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Quantification of Bioactive Acylethanolamides in Rat Plasma by Electrospray Mass Spectrometry

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We developed a high-performance liquid chromatography/mass spectrometry (HPLC/MS) method for the identification and quantification of anandamide, an endogenous cannabinoid substance, and other fatty acid ethanolamides (AEs) in biological samples. Using a mobile-phase system of methanol/water and gradient elution, we achieved satisfactory resolution of all major AEs, including anandamide, palmitylethanolamide (PEA), and oleylethanolamide (OEA). Electrospray-generated quasi-molecular species were used as diagnostic ions and detected by selected ion monitoring (SIM). Synthetic deuterium-labeled AEs were used as internal standards, and quantification was carried out by isotope dilution. A linear correlation ($r^2 = 0.99$) was observed in the calibration curves for standard AEs over the range 0–0.5 nmol. Detection limits between 0.1 and 0.3 pmol per sample and quantification limits between 0.5 and 1.2 pmol per sample were obtained. The method was applied to the quantification of anandamide, PEA, and OEA in plasma prepared from rat blood collected either by cardiac puncture or by decapitation. After cardiac puncture, AE levels were in the low-nanomolar range: anandamide, 3.1 ± 0.6 pmol/ml; PEA, 9.4 ± 1.6 pmol/ml; OEA, 9.2 ± 1.8 pmol/ml (mean \pm SE, $n = 9$). By contrast, after decapitation AEs were dramatically elevated (anandamide, 144 ± 13 pmol/ml; PEA, 255 ± 55 pmol/ml; OEA, 175 ± 48 pmol/ml). Thus, disruptive procedures of blood collection may result in gross overestimates in the concentrations of circulating AEs. © 2000 Academic Press

Key Words: anandamide; fatty acid ethanolamides; cannabinoid receptors; high-performance liquid chromatography; mass spectrometry.

Arachidonylethanolamide (anandamide), the first cannabinoid receptor ligand isolated from mammalian brain (1), is released from neurons during neural activity (2, 3) and is rapidly eliminated by a two-step mechanism consisting of transport into cells (4, 5) followed by enzymatic hydrolysis (6, 7). Anandamide mimics many pharmacological and behavioral effects of cannabinergic agents, including analgesia and inhibition of locomotor activity (8, 9), and may act physiologically to modulate motor signals generated within the basal ganglia (3). Besides the central nervous system, anandamide biosynthesis has been demonstrated in peripheral tissues (10–12), where roles for this compound in the regulation of vascular tone, intestinal motility, pain initiation, and immune responses have been proposed (see, for review, 13).

Saturated and monounsaturated acylethanolamides (AEs³), such as palmitylethanolamide (PEA) and oleylethanolamide (OEA), are produced together with anandamide when brain neurons in culture are stimulated with membrane-depolarizing agents (2, 14, 15). Although these compounds do not activate either of the two cannabinoid receptors identified thus far (CB1 and CB2), several studies suggest that they may participate in cellular signaling. For example, PEA exerts anti-inflammatory effects (16–19) and modulates pain initiation (20, 21), possibly by activating an as yet uncharacterized CB2-like receptor (20). In addition, preliminary studies suggest that the concentrations of anandamide and PEA (but not OEA) in the cerebrospinal fluid of schizophrenic patients may be higher than those of normal subjects, pointing to a possible dysregulation of these compounds in neuropsychiatric disorders (22).

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³ Abbreviations used: AE, acylethanolamide; PEA, palmitylethanolamide; OEA, oleylethanolamide; FIA, flow injection analysis; SIM, selected ion monitoring; TIC, total ion current.

Because of the potential involvement of anandamide and other AEs in the regulation of different physiopathological processes, it is important to develop methods for the accurate identification and quantification of these molecules. Gas chromatography/mass spectrometry (GC/MS) has been generally used for this purpose (23–25). This technique requires, however, partial purification and chemical derivatization of the AEs, resulting in variable recovery and time-consuming sample preparation. High-performance liquid chromatography/mass spectrometry (HPLC/MS) does not suffer from such limitations and may represent, therefore, a valuable alternative to GC/MS (10, 26). To explore this possibility, we developed an isotope dilution HPLC/MS method for the determination and quantification of anandamide and other AEs and evaluated it by measuring the concentration of these bioactive lipids in rat blood plasma.

