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Liquid chromatographic–atmospheric pressure chemical ionization mass spectrometric determination of anandamide and its analogs in rat brain and peripheral tissues

Daisuke Koga, Tomofumi Santa, Takeshi Fukushima, Hiroshi Homma, Kazuhiro Imai*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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Abstract

A simple and selective method for the determination of anandamide (arachidonoylethanolamide), an endogenous cannabinoid receptor ligand, and its analogs with liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) was developed. The calibration curve for standard anandamide was linear over the range 625 fmol–125 pmol per injection (r=0.999) with a precision of 1.0% (C.V.) at 25 pmol. The detection limit attained was 200 fmol per injection at a signal-to-noise ratio of 2. Anandamide and its analogs were extracted from rat brain and peripheral tissues according to the method of Folch, and the recovery of anandamide from rat brain homogenates was 67.0–72.6%. The method was applied to their determination in rat brain and peripheral tissues.

Keywords: Anandamide; Arachidonoylethanolamide; Palmitoylethanolamide; Oleoylethanolamide; Cannabinoid receptor agonist, endogenous

1. Introduction

Anandamide (arachidonoylethanolamide) was identified as an endogenous cannabinoid receptor ligand from porcine brain by Devane et al. [1]. Anandamide produces many of the pharmacological effects – e.g. hypothermia, antinociception, anti-inflammatory – of cannabinoids such as Δ9-tetrahydrocannabinol (Δ9-THC) [2]. The inhibitory effects of anandamide on folskolin-stimulated cAMP production [3,4] and N-type calcium channel current [5] via cannabinoid receptors were also reported. A peripheral cannabinoid receptor (CB2), which is different from the brain receptor (CB1), has also been cloned [6]. In addition, Facci et al. reported that palmitoylethanolamide behaved as an endogenous agonist for the CB2 receptor on mast cells, instead of anandamide [7]. However, the regulation of the synthesis of anandamide and its role in vivo were hard to determine. For a more detailed study on the biosynthetic pathway of anandamide and its biological roles, it is necessary to establish a simple and sensitive method for its determination.

Gas chromatographic–mass spectrometry (GC–MS) is commonly used for lipid analysis. However, anandamide is non-volatile, and thus derivatization, such as trimethylsilylation, was needed, making sample preparation complicated. High-performance liquid chromatography (HPLC) is a popular method for the analysis of various kinds of compounds.

*Corresponding author.

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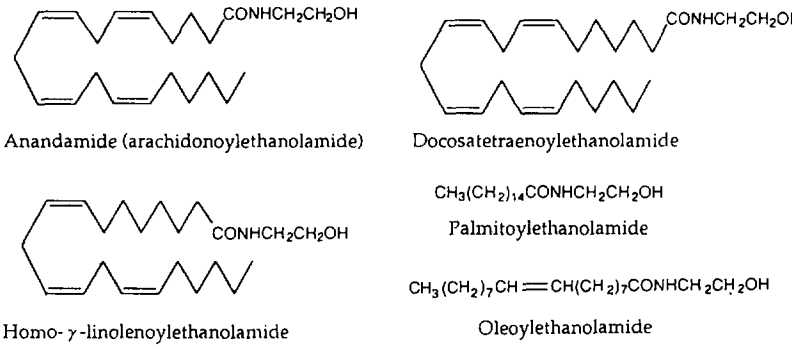

Fig. 1. Chemical structures of anandamide and its analogs.

Since anandamide does not fluoresce and has weak UV absorption, HPLC methods also required derivatization such as fluorometric derivatization with DBD-COCl, as we have previously reported [8]. However, the retention time and fixed wavelengths data obtained by HPLC were not sufficient for the identification and quantitation of anandamide in biological samples, which may contain many structurally related compounds.

Liquid chromatographic–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) [9] is able to ionize and detect anandamide directly. Furthermore, samples are introduced to the mass spectrometer after separation by HPLC, allowing positive identification of anandamide in conjunction with the retention time and molecular mass data. Thus, many samples can be prepared and analysed in a short period of time.

In this study we developed a novel sensitive method for the determination of anandamide by LC–APCI-MS. The method was applied to determine the concentrations of anandamide and its analogs (Fig. 1) in brain regions and peripheral tissues of rats several weeks old.

