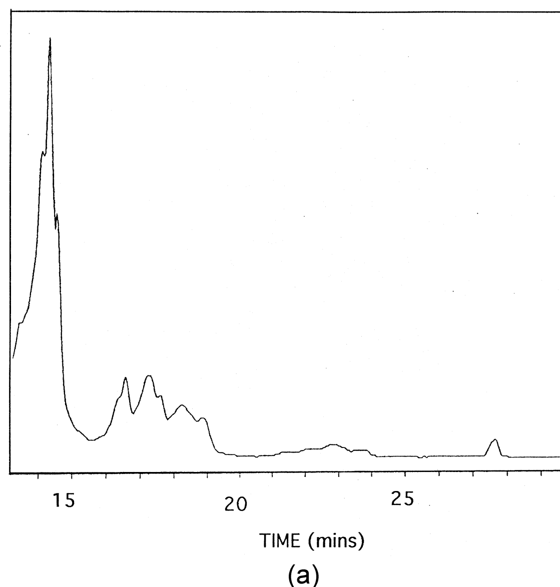


Fig. 2. TLC detection limit of dansyl anandamide. To test the detection limit of pure dansyl anandamide, a series of samples (15–600 fmol) in ethanol (1 μl) was subjected to TLC as described in Section 2.1. After drying with nitrogen, the plate was examined under long-wavelength ultraviolet light.



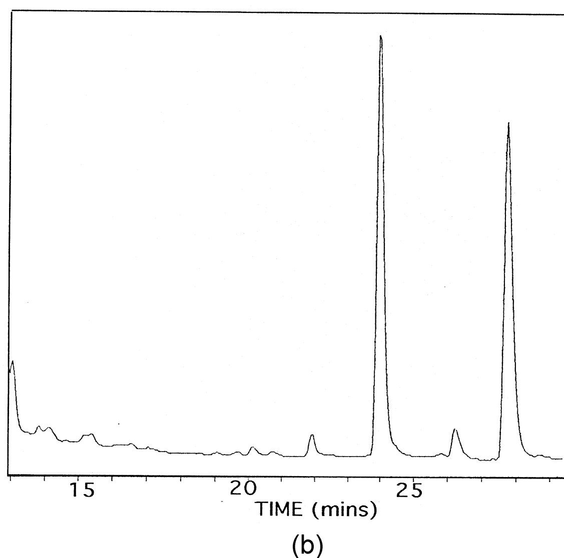


Fig. 3. (a) HPLC of blank cell culture extract; (b) HPLC of blank cell culture extract with added analyte standard and internal standard. Chromatograms were done as described in Experimental. Data shown were from 10 min following injection for a 25-min period and normalized for 100% display. Elution times were: 23.9 min for dansyl anandamide and 27.7 min for dansyl heptadecanoylethanolamide. Absorption of UV light was measured at 255 nm.

as shown schematically in the flow chart (Fig. 4). A calibration curve was constructed using a least-squares linear regression treatment of the data from the analyte/internal standard peak area ratios vs. the concentration of the analyte. The concentrations of anandamide in unknown samples were obtained by interpolation from the daily standard curve. The assay was validated on separate occasions using five different anandamide concentrations within the aforementioned limits in duplicate.

2.5. Application of the assay

The assay has been used to quantify anandamide in hepatocyte culture medium after incubation of 5 mg of anandamide with 10^8 cells in 50 ml of medium at 37°C for 6 h. The media samples were collected at a series of time points following the addition of anandamide, centrifuged at 1500 *g* and stored at −20°C until the analysis. Just prior to the analysis, the samples were brought to 37°C and vortex-mixed for 1 min. The data shown in Fig. 5 are the means of three separate experiments.

3. Results and discussion

Anandamide and heptadecanoylethanolamide were found to react readily with dansyl chloride to yield the corresponding esters (Fig. 1) under the conditions described in the Experimental section. The derivative was shown to be stable when stored in acetone at −20°C for periods up to 18 months. Fig. 2 shows the fluorescence response of dansyl anandamide on a silica gel TLC plate in which the lower limit of detection was less than 15 pmol. This suggests that the limit of any assay using this mode of detection could be extended into the low pmol range when the appropriate purification and instrumentation is applied. Detection on HPLC was made by light absorption at 255 nm. A linear relationship between absorption and mass was found over the range of 1–10 nmol of dansyl anandamide with a correlation factor of 0.986.

