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Isotope dilution GC/MS determination of anandamide and other fatty acylethanolamides in rat blood plasma

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Abstract Anandamide and allied fatty acylethanolamides (AEs) may act as signalling molecules in brain and peripheral tissues. In the present study, we describe an electron-impact gas chromatography/mass spectrometry (GC/MS) method based on isotope dilution, which may be used for the identification and quantification of anandamide and other AEs in biological matrices. The calibration curves for standard AEs were linear over the range 0–1000 pmol ($r^2 = 0.99$) with a coefficient of variation of 4% at 2.5 pmol. Detection and quantification limits were in the high fmol to low pmol range for all AEs. Using this method we measured nanomolar concentrations of three endogenous AEs in deproteinated rat blood plasma (anandamide: 5.2 pmol/ml; palmitylethanolamide: 16.7 pmol/ml; oleylethanolamide: 8.1 pmol/ml). These results are consistent with a regulatory role of anandamide and other AEs in peripheral tissues.

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Key words: Anandamide; Fatty acylethanolamide; Cannabinoid; Plasma; Gas chromatography/mass spectrometry

1. Introduction

Anandamide (arachidonylethanolamide) is an endogenous lipid that binds to cannabinoid receptors with high affinity [1] and mimics the pharmacological effects of cannabimimetic drugs both in vitro and in vivo [2]. Anandamide is released from brain neurons in an activity-dependent manner [3] through a mechanism that involves phospholipase D-mediated cleavage of a membrane phospholipid precursor [4,5]; carrier-mediated uptake into cells followed by enzymatic hydrolysis is thought to terminate its biological effects [6–10]. Though initially isolated from brain, anandamide is widely distributed in peripheral organs and tissues [11], where it may serve broad regulatory functions including control of vascular tone [12–14], intestinal motility [15,16] and immune responses [17].

Anandamide is structurally related to a family of saturated and monounsaturated fatty acylethanolamides (AEs) which have been long recognized as endogenous constituents of animal and plant tissues [18]. The possible physiological roles of these compounds are still largely unexplored, but two lines of evidence suggest that they may participate in cellular signalling. First, saturated and monounsaturated AEs are produced, together with anandamide, when cultured neurons are challenged with Ca^{2+} ionophore or membrane-depolarizing agents [3]. Second, AEs are pharmacologically active. For example, palmitylethanolamide exerts significant antiinflammatory effects when it is administered as a drug in various animal models [19]. Some of these effects may be mediated by the activation of CB2-like cannabinoid receptors [20].

Reliable quantitative methods to measure anandamide and other AEs at trace concentrations are needed to investigate the possible regulatory functions of these compounds. In the present study, we describe an isotope dilution, electron-impact gas chromatography/mass spectrometry (GC/MS) method which may have the sensitivity, selectivity, accuracy and precision required for this analytical task. We applied this assay to the quantification of anandamide and other AEs in rat blood plasma.

2. Materials and methods

2.1. Chemicals

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Supelco (Bellefonte, PA), [3H]anandamide (221 Ci/mmol) from New England Nuclear (Wilmington, DE), fatty acyl chlorides (5,8,11,14-eicosatetraenoylchloride, hexadecanoylchloride and 9-octadecenoylchloride) from Nu-Check Prep (Elysian, MN), [2H4]ethanolamine (isotopic atom enrichment = 98%) from Cambridge Isotope Laboratories (Andover, MA). All solvents were from Burdick and Jackson (Muskegon, MI) and all other chemicals from Sigma (St. Louis, MO).

2.2. Synthesis of standard AEs and [2H4]AEs

Standard AEs were synthesized by the reaction of fatty acyl chlorides with unlabeled or 2H4-labeled ethanolamine [1]. Fatty acyl chlorides in dichloromethane (10 mg/ml) were mixed with 1 equivalent of ethanolamine, and allowed to react for 15 min at 0–4°C. Reactions were stopped by adding water. After vigorous mixing, the upper aqueous phases were discarded to remove unreacted ethanolamine. The organic phases were washed twice with water, concentrated to dryness under a stream of N_2, and the reaction products were reconstituted in methanol. Identity and chemical purity (>98%) of the synthesized AEs and [2H4]AEs were determined by GC/MS (see Section 2.4).