2. Experimental

2.1. Chemicals

Water was purified by a Milli Q reagent system (Millipore, Bedford, MA, USA). Methanol, hexane, isopropanol, acetonitrile, dichloromethane, benzene, diethyl ether, acetone, oxalyl chloride, chloroform and sodium chloride were purchased from Kanto Kagaku (Tokyo, Japan). Methanol, hexane, isopropanol, acetonitrile and dichloromethane were of HPLC grade. Ethanolamine, arachidonic acid, palmitic acid, γ-linolenic acid and oleic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Homo-γ-linolenic acid and docosatetraenoic acid were purchased from Sigma (St. Louis, MO, USA). Anandamide and its analogs, N-acylethanolamines, were synthesized by conversion of the respective acid into its acid chlorides, followed by addition of the acid chloride to excess ethanolamine [1,10].

2.2. Liquid chromatographic–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) systems

The apparatus used was a Hitachi L-6200 HPLC instrument equipped with an analytical column and connected to a Hitachi M-1200H mass spectrometer with an atmospheric pressure chemical ionization (APCI) system (Hitachi, Tokyo, Japan). The separations were carried out on TSKgel ODS 8OTM (150×4.6 mm I.D., 5 μm) (Tosoh, Tokyo, Japan) with a mobile phase of methanol–water (90:10, v/v) or TSKgel Silica-60 (250×4.6 mm I.D., 5 μm) (Tosoh, Tokyo, Japan) with a mobile phase of hexane–isopropanol–methanol (77.5:20:2.5, v/v) at a flow-rate of 1.0 ml/min. The temperatures of the nebulizer and the desolvation region were set at 200 and 400°C, respectively. The drift, focus and multiplier voltages were set at 20 V, 120 V and 1.8 kV, respectively. The needle for a corona discharge was maintained at 3.0 kV. Quasi-molecular ions of anan-

damide and its analogs were monitored for the detection.

2.3. Animal preparation and the extraction of anandamide and its analogs

Male rats (2, 6, 12 and 24 weeks old) of the Sprague–Dawley strain were purchased from Charles River Japan (Yokohama, Japan). Animals were anaesthetized by diethyl ether and dissected after the blood was removed from the abdominal aorta. The determination was performed using rat brains which were divided into four parts, cerebrum (1.03±0.07 g), cerebellum (0.28±0.02 g), hippocampus (0.09±0.03 g) and the remaining part (0.55±0.07 g), and other organs such as heart (1.26±0.14 g), spleen (0.65±0.07 g), thymus (0.55±0.15 g), kidney (2.76±0.13 g), testis (3.3±0.17 g) and liver (1.54±0.18 g) (12-week-old rat, $n=4$).

Anandamide and its analogs were extracted by homogenizing the tissue with 20 volumes of chloroform–methanol (2:1, v/v) in the glass homogenizer according to the method of Folch et al. [11]. The crude extract was mixed with 0.2 volume of 0.73% NaCl solution and separated into two phases. The upper phase was discarded and the lower phase was washed four times with an equal volume of methanol–0.53% NaCl solution–chloroform (48:47:3, v/v). The solvent of the lower phase was evaporated to dryness and dissolved in a small amount of chloroform. Ten volumes of acetone were added to the solution and the mixture was centrifuged at 1000 *g* for 5 min. The supernatant was evaporated to dryness and the residue was dissolved in 50 or 100 μl of benzene, and a 5-μl aliquot of the solution was subjected to LC–MS analysis.

2.4. Calibration and recovery

A calibration curve for peak area vs. six different concentrations of standard solutions of anandamide in benzene between 0.625 and 125 pmol was constructed. Least-squares regression was used for the calculation of the slope, intercept and the correlation coefficient. For the recovery study, rat whole brain homogenates were divided into two groups. Half of the homogenates were spiked with 25, 125 and 250 pmol of anandamide and processed as described above. The peak areas corresponding to spiked anandamide were calculated as the difference of the peak area between anandamide spiked and non-treated homogenates. The recovery was determined by comparing the peak area of spiked anandamide to the standard solution.