MATERIALS AND METHODS

Chemicals. Fatty acyl chlorides (5,8,11,14-eicosatetraenoyl chloride, hexadecanoyl chloride, and 9-(*cis*)octadecenoyl chloride) were from Nu-Check Prep (Elysian, MN); [$^2\text{H}_4$]ethanolamine (isotopic atom enrichment 98%) was from Cambridge Isotope Laboratories (Andover, MA). All solvents were from Burdick and Jackson (Muskegon, MI) and all other chemicals from Sigma (St. Louis, MO).

Synthesis of unlabeled and [$^2\text{H}_4$]-labeled standards. Standard unlabeled and [$^2\text{H}_4$]-labeled AEs were synthesized by the reaction of the corresponding fatty acyl chlorides with unlabeled or [$^2\text{H}_4$]-labeled ethanolamine, respectively (27). Briefly, fatty acyl chlorides were dissolved in dichloromethane (10 mg/ml) and allowed to react with 1 equiv of unlabeled or [$^2\text{H}_4$]-labeled ethanolamine for 15 min at 0–4°C. The reaction was stopped by adding purified water. After vigorous stirring and phase separation, the upper aqueous phase was discarded and the organic phase was washed twice with water to remove unreacted ethanolamine. This reaction results in the quantitative formation of AEs (1, 27), which were concentrated to dryness under a stream of N_2 and reconstituted in chloroform at a concentration of 20 mM. AE solutions were stored at –20°C until used. Identity and chemical purity (>98%) of the synthesized AEs were determined by thin-layer chromatography (TLC) and HPLC/MS.

Collection and preparation of rat plasma. Blood was collected from male Wistar rats anesthetized with methoxyflurane. Two types of studies were performed. In the first, animals were decapitated and blood was collected into glass tubes containing Krebs–Tris buffer plus EDTA (4.5 mM) and aliquoted into 2-ml samples. In the second, 2 ml of blood was collected by cardiac puncture, using a syringe filled with 1 ml of Krebs–Tris

buffer/EDTA (4.5 mM). Samples were centrifuged in Accuspin tubes (Sigma) at 800g for 10 min at 22°C, and the plasma layers were recovered and “spiked” with 500 pmol of [$^2\text{H}_4$]-labeled AEs as internal standards. The standards were added after plasma preparation to avoid variable losses due to high-affinity transport of [$^2\text{H}_4$]anandamide into white blood cells (4, 28). Plasma proteins were precipitated by adding cold acetone (–20°C, 1 volume) and removed by centrifugation at 1000g for 10 min. Residual acetone in supernatants was evaporated under a stream of N_2 . The supernatants were then subjected to lipid extraction with methanol/chloroform (1:2, vol/vol). The chloroform phases were recovered, evaporated to dryness under N_2 , reconstituted in a mixture of chloroform/methanol (1:3, 80 μl), and transferred to 2.0-ml screw top vials with 0.1-ml conical glass inserts to be injected into the HPLC/MS.

HPLC/MS analysis. A HP 1100 Series HPLC/MS system equipped with a Hewlett Packard octadecylsilica (ODS) Hypersil column (100 \times 4.6 mm i.d., 5 μm) was used. Reversed-phase separations were carried out by using linear increases of methanol (B) in water (A) (25% A, 75% B for 2 min; 15% A, 85% B for 3 min; 5% A, 95% B for 20 min; 100% B for 5 min) at a flow rate of 0.5 ml/min. Column temperature was kept at 20°C. Under these conditions, AE standards eluted from the column with the following retention times: anandamide, 15.4 min; PEA, 17.3 min; OEA, 18.4 min. MS analyses were performed with an electrospray ion source set either in the positive or in the negative ionization mode. Capillary voltage (V_{cap}) was set at 3.5 kV, and fragmentor voltage was varied from 80 to 100 V. Nitrogen was used as drying gas at a flow rate of 12 liters/min. The drying gas temperature was set at 350°C and the nebulizer pressure at 50 psi. For quantitative analyses, diagnostic ions (protonated molecular ions $[\text{M} + \text{H}]^+$ and sodium adducts of the molecular ions $[\text{M} + \text{Na}]^+$) were detected in the selected ion monitoring (SIM) mode. Complete system control and data evaluation were done using an online system software (HP Chemstation).

