Analytical Biochemistry **270,** 159–166 (1999)
Article ID abio.1999.4083, available online at http://www.idealibrary.com on IDEAL®

A Gas Chromatographic–Mass Spectral Assay for the Quantitative Determination of Oleamide in Biological Fluids

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Received January 22, 1999

Oleamide is a putative endogenous sleep-inducing lipid which potently enhances currents mediated by $GABA_A$ and serotonin receptors. While a quantitative assay would aid in determining the role of oleamide in physiological processes, most of the available assays are lacking in sensitivity. We now describe a quantitative assay for measuring low nanogram amounts of oleamide in biological fluids using GC/MS in the selective ion-monitoring mode. The internal standard ($^{13}C_{18}$ oleamide) was added to known concentrations of oleamide, which were converted to the *N*-trimethylsilyl or *N*-*tert*-butyldimethylsilyl derivatives before analysis by GC/MS, yielding linear calibration curves over the range of 1–25 ng of oleamide when monitoring the *m*/*z* 338/356 fragments. Using this technique, oleamide levels were determined following solvent extraction of normal rat cerebrospinal fluid and plasma to be 44 and 9.9 ng/ml, respectively. This technique constitutes a sensitive and reliable method for determining low nanogram quantities of oleamide in biological fluids.

Numerous biological functions are regulated by free fatty acids and their derivatives. For example, neurotransmitter- and voltage-gated ion-channel currents are modulated by relatively high (μM) concentrations of long-chain polyunsaturated fatty acids (1, 2). While these functional changes may result from lipid interactions with the membrane microenvironment in which these channels are located, they may also result from their activation of protein kinases (3) which can phosphorylate regulatory sites on the channels (4).

A previously unrecognized brain lipid referred to as "cerebrodiene" (5) in recognition of its site of action and the presence of two unsaturation equivalents may, along with anandamide (6), constitute a new family of unsaturated fatty acid amides which can modulate central nervous system function. This material was isolated from the cerebrospinal fluid (CSF)[2] of sleep-deprived cats (5), where it was reportedly present in very low concentrations (0.1–5 pmol/100 μl = 28 pg–1.4 ng/100 μl). Subsequent structural analysis based on 300 μg of material that, remarkably, was isolated from these same animals identified the compound as oleamide (*cis*-9-octadecenamide, OA) (7). The synthetic material reportedly induces a sleep-like behavioral state when administered intraperitoneally in doses of 5–50 mg or intracerebroventricularly at a dose of 10 nmol (2.8 μg) (7). The discrepancies between the concentrations reported *in vivo* and the amount isolated were said to be due, in part, to severe losses due to surface adsorption (8), but questions remain regarding the potency and specificity of OA as a sleep-inducing agent. These concerns are magnified by the extensive use of fatty acid amides such as OA and erucamide in the plastics industry as slip agents in the production of polyolefin films (9), where they enhance surface migration and facilitate the release of overlying layers during production and use (10). Because of their lubricating and surfactant properties, these compounds dominate the market for refined fatty acid amides in the plastic industry. Thus, they might be common environmental contaminants (11).

While techniques exist for quantifying OA at microgram levels in industrial samples (12–15) and plasma (16), these are not sufficiently sensitive to determine

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[2] Abbreviations used: CSF, cerebrospinal fluid; OA, *cis*-9-octadecenamide; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide; TMS, *N*-trimethylsilyl; BDMS, *N*-*tert*-butyldimethylsilyl.

the levels of OA in CSF and other body fluids of experimental animals in investigations of its physiological and pathophysiological roles. Thus, establishing a procedure that would not just detect, but reliably and quantitatively determine OA concentrations in biological matrices under various conditions is essential. We now describe a modification that allows these techniques (16) to be used for isolating and quantifying low nanogram quantities of OA from biological sources, while reducing the possibility of environmental contamination.

MATERIALS AND METHODS

Chemicals

Oleic acid, oleic acid-$^{13}C_{18}$, and oleic-9,10-D_2 acid were purchased from Sigma Chemicals (St. Louis, MO) and Isotec Inc. (Miamisburg, OH). Oxalyl chloride and trifluoroacetic acid anhydride were obtained from Aldrich Chemicals (Milwaukee, WI). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were procured from Pierce Chemicals (Rockford, IL).