3. Results and discussion

3.1. APCI-MS conditions for anandamide and its analogs

The mass spectra obtained from anandamide and its analogs are shown in Fig. 2. All of them produced the protonated molecular ions as the base peak-ion at *m/z* 348 (anandamide), *m/z* 300 (palmitoylethanolamide), *m/z* 326 (oleoylethanolamide), *m/z* 350 (homo-γ-linolenoyl ethanolamide) and *m/z* 376 (docosatetraenoylethanolamide), so we selected these ions for selected ion monitoring (SIM). To optimize APCI-MS conditions, the effects of the drift voltage and the nebulizer temperature on anandamide ion production were examined in the flow injection analysis (FIA) mode using mobile phases of methanol–water (90:10, v/v) or hexane–isopropanol–methanol (77.5:20:2.5, v/v) at a flow-rate of 1.0 ml/min. At first, the drift voltage was varied over the range 10–80 V with a nebulizer temperature of 200°C (Fig. 3a). The intensity of $[M+H]^+$ decreased as the drift voltage increased, while the intensity of $[M+H-H_20]^+$ increased. When the drift voltage was set at 20 V, we observed the maximum intensity of $[M+H]^+$. We also optimized the nebulizer temperature with a drift voltage of 20 V (Fig. 3b). The temperature for which the $[M+H]^+$ intensity was at a maximum was around 200°C. Similar results were obtained for anandamide analogs.

Anandamide and its analogs were subjected to LC–APCI-MS using an octadecylsilica (ODS) column. Fig. 4 shows the mass chromatograms of standard anandamide and its analogs. Though some of them were not separated from each other, all of them could be determined independently by selecting their protonated molecule ions for detection. We also examined the separation of anandamide and its analogs using a silica column. No separation was achieved, but all of them could be quantified in-

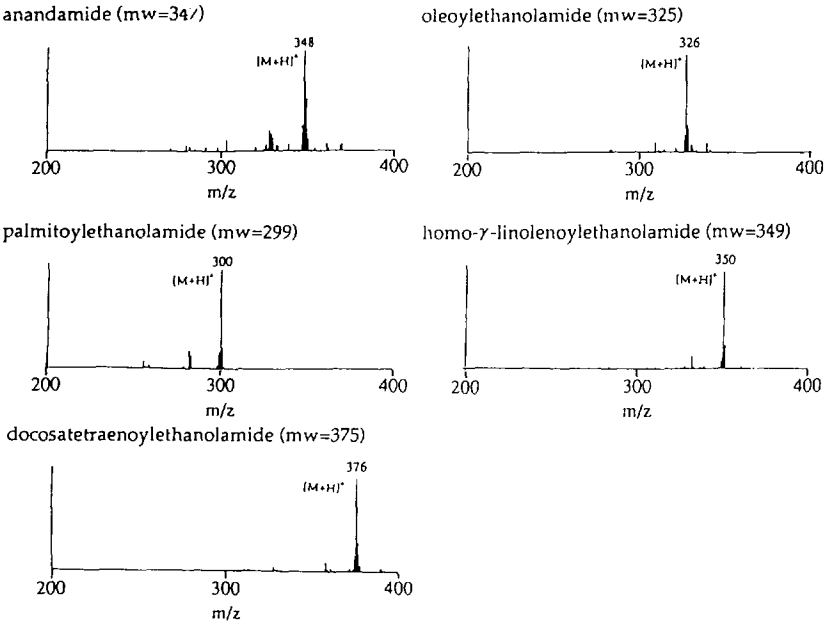

Fig. 2. Mass spectra of anandamide and its analogs. A 125-pmol amount of each compound was injected. Experimental conditions: mobile phase, methanol–water (90:10); flow-rate, 1.0 ml/min; nebulizer temperature, 200°C; drift voltage, 20 V.