Data analysis. Results are expressed as means \pm SE except in Table 1, where they are expressed as means \pm SD. Statistical significance was evaluated using the Student's *t* test.

RESULTS

Mass spectral properties of AEs and [$^2\text{H}_4$]AEs. The spectral characteristics of anandamide and other AEs were examined either in the negative- or in the positive-ion operation modes. Representative negative- and positive-ion electrospray mass spectra obtained with synthetic anandamide are shown in Fig. 1. The negative-ion spectrum is dominated by the deproto-

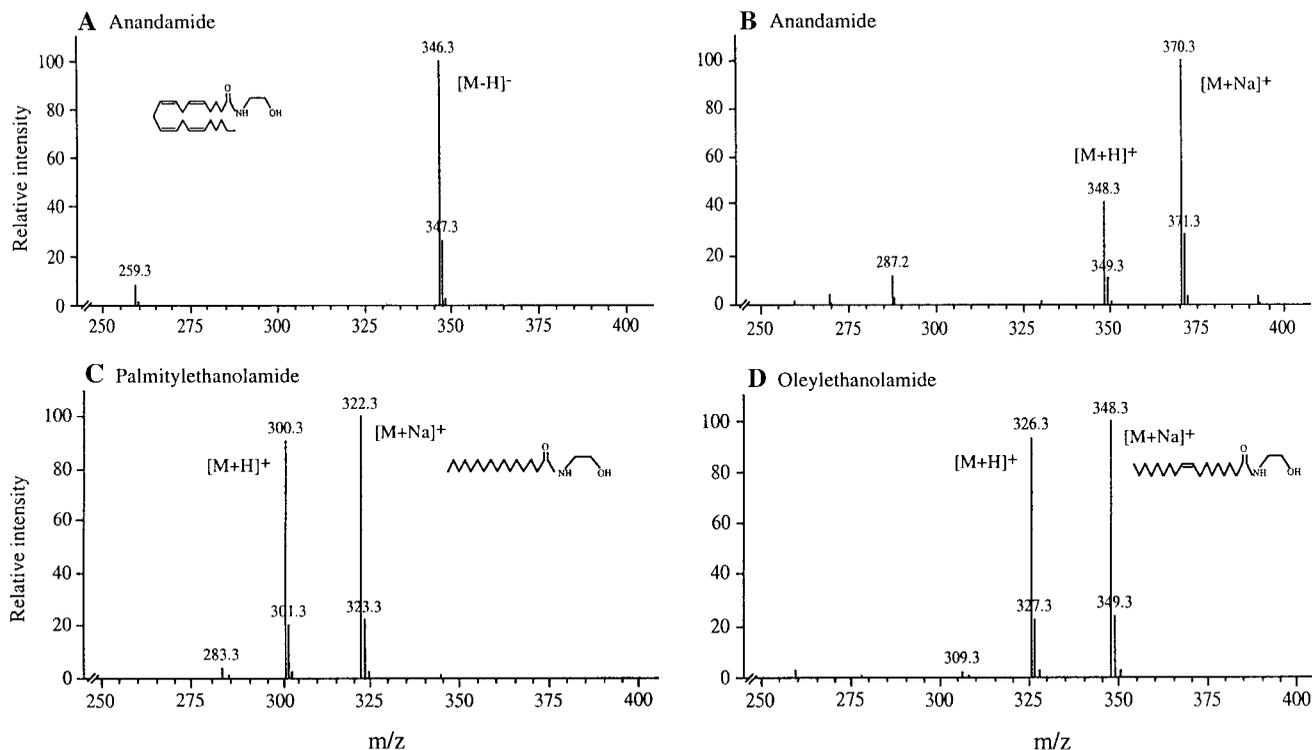


FIG. 1. Electrospray mass spectra of anandamide (A, negative ions; B, positive ions), palmitylethanolamide (C, positive ions), and oleylethanolamide (D, positive ions).