Using the HPLC conditions described in the Experimental section, a separation of retention times of about 3.8 min between the analyte and the internal standard was obtained (Fig. 3b). A chromatogram of a sample cell culture medium that was incubated at

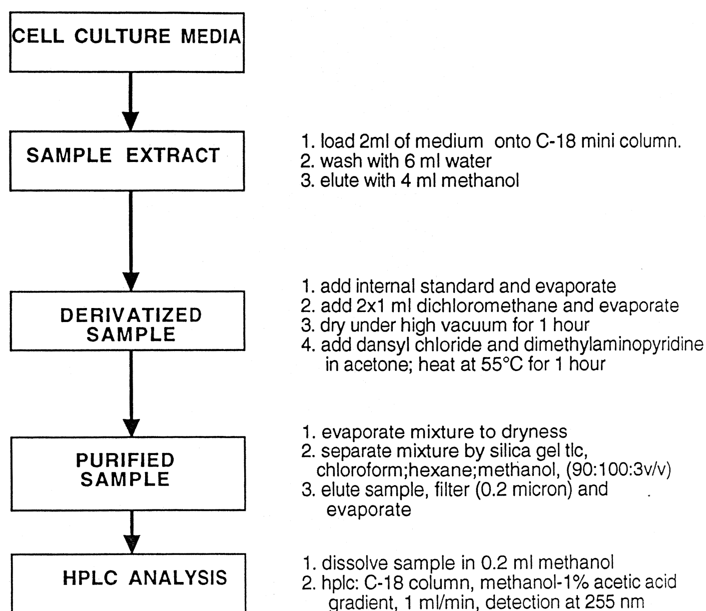


Fig. 4. Schematic outline of the assay procedure.

37°C for 120 min that had been processed as a sample (see Fig. 4) without internal standard showed essentially no interfering substances at the retention times of dansylated anandamide and the internal standard (Fig. 3a). Fig. 3b shows a similar media sample to which anandamide had been added and processed as in Fig. 4 illustrating the feasibility of the method for cell culture analysis.

Fig. 4 is a flow chart of the procedure developed for the measurement of anandamide in tissue culture samples. Anandamide is extracted from the culture medium by passage through a C_{18} reversed-phase mini-column which is then washed with water to remove all polar materials. The anandamide and other lipophyllic substances are then eluted with 100% methanol, internal standard is added and the sample evaporated. After drying, dansyl chloride and dimethylaminopyridine in acetone were added and

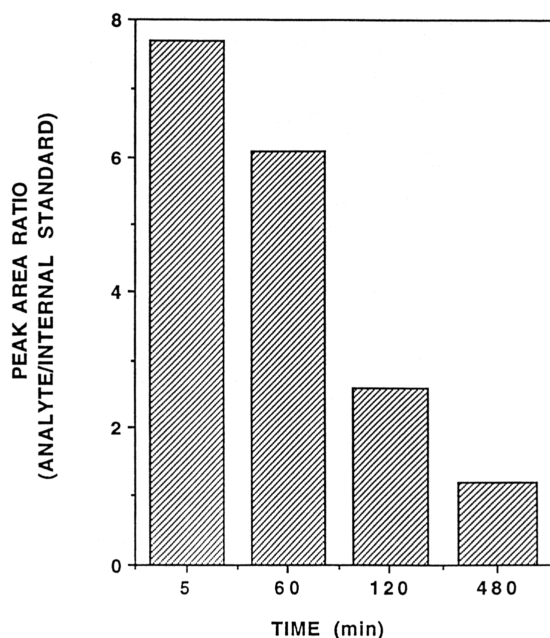


Fig. 5. Time dependent decrease in media level of anandamide in Chang liver cells. The cells were grown in high glucose MEM (50 ml) as monolayer cultures in flasks until 95% confluent. After washing, anandamide was added (5 mg/50 ml of medium) and incubated for the indicated times at 37°C. Anandamide values were obtained on 2.0-ml aliquots of media in duplicate using the dansylation–HPLC method described in Section 2 by interpolation of the peak area ratios from the chromatograms. The results shown are the means of three separate experiments.

the sample was processed as shown in Fig. 4. Aliquots of the sample are analyzed by HPLC and the anandamide quantitated by measurement of peak area ratios of dansylated anandamide and the internal standard at 255 nm. The concentration of anandamide in unknown samples was interpolated from the daily standard curves.

The procedure described above has been applied in a study designed to measure the decrease in media levels of anandamide following its addition to hepatocyte cultures. An experiment showing that anandamide is taken up by Chang liver cells is shown in Fig. 5. In this study the levels of anandamide were monitored over a 6-h time course. A control with no cells present showed essentially no decrease in the levels of anandamide (data not shown), suggesting that non cell related events such as binding to the culture dish do not account for the decreases seen in Fig. 5. The example shown here could be applied to studies of the effects of agents on the transport of anandamide into and out of intact cells. A second possible application under appropriate conditions would be to monitor the actions of agonists for the biosynthesis of anandamide. The method described here could also be used for the measurement of 2-arachidonyl-glycerol, a second putative endocannabinoid [18]. In this case there are two primary hydroxyl groups available for derivatization which should lead to an increase in sensitivity of the assay.

4. Conclusions

(a) The method described here is simple, reproducible, sensitive and relatively inexpensive. (b) Dansyl ethanolamides of long-chain fatty acid are stable for long periods. (c) It is possible that this method can be used to measure the concentrations of endocannabinoids in biological samples such as media from cells in culture.

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