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Analysis of Anandamide, an Endogenous Cannabinoid Substance, and of Other Natural *N*-Acylethanolamines

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ABSTRACT. **Recent reports have suggested that *N*-arachidonoylethanolamine (anandamide) acts as an endogenous ligand for cannabinoid receptors in mammalian brain. Here we describe methods for the extraction, purification and analysis of anandamide and related *N*-fatty acyl-ethanolamines (NAEs). Liquid-phase extraction, silica gel G column chromatography and thin-layer chromatography (TLC) were employed for sample fractionation. Three analytical high-performance liquid chromatography (HPLC) methods for purification of NAEs were developed. Finally, analyses of NAEs by gas chromatography/mass spectrometry (GC/MS) are described. The applications of these analytical methods to the identification of anandamide and related NAEs in cell cultures as well as of artifacts in biosynthetic studies are described.**

INTRODUCTION

The discovery in mammalian brain of a specific membrane receptor for Δ^9-tetrahydrocannabinol (1), a major psychoactive principle in Indian hemp (*Cannabis sativa*, also known as hashish or marijuana), stimulated the search for an endogenous substance with cannabinoid properties. This search led to the isolation from porcine brain of a lipid constituent, identified as *N*-arachidonoylethanolamine (anandamide). In vitro, this novel eicosanoid binds to brain cannabinoid receptors and reduces electrically evoked contractions of mouse vas deferens (2), while, in vivo, it produces a series of behavioural responses typical of cannabinoid drug administration (e.g. catalepsy, hypothermia and analgesia) (3, and, for review, 19). Anandamide was also shown to activate transfected cannabinoid receptors in Chinese hamster ovary cells, thereby triggering cannabinoid-associated transmembrane signalling events, thus further substantiating its role as an endogenous cannabinoid receptor ligand (4, 5, and, for review, 6).

Chemically, anandamide, apart from being, due to its arachidonoyl moiety, an eicosanoid, belongs to a family of fatty acid derivatives, the *N*-acylethanolamines (NAEs) (Fig. 1), long recognized as endogenous components of animal and plant tissues (for reviews, 7, 8). Members of this family, such as *N*-oleoylethanolamine, exert various pharmacological actions on excitable cells (9, 10), and different biochemical mechanisms for their formation in tissues have been proposed (6–8, 11–14, 16). The possibility that, in addition to anandamide, other NAEs, with either saturated or polyunsaturated acyl moieties, are produced by mammalian neurons and

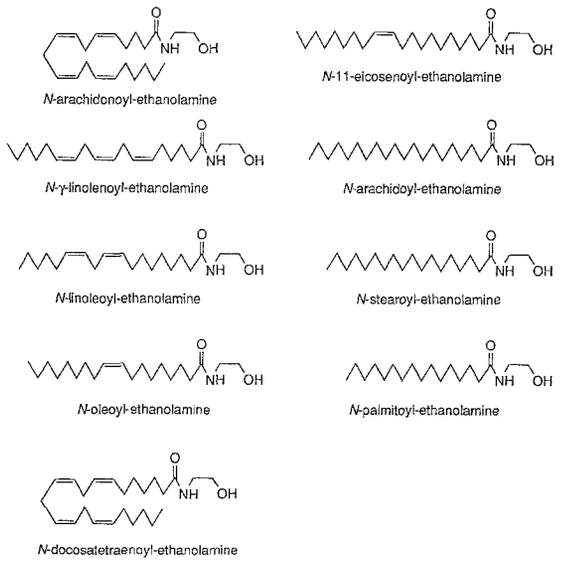

Fig. 1 Chemical structures of some of the NAEs synthesized and analyzed in this study.

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participate in neuronal signalling has been strongly suggested by recent investigations. In these studies, several additional NAEs have been shown, on one hand, to exert cannabinoid-like activities similar to those of anandamide (5, 15) and, on the other, to be produced by intact neurons challenged with various physiological stimuli (16) and to be present in brain tissue (15). Therefore, it can be foreseen that an ever increasing number of efforts will be aimed, in the next years, at fully understanding the physiological role of this important class of lipids, as well as the molecular mechanisms by which their synthesis and inactivation are regulated. Fast and facile methodologies are now needed that would update those applied in the past (2, 11–15) to the large-scale purification and analysis of saturated NAEs, by keeping in mind that: (a) low amounts of polyunsaturated and, therefore, easily oxidized, NAEs are also present in tissues and cells, and (b) several studies will be carried out in the future in cell cultures which can provide only a limited amount of starting material.

In this paper we describe a series of technical approaches for anandamide and NAE extraction, purification and analysis, that fulfill the prerequisites mentioned above.

MATERIALS AND METHODS

Materials

All solvents were purchased from Farmitalia-Carlo Erba, silica gel G thin-layer chromatography (TLC) plates from Merck, silica gel G (230–400 mesh) from Sigma, fatty acid chlorides from Nu-Check Prep (Elysian, MN), ethanolamine and *N,N'*-diisopropylcarbodiimide (DIPCI) from Sigma. High-performance liquid chromatography (HPLC) columns were obtained from Waters, [^{3}H]Ethanolamine (34 Ci/mmol) and [^{3}H]arachidonic acid (AA) (207 Ci/mmol) from Amersham, and [^{14}C]-phosphatidylethanolamine (54 mCi/mmol) and [^{14}C]-*N*-arachidonoylphosphatidylethanolamine from New England Nuclear (53 Ci/mmol).