nated molecular ion (m/z 346.3, $[M - H]^-$) (Fig. 1A). The positive-ion spectrum consists instead of two species (Fig. 1B): the protonated molecular ion $[M + H]^+$, m/z 348.3 and the adduct of the molecular ion with Na^+ ($[M + Na]^+$, m/z 370.3), which constitutes the base peak. Both ions are accompanied by $A + 1$ ^{13}C isotope peaks of expected abundance (29): 25.8% of the ^{12}C $[M + H]^+$ peak (CV, 2.5%, $n = 5$), and 26.3% of the ^{12}C $[M + Na]^+$ peak (CV, 6.5%, $n = 5$). The calculated $A + 1$ ^{13}C isotope contribution for anandamide is 24.2% (29). Positive-ion spectra obtained with synthetic PEA (16:0) and OEA (18:1 Δ^9) displayed the following ions: PEA, m/z 300.3 ($[M + H]^+$) and 322.3 ($[M + Na]^+$) (Fig. 1C); OEA, m/z 326.3 ($[M + H]^+$) and 348.3 ($[M + Na]^+$) (Fig. 1D). In all cases, reproducible and expected ^{13}C patterns were observed for all diagnostic ions. Using flow injection analysis (FIA), we noticed that the total ion current (TIC) obtained in the negative ionization mode was 4 times lower than that obtained with positive ionization. Therefore, to increase the sensitivity of the assay, we selected the positive ionization mode for further analyses.

MS conditions. To optimize the MS conditions, the effects of varying the fragmentor voltage and capillary voltage (V_{cap}) on analyte abundance were examined by FIA. We injected a fixed amount of standard anandamide (100 pmol) using 75% methanol in water as mobile

phase at a flow rate of 0.5 ml/min. The areas of the $[M + Na]^+$ and $[M + H]^+$ peaks were monitored by SIM, setting the V_{cap} at 4000 V and varying the fragmentor voltage from 60 to 200 V. As shown in Fig. 2A, the intensity of the $[M + H]^+$ signal decreased as the fragmentor voltage was increased, whereas the $[M + Na]^+$ reached its maximum intensity at 100 V and declined thereafter. In a second experiment, the V_{cap} was increased from 2500 to 5000 V while the fragmentor voltage was kept constant at 100 V. V_{cap} ramping had no significant effect on the $[M + H]^+$ signal, but it produced an increase in $[M + Na]^+$ ion starting at 3500 V (Fig. 2B). A parallel distribution of $[M + H]^+$ and $[M + Na]^+$ fragments was obtained with deuterated fatty acid ethanolamides (data not shown). No changes in intensity of either ion were produced by modifying the flow rate of drying gas. Our attempts to optimize either signal by adding 0.05% sodium acetate or 0.1% acetic acid to the HPLC mobile phase produced no detectable improvements (data not shown). Similar results were obtained with PEA and OEA (data not shown).

HPLC conditions. AEs were fractionated by reversed-phase HPLC by using an ODS Hypersil column interfaced with the MS. HPLC conditions were optimized for resolution and elution time. A linear gradient of methanol (B) in water (A) (see Materials and Meth-

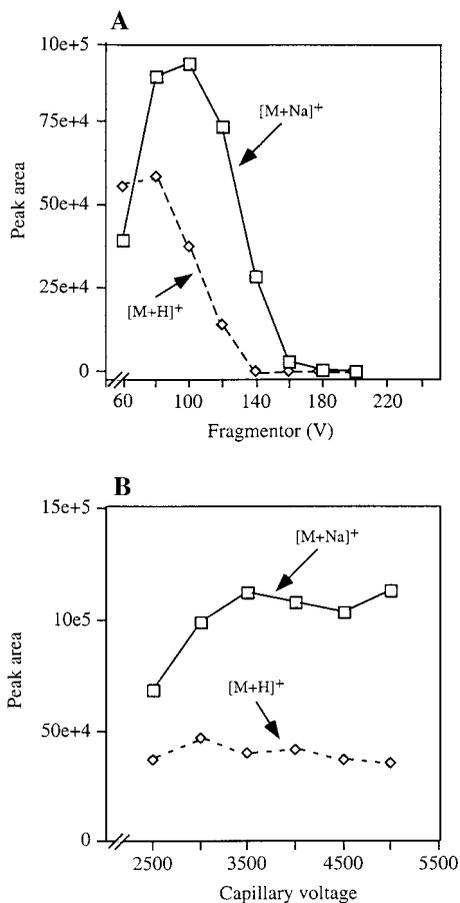


FIG. 2. Effects of varying fragmentor (A) and capillary voltage (B) on the production of anandamide ions $[M + H]^+$ and $[M + Na]^+$.

ods) and a flow rate of 0.5 ml/min allowed satisfactory separations of all AEs tested. Under these conditions, anandamide, PEA, and OEA eluted from the column at 15.4, 17.3, and 18.4 min, respectively. These analyses were carried out at a column temperature of 20°C; increasing temperature above this level resulted in decreased analyte resolution (data not shown). Figure 3 depicts the TIC and SIM recordings for a representative run with synthetic standards.

