Journal of Chromatography B, 878 (2010) 1687–1699

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Validation and application of an LC–MS/MS method for quantitation of three fatty acid ethanolamides as biomarkers for fatty acid hydrolase inhibition in human plasma

Wenying Jian[a,*], Richard Edom[a], Naidong Weng[a], Peter Zannikos[b], Zhiming Zhang[c], Hao Wang[d]

[a] Bioanalysis/DMPK, Johnson & Johnson Pharmaceutical Research and Development, United States
[b] Clinical Pharmacology, Johnson & Johnson Pharmaceutical Research and Development, United States
[c] Analgesics Team, Johnson & Johnson Pharmaceutical Research and Development, United States
[d] Clinical Neuroscience and Pain, Johnson & Johnson Pharmaceutical Research and Development, United States

ARTICLE INFO

*Article history:*
Received 26 February 2010
Accepted 15 April 2010
Available online 22 April 2010

*Keywords:*
Biomarker
Quantitation
LC–MS/MS
Ethanolamides
Fatty acid amide hydrolase

ABSTRACT

Endogenous ethanolamides (fatty acid amides), including arachidonyl ethanolamide (anandamide, AEA), oleoyl ethanolamide (OEA), and palmitoyl ethanolamide (PEA), are substrates of fatty acid amide hydrolase (FAAH). FAAH may play an important role for pain, anxiety/depression, and metabolic disorders. Ethanolamides are considered to be potential pharmacodynamic biomarkers to determine target engagement for FAAH inhibition by novel pharmaceutical agents. A highly selective, sensitive, and high-throughput liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed and validated for simultaneous quantitation of AEA, OEA, and PEA in human plasma. The method employed $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA as "surrogate analytes" to establish the concentration–mass response relationship, i.e. a regression equation. The concentrations of AEA, OEA, and PEA were calculated based on the regression equations derived from the surrogate analytes. This approach made it possible to prepare calibration standard and quality control (QC) samples in plasma devoid of interferences from the endogenous analytes. The analytical methodology required 150 μL of human plasma that was processed via liquid–liquid extraction (LLE) using a 96-well plate format. Chromatographic separation was achieved with a reversed-phase high performance liquid chromatography (HPLC) column using gradient elution, and the run time was 3 min. The method was fully validated and it demonstrated acceptable accuracy, precision, linearity, and specificity. The lower limit of quantitation (LLOQ) was 0.1/0.5/0.5 ng/mL for AEA/OEA/PEA, which was sensitive enough to capture the basal plasma levels in healthy subjects. Benchtop stability in plasma, freeze–thaw stability in plasma, frozen long-term stability in plasma, autosampler stability, and stock solution stability all met acceptance criteria (%Bias within ±12.0%). Characterization of stability in purchased/aged blood indicated that ethanolamides are subject to degradation mediated by intracellular membrane-bound FAAH, which has been shown to be inhibited by phenylmethylsulfonyl fluoride (PMSF). In the presence of PMSF, ethanolamide levels increased slightly over time, suggesting that blood cells release ethanolamides into plasma. Whole blood stability conducted in fresh blood immediately following collection revealed that there was significant elevation of ethanolamide concentrations (~1.3–2.0-fold on ice and ~1.5–3.0-fold at room temperature by 2 h), indicating that *de novo* synthesis and release from blood cells were the predominant factors affecting ethanolamide concentrations *ex vivo*. Accordingly, conditions that ensured rapid separation of plasma from blood cells and consistency in the blood harvesting procedures were established and implemented for clinical studies to minimize the *ex vivo* elevation of plasma ethanolamide concentrations. The variability (intra-subject and inter-subject) of plasma ethanolamide levels was evaluated in healthy subjects during a Phase 0 study (no drug administration) that simulated the design of single-ascending dose and multiple-ascending dose clinical trials in terms of sample collection time points, population, food, and activity. The data indicated there was

*Abbreviations:* AEA, arachidonyl ethanolamide; FAAH, fatty acid amide hydrolase; HPLC, high performance liquid chromatography; HQC, high QC; J&J, Johnson and Johnson; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLE, liquid–liquid extraction; LLOQ, lower limit of quantitation; LQC, low QC; MQC, mid QC; SRM, selected reaction monitoring; NAPE, *N*-acyl-phosphatidylethanolamine; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; PMSF, phenylmethylsulfonyl fluoride; QC, quality control.

* Corresponding author at: Bioanalysis/DMPK, Johnson & Johnson Pharmaceutical Research and Development, 1000 Route 202 South, Raritan, NJ 08869, United States. Tel.: +1 908 927 6584; fax: +1 908 541 0422.
*E-mail address:* wjian@its.jnj.com (W. Jian).

1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2010.04.024

relatively large inter- and intra-subject variation in plasma ethanolamide concentrations. In addition, apparent variations due to time of day and/or food effects were also revealed. Understanding the variability of ethanolamide levels in humans is very important for study design and data interpretation when changes in ethanolamide levels are used as target engagement biomarkers in clinical trials.

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## 1. Introduction

Endogenous ethanolamides (fatty acid amides), including arachidonyl ethanolamide (anandamide, AEA), oleoyl ethanolamide (OEA), and palmitoyl ethanolamide (PEA) are thought to be involved in regulation of multiple physiological and pathophysiological cannabinoid processes, such as feeding, sleeping, reproduction, inflammation, drug addiction, and pain [1–3]. Endogenous ethanolamides are released on demand from cell membrane phospholipid precursors in response to depolarizing agents, hormones and neurotransmitters, and are eliminated by hydrolysis mediated by the intracellular membrane-bound enzyme fatty acid amide hydrolase (FAAH) [1,4–6]. Inhibition of FAAH activity can cause augmented endogenous cannabinoid activity and FAAH inhibitors are being explored as novel therapeutic agents in treatment of diseases such as obesity and neuropathic pain [7]. As the endogenous substrates of FAAH, ethanolamides in plasma are potentially useful biomarkers of target engagement following FAAH inhibitor treatment. Therefore, it was critical to establish reliable bioanalytical methodology to quantify ethanolamides in human plasma.

Bioanalytical methods for quantitation of ethanolamides could suffer from interference caused by the analytes pre-existing in the matrix because they are endogenous compounds. This presents the challenge of constructing calibration curves and quality control samples "on top of" the pre-existing concentrations, which typically causes problems with accuracy and precision particularly at the lower range of quantitation. Further, lot-to-lot variability in the pre-existing concentrations makes the challenge even more difficult. Most of the published methods for quantitation of ethanolamide(s) in biological matrices such as human plasma, rat plasma, and rat brains used saline, organic solvent, or protein solution to prepare standard calibration samples, which are different from the intended matrices [8–12]. The use of proper matrix can be a critical factor for assays based on electrospray ionization mass spectrometry due to differences caused by matrix effects. When alternative matrices are necessary, a good approach is to construct quality control (QC) samples in the true matrix, while generating standard curves in the alternate matrix. The ability to identify concentrations in the QC samples with acceptable accuracy and precision verifies the proper performance of the assay.

In current study, we addressed the issue of endogenous interference using an alternative approach employing a "surrogate analyte" strategy [13,14]. For quantitation of AEA, OEA, and PEA, the stable isotope-labeled compounds $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA were used as "surrogate analytes" to establish the concentration–mass response relationship, i.e. a regression equation. The concentrations of the authentic analytes were calculated based on the regression equations derived from the surrogate analytes. Because the surrogate analytes are not present in human plasma, the assay was free from the interferences caused by endogenous analytes in the matrix, which is a strong bioanalytical advantage.