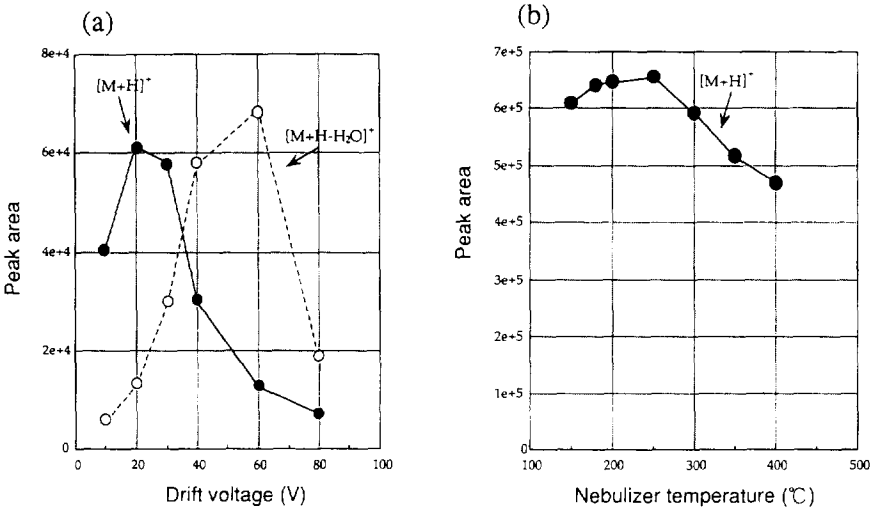

Fig. 3. Effect of drift voltage (a) and neblizer temperature (b) on ion production of anandamide. A 125-pmol amount of anandamide was injected.

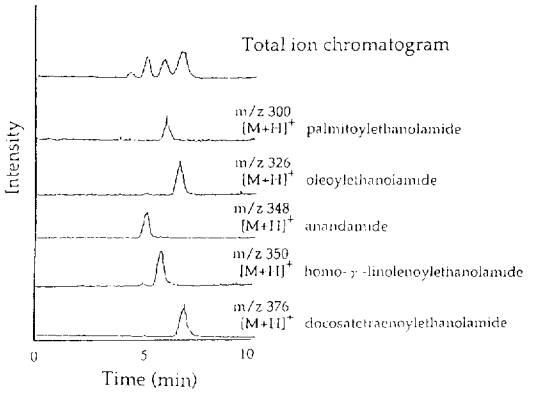

Fig. 4. Mass chromatogram of standard solution of anandamide and its analogs. A 125-pmol of each compound was injected. Experimental conditions: column, TSKgel ODS 80TM; mobile phase, methanol–water (90:10); flow-rate, 1.0 ml/min; nebulizer temperature, 200°C; drift voltage, 20 V.

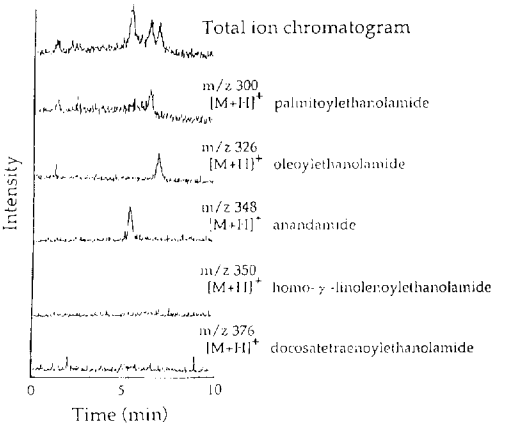

Fig. 5. Typical mass chromatogram obtained from rat kidney extract. Experimental conditions: column, TSKgel ODS 80TM; mobile phase, methanol–water (90:10); flow-rate, 1.0 ml/min; nebulizer temperature, 200°C; drift voltage, 20 V.

dependently by selecting their protonated molecule ions for detection.

3.2. Calibration and recovery

The calibration curve for anandamide was linear over the range 625 fmol–125 pmol per injection (r=0.999). The relative standard deviation values of the peak area for five replicate intra-day assays of anandamide at 625 fmol, 1.25 pmol, 25 pmol and 125 pmol were 8.6, 2.7, 1.0 and 0.8%, respectively. The detection limit attained was 200 fmol per injection at a signal-to-noise ratio of 2. Similar results were obtained for anandamide analogs. The recoveries from rat brain homogenates spiked with 25, 125 and 250 pmol anandamide were 68±12, 67±9.0 and 72.6±8.9% (n=3), respectively.