Synthesis of Oleamide and Its Derivatives

Oleamide, oleamide-$^{13}C_{18}$, and oleamide-9,10-D_2 were prepared from oleic acid, oleic acid-$^{13}C_{18}$, and oleic-9,10-D_2 acid, respectively, using a modification of previous techniques (17). Three equivalents of oxalyl chloride (1.062 mmol or 0.532 ml of a 2 M solution in methylene chloride) were added through a septum to 100 mg of oleic acid or one of its isotopomers (0.355 mmol 1 equiv) in 3.5 ml dry methylene chloride (dried over 5-Å molecular sieves) in a Reactivial (Pierce Chemicals) and maintained under nitrogen. The solution was stirred at room temperature with a magnetic stirrer and protected from light with aluminum foil for 4 h and then evaporated under a stream of nitrogen. The resulting acid chloride was dissolved in 0.7 ml methylene chloride, cooled to 0°C and ammonium hydroxide [20–22% (w/w), ca. 0.2 ml] was added dropwise under a nitrogen atmosphere. After stirring for 15 min, 5 ml methylene chloride was added and the mixture extracted with water until the pH of the washes was neutral. The methylene chloride layer was then evaporated and methanol added to dissolve the solid residue. The methanol was then slowly evaporated under a nitrogen stream until crystallization began. After overnight storage in a refrigerator, the crystals were filtered from the mother liquor and washed well with cold methanol/water (1:1) to give a 90–97% yield with a melting point of 73–74°C. Elemental analysis of the normal isotopomer was as follows: calculated C 76.80, H 12.53, N 4.97; found C 76.78, H 12.46, N 4.97.

Preparation of Oleamide Derivatives

In all cases, OA standards and samples potentially containing OA were handled with minimal exposure to plastics. For example, standard solutions were measured using gas-tight capillary syringes, and all materials were stored, reacted, and otherwise handled in glass vessels capped with Teflon-lined lids. Standard solutions of *N*-trimethylsilyl oleamide and *N*-t-butyldimethylsilyl oleamide were prepared by evaporating 10 μl of a 10 ng/μl solution of the desired OA isotopomer in 200-μl crimp-top glass injection vials and heating it with 10 μl of BSTFA or MTBSTFA, respectively, at 95°C for 5 min. From these vials, 1 μl of the resulting mixture was analyzed by GC/MS. Oleonitrile was prepared by combining the same quantities of OA with 10 μl of trifluoroacetic anhydride and heating for 30 min at 95°C. The solution was subsequently evaporated and redissolved in 10 μl of dry chloroform and 1 μl was injected.

Statistical analysis of precision and accuracy was performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Measurement of Oleamide in Rat Plasma and Cerebrospinal Fluid

Adult (250 g), male Sprague–Dawley rats (Taconic Farms, Germantown, NY) were maintained on a 12-h day/night cycle (lights on at 0700) with free access to food and water according to NIH–AAALAC guidelines. Samples of cerebrospinal fluid were obtained by anesthetizing the rat with 80 mg/kg of sodium pentobarbital and then mounting it in a stereotaxic instrument. The head was tilted downward at a 60° angle, and an incision was made through the skin overlying the foramen magnum. The biventer cervicis, longissimus capitis, and rectus capitis muscles were released from the base of the skull, exposing the fascia over the foramen magnum. A glass capillary tube (sharpened by a micropipet puller) was connected to a 1-ml syringe by methanol-washed Teflon tubing and inserted through the membrane into the foramen. Slight suction was applied and 150–200 μl of CSF obtained. The sample was transferred to a 200-μl crimp-top glass microvial and centrifuged at 1000*g* for 5 min. The supernatant was then transferred to a fresh microvial, capped, and frozen at −70°C until used. After the CSF tap, blood was obtained via the jugular vein using heparinized glass Pasteur pipets. The tubes were then spun at 1000*g* for 5 min, the plasma removed and stored at −70°C until used.

The OA content of each sample was determined by adding 25 ng of $^{13}C_{18}$-oleamide as an internal standard and then extracting the mixture three times against an equal volume of hexane:ethyl acetate (3:1) by vortexing five times for 3 s. The hexane:ethyl acetate layer was removed, the volume reduced by evaporation under