Using this unique surrogate analyte approach in the current study, a highly selective, sensitive, and high-throughput liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed and fully validated for simultaneous quantitation of AEA, OEA, and PEA in human plasma. The fully validated method was used in the investigation of the whole blood stability of the ethanolamides in order to establish sample collection procedures to effectively minimize the variability due to *ex vivo* change of plasma ethanolamide concentrations in clinical studies.

It is critical to understand the biological variability of biomarkers, especially for those expected to exhibit moderate changes with drug treatment, which is the case for ethanolamides. Naturally occurring variability may mask the effect of drug treatment and complicate data interpretation. To study the variability of plasma ethanolamides between subjects (inter-subject) and within subject (intra-subject), we conducted a single-center Phase 0 study that simulated typical first-in-human studies. Plasma samples were collected from healthy subjects at predefined time points over two days for the measurement of basal plasma ethanolamide concentrations. This is the first time that inter-/intra-subject variations, at different times of the day, were systematically investigated in humans. The results elucidated in this study could be used to facilitate the design of clinical studies for which plasma ethanolamide levels can be used as biomarkers of target engagement for FAAH inhibition and other related targets.

## 2. Experimental

### 2.1. Chemicals and reagents

AEA, OEA, PEA, $D_4$-AEA, $D_8$-AEA, $D_2$-OEA, and $D_4$-PEA were purchased from Cayman Chemicals (Ann Arbor, MI). $D_4$-OEA and $^{13}C_2$-PEA were synthesized internally by the radio-synthesis group of J&J Pharmaceutical Research and Development, LLC (Raritan, NJ). HPLC grade acetonitrile and isopropanol were obtained from EMD Chemicals Inc. (Gibbstown, NJ). Ammonium acetate, HPLC grade absolute ethanol (>99.5%), n-hexane, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade dimethyl sulfoxide was purchased from Burdick and Jackson (Morristown, NJ). Control human plasma was obtained from Bioreclamation (Hicksville, NY).

### 2.2. Calibration standard and quality control sample preparation

Standard stock solutions of $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA were prepared individually at 1.00 mg/mL in absolute ethanol in amber glass vials. A combined standard spiking solution of $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA at 1000/10,000/5000 ng/mL in 50/50: acetonitrile/dimethyl sulfoxide (v/v) was prepared in an amber glass vial by diluting from the standard stock solutions. The stock solutions and the spiking solution were stored at −20 °C. Calibration standard samples containing $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA at concentrations of 0.1/1.0/0.5, 0.2/2.0/1.0, 0.5/5.0/2.5, 1.0/10/5.0, 2.0/20/10, 5.0/50/25, 8.0/80/40, and 10/100/50 ng/mL were prepared fresh daily by serially diluting the standard spiking solution in blank human plasma.

QC stock solutions of $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA were prepared individually at 1.00 mg/mL in absolute ethanol in amber glass vials. A combined QC spiking solution of $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA at 10,000/100,000/50,000 ng/mL in 50/50: acetonitrile/dimethyl sulfoxide (v/v) was prepared in an amber glass vial by diluting from the QC stock solutions. The QC samples containing $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA at concentrations of 0.3/3.0/1.5 (LQC), 5.0/50/25 (MQC), and 8.0/80/40 (HQC) were prepared by diluting the QC spik-

ing solution in blank human plasma. The QC samples were stored at −20 °C.

### 2.3. Sample preparation procedure

The plasma samples were processed using liquid–liquid extraction (LLE) as follows. An aliquot (150 μL) of each sample was pipetted into the wells of a 1 mL 96-well polypropylene plate. Then, 20 μL of internal standard spiking solution containing $D_8$-AEA/$D_2$-OEA/$D_4$-PEA at 100/100/1500 ng/mL in acetonitrile was added to each well. The plate was vortexed at a setting of 1000 rpm for 1 min, followed by addition of 500 μL of 90/10: n-hexane/isopropanol (v/v) to each well using a Tomtec liquid handling robot (Tomtec Inc. Hamden, CT). The plate was sealed, vortexed on a plate shaker at a setting of 1600 rpm for 10 min, and then centrifuged to separate the phases. The organic layer (400 μL) was immediately transferred to a new 96-well plate using the Tomtec. The samples were evaporated to dryness under a gentle flow of nitrogen at ambient temperature and reconstituted using 100 μL of ethanol. The plate was vortexed at a setting of 1000 rpm for 1 min and placed into the autosampler set at 10 °C. The injection volume was 10 μL.

### 2.4. Liquid chromatography and mass spectrometry

The HPLC system consisted of Shimadzu LC-20AD pumps and a SIL-HTC autosampler (Columbia, MD). A Zorbax Eclipse XDB C18 column (2.1 mm × 50 mm, 5 μm, Agilent, Santa Clara, CA) was employed. HPLC mobile phase A was 10 mM ammonium acetate in water (pH unadjusted), mobile phase B was 10 mM ammonium acetate in 90/10: acetonitrile/water (v/v), and mobile phase C was 10 mM ammonium acetate in 50/50: acetonitrile/water (v/v). The autosampler needle rinse solvent was 0.1% trifluoroacetic acid in 50/50: acetonitrile/water (v/v). The gradient started at 40% B, was linearly increased to 100% B over 1.2 min, held at 100% B for 0.8 min, and then returned to 40% B in 0.1 min. The run time was 3 min and the HPLC flow rate was 0.6 mL/min. The post-column flow was delivered to the mass spectrometer from 1.5 to 2.8 min, and to waste for the remaining run time. Pump C delivered make-up flow (0.6 mL/min) to the mass spectrometer when the flow from the column was diverted to waste.

The HPLC system was interfaced with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) operated in positive Turbo-Ionspray® mode. The operating parameters were optimized and set at following values: curtain gas 30 psi, GAS1 50 psi, GAS2 50 psi, Ionspray® voltage 5000 V, source temperature 500 °C, and CAD gas 4 (arbitrary units). Selected reaction monitoring (SRM) for the 3 authentic analytes (AEA, OEA, and PEA), the 3 surrogate analytes ($D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA) and the 3 internal standards ($D_8$-AEA, $D_2$-OEA, and $D_4$-PEA) was performed using: AEA, *m/z* 348 → 62, OEA, *m/z* 326 → 62, PEA, *m/z* 300 → 62, $D_4$-AEA, *m/z* 352 → 66, $D_4$-OEA, *m/z* 330 → 66, $^{13}C_2$-PEA, *m/z* 302 → 62, $D_8$-AEA, *m/z* 356 → 63, $D_2$-OEA, *m/z* 328 → 62, $D_4$-PEA, *m/z* 304 → 62. Declustering potential (DP) was 50 V for AEA, $D_4$-AEA and $D_8$-AEA, 62 V for OEA, $D_4$-OEA and $D_2$-OEA, and 60 V for PEA, $^{13}C_2$-PEA and $D_4$-PEA. Collision energy (CE) was 32 V for AEA, $D_4$-AEA and $D_8$-AEA, 32 V for OEA, $D_4$-OEA and $D_2$-OEA, and 30 V for PEA, $^{13}C_2$-PEA and $D_4$-PEA. Entrance potential (EP) was 10 V for all molecular ions, and collision cell exit potential (CXP) was 5 V for all product ions.