3.3. Anandamide and its analogs from rat tissues

With this method, we determined anandamide and its analogs such as palmitoylethanolamide, oleoylethanolamide, homo-γ-linolenoylethanolamide and docosatetraenoylethanolamide simultaneously. The ions monitored were m/z 348, 300, 326, 350 and 376, respectively. The determination was mainly performed with LC–APCI-MS in the SIM mode with the ODS column (Fig. 5), and LC–APCI-MS with the silica column was used for confirmation. The concentrations of anandamide, palmitoylethanolamide and oleoylethanolamide are shown in Tables 1–3. In this study, homo-γ-linolenoylethanoamide and docosatetraenoylethanolamide, which were identified as constituents binding to the cannabinoid receptor [12], were not detected in any tissue. Anandamide was present in all brain regions. This result does not contradict the reports about the localization of the cannabinoid receptor in the brain [13,14]. The difference in the concentrations of anandamide among various week-old rats was demonstrated. The concentrations of both palmitoylethanolamide and oleoylethanolamide changed similarly. These results suggested that palmitoylethanolamide biosynthesis was regulated simultaneously with oleoylethanolamide in physiological states. Schmid et al. [15] reported that N-acylethanolamines consisting of rather saturated fatty acids, were produced by metabolism of N-acylethanolamine phospholipids by a phosphodiesterase of the phospholipase D type. Their results were comparable to ours. Biphasic changes of the concentrations of both palmitoylethanolamide and oleoylethanolamide, observed only in hippocampus, may aid in the determination of a role for N-acylethanolamines in the hippocampus.

Table 1
Concentrations of anandamide in rat brain and peripheral regions (pmol/g wet tissue)

	2-week-old rats (n=3)	6-week-old rats (n=3)	12-week-old rats (n=4)	24-week-old rats (n=3)
Cerebrum	N.D.	73.4±26.7	77.0±21.8	11.8±8.1
Cerebellum	10.5±9.5	50.2±27.0	73.1±17.9	29.3±25.2
Hippocampus	N.D.	32.9±12.8	201±110	76.2±6.8
Others	19.8±8.3	48.4±2.9	73.4±24.0	35.2±5.1
Kidney	N.D.	63.7±3.2	164±16.6	34.9±15.6
Testis	N.D.	43.5±5.6	2.9±10.2	11.2±3.7
Liver	N.D.	19.7±5.5	77.1±86.0	30.5±10.2
Heart	N.D.	126±20.5	25.3±21.2	21.2±5.8
Thymus	N.D.	40.6±22.8	137±49.3	N.D.
Spleen	N.D.	2.1±3.6	325±50.1	76.7±0.9

All data were expressed as mean±S.D..

Table 2
Concentrations of palmitoylethanolamide in rat brain and peripheral regions (pmol/g wet tissue)

	2-week-old rats (n=3)	6-week-old rats (n=3)	12-week-old rats (n=4)	24-week-old rats (n=3)
Cerebrum	27.3±19.3	60.1±15.2	263±31.5	334±40.4
Cerebellum	119±45.4	541±133	454±16.5	581±40.0
Hippocampus	242±179	60.0±6.9	7.53±2.4	544±19.9
Others	103±5.5	521±133	604±152	929±171
Kidney	147±34.0	134±26.3	129±21.2	174±24.9
Testis	N.D.	36.5±3.4	92.6±42.6	78.6±32.4
Liver	98.7±3.0	90.6±12.7	68.8±26.2	78.9±6.8
Heart	135±37.5	233±3.0	114±29.8	56.1±4.3
Thymus	N.D.	139±39.1	132±50.5	106±20.2
Spleen	394±134	59.0±3.3	325±14.4	102±7.9

All data were expressed as mean±S.D..

4. Conclusion

We established a sensitive and selective method for determining anandamide and its analogs with HPLC–APCI-MS. It requires only simple pre-treatment of samples without any derivatization procedure and is therefore superior to GC–MS and fluorometric detection methods. This present method

Table 3
Concentrations of oleoylethanolamide in rat brain and peripheral regions (pmol/g wet tissue)

	2-week-old rats (n=3)	6-week-old rats (n=3)	12-week-old rats (n=4)	24-week-old rats (n=3)
Cerebrum	36.9±26.4	46.1±7.3	165±16.5	167±25.2
Cerebellum	110±73.4	485±85.9	321±47.1	281±52.2
Hippocampus	262±130	55.9±6.5	N.D.	262±55.8
Others	118±1.5	415±11.4	375±69.0	433±102
Kidney	85.0±113	147±10.8	164±26.2	179±26.8
Testis	N.D.	39.5±6.7	38.7±3.9	19.3±11.5
Liver	34.7±30.0	29.3±5.2	64.5±40.2	66.4±4.9
Heart	155±28.8	232±17.4	92.2±18.8	52.5±6.2
Thymus	N.D.	169±44.7	152±60.5	104±18.1
Spleen	129±22.3	59.4±7.9	245±24.4	107±2.5

All data were expressed as mean±S.D..

should provide a strong tool for determining the biosynthetic pathway of anandamide and its biological roles.

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