Synthesis of radiolabelled and unlabelled NAEs

NAEs were synthesized either by direct condensation with ethanolamine and acyl chloride (2) or by the carbodiimide method. In this case, *N,N*-di-isopropyl-carbodiimide (1 equiv.) was added under stirring at 0°C to a solution of the appropriate fatty acid (1.5 equiv.) in dichloromethane (1 ml). After stirring for 10 min, ethanolamine (1 equiv.) was added dropwise to the cooled (0°C) solution. The reaction was then allowed to warm up to room temperature and stirred for a further 12 h, after which the homogenous reaction mixture was diluted with methanol (1 ml), and dried under nitrogen. Synthetic NAEs were purified by analytical TLC in chloroform:methanol:ammonia (85/15/1) (R_f = 0.8).

Extraction and fractionation by column chromatography

[^{3}H]Anandamide (10 000 dpm/ml, 0.60 mCi/mmol, prepared using the carbodiimide method), was added to Dulbecco's modified Eagle medium (DMEM) and extracted with various organic solvents. The organic phases were removed, evaporated under reduced pressure and the residues fractionated by silica gel column chromatography. A slurry of silica gel in chloroform was poured into Pasteur pipettes (final volume = 0.2–0.4 mL). Samples were dissolved in chloroform, loaded onto columns and eluted with a step gradient of methanol in chloroform (from 0–55%).

TLC

Samples from open-bed chromatography were dissolved in 20–50 µl of chloroform/methanol (9:1) and applied to analytical silica gel G TLC plates. Plates were developed with a solvent system of chloroform/methanol (9:1 or 95:5) or chloroform/methanol/ammonia (85:15:1). Different NAEs migrated with similar R_f under these conditions, irrespective of carbon chain length and degree of unsaturation. After visualizing the lipids by exposure to iodine vapours, bands were scraped off, dissolved in chloroform/methanol (1:1), and evaporated under nitrogen.

HPLC

NAEs fractionated by silica gel column chromatography and/or TLC were purified by normal-phase HPLC, using a Resolve silica column (4.6 mm × 15 cm, 5 µm, Waters) eluted with a gradient consisting of 2-propanol in n-hexane (from 0–20% over 20 min) at a flow rate of 1.5 ml/min. All NAEs were eluted from the column at a retention time of 12–13 min. Further fractionation was achieved by reversed-phase HPLC, using a Nova-pak C18 column (4.6 mm × 15 cm, 4 µm, Waters), eluted with a gradient of methanol in water (75–100% over 50 min) at a flow rate of 1.0 ml/min. Because this system did not resolve *N*-γ-linoleoyl-ethanolamine from anandamide, an additional HPLC separation procedure was developed. A Free Fatty Acid HP column (4.6 mm × 15 cm, 5 µm, Waters) was eluted isocratically with a solvent of 70% acetonitrile/tetrahydrofuran/water (45:20:35) in water, at a flow rate of 1.5 ml/min.

HPLC analyses were carried out with two Waters 510 pumps controlled by a Waters Automatic Gradient Controller, and UV absorbance was monitored at 214 nm with a multiwavelength 486 Waters detector. Fractions were collected in polypropylene tubes and brought to dryness under reduced pressure immediately after collection.

GC/MS

NAEs were acetylated by allowing them to react overnight with acetic anhydride in pyridine (1:4) at room

temperature in an atmosphere of nitrogen. The reactions were stopped by adding excess methanol, and the samples evaporated under a stream of nitrogen. *O*-Acetyl-NAEs were dissolved in chloroform (5 µl) and analyzed by GC, using a Carlo Erba chromatograph equiped with a OV-1 capillary column or by GC/MS using a Shimadzu apparatus equipped with a OP-15 capillary column, with a temperature gradient from 220–300°C at 4°C/min (temperature of injector, transfer line and detector were set at 300°C). Electron voltage was set at 70 eV.

RESULTS

NAE extraction from incubation media

Several organic solvents were compared for their ability to extract [^{3}H]Anandamide from samples of incubation media. The most effective extraction of [^{3}H]Anandamide was obtained by using a solvent system of chloroform/methanol (2:1) (recovery of 83.2 ± 7.8%, mean ± SEM, n = 3). We obtained lower recoveries using diethyl ether (70.4 ± 1.6%) or ethyl acetate (59.6 ± 3.1%), two solvents that are often used to extract AA derivatives from tissue samples.

Column chromatography

We compared the chromatographic behaviours of various ethanolamine-containing lipids on mini-columns of silica gel G eluted with increasing concentrations of methanol in chloroform (Fig. 2). [^{3}H]Anandamide was eluted from the column with 10% methanol, *N*-arachidonoylphosphatidylethanolamine (suggested to serve as the precursor of anandamide in central neurons [16]) with 30% methanol, and phosphatidylethanolamine with ≥60% methanol.