Chromatographic peak areas for the analytes and internal standards were obtained using Analyst® version 1.4.1. software (Applied Biosystems, Foster City, CA). The peak areas were imported into a Watson LIMS System version 7.1.0.01 (Thermo Fisher Scientific, Philadelphia, PA) for linear regression analysis. For each calibration standard, the peak area ratios of $D_4$-AEA/$D_8$-AEA, $D_4$-OEA/$D_2$-OEA, and $^{13}C_2$-PEA/$D_4$-PEA were determined. A linear regression with a weighing of $1/x^2$ was then calculated using the regression equation: $y = m(x) + b$, where "$y$" is equal to the peak area ratio of analyte/internal standard, "$m$" is equal to the slope of the calibration curve, "$x$" is equal to the concentration of analyte, and "$b$" is equal to the $y$-intercept of the calibration. For determination of the concentrations of the authentic analytes, the peak area ratio of AEA/$D_8$-AEA, OEA/$D_2$-OEA, and PEA/$D_4$-PEA were determined and the concentrations were calculated using the equations derived from the surrogate analytes.

### 2.5. Validation procedure

Validation of the assay was conducted using calibration standards and QC samples prepared by fortifying human plasma with the surrogate analytes. In the validation, the surrogate analytes were regarded as the analytes and concentration and stability data were generated from them.

The linearity, accuracy, and precision of the assay were assessed in three separate analytical batch runs. Each batch included a standard curve and six replicates of each level of low (LQC), mid (MQC), and high QC (HQC). The nominal concentrations were the fortified levels of the surrogate analytes. The standard curve from each batch was regressed separately and the values for the QCs were calculated. Specificity of the assay was confirmed by analyzing 6 different lots of blank human plasma for chromatographic interference. Any chromatographic peak at the retention time of the analyte in its SRM channel with an area greater than 20% (5% for internal standard) of the LLOQ sample was considered significant interference.

Bench-top stability samples were prepared in six replicates at LQC and HQC levels and kept at room temperature for 6 h before extraction. Mean concentrations were calculated and compared against the nominal concentrations. Freeze–thaw stability was evaluated at LQC and HQC levels (six replicates each) by freezing the samples completely at −20 °C then allowing them to thaw completely at room temperature for three cycles. The samples were extracted with freshly prepared standard curves and QC samples. The mean concentrations of the tested samples were calculated and compared against the nominal concentrations. The processed sample/autosampler stability was evaluated by re-injecting extracted LQC, MQC, and HQC samples (in six replicates each) that had been kept in the autosampler for 72 h. The mean concentrations of the re-injected QCs were calculated using a freshly prepared standard curve and were compared against the nominal concentrations. The long-term storage stability of each analyte in human plasma at −20 °C was evaluated up to 132 days. The LQC and HQC were extracted in six replicates with a freshly prepared standard curve. The mean determined concentrations for the QCs were compared against the nominal concentrations.

The stability of 1.0 mg/mL stock solutions in ethanol stored at −20 °C was evaluated for up to 167 days for $D_4$-OEA and 168 days for $^{13}C_2$-PEA. The previously prepared and stored solutions were compared against solutions freshly prepared from a new weighing of the powder. For $D_4$-AEA, the purchased compound was delivered as a 1.00 mg/mL solution in ethanol. The stock solution stability of $D_4$-AEA was evaluated by comparing the stock solution stored at −20 °C for 71 days against a newly purchased lot. Six replicate injections at 1000 ng/mL for each respective analyte and 100 ng/mL for their internal standards were made for each stock solution. The mean peak area ratios of the samples from the tested stocks were compared with those from the freshly prepared stocks.

Relative matrix effect was determined by spiking $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA at low (0.3/3.0/1.5 ng/mL) and high (8.0/80/40 ng/mL) concentrations together with the internal standards into extracted matrix blanks from 6 individual plasma lots.

The percent coefficient of variation (%CV) was calculated for the peak area ratio of each analyte at each concentration across the lots of plasma. A %CV ≤15% was considered acceptable. Recovery was determined at three concentration levels (0.3/3.0/1.5, 5.0/50/25, and 8.0/80/40 ng/mL for $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA) in six replicates. It was calculated by comparing the peak area ratios of the analyte/internal standard in the extracted samples against that from matrix blanks spiked post-extraction. Internal standards were post-spiked into both the extracted samples and the extracted matrix blanks.

### 2.6. Whole blood stability evaluation procedure

#### 2.6.1. Whole blood stability evaluation in purchased blood

Whole blood stability was evaluated by monitoring the surrogate analytes spiked into $K_2$EDTA blood. The endogenous analytes in the same samples were also monitored. The blood was purchased from Bioreclamation. Whole blood pools were prepared by spiking $D_4$-AEA/$D_2$-OEA/$D_4$-PEA at low (0.3/3.0/3.0 ng/mL) and high concentrations (8.0/80/80 ng/mL) into blank whole blood in the absence or presence of PMSF. The PMSF stock solution was prepared at a concentration of 250 mM in ethanol and a 1–100 dilution in the whole blood achieved a final concentration of 2.5 mM. The whole blood pools were split and kept at room temperature and in an ice-bath. A sample was taken at time intervals of 0 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h, and immediately processed in a refrigerated centrifuge to generate the plasma. Each sample was extracted and analyzed in triplicate. The mean peak area ratio for the surrogate analytes at each time point was compared to that of the time 0 sample. In addition to the surrogate analytes, the SRM channels for the authentic analytes were also monitored and the peak area ratios were used to evaluate the stability of the authentic analytes in the whole blood.

#### 2.6.2. Whole blood stability evaluation in fresh blood

Stability in fresh whole blood was evaluated by monitoring the authentic analytes in blood freshly collected from healthy volunteers. Blood samples (~20 mL) were collected from each of the 6 donors. Within 5 min of collection, 1 mL of blood was transferred from each donor to generate plasma by centrifuging at 2500 × *g* and 4 °C for 5 min. The rest of the blood from each donor was split equally and kept at room temperature and in an ice-bath. A sample was taken at time intervals of 15 min, 30 min, 1 h, and 2 h, and immediately processed to generate plasma [the time points are relative to time-zero ($t = 0$) when the first plasma sample was separated]. After centrifugation, the plasma samples were immediately transferred into suitable storage tubes and placed on dry ice until frozen. The samples were stored frozen at −20 °C until quantitative analysis. The samples were extracted in singlet along with calibration standard and QC samples prepared using surrogate analytes. The concentrations of the authentic analytes were calculated against the calibration standard curves established using surrogate analytes.