2.3. Extraction and fractionation of rat plasma AEs

5 ml of blood was collected from the heart of anesthetized male Wistar rats using a syringe filled with 2 ml Krebs-Tris buffer (NaCl 136 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM, glucose 10 mM, Trizma base 20 mM; pH 7.4) containing EDTA 4.5 mM. Blood samples were centrifuged in Accuspin tubes (Sigma) for 10 min at 18°C ($800 \times g$) and the plasma layers were carefully recovered and spiked with [2H4]AEs (600 pmol each). After acetone precipitation of plasma proteins, the supernatants were collected and subjected to lipid extraction with methanol/chloroform. Enough of each solvent was added to reach a final ratio buffer/methanol/chloroform of 1:1:2 (v/v/v). The chloroform phases were recovered, evaporated to dryness under N_2, reconstituted in chloroform (50 μl) and injected into the HPLC. HPLC fractionations were performed on a Hewlett-Packard 1090 Liquid Chromatograph, equipped with a normal-phase Resolve Silica column (3.9 mm×15 cm, 5 μm, Waters Associates), eluted with a gradient of isopropyl alcohol (B) in *n*-hexane (A) (100% A initial; 90% A, 10% B for 1 min; 60% A, 40% B for 7 min, 50% A, 50% B for 12 min) at a flow rate of 1.7 ml/min. Under these conditions, all AEs were eluted from the HPLC column between 4.7 and 5.3 min. The AE-containing fractions were collected in glass reaction vessels (Supelco), dried under N_2 and subjected to chemical derivatization (see Section 2.4). To estimate recoveries, in some experiments

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[³H]anandamide (20 000 dpm) was added to an aqueous solution (1 ml) of non-radioactive anandamide (100 nM), extracted in chloroform/methanol and subjected to HPLC fractionation. The AE-containing fractions were concentrated to a volume of 1 ml under N_2, and radioactivity was determined by liquid scintillation counting.

2.4. GC/MS analysis

The AEs were derivatized by treatment with BSTFA for 15 min at room temperature. The trimethylsilylether (TMS) derivatives produced in this reaction were dried under N_2, reconstituted in *n*-hexane and injected in the splitless mode into a Hewlett-Packard 5890 GC equipped with an HP-5MS capillary column (30 m; internal diameter, 0.25 mm) and interfaced with a Hewlett-Packard 5972 MS. Starting one minute after the injection, the oven temperature was increased from 150°C to 280°C at a rate of 8°C/min. The injector temperature was kept at 250°C and helium was used as carrier gas. The ion energy was 25 eV and the accelerating voltage was 1.6 V. Under these conditions, the TMS derivatives of [²H₄]AE standards were eluted from the GC at the following retention times: [²H₄]anandamide: 18.6 min; [²H₄]palmitylethanolamide: 15.6 min; [²H₄]oleylethanolamide: 17.3 min. Because of GC isotope discrimination, unlabeled AEs had retention times approximately 0.02 min longer than the corresponding [²H₄]AEs. We monitored, by selected-ion monitoring (SIM), AE fragments produced by the loss of one methyl group ([M-15]⁺).

3. Results and discussion

3.1. Mass spectral properties of AEs and [²H₄]AEs

The electron-impact mass spectra of synthetic unlabeled anandamide (*cis*20:4), palmitylethanolamide (16:0) and oleylethanolamide (*cis*18:1) are shown in Fig. 1. Diagnostic fragments for all compounds were found in the high mass range. They included molecular ions ([M]⁺) as well as ions produced by the loss of one methyl group ([M-15]⁺). Additional informative and prominent fragments were: [M-43]⁺ (loss of one propyl group); [M-90]⁺ (loss of TMSOH group; this fragment was absent from the spectrum of anandamide-TMS which contained instead a fragment at *m/z* 328, [M-91]⁺); and *m/z* 175/179 (possibly corresponding to [H₂C=COH–NH–CH₂–CH₂–O–TMS]⁺, which may be produced through McLafferty rearrangement).