Class fractionation of NAEs

Analytical and semi-preparative TLC silica gel plates and normal-phase HPLC were used to purify NAEs as a class. Using a mixture of chloroform/methanol as solvent system, the R_fs of synthetic NAEs were 0.3 (90:10) and 0.5 (95:5). Using the chloroform:methanol:ammonia (85/15/1) system, the R_f was 0.8, and a better resolution between NAEs and AA (R_f = 0.4) was obtained. The yield of [^{3}H]Anandamide after elution from TLC plates ranged from 50–75% of the amount spotted. Higher recovery was obtained when using normal-phase HPLC (98 ± 6.6%). With the n-hexane/2-propanol gradient described under Materials and Methods, all NAEs were eluted at a retention time of 12–13 min (corresponding to 93% n-hexane) (Fig. 3).

Purification of individual NAEs

We used reverse-phase HPLC to resolve individual

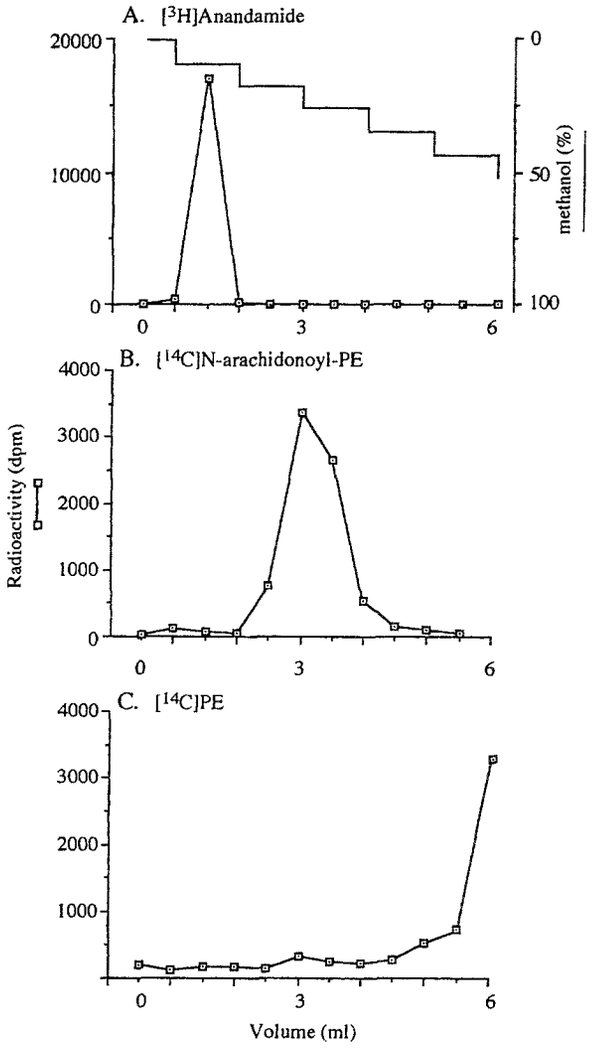


Fig. 2 Radioactivity elution profile of [^{3}H]Anandamide (A), [^{14}C]*N*-arachidonoylphosphatidylethanolamine (PE) (B) and [^{14}C]PE (C) from silica gel G mini-columns eluted with a solvent system consisting of increasing percentages of methanol (–) in chloroform.

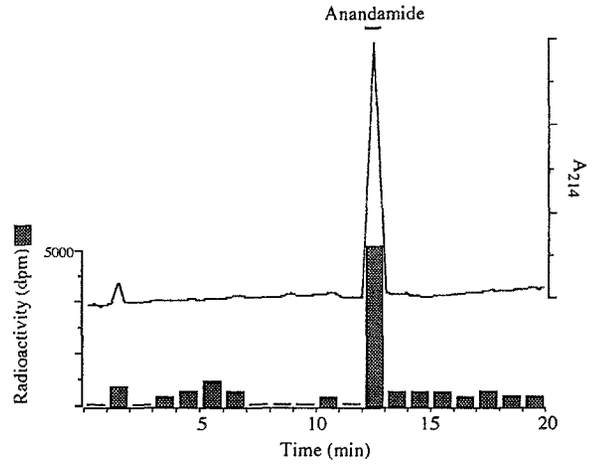


Fig. 3 UV (214 nm, –) and radioactivity (dpm) profiles of a typical normal-phase HPLC analysis of a mixture of synthetic, unlabelled anandamide (40 nmol) and [^{3}H]Anandamide (6000 dpm). All other NAEs tested were eluted from the HPLC column at the same retention time. Chromatographic conditions are described under Materials and Methods. For a typical radioactivity profile of an analogous HPLC analysis of NAE from central neurons see Ref. 16.

NAEs, based on the length of their acyl chain and on their degree of unsaturation. When injected into a Nova-Pak (Waters) analytical column eluted with a methanol/water gradient, NAEs differing in two methylene groups were separated by at least 5.5 min, while those differing in one double bond were separated by at least 2.5 min (Fig. 4A). As a rule, we observed that the longer the acyl chain and the lower its degree of unsaturation, the longer was the retention time of the NAE. Recovery of [^{3}H]-Anandamide after reverse-phase HPLC was 84 ± 8.7%.