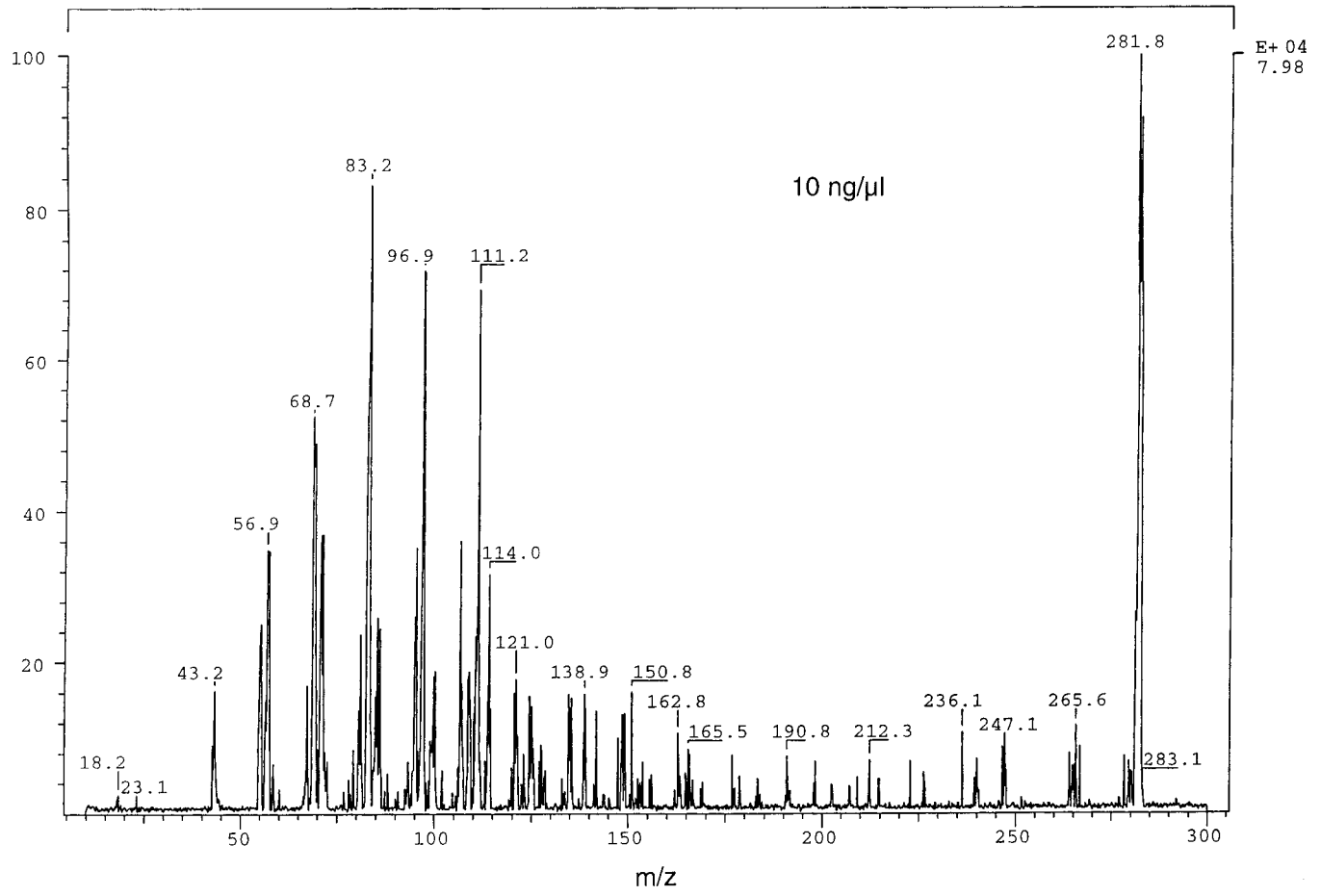

FIG. 1. MS/MS spectrum of $(M + H)^+$ from 10 ng of oleamide.

nitrogen, and the remainder placed into a 200-μl crimp-top tube and evaporated to dryness. To this residue was added 10 μl of BSTFA. The tube was sealed and heated to 95°C for 5 min, and then 1 μl of the resulting mixture was analyzed by GC/MS.

Instrumentation

A Hewlett–Packard 5973 series mass spectrometer in the electron-impact mode was used with a Vectra Chemstation and a 6890 series gas chromatograph fitted with a 30 m × 0.25-mm (i.d.) × 0.25-μm (nominal) 5% phenylmethylsiloxane (HP-5MS) capillary column. The instrument settings used were as follows: initial temperature, 100°C; initial time, 4.00 min; rate, 20.0°C/min; final temperature, 250°C; final time, 18.0 min; total time, 30.0 min; injection port, 250°C; detector transfer line, 280°C; carrier gas, He; flow rate, 23.9 ml/min.