### 2.7. Phase 0 study

A single-center Phase 0 study in eight healthy young male subjects was conducted to determine the diurnal variations and food effects of plasma ethanolamide levels. This study was approved by the MDS Pharma Service Institutional Review Board (Lincoln, NE) and was conducted following Good Clinical Practice guidelines. After giving written informed consent, subjects were screened within 14 days prior to Day 1 to determine their eligibility according to the inclusion and exclusion criteria. The exclusion criteria were generally the same as those of a standard Phase I clinical study. Examples of the exclusion criteria are: use of any prescription or over-the-counter medication, herbal medication (including garlic extract and herbal teas), vitamins, or mineral supplements within 14 days or 5 half-lives, whichever is longer, prior to Day 1; positive screen for alcohol and/or drugs of abuse; drinks, on average, more than 5 cups (8 oz) of tea/coffee/cocoa/cola per day; history of or current significant medical illness; psychological and/or emotional problems, smoker for within 6 months prior to Day 1 based on medical history, and so on. Eligible subjects were asked to enter the clinical research unit (CRU) on the evening of Day −1 and remain in the clinic for two nights. On Day 1, blood samples were collected under fasting conditions, which were defined as at least 10 h without food or drink prior to Day 1 procedures, with the exception of water. Blood samples were collected at 0, 1, 2, 3, 4, 6, 8, 10, 14, 18, and 22 h, where 0 h was to occur around 8:00 a.m. Subjects were given a standardized meal immediately after the 4 h blood sample was obtained. A standardized evening meal was provided at 10 h post 0 h, immediately after the 10 h blood sample was obtained. On the evening of Day 1, the subjects fasted overnight for at least 10 h. On Day 2, blood samples were collected at 0, 1, 2, 3, 4, 6, and 8 h, where 0 h was to occur around 8:00 a.m. Subjects were given a standardized meal immediately after the 4 h blood sample was obtained. Subjects were discharged from the CRU on Day 2 after completion of the end-of-study procedures.

At each time point, 3 mL of blood was collected into 4 mL $K_2$EDTA blood collection tubes, which had been pre-chilled on ice. The maximal time from blood collection to the start of centrifugation was less than 3 min from start time of collection. Plasma was generated by centrifuging blood samples in a refrigerated centrifuge (4 °C) at 2500 × *g* for 5 min. The plasma was transferred immediately to pre-chilled storage tubes and put on dry ice until frozen. The time between blood collection and freezing the plasma did not exceed 15 min. The samples were shipped frozen and stored at −20 °C until quantitative analysis. The samples were extracted in singlet along with calibration standard and QC samples prepared using the surrogate analytes. The concentrations of authentic analytes were calculated against the calibration standard curves established using surrogate analytes.

## 3. Results and discussion

### 3.1. Analytical strategy

For bioanalytical method validation and sample analysis, calibration standard and QC samples ideally should be prepared in the same matrix as the intended sample. This is especially true when electrospray ionization mass spectrometry is used because components in the sample can lead to matrix effects such as enhancement or suppression of ionization. Matrix effects occurring between different matrices can lead to bias in the method and therefore affect the results. In the case of ethanolamine quantitation, there is no true "blank" plasma available because the analytes are endogenous. In some previously established quantitation methods for ethanolamine(s), calibration standard samples have been reported to be prepared in saline, organic solvent, or BSA (bovine serum albumin) solution, which are different from the intended matrix [8–12]. Another option is to screen multiple lots of human plasma to identify a few lots that contain none of the intended analytes. However, this strategy is time- and resource-consuming, and it may not be possible to find a truly blank lot. Even if found, it could be questioned whether these "blank lots" are representative of the real matrix. Alternatively, plasma could be depleted of the intended analytes using methods such as charcoal stripping, acid or alkaline hydrolysis, or affinity chromatography. For example, Palandra used charcoal-stripped human plasma to prepare standard curve samples in their report on the

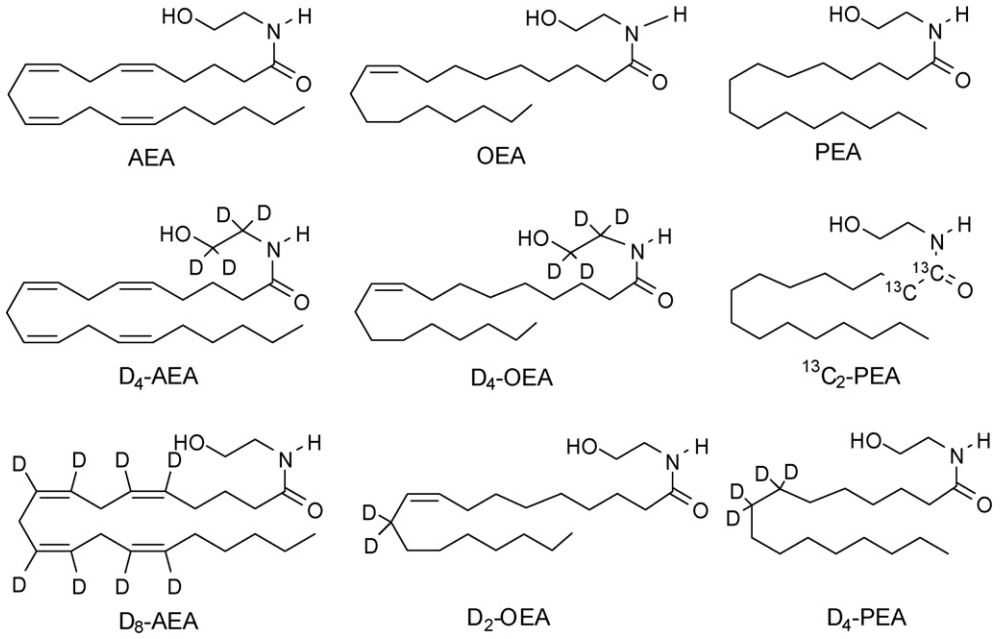


**Fig. 1.** Structures of ethanolamides and the stable isotope-labeled ethanolamides used in this study.

quantitation of endogenous ethanolamides in human plasma [15]. However, the depletion process could be labor-intensive and only partially successful. In addition, the depleted plasma could be significantly different from natural plasma and therefore it may defeat the purpose of using identical matrix. On the other hand, if an alternative matrix could be shown to quantify QC samples made in the true matrix with acceptable accuracy and precision, this approach to quantification of endogenous analytes can be practical.

In the current work, a "surrogate analyte" approach was employed for quantitation of ethanolamides, free from endogenous analyte interferences. The concept was to generate calibration curves using the mass spectrometry response derived from the surrogate analyte to calculate the concentration of the authentic analyte based on the regression equation of the surrogate [13,14]. In this work, the surrogate analytes were the stable isotope-labeled ethanolamides, namely $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA (structures shown in Fig. 1), which theoretically exhibit identical liquid chromatographic behavior and mass spectrometry response as the non-labeled, authentic ethanolamides, AEA, OEA, and PEA. The stable isotope-labeled ethanolamides are not present in human plasma and therefore provide response–concentration equations free of interference from the endogenous analytes. Another version of stable isotope-labeled ethanolamides ($D_8$-AEA, $D_2$-OEA, $D_4$-PEA, structures shown in Fig. 1) was added during sample extraction and served as internal standards, as usually used in quantitative stable isotope dilution LC–MS/MS methodologies. Careful consideration was taken in deciding which isotope-labeled compounds were to be used as surrogate analytes, and which as internal standards. Both $D_4$-AEA and $D_8$-AEA are commercially available. $D_4$-AEA was chosen as a surrogate analyte because it was expected to be more chemically and physically similar to authentic AEA than $D_8$-AEA due to less deuterium labeling. For OEA and PEA, only one isotope-labeled version was commercially available: $D_2$-OEA and $D_4$-PEA. Therefore, another version of isotope-labeled compounds, $D_4$-OEA and $^{13}C_2$-PEA, was internally synthesized. They were chosen as surrogate analytes because it is easier to guarantee consistent quality and a constant supply from our internal source, which is very important in support of clinical studies for a potentially large number of samples and a long duration of time.