Reverse-phase HPLC on Nova-Pak columns did not allow us to resolve anandamide from *N*-linoleoyl-ethanolamine. To achieve this separation we used a Free Fatty Acid HP column (Waters), eluted under isocratic conditions (Fig. 4B). Overall recovery of [^{3}H]Anandamide, after the two consecutive reverse-phase HPLC steps, was 60 ± 2.6%. Using either HPLC systems, we could detect amounts of NAEs ranging from 4 nmol (for anandamide) to 200 nmol (for *N*-palmitoylethanolamine), when monitoring UV absorbance at 214 nm.

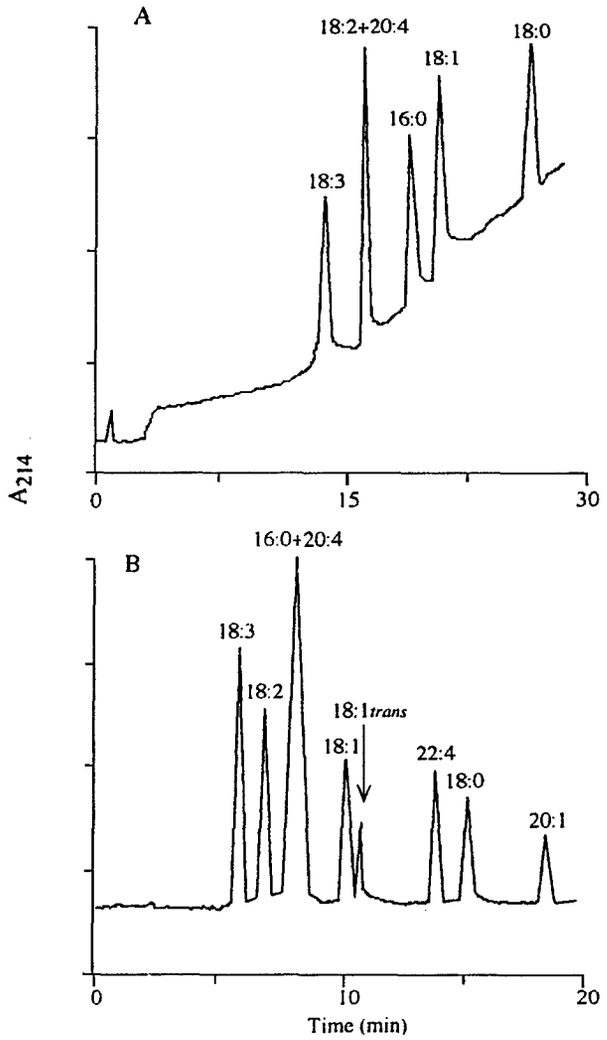


Fig. 4 UV (214 nm) profiles of reversed-phase HPLC analyses of various synthetic NAEs on (A) Nova-pak C-18 column or (B) Free Fatty Acid HP column. Chromatographic conditions are described under Materials and Methods. Notations over each peak indicate the length and unsaturation of the fatty acyl chain on each NAE analyzed. For a typical radioactivity profile of an analogous HPLC analysis of a mixture of NAEs from stimulated central neurons see Ref. 16.

Application of HPLC to reveal artifactual 'anandamide formation'

We employed the first reversed-phase HPLC method to uncover an artifact in a previous study, which reported formation of anandamide in rat brain homogenates (17). Rat brain homogenates (10 mg of protein) were incubated in Tris-HCl buffer (0.1 M, pH = 9.0, 10 ml) for 60 min at 37°C, in the presence of AA (1 mM), [^{3}H]ethanolamine (1.6 mM, 1 μCi), and phenylmethylsulphonyl fluoride (PMSF, 1.5 mM), a common protease inhibitor added to prevent anandamide degradation (17). The reaction was terminated by adding 2 vol of chloroform/methanol (1:1). After extraction, the organic phase was evaporated and analyzed by normal-phase HPLC or TLC. TLC analysis was carried out using either the solvent system described above or the organic layer of ethyl acetate/hexane/acetic acid/water (100:50:20:100), as described (17). As reported, a radioactive component with the chromatographic behaviour of authentic anandamide on both TLC and normal-phase HPLC was formed during these incubations (17). However, when this component was scraped off the TLC plate and subjected to reversed-phase HPLC on Nova-Pak column, no radioactive component was found to co-elute with anandamide (16 min, Fig. 4A), and the only radioactivity peak found eluted from the column with a retention time of 5 min, and displayed a strong UV absorbance at 260 nm, unexpected in a NAE. Analysis of this compound by proton NMR ($CDCl_3$, 500 MHz) provided a spectrum with few, but highly diagnostic signals (δ = 7.38, 5H, multiplet, aromatic protons; δ = 5.26, 1H, broad singlet, sulphonamide proton; δ = 4.26, 2H, sharp singlet, phenylmethylene protons; δ = 3.54, 2H, doublet doublet, ethanolamine α-methylene protons; δ = 3.01, 2H, broad doublet, ethanolamine β-methylene protons; δ = 2.86, 1H, broad singlet, hydroxylic proton). Assignment of these signals demonstrated unambiguously that the product formed was neither anandamide nor any other NAE (2, 15), but in fact an adduct of PMSF with ethanolamine (*N*-phenylsulphonylethanolamine, see Fig. 5) whose condensation at basic pH can be predicted.