Isotope dilution assay. Standard calibration curves were constructed by adding a constant amount of deuterium-containing standards (0.5 nmol) to increasing amounts of the corresponding unlabeled AEs, followed by analysis of the $[M + Na]^+$ or $[M + H]^+$ ions in the SIM mode. SIM peaks were integrated, and the ratios of the area of unlabeled and $[^2H_4]$ -labeled ions were plotted against the amount of spiked AEs. We observed the following unlabeled/labeled ratios in deuterium-containing AEs: 0.011 for anandamide and 0.010 for PEA and OEA. These values were plotted as corresponding to 0 pmol. For all compounds, the MS responses were linear over the range 0–500 pmol, and

the correlation coefficient values (r^2) of the regression lines were greater than 0.99 ($n = 5$).

Accuracy and precision. We assessed the accuracy and precision of the method by measuring the recovery of known amounts of AEs in the presence of 0.5 nmol of deuterium-labeled standards. Table 1 summarizes the estimates obtained in five independent determinations. Accuracy was expressed as the ratio and/or difference between the actual and nominal values observed. Precision was expressed as percent coefficient of variation (CV), by dividing the standard deviation by the sample mean and multiplying the resulting value by 100.

Limits of detection and quantification. The limit of detection, that is, the lowest amount that produces a peak height of three times the signal-to-noise ratio, was 0.3 pmol for anandamide, 0.1 pmol for PEA, and 0.2 pmol for OEA. The limit of detection obtained by isotope dilution GC/MS was 0.4 pmol for anandamide and 0.1 pmol for PEA and OEA (25). The limit of quantification (LOQ), defined as the lowest quantity

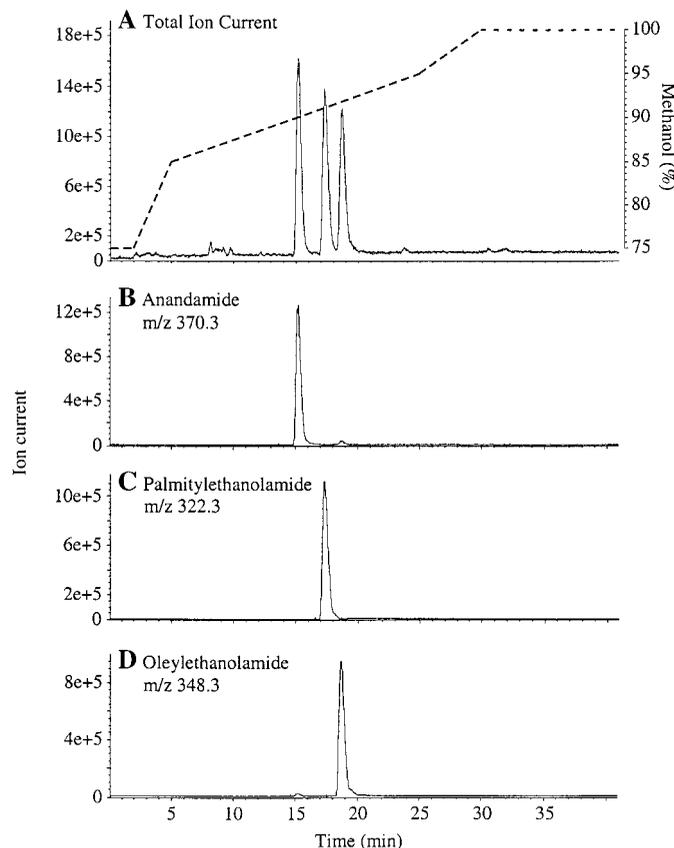


FIG. 3. Total ion current (A) and selective ion recordings of standard anandamide (B), palmitylethanolamide (C), and oleylethanolamide (D). Fatty acid ethanolamides were separated by reversed-phase HPLC using a linear gradient of 75–100% methanol in water (dashed line).