Electrospray mass spectra were obtained using a Finnigan TSQ700 spectrometer equipped with a fluid junction electrospray system as described previously (18). OA standard solutions were prepared in concentrations ranging from 100 μg/μl to 10 pg/μl in 10% acetic acid/methanol. Fragmentation of the $(M + H)^+$ ion was performed with a collision voltage in Q2 of −29 V and an argon gas pressure of 0.8 torr.

RESULTS

The possibility of analyzing OA solutions without chromatography was briefly investigated using selected ion monitoring of the $(M + H)^+$ ion at *m/z* 282 obtained by electrospray mass spectrometry. However, considerable signal was present at this mass even in the blank. Better results were obtained by observing the MS/MS of this ion. Serial dilutions were made in 10% acetic acid/methanol and spectra with sufficient detail to give confidence in identifying this compound were obtained at concentrations as low as 10 ng/μl (Fig. 1). While this approach is still under consideration, the many compounds expected to be present in direct extracts of body fluids militated against its continued use. Subsequently, a gas chromatographic method was investigated.

While OA itself can be successfully chromatographed on silicone columns (9–13), tailing due to the NH groups hinders quantitation at the low concentrations

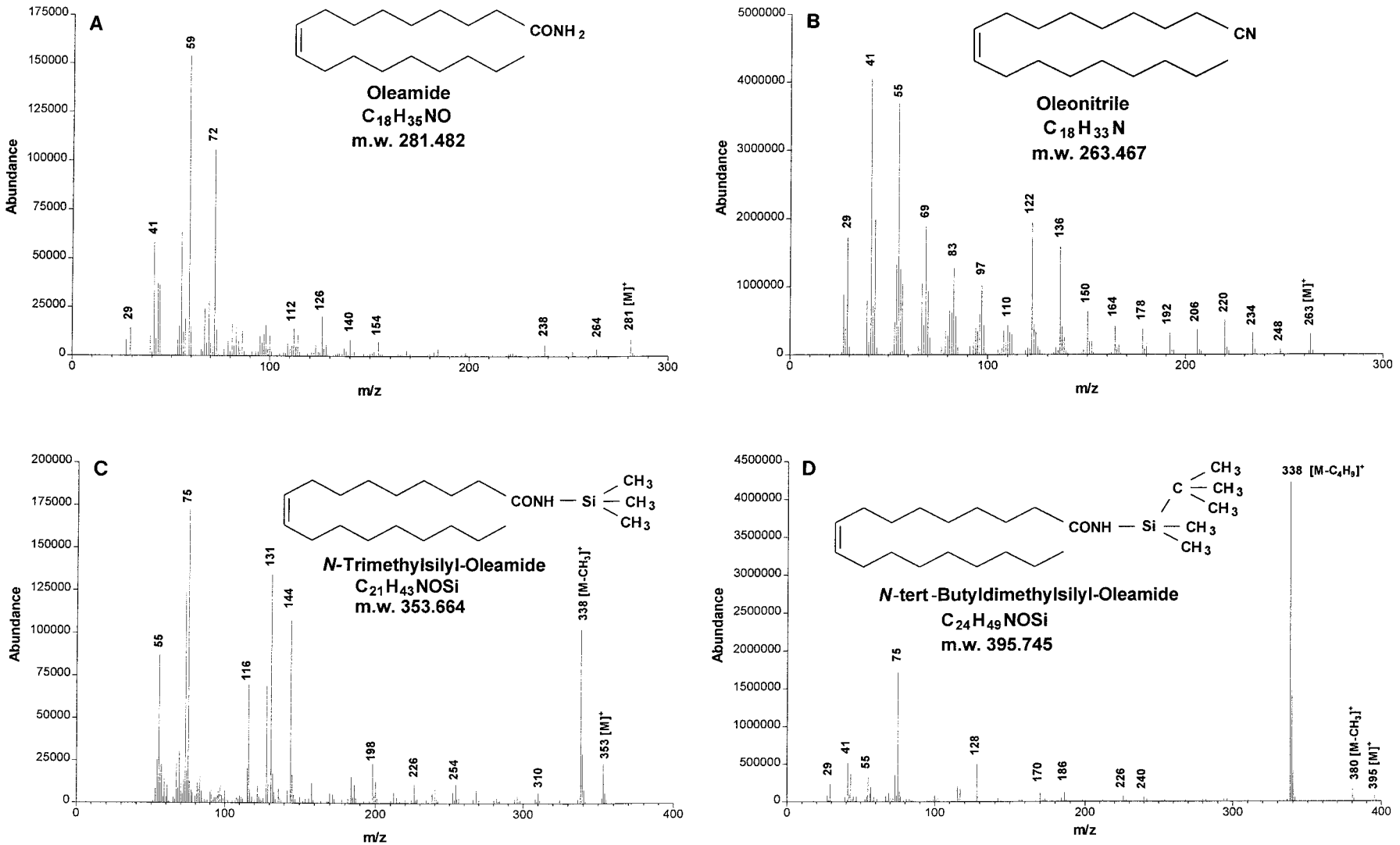

FIG. 2. Electron-impact mass spectra of oleamide and its derivatives. While low levels of the molecular ion of oleamide can be noted (*m/z* 281, A), the predominant fragment peaks are found at lower *m/z* (41, 59, and 62) where they may be substantially interfered with by the contents of biological samples. Derivatizing oleamide to the nitrile (B) improves chromatographic performance, but still yields low-molecular-weight fragments. However, converting oleamide to either the *N*-trimethylsilyl (C) or *N-tert*-butyldimethylsilyl (D) reliably yielded measurable amounts of a fragment with *m/z* 338.

TABLE 1

Retention Times for Oleamide and Its Derivatives

Compound	Retention time (min)
Oleonitrile (OA-CN)	11.7
Oleamide (OA)	13.0
N-Trimethylsilyl ester of oleamide (OA-TMS)	13.9
N-tert-Butyldimethylsilyl ester of oleamide (OA-BDMS)	16.6