GC of *O*-acetyl-NAEs

Capillary GC separation of NAEs, as *O*-acetyl derivatives, was also carried out. Applying a slow temperature gradient, NAEs with saturated even acyl chains (from C-12 to C-22) were separated at intervals of 2.5–2.9 min (Fig. 6). Unsaturated NAEs were eluted earlier than the parent saturated compounds. The detection limit, using a flame ionization detector, was 0.05–0.1 nmol of injected NAE.

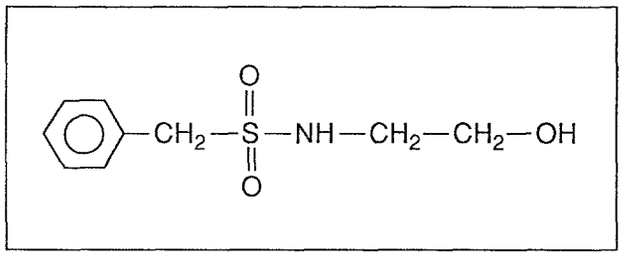


Fig. 5 Structure of phenylmethylsulphonylethanolamine, a non-enzymatic adduct produced in incubations of brain tissue homogenates at pH 9 with ethanolamine (1.6 mM) and PMSF (1.5 mM).

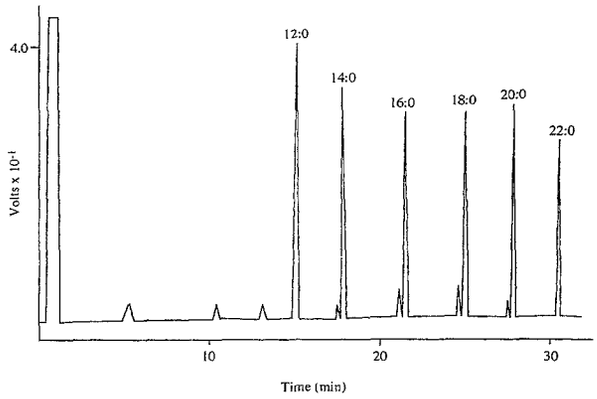


Fig. 6 Typical GC elution profile of a mixture of synthetic acetylated NAEs. Sample derivatization and chromatographic conditions are described under Materials and Methods. Products eluting from the GC were detected by flame ionization.

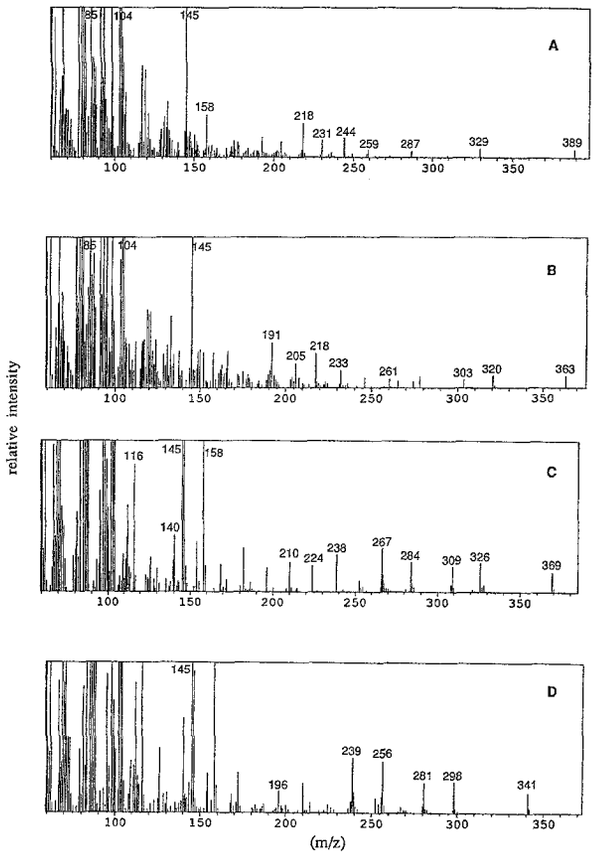


Fig. 7 Electron-impact mass spectra of anandamide (A), *N*-γ-linolenoylethanolamine (B), *N*-stearoylethanolamine (C) and *N*-palmitoylethanolamine (D). The *m/z* of some diagnostic fragments are shown.

Structural characterization of NAEs

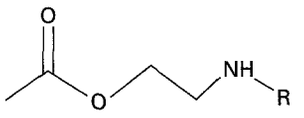
We used electron-impact GC/MS for structural characterization of *O*-acetyl-NAEs. Full mass spectra and characteristic fragmentation patterns for various NAEs are shown in Fig. 7 and Table 1. Fragments diagnostic of individual NAEs are found in the high mass range. These include the molecular ions (M^+) as well as ions formed by the loss of $CH_3CO^\bullet$ (–43), CH_3COOH (–60), $CH_3COO(CH_2)_2NH^\bullet$ (–102) and $CH_3COO(CH_2)_2NHCO^\bullet$ (–130) (Figs 7 and 8). Fragments common to most NAEs include *m/z* 85 and 145, likely produced by β-cleavages accompanied by McLafferty rearrangement, and *m/z* 98 and 158, produced by γ-cleavages (Figs 7 and 8). In addition, marked aliphatic fragmentation occurs, especially for saturated NAEs (Fig. 7).

