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Simultaneous quantitative analysis of *N*-acylethanolamides in clinical samples

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ABSTRACT

A simple and rapid analytical method is described for the simultaneous quantitative analysis of three different *N*-acylethanolamides in human biological samples: anandamide (AEA), oleoylethanolamide (OEA), and palmitoylethanolamide (PEA). The method is based on a new hybrid solid phase extraction–precipitation technology followed by ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) analysis using d_4-AEA as the internal standard. The method is linear up to 100 ng/ml with a limit of quantitation of 50 pg/ml for AEA and 100 pg/ml for OEA and PEA. Good reproducibility, accuracy, and precision were demonstrated during the method validation. Application of this new methodology to the analysis of clinical study samples is presented.

Fatty acid ethanolamides (or *N*-acylethanolamides) belong to a family of lipid signaling molecules widely distributed in plants and animal tissues [1,2]. They play important roles as physiological modulators of biological processes [3].

Within this family, anandamide (arachidonoylethanolamide [AEA])[1] has raised widespread interest since its isolation and characterization in 1992 [4]. Anandamide is an endogenous cannabinoid neurotransmitter that exerts most of its biological actions via activation of the cannabinoid receptor CB1. Although it also binds to CB2, the affinity is low and may act as an antagonist [5,6]. Several studies have also indicated the interaction between AEA and the capsaicin receptor TRPV1. Anandamide has been involved in regulation of physiological functions such as eating and sleeping patterns, energy metabolism, reproduction, cardiovascular modulation, and pain relief.

Other naturally occurring *N*-acylethanolamides are oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), both of which are structurally related to AEA but devoid of affinity for cannabinoid receptors. Oleoylethanolamide, formed from oleic acid and phosphatidylethanolamine, is known to possess anorexigenic properties in animal models [7]. OEA is synthesized in the small intestine of various vertebrates species, where its levels vary with food intake. Several biological functions have been attributed to OEA. In animals, OEA controls food intake through activation of the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) and stimulates the vagal nerve through TRPV1. OEA also promotes lipid use and modulates lipid storage in liver and circulating plasma lipids [8].

PEA is a saturated *N*-acylethanolamide containing the palmitoyl moiety. The role of PEA in inflammation and nociception via a variety of molecular mechanisms has been largely documented [9], and several studies have demonstrated the involvement of PPAR-α [10,11] and possibly GPR55 [12] in the anti-inflammatory activity of this molecule [13]. Recent investigations have demonstrated the capability of PEA to inhibit the secretion of tumor necrosis factor-α (TNF-α) in human adipose tissue, indicating a possible role in obesity-associated insulin resistance [14].

Both OEA and PEA also possess neuromodulatory properties as endogenous ligands of PPAR-α in the brain [15]. Anandamide and other endocannabinoids are produced on demand from membrane phospholipids by a series of intracellular enzymes and released from cells, followed by immediate action as signaling molecules. Following their release onto cannabinoid receptors, endocannabinoids are removed from the extracellular space by membrane transport and then degraded by intracellular enzymatic hydrolysis.

Fatty acid amide hydrolase (FAAH) is the major enzyme responsible for the catabolism of AEA and other noncannabinoid fatty acid amides, including PEA and OEA [16]. FAAH is an integral membrane enzyme that degrades *N*-acylethanolamides though a two-step

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[1] *Abbreviations used:* AEA, arachidonoylethanolamide (anandamide); OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PPAR-α, peroxisome proliferator-activated receptor-α; TNF-α, tumor necrosis factor-α; FAAH, fatty acid amide hydrolase; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; ESI, electrospray ionisation; SIM, selected ion monitoring; UPLC, ultra-performance liquid chromatography; SRM, selected reaction monitoring; CID, collision-induced dissociation; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; *S/N*, signal/noise.

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process consisting of transport into cells followed by intracellular hydrolysis to the corresponding fatty acid and ethanolamine. This critical role of FAAH as regulator of the endogenous levels of *N*-acylethanolamides makes this enzyme an attractive therapeutic target for the treatment of different human disorders. In particular, FAAH inhibitors constitute a promising new generation of therapeutics of potential value for the treatment of pathologies of the central nervous system and of peripheral tissues [17].

FAAH inhibitors produce anti-inflammatory and antihyperalgesic effects in a wide range of animal models. These beneficial effects caused by FAAH blockade are mediated predominantly through the activation of CB1 and CB2 receptors, although non-cannabinoid mechanisms of action can also play a fundamental role. The observation that FAAH inhibitors produce antinociception without eliciting general cannabimimetic effects, together with their lack of abuse potential, makes them good candidates for drug development purposes [18]. Monitoring the concentrations of *N*-acylethanolamides is important during the development of FAAH inhibitors for therapeutic applications.