Note. Oleamide (100 ng) was added to trifluoroacetic anhydride (OA-CN), BSTFA (OA-TMS), or MTBSTFA (OA-BDMS), and then heated at 95°C for 30 (OA-CN) or 5 min (OA-TMS, OA-BDMS). One microliter (≈10 ng OA derivative) of the resulting material was then injected neat onto the GC with an HP-5MS capillary column and run according to the program described under Materials and Methods.

expected in body fluids. To overcome this problem, OA (Fig. 2A) was converted to the corresponding nitrile (Fig. 2B) or either the *N*-trimethylsilyl (TMS, Fig. 2C) or *N-tert*-butyldimethylsilyl (BDMS, Fig. 2D) derivatives for quantitation by GC/MS. The retention times of OA, oleonitrile (OA-CN), *N*-trimethylsilyl oleamide (OA-TMS), and *N-tert*-butyldimethylsilyl oleamide (OA-BDMS) on an HP-5MS capillary column are listed in Table 1. These derivatives were then evaluated by averaging three independent determinations made by combining 10 ng of OA together with 25 ng of the corresponding $^{13}C_{18}$-oleamide and appropriate reagents. The fragment ions monitored (oleamide derivative/$^{13}C_{18}$-oleamide derivative) were, for OA-CN: 122/130 $(C_8H_{12}N)^+$, 136/145 $(C_9H_{14}N)^+$, and 220/235 $(M\text{-}C_3H_7)^+$; for OA-TMS: 338/356 $(M\text{-}CH_3)^+$ and 353/371 (M^+); and for OA-BDMS: 338/356 $(M\text{-}C_4H_9)^+$, 380/398 $(M\text{-}CH_3)^+$, and 395/413 (M^+) (Fig. 2, Table 2). Except for the 220/235 fragments, all of these combinations provided comparable quantitative precision (0.28–2.96%) and accuracy (99.5–104.4%). The limit of OA detection using the above derivatives was determined in full-scan and selected ion-monitoring modes using solutions of pure OA. Thus, 1 pg of OA converted to OA-CN can be detected in full-scan mode, and 100 fg of OA derivatized to OA-TMS (Fig. 3A) or to OA-TBDMS (Fig. 3B) could be detected in selected ion-monitoring mode using *m/z* 338. For purposes of quantifying OA as OA-CN (*m/z* 136), OA-TMS (*m/z* 338), or OA-TBDMS (*m/z* 338), calibration curves were developed plotting the ratio of the concentration of OA and $^{13}C_{18}$-OA versus the concentration of OA added (1 to 25 ng, Fig. 4). These calibrations were linear over the entire range of OA concentrations tested. The respective correlation coefficients (r^2) and the *x*-intercepts (x_i) for the lines fitted to the data from the derivatives are as follows: OA-CN (0.999, −0.31, Fig. 4A), OA-TMS (0.998, −0.08, Fig. 4B), and OA-BDMS (0.992, −1.34, Fig. 4C).