3.2. Linearity of the isotope dilution assay

Monitoring the [M-15]⁺ fragments, which are reasonably abundant in the AE-TMS spectra, we observed MS responses that were linear ($r^2 = 0.99$) when amounts of unlabeled AEs ranging from 0 to 1000 pmol were injected into the GC/MS together with a fixed amount of ²H₄-labeled standards (600 pmol).

3.3. Precision and accuracy

Precision and accuracy of the method were assessed in five independent determinations by injecting 2.5, 10 or 100 pmol of each AE together with 600 pmol of the corresponding
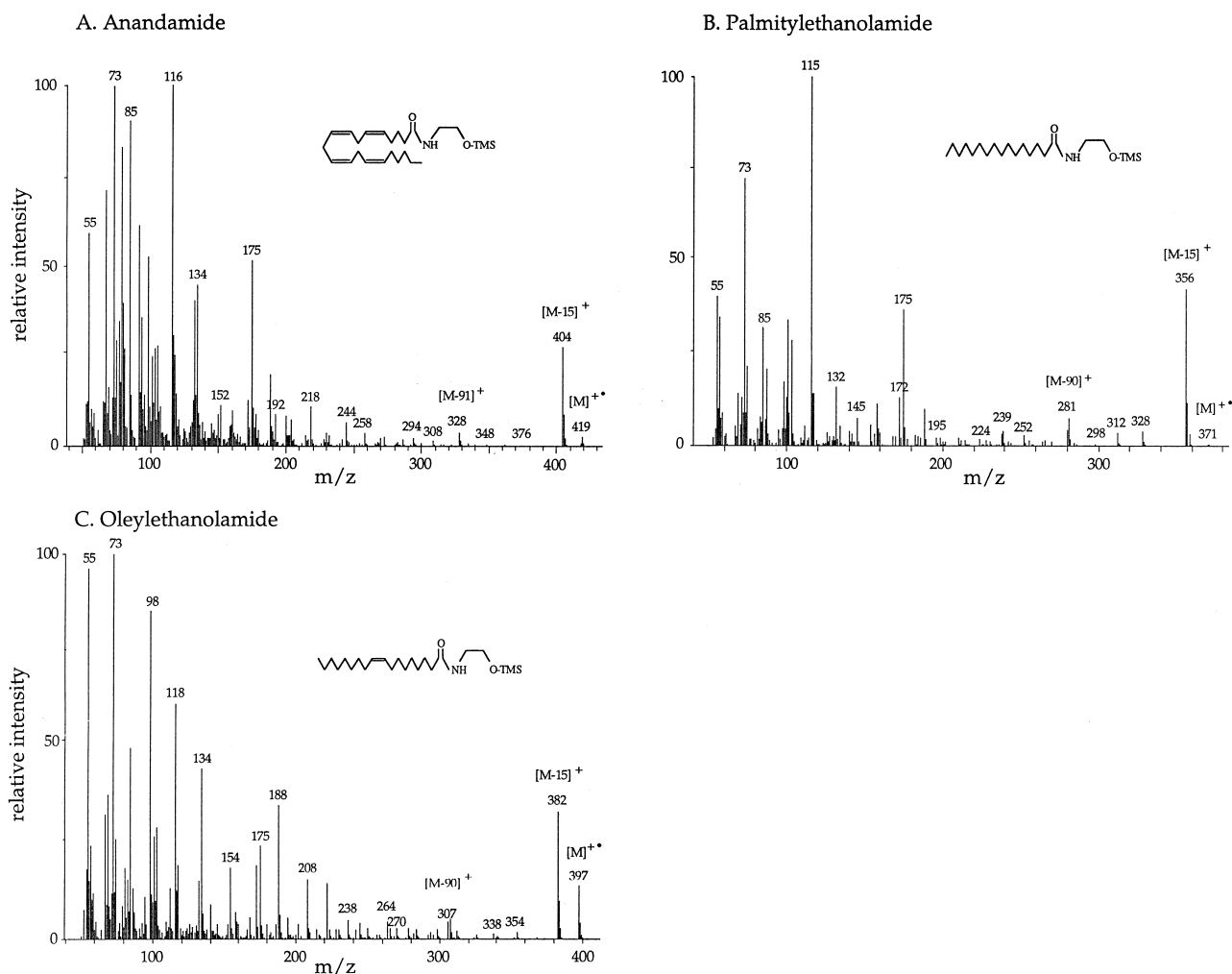