Different analytical methods have been reported in the literature for the analysis of different *N*-acylethanolamides, especially anandamide. Initial methodologies were based on gas chromatography/mass spectrometry (GC/MS) [19] requiring laborious and time-consuming derivatization steps. Methods employing chromatographic separation followed by fluorescence detection have also been reported [20] with the same pitfalls, limiting the analysis to the quantification in tissue samples.

Later on, to gain enough sensitivity for the analysis of biological fluids, liquid chromatography (LC)/MS methods were developed using electrospray ionization (ESI) and quantification performed by isotope dilution [21] and in selected ion monitoring (SIM) mode. Also, more original methodologies, such as the use of silver cation coordination, have been reported by Schreiber and coworkers [22]. During the past couple of years, even more sensitive methods have become available using more sophisticated instrumentation and tandem MS [23,24].

All of these methods have the limitation of using laborious liquid–liquid extraction for biological sample preparation. Slight improvements were recently reported by Marczylo and coworkers [25] employing solid phase extraction for anandamide analysis and also by Palandra and coworkers [26] using a simplified 96-well protein precipitation technology.

The aim of the current work was to develop a simple and high-throughput bioanalytical method suitable for the routine simultaneous quantitative determination of AEA, OEA, and PEA in clinical samples and to validate the assay according to regular pharmaceutical standards [27].

Materials and methods

Materials and reagents

O-AEA (virodhamine) and d_4-AEA were purchased from Cayman Chemicals (Ann Arbor, MI, USA). *N*-AEA, OEA, and PEA, as well as albumin from bovine serum, were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands).

Solid phase extraction–precipitation cartridges (HybridSPE–Precipitation Cartridges, 30 mg/ml) were purchased from Supelco (Bellefonte, PA, USA). Methanol, *acetonitrile,* and *water* were of ultra-performance liquid chromatography (*UPLC)*/MS quality and were obtained from *Biosolve* (Valkenswaard, The Netherlands). Ammonium formate, formic acid, sodium chloride, chloroform, and 2-propanol were purchased from Merck (Darmstadt, Germany).

Plasma samples

Human plasma samples were obtained from different healthy human volunteers who were enrolled in a clinical trial focused on the study of the biological effects of FAAH inhibition. The study protocol was approved by the local ethics committee. All of the subjects gave written informed consent.

Subjects were men and women, ages 18–55 years, without any clinically significant history of any disease or disorder. Subjects did not have any history of significant psychiatric disorder or drug abuse (including the use of cannabis) or addiction.

The study was conducted in accordance with the principles of the Declaration of Helsinki and International Conference on Harmonization/World Health Organization Good Clinical Practice guidelines.

Blood (5 ml) was collected for plasma preparation in commercially available blood collection tubes. After collection, blood samples were gently and carefully mixed and immediately cooled on melting ice. All sample tubes were centrifuged within 30 min of collection for 10 min at 1500*g* at 4 °C. The plasma was harvested and stored in a freezer maintained between –60 and −80 °C until analysis.

High-performance liquid chromatography/mass spectrometry

Chromatographic separation was carried out in an Acquity UPLC system (Waters, Etten-Leur, The Netherlands) equipped with a reverse-phase C18 column (Acquity UPLC BEH, 2.1 mm i.d. × 50 mm length, 1.7 μm). Mobile phase A consisted of 10 mM ammonium formate in UPLC-grade water. Mobile phase B was UPLC-grade acetonitrile. The total run time was 4 min. The gradient steps were as follows: 0–2.5 min, linear gradient from 50 to 85% solvent B; 2.5–2.6 min, linear gradient from 85 to 97% solvent B; 2.6–3.0 min, isocratic at 97% solvent B; 3.0–3.1 min, linear gradient from 97 to 50% solvent B; 3.1–4.0 min, isocratic at 50% solvent B. The flow rate employed was 700 μl/min. The column temperature was kept constant at 50 °C, and the samples were maintained at 5 °C in the autosampler.

The analytes were quantified with a 4000 Q-Trap triple quadrupole linear ion trap mass spectrometer equipped with a TurboIonSpray interface (Applied Biosystems, Foster City, CA, USA) operated in the positive ion mode. The instrument was tuned individually for each compound to give the highest sensitivity. The most important optimized selected reaction monitoring (SRM) parameters are summarized in Table 1.

Under full scan analysis, the mass spectrum was dominated by the protonated molecular ion $[M + H]^+$ for all of the

Table 1
Mass spectrometric parameters used for the quantitative analysis of the different *N*-acylethanolamides.

N-acylethanolamide	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (ms)	Declustering potential (eV)	Collision energy (eV)
Virodhamine	1.58	348.2	287.2	50	80	−17
AEA	1.71	343.2	287.2	50	80	−17
PEA	1.95	300.3	62.3	50	80	−33
OEA	2.12	326.3	62.3	50	80	−33