The derivatization techniques described above were then used to quantify OA in CSF and plasma taken from normal rats at approximately 10:00 AM. The $^{13}C_{18}$-OA internal standard (25 ng) was added to 1-ml samples of rat plasma that were subsequently extracted, derivatized, and quantified by GC/MS (Table 3). Conversion of OA to the nitrile gave the lower value of 12.6 ng/ml, while TMS derivatization yielded a quantity of 14.9 ng/ml OA. Varying the concentrations of the $^{13}C_{18}$-OA internal standard from 50 to 100 ng had little effect on the determination of OA (OA-BDMS using fragments *m/z* 338/356) in rat plasma, yielding levels of 13.0 and 12.2 ng OA/ml with 50 and 100 ng $^{13}C_{18}$-OA. OA (as OA-BDMS) levels in CSF and plasma from four rats were subsequently determined using 25 ng of $^{13}C_{18}$-OA as the internal standard. Plasma and CSF OA levels in normal rats, determined using the OA-TMS derivative, fragment 338/356, were found to

TABLE 2

Precision and Accuracy of the Method for Quantifying Oleamide

Derivative	Measured fragments	Injected amount (ng)	Measured amount (ng ± SD)	Precision (CV, %)	Accuracy	
					Actual/nominal (%)	Actual − nominal (ng)
OA-CN	122/130	10	10.44 ± 0.05	0.51	104.4	0.44
	136/145	10	10.30 ± 0.19	1.94	103.0	0.30
	220/235	10	11.12 ± 0.65	5.89	111.2	1.12
OA-TMS	338/356	10	10.20 ± 0.08	0.80	102.0	0.20
	353/371	10	10.19 ± 0.10	1.00	101.9	0.19
OA-BDMS	338/356	10	9.95 ± 0.03	0.38	99.5	−0.05
	380/398	10	10.13 ± 0.02	0.28	101.3	0.13
	395/413	10	10.16 ± 0.30	2.96	101.6	0.16

Note. Oleamide (100 ng) was added to trifluoroacetic anhydride (OA-CN), BSTFA (OA-TMS), or MTBSTFA (OA-BDMS) and then heated at 95°C for 30 (OA-CN) or 5 min (OA-TMS, OA-BDMS). One microliter (≈10 ng OA derivative) of the resulting material was then injected neat onto the GC with an HP-5MS capillary column and run according to the program described under Materials and Methods. The amount of oleamide in each sample was determined by measuring the indicated fragments by electron impact mass spectroscopy in selected ion-monitoring mode.

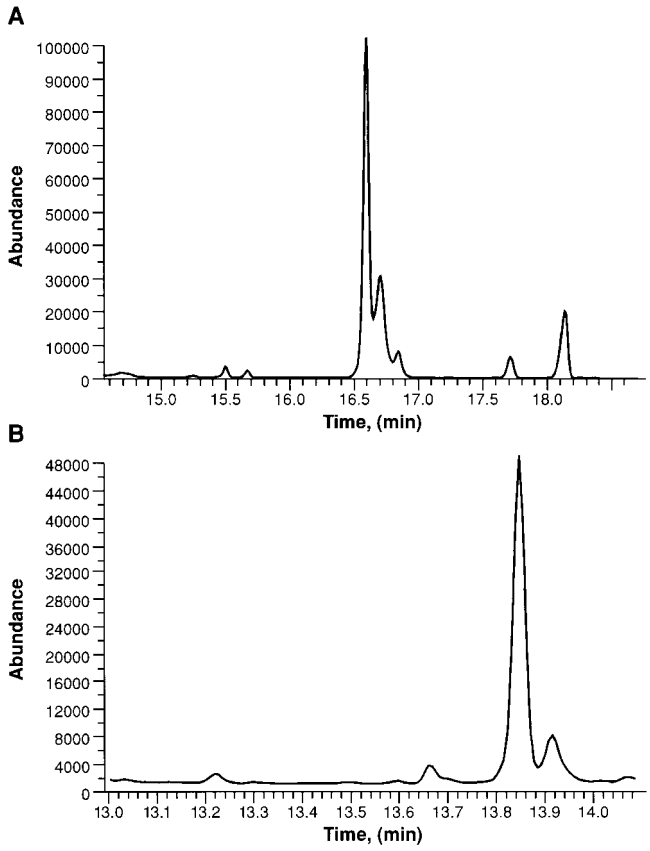

FIG. 3. Chromatograms of the silanized derivatives of oleamide. The *m/z* 338 fragments of 100 fg of *N-tert*-butyldimethylsilyl oleamide (A) and 100 fg of *N*-trimethylsilyl oleamide (B) were monitored in selected ion mode.

be 9.9 ± 1.0 ng/ml ($n = 31$) and 44 ± 3.0 ng/ml ($n = 27$), respectively.