TABLE 1
Accuracy and Precision of the Method

Injected amount (pmol)	Measured amount (mean \pm SD)	Accuracy		Precision (CV, %)
		Actual/nominal (%)	Actual - nominal (pmol)	
Anandamide				
2.5	2.7 \pm 0.1	108	0.2	3.7
5	5.1 \pm 0.3	102	0.1	5.9
25	25.7 \pm 2.2	102.8	0.7	8.6
Palmitylethanolamide				
2.5	2.3 \pm 0.2	92	-0.2	8.7
5	5.1 \pm 0.5	102	0.1	9.8
25	25 \pm 2.2	100	0	8.8
Oleylethanolamide				
2.5	2.4 \pm 0.3	96	-0.1	12.5
5	5.3 \pm 0.4	106	0.3	7.5
25	25.1 \pm 0.7	100.4	0.1	2.8

that can be measured with acceptable accuracy (arbitrarily set at CV < 20%), was determined by injecting into the HPLC/MS varying amounts of AEs in the presence of 0.5 nmol of deuterium-containing standards. The resulting LOQ values were 1.25 pmol for anandamide (CV = 15.8%, $n = 4$), 0.5 pmol for PEA (CV = 17%, $n = 4$), and 0.5 pmol for OEA (CV = 14%, $n = 4$). The GC/MS LOQ was 2 pmol for anandamide, 0.6 pmol for PEA, and 1.25 pmol for OEA (25).

Identification and quantification of AEs in rat blood plasma. To limit the possible influence of circadian fluctuations on AE levels, all blood samples were collected between 1400 and 1500 hours. Plasma samples, obtained by either decapitation or cardiac puncture, were spiked with synthetic [$^2\text{H}_4$]-labeled AEs and immediately subjected to acetone precipitation of protein. After sample centrifugation, the supernatants were subjected to chloroform/methanol extraction and were analyzed by HPLC/MS. Identification and quantification of anandamide, PEA, and OEA were carried out by monitoring the following diagnostic ions: m/z 370.3 and 374.3 for anandamide and [$^2\text{H}_4$]-anandamide; m/z 322.3 and 326.3 for PEA and [$^2\text{H}_4$]-PEA; and m/z 348.3 and 352.3 for OEA and [$^2\text{H}_4$]-OEA, respectively. In agreement with an earlier report (25), all three AEs were found in rat plasma (Fig. 4).

As shown in Fig. 5, when blood was collected by cardiac puncture, we measured 3.1 ± 0.6 pmol/ml of anandamide, 9.4 ± 1.6 pmol/ml of PEA, and 9.2 ± 1.8 pmol/ml of OEA ($n = 9$). Such low concentrations are well below those needed to activate CB1 cannabinoid receptors (1) and are in good agreement with previous GC/MS determinations (25) (Fig. 5). By contrast, when blood was collected by decapitation, the levels of anandamide and other AEs were dramatically elevated, reaching high nanomolar concentrations at which anandamide is expected to be biologically active (Fig. 5).

DISCUSSION

We have developed an HPLC/MS method for the quantitative analysis of anandamide and other AEs that may overcome several limitations of available GC/MS methodologies (23, 24, 27). The elimination of chemical derivatization (necessary in GC/MS but not in HPLC/MS) reduces the time required for sample preparation and obviates contamination by reaction side products. Moreover, the mild ionization conditions of electrospray ionization yield simple mass spectra dominated by diagnostic ions of high mass ($[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$), providing considerable sensitivity without a substantial loss of selectivity. Additional structural information may be readily obtained by comparing