Ideally, the instrument response of the surrogate analyte should be identical to the authentic analyte. To compensate for any potential response difference caused by an isotope effect or differences in ionization efficiency, a correction factor could be used, which is determined by dividing the response of the surrogate analyte by that of the authentic analyte at equivalent concentrations. In this work, correction factors were determined to be 1.08, 1.09, and 0.91 for AEA, OEA, and PEA, respectively. They were not applied because they are very close to theoretical value 1.00. This means that the concentration values reported in the current work should be interpreted with some caution in terms of the absolute value. However, it does not compromise the performance of the assay and fully fits the intended purpose of the study, for which the *relative* changes in concentration are the important consideration [16–18].

Using the surrogate analyte approach, the advantage of being able to prepare calibration standards in the exact intended matrix is accompanied by the disadvantages of the difficulty of synthesizing, or the expense of purchasing the stable isotope-labeled surrogate analytes. In contrast, using an alternative matrix such as saline or protein solution is cost effective, but it can be difficult to establish the accuracy and precision of the assay due to the significant difference from the biological matrix.

In application of the surrogate analyte approach, it is critical to ensure the SRM channels of the authentic analytes, surrogate analytes, and internal standards do not interfere with each other. Since ethanolamides consist only of C, H, O, and N, the possibility of interference caused by abundant heavy isotopes is minimal when two compounds differ by 3 mass units or more. In cases when two compounds are 2 mass units apart, the potential interference has no significant impact either. For example, contribution from PEA to the SRM channel of $^{13}C_2$-PEA due to natural heavy isotopes is theoretically calculated to be about 2%. Since endogenous PEA concentration is low (maximum concentration determined in the current study is 2.48 ng/mL), the contribution from endogenous PEA to $^{13}C_2$-PEA signal would be negligible. In addition, with careful selection of internal standard concentrations, the impact from $D_2$-OEA to $D_4$-OEA was minimized, so was that from $^{13}C_2$-PEA to $D_4$-PEA. Another critical factor is the isotopic purity. During method development for the current work, the isotopic purity was determined for all three surrogate analytes and three internal standards

to make sure that they do not interfere with each other, nor with the authentic analytes ($d_0$ content). Full incorporation of the label is also an important consideration. For example, if $D_4$-AEA was to contain only 70% full incorporation, with the remainder being $D_3$-, or $D_2$-, etc., this would produce systematic bias in the surrogate analyte approach. We determined the label incorporation for $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA to be greater than 99% (data not shown). This was considered acceptable for use and a correction factor was not applied.

Endogenous ethanolamides circulate at low concentrations in human plasma. In addition, there is large inter-subject variability (up to 10-fold) in plasma ethanolamide concentrations as reported in the literature [11,19]. In order to establish a lower limit of quantitation (LLOQ) sensitive enough to capture all the basal concentrations in the intended studies, twenty lots of commercial human plasma were screened using preliminary calibration curve ranges to measure their endogenous AEA, OEA, and PEA concentrations. It was found that the endogenous concentrations ranged from 0.11 to 0.37 ng/mL for AEA, from 1.59 to 5.81 ng/mL for OEA, and from 0.76 to 2.48 ng/mL for PEA. Accordingly, with the consideration of a potential several-fold increase with drug treatment, the standard calibration range was established as 0.1/1.0/0.5–10/100/50 ng/mL for AEA/OEA/PEA. The assay was fully validated using this range. Subsequently, in the application of the assay in a clinical study, it was found that the LLOQ of OEA was not low enough to capture all the basal concentrations at different time points of the day. Therefore, the calibration curve range was modified to 0.1/0.5/0.5–10/50/50 ng/mL and appropriate validation experiments were repeated (data not shown).

### 3.2. Validation results

Even though there is no specific regulatory requirement regarding biomarker method validation, a "fit-for-purpose" strategy has been the consensus among the biomarker community. Thus, an increase in the rigor of biomarker method validation is expected as biomarkers are used for increasingly advanced drug development stages [16–18]. In current study, the intended use of the assay was for measuring the ethanolamides as biomarkers in subjects entered in clinical trials and the data will be used for critical decision-making during the development of new drug candidates. To ensure high quality of the data, a rigorous full validation was conducted to extensively evaluate the performance of the assay.

As mentioned in Section 2, the validation data were generated using surrogate analytes. Since the surrogate analytes are theoretically identical to the authentic analytes, the results of the validation reflect the performance of the assay for the measurement of the authentic analytes.

A chromatogram of an extracted blank plasma sample is illustrated in Fig. 2A. The endogenous AEA, OEA, and PEA showed as distinct peaks eluted at a retention time of 1.84, 2.10, and 2.01 min, respectively. There were no peaks in the SRM channels of the surrogate analytes and internal standards at the expected retention time except for a small peak for $^{13}C_2$-PEA, which was negligible (<10%) when compared to the peak of $^{13}C_2$-PEA in LLOQ sample (Fig. 2B). Investigations into the identity of this peak failed due to its weak intensity. However, it was persistent in the assay procedure at intensities typical of this example. It is speculated as a carryover because the peak was absent when the assay was run on a different LC–MS system for the first time. As mentioned above, it posed no threat to the integrity of the assay due to its low intensity relative to the LLOQ. The specificity experiment described in Section 2.5 using six separate lots of blank human plasma indicated that there was no significant interference at the retention times of any of the surrogate analytes or internal standards other than the small peak for $^{13}C_2$-PEA.

The specificity for the authentic analytes was more challenging to determine. The analytes are present in every plasma sample due to their endogenous nature, so obtaining a true matrix blank was not possible. Traditional techniques to establish selectivity involve the careful examination of chromatographic peak shape between injections of the neat analyte and extracted, authentic samples. Differences may suggest co-eluting isobaric interferences. In addition, several SRM channels can be simultaneously monitored for each analyte to confirm the relative product ion intensities match between neat and extracted samples. In addition to these two approaches, we examined the specificity using the advanced scanning functions of an API 4000 QTrap mass spectrometer in which very sensitive information-dependent acquisition (IDA) of product ion spectra can be obtained. It was found that the multiple reaction monitor (MRM) triggered product ion mass spectra from neat solutions of AEA, OEA, and PEA were identical to those obtained from the chromatographic peaks eluting at their respective retention times from the extracted blank plasma. The absence of any unassigned product ions suggested that the chromatographic peaks were solely due to the authentic analytes and not due to any significant interference from the matrix.

Fig. 2B shows the chromatogram of an extracted LLOQ sample (0.1/0.5/0.5 ng/mL for $D_4$-AEA/$D_4$-OEA/$^{13}C_2$-PEA). It is worth noting that the peak height for each surrogate analyte was lower than that of the corresponding authentic analyte for each compound (~2–3-fold), indicating that the quantitation limit was low enough to cover the endogenous concentrations.

The linearity of the calibration curves was determined over the specified range shown in Table 1. For each compound, the calibration curve showed a coefficient of determination ($R^2$) greater than 0.994. The %CV of the standards was ≤4.9% and the %Bias was within ±13.0% for all three compounds at all concentrations (Table 1). Intra-assay and inter-assay accuracy and precision were assessed over three separate days by preparing a daily standard calibration curve along with six replicates of LQC, MQC, and HQC samples. ANOVA analysis of the accuracy and precision is presented in Table 2. The Between Run, Within Run, and Total %CV were ≤5.3, 7.1, and 8.8%, respectively, for all three compounds at all concentrations. The %Bias was within ±7.0% in each case. In addition, the concentrations of the endogenous analytes in the QC samples were calculated for the above three analytical runs (Table 3). The endogenous analytes were found to be quantified in a highly reproducible manner on the daily basis. Overall, the %CV of the calculated concentrations from the three runs was ≤7.8% for all three compounds, demonstrating excellent reproducibility when the assay was applied for the measurement of the endogenous analytes.