Finally, because both oleic acid and ammonia may be found in plasma concentrations of 50–100 and 0.5–2.0 mM, respectively, in pathophysiological states such as liver failure (19, 20), we investigated the formation of OA from oleic acid and ammonia at the high temperature of the GC injector. To this end, 2.8 mg oleic acid was mixed with 39 μl ammonium hydroxide (30%), evaporated to dryness, and dissolved in 50 μl hexane: ethyl acetate (3:1) and 1 μl was injected directly on the column with the injector at 290°C. OA was formed under these conditions but with a yield of only 0.4%. When a similar mixture was first combined with either of the aforementioned silanizing reagents, OA was not detected.

DISCUSSION

Numerous unsaturated fats and their derivatives are biologically active messengers, capable of directly or indirectly modulating the function of neurotransmitter receptors and ion channels (1–4). OA is a representative member of a potentially new class of such biologically active lipids, the monounsaturated fatty acid amides, and may be a novel, sleep-inducing agent (5, 7, 8). However, OA is also widely used in the plastics

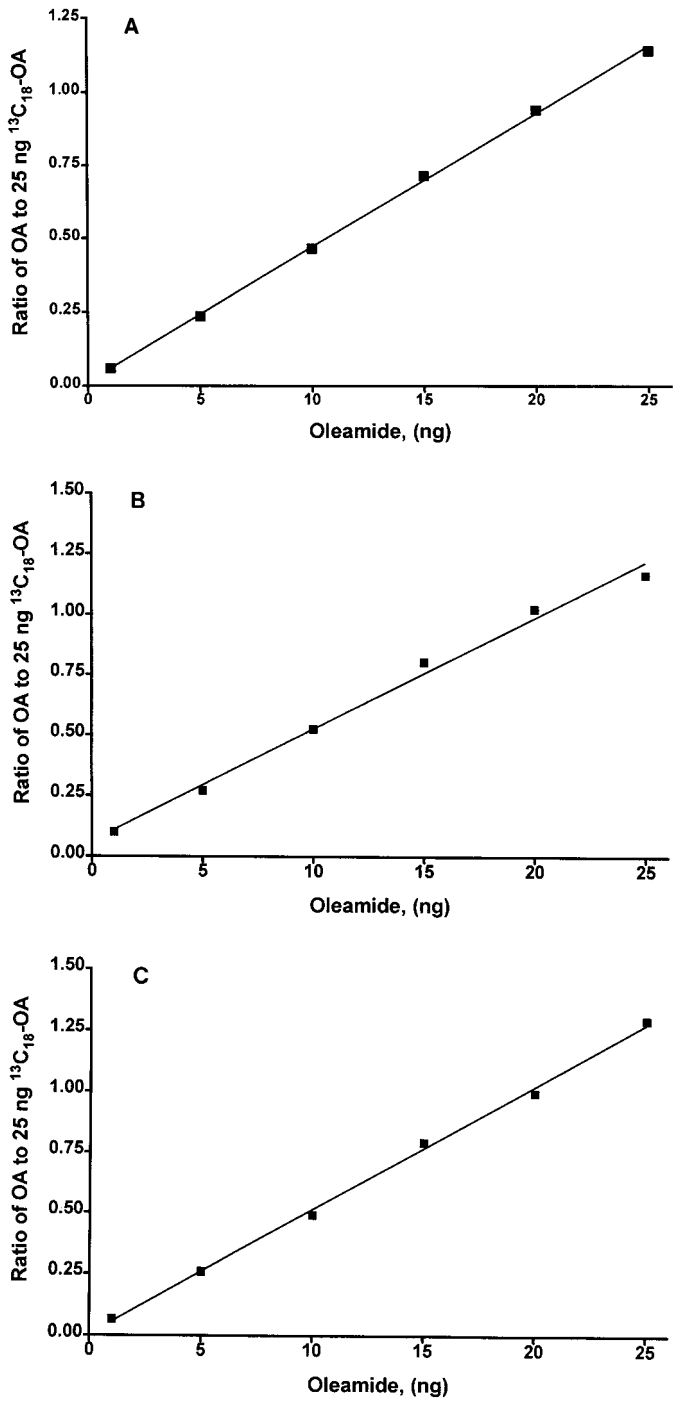

FIG. 4. Calibration curves for oleamide derivatives. Oleamide levels were determined by monitoring the 136/145 *m/z* fragment of oleonitrile (A) and the 338/356 *m/z* fragments of either *N*-trimethylsilyl-oleamide (B) or *N-tert*-butyldimethylsilyl oleamide (C). The ratio of the amount of oleamide to 25 ng of the internal standard, $^{13}C_{18}$-oleamide, was plotted against the known concentration of oleamide added, and a line was fitted through the points by linear regression.