Fig. 1. Electron impact mass spectra of the TMS derivatives of anandamide (A), palmitylethanolamide (B), and oleylethanolamide (C).
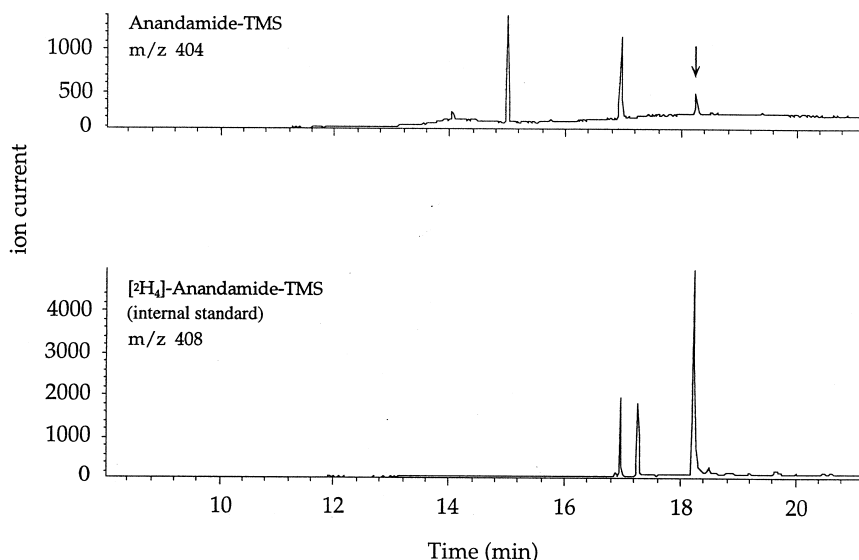
Fig. 2. Representative selected ion recording of a rat blood plasma sample showing the presence of components with the chromatographic properties of anandamide. Anandamide was partially purified by normal-phase HPLC and analyzed by GC/MS in the SIM mode as TMS derivative. The arrow indicates the retention time of authentic standard. Results are from one experiment, representative of six.

standard. The estimates obtained in these analyses are reported in Table 1. Percent coefficients of variation (CV) were calculated dividing the standard deviation by the sample mean and multiplying the resulting value by 100.

3.4. Limit of detection (LOD) and limit of quantification (LOQ)

When monitoring the $[M-15]^+$ fragments of $^2H_4$-labeled AE standards by SIM, we obtained the following labeled/unlabeled ratios: 0.012 for anandamide, 0.0009 for palmitylethanolamide and 0.0087 for oleylethanolamide. These blank values were plotted in calibration curves as corresponding to 0 pmol. The LOD, i.e. the injected quantity which produced a signal corresponding to an average blank plus 3 standard deviations, was 0.4 pmol for anandamide, 0.1 pmol for palmitylethanolamide, and 0.1 pmol for oleylethanolamide.

The LOQ, i.e. the lowest quantity which could be measured with acceptable accuracy (arbitrarily set at a CV < 20%), was determined by injecting into the GC/MS varying amounts of AEs together with 600 pmol of the corresponding $^2H_4$-labeled standards. The lowest quantifiable mass was 2 pmol for anan-

damide (CV = 4%; $n = 5$), 1.25 pmol for oleylethanolamide (CV = 8.5%; $n = 3$), and 0.6 pmol for palmitylethanolamide (CV = 9.6%; $n = 3$).