From the knowledge of these typical fragmentation patterns it is possible to use GC/MS analysis of unknown samples, purified by HPLC and subsequently acetylated, in the selected ion monitoring (SIM) mode. Using this method, very low amounts of NAEs (down to 5 pmol) can be detected, and their structure assigned with confidence.

Application of the methods described here to studies of anandamide biosynthesis

The methods described above were used to study the mechanisms underlying anandamide biosynthesis in primary cultures of striatal and cortical rat neurons (16). In these cultures, basal levels of NAEs are very low and can be detected only by labelling the cells overnight with [^{3}H]ethanolamine, by extracting the cells with chloroform/methanol (2:1) and by submitting the extract to the open bed chromatography and normal-phase HPLC procedures described above. It is possible to detect a radioactivity peak with the same retention time as NAEs (16), but it is then difficult to fractionate this peak further, due to its low amounts, by reversed-phase HPLC. Conversely, if radiolabelled cells are stimulated with substances which depolarize neurons and provoke Ca^{2+} influx into the cell, the amount of radioactivity (per Petri dish of cells, i.e. per 2.5×10^7 cells) incorporated into the above peak allows one to carry out, in sequence, both reversed-phase HPLC steps described in this study, and therefore to characterize the fatty acyl composition of the NAEs produced by stimulated neurons (Table 2, Ref. 16). Moreover, it has been possible to push further the chemical characterization of NAEs of neuronal origin, by stimulating unlabelled neurons contained in about 10 Petri dishes (i.e. 2.5×10^8 cells) with either ionomycin or kainate, and by applying in sequence the extraction, column chromatography, normal-phase HPLC, acetylation and GC/MS procedures

Table 1 Selected GC/MS data for various NAEs. Retention times (T_r) and most significant electron impact (EI, 70 eV) fragment ions, with their relative abundance in parentheses, are indicated



R-	T_r	EI ion peaks (*m/z*)
arachidonyl - 20:4 Δ5,8,11,14	15.75 min	389 (3), 329 (3), 287 (5), 259 (15), 244 (10), 218 (10), 158 (90), 145 (70), 104 (85), 98 (85), 85 (100);
11-eicosenoyl - 20:1, Δ11	16.78 min	395 (10), 352 (10), 335 (7), 293 (15), 265 (10), 237 (25), 158 (22), 145 (85), 116 (60), 98 (100), 85 (90);
linoleoyl - 18:2, Δ9,12	13.97 min	365 (10), 323 (8), 305 (15), 263 (20), 207 (26), 194 (30), 145 (35), 98 (90), 85 (100);
γ-linolenoyl - 18:1, Δ 6,9,12	13.47 min	363 (5), 320 (6), 303 (6), 278 (3), 261 (10), 233 (18), 218 (15), 205 (10), 191 (18), 145 (60), 104 (90), 98 (90), 85 (90), 55 (100);
lignoceroyl 24:0	22.37 min	453 (10), 410 (7), 393 (7), 368 (7), 351 (10), 323 (10), 158 (50), 145 (100), 98 (85), 85 (90);
behenoyl 22:0	19.70 min	425 (5), 382 (8), 365 (15), 340 (8), 323 (12), 295 (10), 280 (20), 158 (80), 145 (100), 98 (90), 85 (90);
arachidoyl - 20:0	16.87 min	397 (5), 354 (10), 337 (10), 312 (15), 295 (10), 267 (8), 252 (7), 158 (90), 145 (100), 98 (90), 85 (95);
stearoyl - 18:0	13.97 min	369 (7), 326 (12), 309 (15), 284 (15), 267 (15), 238 (10), 224 (8), 210 (9), 158 (80), 145 (100), 140 (15), 112 (20), 98 (86), 85 (90);
palmitoyl 16:0	11.10 min	341 (5), 298 (8), 281 (7), 256 (15), 239 (17), 210 (8), 196 (7), 158 (80), 145 (100), 140 (20), 112 (25), 85 (95);
miristoyl 14:0	8.30 min	313 (8), 270 (20), 253 (8), 228 (20), 211 (40), 168 (10), 145 (95), 98 (90), 85 (100);
lauroyl 12:0	5.83 min	285 (5), 242 (13), 225 (7), 200 (40), 183 (50), 158 (40), 145 (90), 98 (90), 85 (100);