The stability results are presented in Table 4. Overall, $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA demonstrated excellent stability under the tested conditions, including on the bench-top (in plasma) for 6 h, freeze–thaw (in plasma) for 3 cycles, in the autosampler (10 °C) for 72 h, and frozen (−20 °C, in plasma) for up to 132 days. The %Bias from nominal concentration was within ±12.0% for all three compounds at all concentrations under all tested conditions. In addition to quantifying the concentrations of the spiked surrogate analytes, the concentrations of the endogenous analytes in the stability QC samples were also measured and compared to the values determined on Day 0. It was found that the %Change of the concentrations of AEA, OEA, and PEA from those of Day 0 were all within ±16.2% for the three different tested conditions (bench-top 6 h, freeze–thaw 3 cycles, frozen 132 days, Table 5). This further confirmed that the stability of the surrogate analytes could reliably reflect the stability of the authentic analytes.

Stock solutions in ethanol at 1.00 mg/mL stored at −20 °C were also evaluated for their stability. The %Change of the original stock solutions from the freshly prepared (freshly purchased in the case of

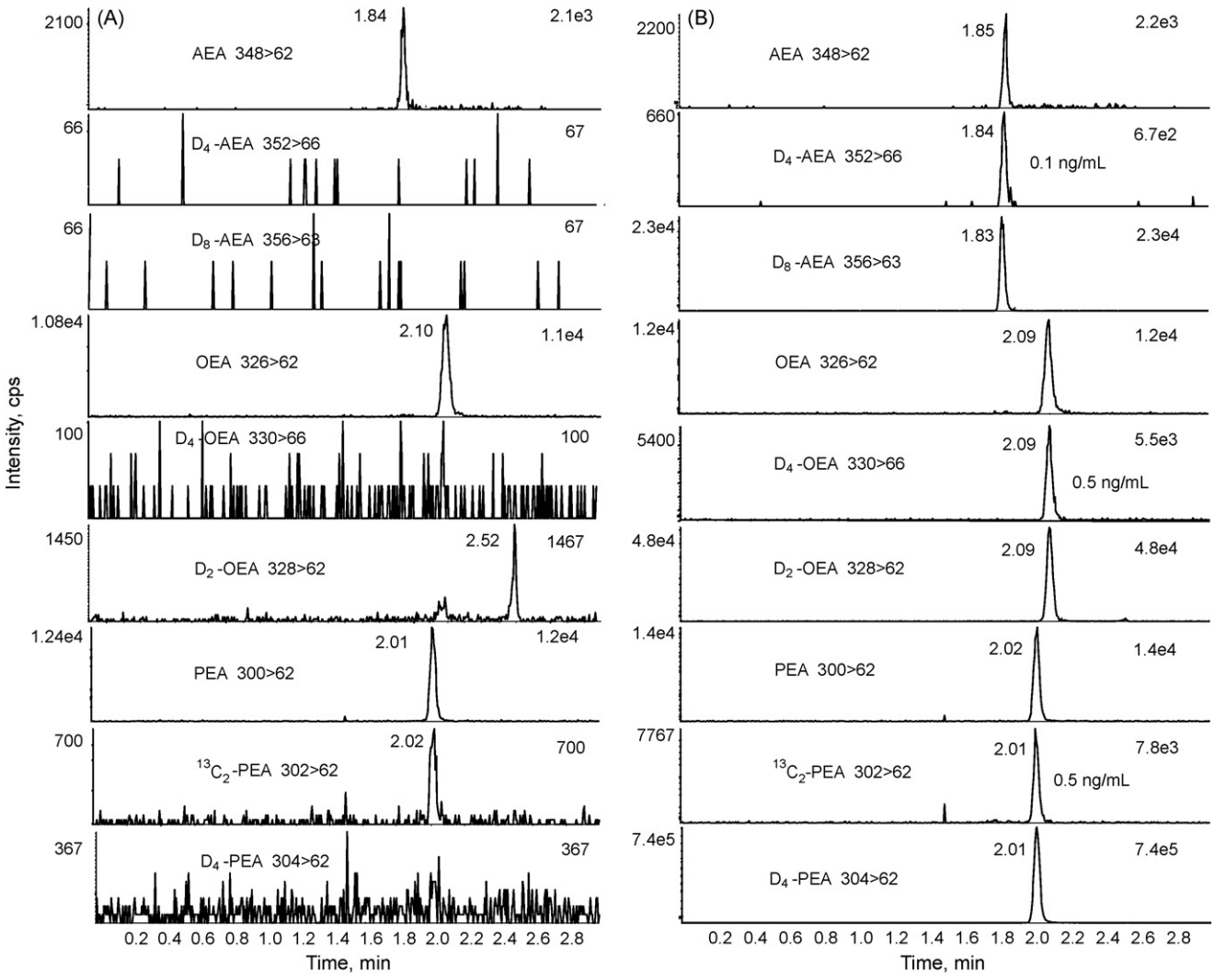


**Fig. 2.** LC–MS/MS chromatograms of (A) an extracted blank human plasma sample without internal standards; (B) extracted human plasma spiked with $D_4$-AEA (0.1 ng/mL), $D_4$-OEA (0.5 ng/mL), and $^{13}C_2$-PEA (0.5 ng/mL) at LLOQ with internal standards.

**Table 1**
Linearity, accuracy and precision of calibration standards (3 analytical runs).

| | STD1_0.10 | STD2_0.20 | STD3_0.50 | STD4_1 | STD5_2 | STD6_5 | STD7_8 | STD8_10 | Slope | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| $D_4$-AEA | | | | | | | | | | |
| Overall mean | 0.107 | 0.174 | 0.487 | 1.00 | 2.09 | 5.13 | 8.05 | 10.1 | 0.297084 | 0.99439 |
| S.D. | 0 | 0.00115 | 0.0125 | 0.0396 | 0.0451 | 0.105 | 0.325 | 0.287 | 0.004648 | 0.00043 |
| %CV | 0 | 0.7 | 2.6 | 4.0 | 2.2 | 2.0 | 4.0 | 2.8 | 1.6 | 0 |
| %Bias | 7.0 | −13.0 | −2.6 | 0 | 4.5 | 2.6 | 0.6 | 1.0 | | |
| | STD1_1 | STD2_2 | STD3_5 | STD4_10 | STD5_20 | STD6_50 | STD7_80 | STD8_100 | Slope | $R^2$ |
| $D_4$-OEA | | | | | | | | | | |
| Overall mean | 1.03 | 1.87 | 5.01 | 10.1 | 20.0 | 50.1 | 82.1 | 99.4 | 0.208528 | 0.99789 |
| S.D. | 0.0265 | 0.0854 | 0.243 | 0.171 | 0.200 | 1.500 | 0.458 | 3.460 | 0.001373 | 0.00181 |
| %CV | 2.6 | 4.6 | 4.9 | 1.7 | 1.0 | 3.0 | 0.6 | 3.5 | 0.7 | 0.2 |
| %Bias | 3.0 | −6.5 | 0.2 | 1.0 | 0 | 0.2 | 2.6 | −0.6 | | |
| | STD1_0.5 | STD2_1 | STD3_2.5 | STD4_5 | STD5_10 | STD6_25 | STD7_40 | STD8_50 | Slope | $R^2$ |
| $^{13}C_2$-PEA | | | | | | | | | | |
| Overall mean | 0.508 | 0.957 | 2.57 | 5.02 | 10.1 | 25.5 | 39.8 | 48.5 | 0.010357 | 0.99866 |
| S.D. | 0.00802 | 0.0291 | 0.100 | 0.0231 | 0.121 | 0.115 | 1.310 | 0.361 | 0.000222 | 0.00074 |
| %CV | 1.6 | 3.0 | 3.9 | 0.5 | 1.2 | 0.5 | 3.3 | 0.7 | 2.1 | 0.1 |
| %Bias | 1.6 | −4.3 | 2.8 | 0.4 | 1.0 | 2.0 | −0.5 | −3.0 | | |