3.5. Identification and quantification of AEs in rat blood plasma

Synthetic $[^2H_4]$AEs (600 pmol each) were added to plasma samples, proteins were precipitated with acetone, and lipids in the supernatant were extracted with chloroform/methanol. Preliminary tests indicated that the acetone precipitation step was necessary to assure the elimination of contaminating components present in plasma and to obtain a satisfactory chromatographic fractionation. The lipid extracts were subjected to normal-phase HPLC using a method previously published by our laboratory [21], but modified to require a shorter elution time (see Section 2). Under these conditions, all AEs were eluted together from the column at a retention time comprised between 4.7 and 5.3 min. Considering the time necessary to re-equilibrate the column, the fractionation of one sample was completed in approximately 20 min. Although time-consuming, this HPLC step was essential, as it signifi-

Table 1
Precision and accuracy of the method

Injected amount (pmol)	Measured amount (pmol ± S.D.)	Precision (CV, %)	Accuracy	
			Actual/nominal (%)	Actual−nominal (pmol)
Anandamide				
2.5	2.5 ± 0.1	4	100	0
10	9.8 ± 0.1	1	98	−0.2
100	100.8 ± 1.4	1.4	100.8	0.8
Palmitylethanolamide				
2.5	2.5 ± 0.1	4	100	0
10	10.2 ± 0.2	1	103	0.3
100	100.1 ± 3,0	3	100.1	0.1
Oleylethanolamide				
2.5	2.5 ± 0.1	4	100	0
10	10.3 ± 0.1	1	103	0.3
100	100.0 ± 1.6	1.6	100	0

cantly improved GC resolution and prolonged GC column life. The overall recovery of the extraction and fractionation procedures, measured in six independent experiments by using [^{3}H]anandamide as a tracer, was $68.0 \pm 2.5\%$.

In plasma samples from six rats, we observed components which were eluted from the GC at the retention times expected for anandamide, palmitylethanolamide and oleylethanolamide (Fig. 2 and data not shown). These components coeluted with the corresponding ^{2}H$_4$-labeled AE standards, and displayed the following diagnostic fragments: anandamide, m/z 419, [M]$^+$ and m/z 404, [M-15]$^+$; palmitylethanolamide, m/z 356, [M-15]$^+$; oleylethanolamide, m/z 382, [M-15]$^+$ (Fig. 2 and data not shown). These results indicate that deproteinated rat plasma contains detectable levels of the three endogenous AEs. A quantitative estimate of AE plasma concentrations, obtained in 12 independent determinations from six rats, revealed 5.2 ± 1 pmol/ml of anandamide, 16.7 ± 2.7 pmol/ml of palmitylethanolamide and 8.1 ± 1.2 pmol/ml of oleylethanolamide.

4. Conclusions

Quantitative measurements of anandamide and other AEs in biological samples are hindered by the low concentrations of these compounds in tissues and by their high adsorption to glass and plastic surfaces. The isotope dilution GC/MS method described in the present study should help overcome such difficulties. This method, in combination with other recently published ones [22–24], may be applied in future studies aimed at determining the changes in AE content occurring in blood plasma, cerebrospinal fluid or brain microdialysis perfusates under various physiological and pathological conditions, or during drug administrations. Here, we have used it to determine AE levels in rat blood plasma. Our results show that low nanomolar concentrations of AEs may be readily measured in plasma samples which had been deproteinated by acetone treatment. This result is at variance with a previous study in which anandamide could not be detected in human plasma by using a HPLC/MS/MS method [11]. Possible explanations for this discrepancy include differences in sample preparation (e.g. deproteination), extraction or fractionation. The concentration of anandamide in plasma reported here is well below those considered sufficient to activate cannabinoid receptors of either the CB1 or CB2 subtype [1,25], particularly when taking into account that anandamide may be extensively bound to serum proteins. Therefore, our results do not support a role of anandamide as a circulating hormone. However, in light of the low concentrations of anandamide in brain [4,5,11] and of its short half-life in vivo (5–15 min) [26], the results do suggest that peripheral tissues represent a significant source of circulating anandamide. In keeping with this possibility, anandamide formation was recently described in stimulated macrophages [24], and was suggested to participate in the life-threatening hypotension that accompanies hemorrhagic shock [27].

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