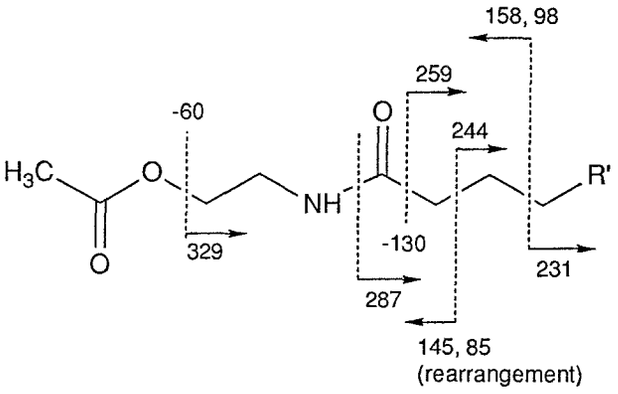


Fig. 8 Electron-impact GC/MS (70 eV) fragmentation pattern of *O*-acetyl-NAEs. The *m/z* of some fragment ions, diagnostic of all NAEs or of anandamide, used for mass fragmentographic (selected ion monitoring) analyses of NAEs from stimulated central neurons, are shown.

described in this article (16). Treatment of neurons with these stimuli led to the formation of amounts of anandamide that could be estimated to be around 20 pmols since the whole work-up has an average yield of about 65%, and the amounts found for this polyunsaturated NAE by GC/MS (using the SIM mode) were slightly above the detection limit of this technique (5 pmol). Anandamide was the minor component of the NAE mixture produced by stimulated central neurons, the most abundant NAEs being *N*-palmitoyl-, *N*-stearoyl-, *N*-linoleoyl- and *N*-oleoyl-ethanolamines (Table 2, Ref. 16).

Table 2 Percent composition (means ± SEM, $n = 3$) of NAEs from cortical neurons prelabelled with [^{3}H]ethanolamine and stimulated with 2 μM ionomycin (Calbiochem)

[^{3}H]AnNH	% composition intact neurons (ionomycin stimulation)
[arachidonoyl]	4.9 ± 1.1
[linolenoyl]	10.6 ± 1.1
[linoleoyl]	1.1 ± 0.3
[oleoyl]	31.3 ± 1.7
[stearoyl]	21.7 ± 2.7
[palmitoyl]	25.7 ± 2.2

Data are expressed as percent of total recovered [^{3}H]NAEs ± SEM ($n = 3$). After the stimulation, neurons were extracted with chloroform/methanol (2:1 v/v), and the extract was fractionated by silica G open bed chromatography, followed by normal-phase HPLC and by reversed-phase HPLC-1 (Nova-pak column). 1 min HPLC fractions were collected and the radioactivity associated with 100 μL aliquots counted after adding 5 ml scintillation liquid. HPLC fractions corresponding to anandamide plus *N*-linoleoylethanolamine (retention time = 16 min) were brought to dryness and analyzed by reversed-phase HPLC-2 (Free Fatty Acid column), to determine the relative composition of these two NAEs. Radiolabelled NAEs derive from the hydrolysis of the corresponding radiolabelled *N*-acylphosphatidylethanolamines (16) in which the incorporation of [^{3}H]ethanolamine can be assumed to be the same for each species. Therefore, measure of radioactivity can be used to provide an evaluation of the % composition of each NAE. Accordingly, the data shown in the table were confirmed by GC analyses carried out as described in the text

The techniques described in this study were also applied to isolate and identify NAEs in stimulated neuroblastoma × glioma NG108–15 cells, where, although much lower amounts of NAEs were detected, the percent composition of the various species was similar to that described in Table 2.

DISCUSSION

In this report we describe a series of technical approaches that should permit one to tackle the analytical problems expected to rise during studies on the biosynthesis of anandamide and related NAEs in cell cultures.

We find that extraction with chloroform/methanol (as opposed to other commonly used organic solvents) and chromatography on mini-columns of silica gel G, are rapid, inexpensive and efficient fractionation procedures that allow the separation of NAEs from many potential contaminants (such as ethanolamine metabolites and ethanolamine-containing phospholipids). In our experience, these procedures represent two very useful, if not essential, clean-up steps before HPLC and GC/MS analyses. Alternatively, NAEs may be fractionated by TLC, a method that provides greater resolution than open-bed chromatography (for instance, when using the chloroform:methanol:ammonia (85/15/1) method, it resolves NAEs from free AA), while being relatively inefficient if quantitation is needed (in our hands, yields of fractionated NAEs after scraping from the plate and eluting off the silica gel varied between 50 and 75%).

We obtained greater accuracy and recovery when we fractionated anandamide and other NAEs by normal-phase HPLC. Like TLC, the normal-phase HPLC fractionation described here may be applied to fractionate NAEs as a class, but it has not proven itself suitable to resolve individual NAEs. For this purpose, we developed instead two distinct reversed-phase HPLC protocols that, when used in a sequential fashion, ensure a confident chromatographic identification and/or purification of most naturally occurring NAEs. This approach is likely to be useful especially when trace amounts of radioactive NAEs are under study, such as those found in NG108–15 cells.