**Table 2**
Accuracy and precision of QC samples (ANOVA analysis of 3 analytical runs).

| | Nominal conc. (ng/mL) | Mean observed conc. (ng/mL) | %Bias | Between run precision (%CV) | Within run precision (%CV) | Total variation (%CV) |
|---|---|---|---|---|---|---|
| $D_4$-AEA | | | | | | |
| LQC | 0.300 | 0.321 | 7.0 | 5.3 | 7.1 | 8.8 |
| MQC | 5.00 | 4.93 | −1.4 | 2.0 | 5.5 | 5.8 |
| HQC | 8.00 | 8.31 | 3.9 | 1.4 | 3.2 | 3.5 |
| $D_4$-OEA | | | | | | |
| LQC | 3.00 | 2.94 | −2.0 | 1.3 | 3.0 | 3.3 |
| MQC | 50.0 | 49.4 | −1.2 | 0.0 | 4.9 | 4.6 |
| HQC | 80.0 | 80.6 | 0.8 | 0.0 | 3.6 | 3.3 |
| $^{13}C_2$-PEA | | | | | | |
| LQC | 1.50 | 1.49 | −0.7 | 3.5 | 5.6 | 6.6 |
| MQC | 25.0 | 25.2 | 0.8 | 2.1 | 3.4 | 4.0 |
| HQC | 40.0 | 41.5 | 3.8 | 0.0 | 2.9 | 2.8 |

$D_4$-AEA) was within ±2.66% for $D_4$-AEA stored for 71 days, $D_4$-OEA stored for 167 days, and $^{13}C_2$-PEA stored for 168 days (Table 6).

The relative matrix effect was evaluated by calculating the %CV of the peak area ratio of the surrogate analyte/internal standard spiked at low and high concentrations within the calibration curve range in six extracted individual lots of blank human plasma. Since the authentic ethanolamides have almost identical retention times as the surrogate analytes, and their chemical properties are nearly identical, it was presumed their matrix effects would be the same as the surrogates. The results showed a %CV ≤12.71% for $D_4$-AEA, ≤3.96% for $D_4$-OEA, and ≤3.75% for $^{13}C_2$-PEA at both low and high concentrations, indicating acceptable matrix effect. Any ionization suppression or enhancement potentially caused by matrix components was also evaluated during method development by post-column infusion [20] and phospholipid profiling [21]. The post-column infusion revealed that $D_4$-AEA and $^{13}C_2$-PEA eluted at a plateau, while $D_4$-OEA eluted near the slope of an ion suppression region in the profile (data not shown). This explained the poor performance of the assay when initially $D_8$-AEA was used as the internal standard for $D_4$-OEA. $D_8$-AEA did not elute at the same retention time as $D_4$-OEA and its instrument response did not track that of $D_4$-OEA. When $D_2$-OEA was later available and used as the internal standard for $D_4$-OEA, it proved to reliably compensate for the ion suppression and it ensured good performance of the assay. Major phospholipids were monitored using an in-source collision induced dissociation (CID) approach of SRM *m/z* 184 → 184. The results indicated the major phospholipids all eluted before 1.5 min on each chromatographic run, and therefore had no interference with any of the analytes which all eluted after 1.8 min (data not shown).

During method development, different combinations of organic solvent were evaluated for their extraction efficiency for the ethanolamides from human plasma. Among the tested conditions, different percentages of ethyl acetate in n-hexane (10–90%) gave acceptable recovery (~65–80%), while ethyl acetate, diethyl ether, and 5% methanol in n-hexane showed recovery less than 50%. Overall, 10% isopropanol in n-hexane provided best recovery, ranging from 86.8 to 111.5% for $D_4$-AEA, $D_4$-OEA, and $^{13}C_2$-PEA at low, mid and high concentrations as determined in the validation.

**Table 3**
Mean ($n$ = 6) calculated concentrations (ng/mL) of endogenous analytes in QC samples in three analytical runs.

| | Run 1 | Run 2 | Run 3 | Average | %CV |
|---|---|---|---|---|---|
| AEA | | | | | |
| LQC | 0.275 | 0.276 | 0.290 | 0.280 | 3.0 |
| MQC | 0.264 | 0.274 | 0.304 | 0.281 | 7.4 |
| HQC | 0.269 | 0.264 | 0.304 | 0.279 | 7.8 |
| OEA | | | | | |
| LQC | 1.735 | 1.790 | 1.849 | 1.791 | 3.2 |
| MQC | 1.719 | 1.766 | 1.832 | 1.772 | 3.2 |
| HQC | 1.720 | 1.723 | 1.862 | 1.768 | 4.6 |
| PEA | | | | | |
| LQC | 1.832 | 1.687 | 1.920 | 1.813 | 6.5 |
| MQC | 1.826 | 1.670 | 1.779 | 1.758 | 4.6 |
| HQC | 1.815 | 1.617 | 1.846 | 1.759 | 7.1 |

### 3.3. Whole blood stability

It was critical to evaluate the stability of the ethanolamides in whole blood in order to establish a reliable sample collection and processing procedure to minimize any *ex vivo* changes.

Whole blood stability was initially evaluated in purchased human blood. $D_4$-AEA, $D_2$-OEA, and $D_4$-PEA were spiked at low and high concentrations (within the calibration range) into the blank whole blood and incubated at room temperature or on ice for different periods of time before plasma was generated, extracted and analyzed. Fig. 3A–C shows the abundance–time curve of $D_4$-AEA, $D_2$-OEA, and $D_4$-PEA. The concentrations at 0 min were deemed as 100% and the concentrations of the other time points were expressed as % of that 0 min value. Time- and temperature-dependent decreases (~20–25%) were observed for the three analytes during the course of the 4 h incubation. The analytes diminished gradually at room temperature while remaining stable on ice. When PMSF was present in the whole blood, the decrease was prevented and the analytes remained stable at both room temperature and on ice, as shown in Fig. 3D–F. Ethanolamides have been shown to be subject to hydrolysis by intracellular membrane-bound FAAH in blood cells [5,6]. Schmidt made the observation that AEA levels were 2.3-fold higher in plasma derived from blood treated with PMSF for 24 h than that from the non-treated blood [22]. In addition, Arai found that the brain concentration of AEA in rats dosed with PMSF was ~16-fold higher than those not dosed with PMSF [12]. Our observations in this study confirmed the existence of hydrolase activity in whole blood and that the degradation of the ethanolamides was temperature-dependent and was blocked by PMSF.