TABLE 3

Comparability of Derivatization Procedures for Determining Oleamide Levels in Rat Plasma

Derivative	Fragment measured (*m*/*z*)	Result (ng/ml)
OA-CN	136/145	12.6
OA-TMS	338/356	14.9
OA-BDMS	338/356	13.0

Note. The $^{13}C_{18}$-OA internal standard (25 ng) was added to 1-ml samples of rat plasma, which was subsequently extracted, derivatized with trifluoroacetic anhydride (OA-CN), BSTFA (OA-TMS), or MTBSTFA (OA-BDMS), and then quantified by GC–MS using the techniques described under Materials and Methods.

industry as a lubricant and surfactant (9, 10) and may be a significant environmental contaminant. Despite this widespread industrial use, several lines of evidence suggest that OA may play physiologically relevant roles. OA can be synthesized endogenously, as indicated by studies using brain microsome preparations as synthetic enzyme sources (21), and by cultured neuroblastoma cell lines (22). A hydrolase responsible for the degradation of fatty acid amides, including anandamide and OA, has also been isolated from rodent and human brain and liver, cloned, and expressed (23, 24). Moreover, OA has been found to selectively and potently enhance the amplitude of currents mediated by $GABA_A$ and $5HT_{2A,C}$ receptors (25, 26), with an $EC_{50} \approx 500$ nM. This evidence strongly suggests that OA is a physiologically relevant messenger lipid. However, investigations into its potential biological function have been hampered by the lack of a method to accurately quantify this substance. While a number of methods exist for quantifying OA at the levels encountered in industrial situations (12–16), no previously described technique is sufficiently sensitive to quantify OA at the low levels expected to be encountered in biological specimens.

Our first attempts to develop a useful technique for quantifying OA in biological samples used electrospray mass spectrometry, which indicated that OA might be analyzable at nanogram levels without chromatographic separation. While we are still investigating the feasibility of this technique, chromatographic isolation of OA from the wide range of potential contaminants present in biological samples is straightforward, and a GC/MS method was developed based in part upon previously reported techniques (16). Initially, OA was converted by dehydration to nitrile (OA-CN) by heating with trifluoroacetic anhydride with better than 90% efficiency. While this derivative chromatographed well without tailing and was readily detected by EI/MS, its low molecular weight brought its mass into a region where other substances could interfere with its detection. Derivatizing OA with either of the silylating reagents, BSTFA or MTBSTFA, increased the analytical mass into the range of 338–395 Da. Moreover, each reagent provided three sets of analyte ions which could be reliably used for quantitation using the $^{13}C_{18}$ internal standard. Calibration curves were subsequently obtained that are linear well into the range of a few nanograms and have the potential for extension into the high femtogram range. By converting OA to the TMS derivative, plasma and CSF levels of OA were determined in normal rats and ranged from approximately 30 to 170 nM, respectively.

The concentrations of OA determined in rat body fluids have several ramifications. These nanomolar concentrations suggest that under normal conditions OA levels are below those expected to enhance the function of GABAergic and serotonergic systems (25, 26). However, in conditions such as sleep deprivation, CSF levels reach approximately 450 nM (27), which is sufficient to enhance the function of these neurotransmitter systems. In addition, the nanogram amounts of plasma and CSF OA measured in this study are two to three orders of magnitude lower than reported earlier. Concentrations of OA and other fatty acid amides were previously determined to range from 2 to 32 μg/ml in the plasma of normal and luteal-phase women (16). While these levels may result from biological processes, given the ubiquity of OA in plastic products it is possible that they reflect contamination occurring during sample procurement and preparation. Thus, care should be taken to minimize sample contact with plastic articles.

In summary, we have provided a simple and robust technique for absolute measurement of nanogram quantities of OA obtained from biological matrices. This technique involves derivatization of OA using silanizing agents and simple solvent extraction, followed by GC and electron-impact mass spectrometry. Absolute quantitation of OA using this method should provide substantial insights into its physiological and pathophysiological functions in mammalian systems.

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