Unambiguous identification of NAEs produced in biological tissues can be best provided by GC/MS. *O*-Acetyl-NAEs can be readly prepared by acetylation of fractionated biological samples and are well resolved on capillary GC columns using standard conditions for the separation of fatty acid methyl esters. We carried out GC/MS analyses of acetylated NAEs by using a quadrupole mass spectrometer equipped with an electron impact source. As expected, electron-impact MS produces mass spectra with richer fragmentation patterns, at both ends of the molecule, than those displayed by the chemical ionization technique (15). This fragmentation pattern exhibits at the same time significant molecular ion peaks, thus ensuring a great diagnostic power. Moreover, the presence of very intense fragmentation peaks, such as those at *m/z* 85, 98, 104, 145 and 158, common to all NAEs, makes the GC/MS technique, when detecting the metabolites eluted from the GC column by mass fragmentography (selected ion monitoring, SIM) a very sensitive method to identify NAEs (16). The choice of more diagnostic, albeit less intense, MS peaks, coupled to the high resolving capacity of GC capillary columns, should allow one to determine the structure of most naturally occurring NAEs.

We have applied the analytical procedures described here to a series of biochemical studies on the formation, release, uptake and degradation of anandamide in brain neurons in primary culture (16) and brain tissue (18). In those studies we have applied the GC/MS technique described here to the characterization of trace amounts of anandamide, and we have shown the stimulus-induced, neuron-specific, formation of a family of NAEs, likely derived from phosphodiesterase-catalyzed hydrolysis of the phospholipid precursor, *N*-acylphosphatidylethanolamine. In the present report, we have shown how the sequential use of normal-phase and reversed-phase HPLC can be used to calculate the percent amounts of each NAE in mixtures of neuronal origin, where both saturated and unsaturated forms of these compounds are present in low amounts (15, 16). Moreover, we have used some of the same techniques to show how an incomplete chromatographic characterization of biologically derived NAE may lead to erroneous identifications. As additional, novel methods to study NAEs metabolism become available in the literature, a higher investment of technologies and energies in this new and exciting area of neurochemistry may be foreseen.

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References

1. Mechoulam R. In: Mechoulam R, ed. Cannabinoids as therapeutic agents. Boca Raton: CRC Press, 1986: 1–19.
2. Devane W A, Hanus L, Breuer A et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992; 258: 1946–1949.
3. Fride E, Mechoulam R. Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. Eur J Pharmacol 1993; 231: 313–314.
4. Vogel Z, Barg J, Levy R, Saya D, Heldman E, Mechoulam R. Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. J Neurochem 1993; 61: 352–355.
5. Felder C C, Briley E M, Axelrod J, Simpson J T, Mackie K, Devane W A. Anandamide, an endogenous cannabinomimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. Proc Natl Acad Sci USA 1993; 90: 7656–7660.
6. Di Marzo V, Fontana A. Anandamide, an endogenous cannabinomimetic eicosanoid: 'killing two birds with one stone'. Prostaglandins Leukot Essent Fatty Acids 1995; 53: 1–11.
7. Schmid H H O, Schmid P C, Natarajan V. *N*-Acylated glycerophospholipids and their derivatives. Prog Lipid Res 1990; 29: 1–43.
8. Chapman K D, Moore T S Jr. *N*- Acylphosphatidylethanolamine synthesis in plants: occurrence, molecular composition, and phospholipid origin. Arch Biochem Biophys 1993; 301: 21–33.
9. Epps D E, Mandel F, Schwartz A. The alteration of rabbit

skeletal sarcoplasmic reticulum function by *N*-acylethanolamine, a lipid associated with myocardial infarction. Cell Calcium 1982; 3: 531–543.
10. Gulaya N M, Melnik A A, Balkov D I, Volkov G L, Vysotsky M V, Vaskosky V E. The effect of long-chain *N*-acylethanolamines on some membrane-associated functions of neuroblastoma C1300 N18 cells. Biochim Biophys Acta 1993; 1152: 280–288.
11. Bachur N R, Udenfriend S. Microsomal synthesis of fatty acid amides. J Biol Chem 1966; 241: 1308–1313.
12. Natarajan V, Reddy P V, Schmid P C, Schmid H H O. On the biosynthesis and metabolism of *N*-acylethanolamine phospholipids in infarcted dog heart. Biochim Biophys Acta 1981; 664: 445–448.
13. Schmid P C, Reddy P V, Natarajan V, Schmid H H O. Metabolism of *N*-acylethanolamine phospholipids by a mammalian phosphodiesterase of the phospholipase D type. J Biol Chem 1983; 258: 9302–9306.
14. Natarajan V, Schmid P C, Reddy P V, Schmid H H O. Catabolism of *N*-acylethanolamine phospholipids by dog brain preparations. J Neurochem 1984; 42: 1613–1619.
15. Hanus L, Gopher A, Almog S, Mechoulam R. Two new unsaturated fatty acid ethanolamides in brain that bind to the cannabinoid receptor. J Med Chem 1993; 36: 3032–3034.
16. Di Marzo V, Fontana A, Cadas H et al. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 1994; 372: 686–691.
17. Deutsch D G, Chin S A. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem Pharmacol 1993; 46: 791–796.
18. Désarnaud F, Cadas H, Piomelli D. Anandamide amidohydrolase activity in rat brain microsomes, identification and partial characterization. J Biol Chem 1995; 270: 6030–6035.
19. Mechoulam R, Hanus L, Martin B R. Search for endogenous ligands of the cannabinoid receptor. Biochem Pharmacol 1994; 48: 1537–1544.