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Journal of Chromatography A, 1101 (2006) 278–285
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Sample preparation and gas chromatography of primary fatty acid amides

Tamanna Sultana, Mitchell E. Johnson *

Department of Chemistry and Biochemistry, Duquesne University, Pittsburgh, PA 15282, USA

Received 5 August 2005; received in revised form 7 October 2005; accepted 11 October 2005
Available online 2 November 2005

Abstract

A method for the isolation of bio-active primary fatty acid amides (PFAM's) from total lipid extract by solid-phase extraction (SPE) was developed and validated. The lowest mass of amide to be loaded and recovered by this method was detected as 0.5 μg using 500 mg of normal phase adsorbent. The isolated PFAM's were separated and quantified by GC/MS and percent recoveries were calculated. An HP-5MS column was able to provide base line separation between the saturated and unsaturated PFAM's whereas clear resolution between geometric and positional isomers having the same number of carbons was obtained using a BPX70 column. The separated amides were all 18 carbon analogs of *cis*-9-octadecenoamide (oleamide). Detection limits in the single ion monitoring mode were found to be on the order of 10 pg in a 1 μl injection. Solid phase extraction of amides from total lipid extract before GC/MS analysis provides clean detection and interference free analysis.

Keywords: Solid phase extraction; Sample preparation of primary fatty acid amides; $N_{18}TG_2$ cells; GC/MS; BPX70 column; Separation of unsaturated primary fatty acid amides

1. Introduction

Fatty acid amides comprise one of the important classes of bioactive lipids found in mammals. *N*-Acylethanolamines (NAEs) and primary fatty acid amides (PFAMs) are two important families of this class [1]. Members of both of these families consisting of long chain saturated and unsaturated acyl chains are considered to be bioregulators due to their hormone-like activities [2]. They became recognized as a biological signaling class of lipids when anandamide (*N*-arachidonylethanolamine) was first identified as an endogenous compound that binds to the CB1 cannabinoid receptor in the brain [3]. It was found to mimic all the activities of the active ingredient of marijuana and was thought to be a potent neuromodulator besides its other hormonal activities. Within 3 years researchers discovered oleamide, a primary fatty acid amide, to be accumulated in the cerebrospinal fluid of sleep deprived cats [4]. Since then oleamide has been studied widely in various biochemical and pharmacological ways. While the functions and metabolic pathways of NAEs are well established [5–9], very little is known about the biosynthesis and biodegradation pathways of PFAMs. They were first identified in human luteal phase plasma [10] even though neither their functions nor their bio-synthetic pathways were recognized at the time. Oleamide has also been found to modulate serotonin neurotransmissions [11], to increase affinity of γ-aminobutyric acid for its receptors [12], to inhibit lymphocyte proliferation [13], to prevent gap junction communication in glial cells [14,15], and to inhibit human synovial fluid phospholipase A_2 [16]. Of the other amides, erucamide may simulate angiogenesis [17] and regulate fluid imbalance [18]; arachidonamide inhibits human synovial phospholipase A_2 [16]; linoleamide increases Ca^{2+} flux [19] and inhibits *erg* current in pituitary cells [20]. Even though the actions of most of the other amides are yet not known, it is certain that fatty acid amides as a class have interesting biological activity in mammals.

In order to better understand the hormonal role of PFAM's as a class it is crucial that the PFAM's are isolated for further analysis. Unfortunately, most published analytical studies revolve almost exclusively around oleamide with occasional attendance to other PFAM's. It is evident from various studies that this lipid class might regulate biological activities in mammals in a fashion indirectly related to endocannabinoids. Sample prepa-

* Corresponding author. Fax: +1 412 396 5683.
E-mail address: johnsonm@duq.edu (M.E. Johnson).

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doi:10.1016/j.chroma.2005.10.027

ration of PFAM's from biological cells and tissues, such as by SPE, could allow the isolation of these lipids from the total lipid extract for interference-free instrumental detection. Isolation by SPE before analysis can provide preconcentration, higher sensitivity, and a lower limit of detection with excellent chromatographic separation. The use of aminopropyl bonded columns instead of silica columns is favored in lipid analysis because these are comparatively less polar than silica and therefore are less affected by moisture in their capacity for lipid adsorption [21]. Kaluzny et al. [22] were among the first to use the aminopropyl bonded columns for the separation of seven different neutral lipid classes by piggybacking multiple columns, but unfortunately their method was not found to be reproducible by this laboratory. The reproducibility of a given SPE method largely depends on the SPE phase, sorbent conditioning, amount of sample loaded, amount of each mobile phase used for elution as well as the flow rate of the mobile phase. Therefore, it is necessary to describe a developed SPE method in detail in order for others to be able to reproduce the method. Various modifications of Kaluzny method have been reported which were found to be better at isolating any particular lipid class [23] or simple lipids [24]. Very recently Madl and Mittelbach [25] reported solid phase extraction of PFAMs using a nonpolar solvent hexane with a nonpolar C18 packing [26]. The authors reported 93 and 95% recoveries from tallow and fatty acid methyl ester (biodiesel) matrices, respectively. However, primary fatty acid amides were not completely isolated from other lipid classes.