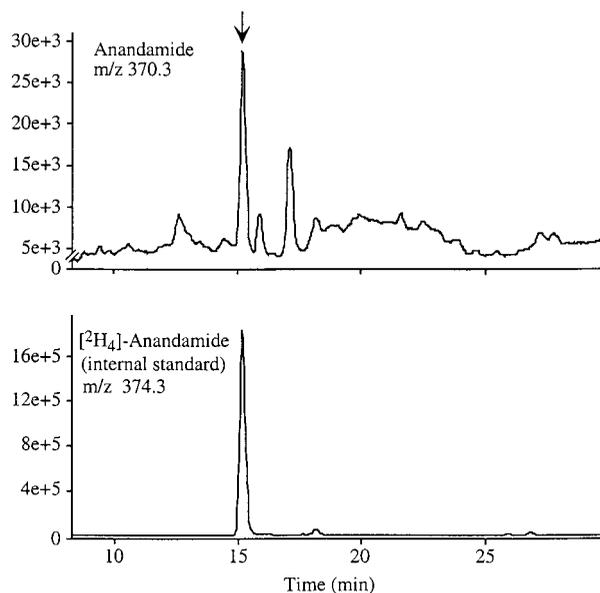


FIG. 4. Representative selected ion chromatogram of a rat blood plasma sample, showing a component that elutes with the retention time of anandamide (arrow).

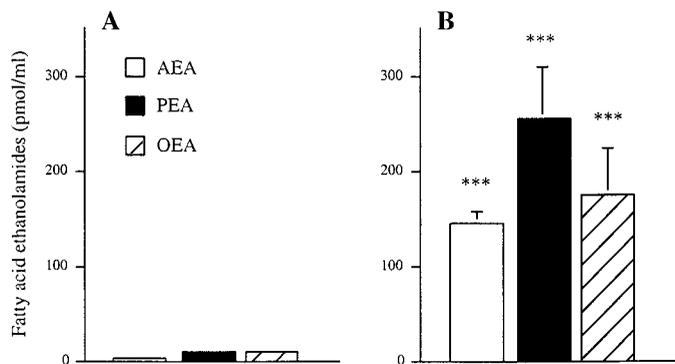


FIG. 5. Fatty acid ethanolamide concentrations in rat blood plasma collected by cardiac puncture (A) or by decapitation (B). Results are expressed as the mean \pm SE of 6–8 independent experiments. *** $P < 0.001$ compared to cardiac puncture, Student's t test.

spectra obtained in the positive and negative ionization modes (Fig. 1) or by increasing appropriately the fragmentor voltage. Operating at fragmentor voltages higher than 120 V results, indeed, in an overall loss of signal intensity but also in a greater molecular fragmentation. For example, in the case of anandamide, informative fragments produced in the ES negative ionization mode operating at a fragmentor voltage of 150 V included m/z 346.3 ($[M - H]^-$), 329.2 ($[M - H_2O]^-$), 316.3 ($[M - 31]^-$, possible loss of CH_2OH), 301.2 ($[M - 45]^-$, possible loss of CH_2CH_2OH), and 259.3 ($[M - 87]^-$, possible loss of $CONH(CH_2)_2OH$).

The present method compares positively with that of Koga *et al.*, who used HPLC coupled to atmospheric pressure chemical ionization to determine anandamide levels in various rat tissues, by comparison with an external standard (26). In particular, the isotope dilution procedure developed here should greatly facilitate AE analysis and improve its reliability. In addition, with our instrumentation, atmospheric pressure chemical ionization produced greater fragmentation and lower signal intensity than electrospray ionization, thus reducing assay sensitivity (data not shown).

Since its development, we have applied the present method to the analysis of anandamide and related AEs in a variety of biological fluids, including rat blood plasma (present study), human seminal plasma (A. Giuffrida, D. Piomelli, and H. Schuel, in preparation), human cerebrospinal fluid, and human blood serum (A. Giuffrida *et al.*, in preparation). The results obtained in our analyses of rat plasma are noteworthy for two reasons. First, they support the conclusion, suggested by earlier work (25), that precipitation of soluble proteins is necessary for the accurate measurement of anandamide and allied AEs in biological fluids. Thus, a substantial underestimate of plasma AE levels may be obtained by HPLC/MS if deproteination is not carried out before lipid extraction (10). Second, the present results demonstrate that AE concentrations in plasma

may be affected by the method used for blood collection. In particular, we found that disruptive procedures such as decapitation yield dramatically higher AE levels in plasma than relatively unintrusive procedures such as cardiac puncture. The reasons for these differences are currently under study, but possible explanations include tissue damage and vascular hypotension, which were proposed to elicit AE generation from precursor *N*-acyl phosphatidylethanolamines (23, 30).

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