Even though most of the gas chromatographic separation analysis of PFAM's mentioned in the literature required prior derivatization, these amides can be analyzed by some nonpolar columns (such as HP-5MS) with high sensitivity without derivatization provided a clean sample is available [27]. Using this methodology, derivatization could be avoided, but unfortunately the separation is limited to the baseline resolution between the saturated and unsaturated PFAM's. The separation of derivatized PFAM's in terms of the number of carbon has been achieved using nonpolar columns [10,25,28–29]. In some cases, separation by the number and position of unsaturation was achieved [30,31] but separation in terms of geometrical and most positional isomers has yet to be accomplished. The use of a polar column for the separation of PFAM's has also been described, but only a few unsaturated amides were separated, probably due to the poor column performance [32]. Madl and Mittelbach reported the use of APCI/MS for the analysis of PFAMs without derivatization with detection limit of 18 fmol, but only a few amides were included in the study.

In this paper, a method for the isolation of PFAMs as a lipid class by SPE has been demonstrated and the validation of this method by lipid extracts from $N_{18}TG_2$ mouse neuroblastoma cells has been described in detail for ease of reproducibility. It is important to point out here that these cells have the necessary catalytic activity to produce PFAMs and are used as model cell line for PFAM biosynthesis. Separation of 15 different C12–C22 underivatized amides was obtained using an HP-5MS column. Employing a polar, bonded column, BPX70, it was possible to achieve baseline resolution between the derivatized 18 carbon analogs of oleamide.

2. Reagents and materials

Oleamide (OM), oleic acid (OA), tristearin (TS), monooleoylglycerol (MOG), dipalmitin (DP), phosphatidylcholine (PC), *N*-oleoylglycine (NOG), *N*-oleyolethanolamine (NOE), sphingomyelin (Sph) and bovine brain gangliosides (G) were purchased from Sigma (St. Louis, MO, USA). Cholesterol (Ch) was purchased from EM Science (Darmstadt, Germany) and cholesterol palmitate (ChP) from Janssen Chemical (Geel, Belgium). The solvents were purchased as follows: ACS reagent grade hexanes, methanol and glacial acetic acid from Fisher Scientific (Fair Lawn, NJ, USA); acetone from Acros Organics (Geel, Belgium); chloroform and diethyl ether from Aldrich (Milwaukee, WI, USA). All the primary fatty acid amides (except oleamide) were synthesized from the corresponding acids in our laboratory modifying a published protocol [33]. All the free fatty acids were purchased from Sigma unless otherwise mentioned.

The undifferentiated $N_{18}TG_2$ cells grown in oleic acid were used, because only this cell type was available in considerable amount to carry out this study. These cells were donated by David and Kathy Merkler of University of South Florida. The cell description is as follows: $N_{18}TG_2$ is a neuroblastoma cell line that was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The $N_{18}TG_2$ cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Cellgro, Herndon, VA, USA) supplemented with 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA), 10% fetal bovine serum (Atlanta Biological, Atlanta, GA, USA), and 100 mM 6-thioguanine (Sigma) at 37 °C and 5% CO_2 atmosphere. Undifferentiated cells that were at 60% confluency were treated with DMEM + 200 μM oleic acid for 2 days and the cells were detached from the tissue culture flask using a cell scraper. The cells were then centrifuged ($250 \times g$) and washed three times with Dulbecco's Phosphate Buffered Saline. The pellets were flash frozen in a dry ice/methanol bath and stored at −80 °C.

Discovery DSC-Si and Discovery DSC-NH_2 SPE phases were purchased from Supelco (Bellefonte, PA, USA). Irregularly shaped and acid washed base silica possessed following properties: 50 μm particle size, 70 Å pore diameter, 480 m^2/g specific surface area and 0.9 cm^3/g pore volume. DSC-Si is the unbonded silica phase, which is basically used as a normal phase adsorbent whereas DSC-NH_2 is the aminopropyl bonded silica which can be used as either normal phase or the ion-exchange adsorbent. Polypropylene SPE tubes (empty) of 3 ml volumes with polyethylene frits were also purchased from Supelco. Five hundred milligrams of the phases were packed into the tubes between two frits with hand pressure in order to carry out SPE. High performance thin layer chromatography plates (HPTLC) from Analtech (Newark, DE, USA) were used for viewing the lipid contents in different SPE fractions. These plates were of 10 cm × 10 cm, 200 μm film thickness, with organic binder and no fluorescence indicator incorporated. Primuline dye (CI 49,000; direct yellow 59) used for spraying the HPTLC plates was obtained from Aldrich (Milwaukee, WI, USA).