In addition to monitoring $D_4$-AEA, $D_2$-OEA, and $D_4$-PEA, the levels of the endogenous analytes AEA, OEA, and PEA were also measured and plotted against time. Interestingly, AEA, OEA, and PEA all appeared to be relatively more stable than the surrogate analytes in whole blood both at room temperature and on ice during 4 h of incubation (Fig. 4A–C). When PMSF was present in the whole blood, an upward trend in the levels of AEA, OEA, and PEA was revealed, which was more obvious at room temperature (Fig. 4D–F). AEA is known to be released from whole blood cells *ex vivo* due to mobilization of pre-existing AEA from cellular structures of blood cells, from *de novo* synthesis and exocytosis, or from

**Table 4**
Bench-top, freeze–thaw, autosampler, and long-term plasma stability.

| | Bench-top, 6 h | | Freeze–thaw, 3 cycles | | Autosampler, 72 h (10 °C) | | | Long-term, 132 days (−20 °C) | |
|---|---|---|---|---|---|---|---|---|---|
| | LQC | HQC | LQC | HQC | LQC | MQC | HQC | LQC | HQC |
| $D_4$-AEA | | | | | | | | | |
| Nominal conc. (ng/mL) | 0.300 | 8.00 | 0.300 | 8.00 | 0.300 | 5.00 | 8.00 | 0.300 | 8.00 |
| Observed conc. (ng/mL) | 0.300 | 7.83 | 0.286 | 8.07 | 0.307 | 5.03 | 8.49 | 0.264 | 8.11 |
| %Bias | 0.0 | −2.1 | −4.7 | 0.9 | 2.3 | 0.6 | 6.1 | −12.0 | 1.4 |
| $D_4$-OEA | | | | | | | | | |
| Nominal conc. (ng/mL) | 3.00 | 80.0 | 3.00 | 80.0 | 3.00 | 50.0 | 80.0 | 1.50 | 40.0 |
| Observed conc. (ng/mL) | 2.65 | 79.7 | 2.75 | 81.0 | 3.02 | 50.8 | 82.8 | 1.59 | 43.9 |
| %Bias | −11.7 | −0.4 | −8.3 | 1.3 | 0.7 | 1.6 | 3.5 | 6.0 | 9.8 |
| $^{13}C_2$-PEA | | | | | | | | | |
| Nominal conc. (ng/mL) | 1.50 | 40.0 | 1.50 | 40.0 | 1.50 | 25.0 | 40.0 | 1.50 | 40.0 |
| Observed conc. (ng/mL) | 1.39 | 40.3 | 1.43 | 40.8 | 1.48 | 25.9 | 41.5 | 1.46 | 39.5 |
| %Bias | −7.3 | 0.8 | −4.7 | 2.0 | −1.3 | 3.6 | 3.8 | −2.7 | −1.3 |

**Table 5**
Stability of the endogenous analytes in the stability QC samples.

| | Bench-top, 6 h | | Freeze–thaw, 3 cycles | | Long-term, 132 days (−20 °C) | |
|---|---|---|---|---|---|---|
| | LQC | HQC | LQC | HQC | LQC | HQC |
| AEA | | | | | | |
| Day 0 conc. (ng/mL) | 0.276 | 0.264 | 0.275 | 0.269 | 0.237 | 0.228 |
| Observed conc. in stability QCs (ng/mL) | 0.293 | 0.257 | 0.275 | 0.273 | 0.227 | 0.235 |
| %Bias | 6.2 | −2.7 | 0.0 | 1.5 | −4.2 | 3.1 |
| OEA | | | | | | |
| Day 0 conc. (ng/mL) | 1.790 | 1.723 | 1.735 | 1.720 | 1.332 | 1.298 |
| Observed conc. in stability QCs (ng/mL) | 1.730 | 1.728 | 1.728 | 1.786 | 1.464 | 1.469 |
| %Bias | −3.4 | 0.3 | −0.4 | 3.8 | 9.9 | 13.2 |
| PEA | | | | | | |
| Day 0 conc. (ng/mL) | 1.687 | 1.617 | 1.832 | 1.815 | 1.848 | 1.839 |
| Observed conc. in stability QCs (ng/mL) | 1.709 | 1.629 | 1.683 | 1.631 | 1.592 | 1.542 |
| %Bias | 1.3 | 0.7 | −8.1 | −10.1 | −13.9 | −16.2 |

a combination of both [4]. Overall, there are two opposite forces that affect ethanolamide concentrations in whole blood, one being the FAAH activity that causes the ethanolamide levels to decrease, the other being the release from blood cells or *de novo* synthesis that cause an increase. The apparent "stability" observed for AEA, OEA, and PEA in whole blood in the absence of PMSF might be due to a combined and balanced result of these two forces. When PMSF was added to inhibit the enzyme activity of FAAH, the balance was broken and release or *de novo* synthesis became the predominant factor affecting the ethanolamide concentrations, and therefore an increase was observed. In contrast to the authentic (endogenous) analytes, and consistent with the fact that *de novo* synthesis or release was not possible for the surrogate analytes, no increase was observed for surrogate analytes in the presence of PMSF. These observations in this experiment confirmed the release of AEA, OEA, and PEA from blood cells and were consistent with previously reported observations of *ex vivo* increase of AEA in whole blood [11,23].

The enzymes and/or transporters involved in the release of ethanolamides remain poorly characterized [4]. It is known that ethanolamides are synthesized from their precursors *N*-acyl-phosphatidylethanolamines (NAPE) via multiple pathways involving hydrolysis by phospholipase D, which is a membrane enzyme located on blood cells [24,25]. FAAH, the enzyme involved in catabolism of ethanolamides is also a membrane enzyme in blood cells [1,5]. Therefore, to prevent *ex vivo* change of ethanolamide levels, it is critical to separate the plasma from blood cells as soon as possible so that the enzymes/transporters involved in the biosynthesis and catabolism of ethanolamides are removed. Once blood cells are separated, ethanolamides remain stable in plasma, as demonstrated in the plasma stability experiments in the assay validation shown in Section 3.2 (Tables 4 and 5). The stability of the authentic, endogenous ethanolamides and the spiked surrogate ethanolamides indicated that there was no FAAH activity in the plasma. In addition, plasma stability of NAPE, the biosynthetic precursor of AEA, was investigated. It was found that NAPE spiked in plasma was stable for at least 24 h at room temperature at natural pH or under acidified conditions (pH ~3), and for at least three freeze–thaw cycles (data not shown). This further confirmed the biosynthesis enzymes of ethanolamides are not present in plasma, and once separated from blood cells, plasma is deficient of *ex vivo* biosynthesis of ethanolamides.

Because there is potential *ex vivo* change of the concentrations of ethanolamides in whole blood, it was critical to establish a sample collection procedure to obtain data that truly reflects the *in vivo* concentrations. The rate and trend of change in the concentrations of ethanolamides in whole blood are determined by the enzyme activities, which may vary significantly between aged, purchased blood versus freshly collected blood. In order to mimic the conditions in clinical studies, stability experiments were conducted in blood freshly collected from healthy donors. Blood samples were

**Table 6**
Stability of stock solutions in ethanol stored at −20 °C.

| | Days stored | %Change from stock solutions freshly prepared |
|---|---|---|
| $D_4$-AEA[a] | 71 | 0.35 |
| $D_4$-OEA | 167 | −2.66 |
| $^{13}C_2$-PEA | 168 | −0.23 |

[a] Stability of D4-AEA was tested only up to 71 days due to limited availability of fresh $D_4$-AEA.

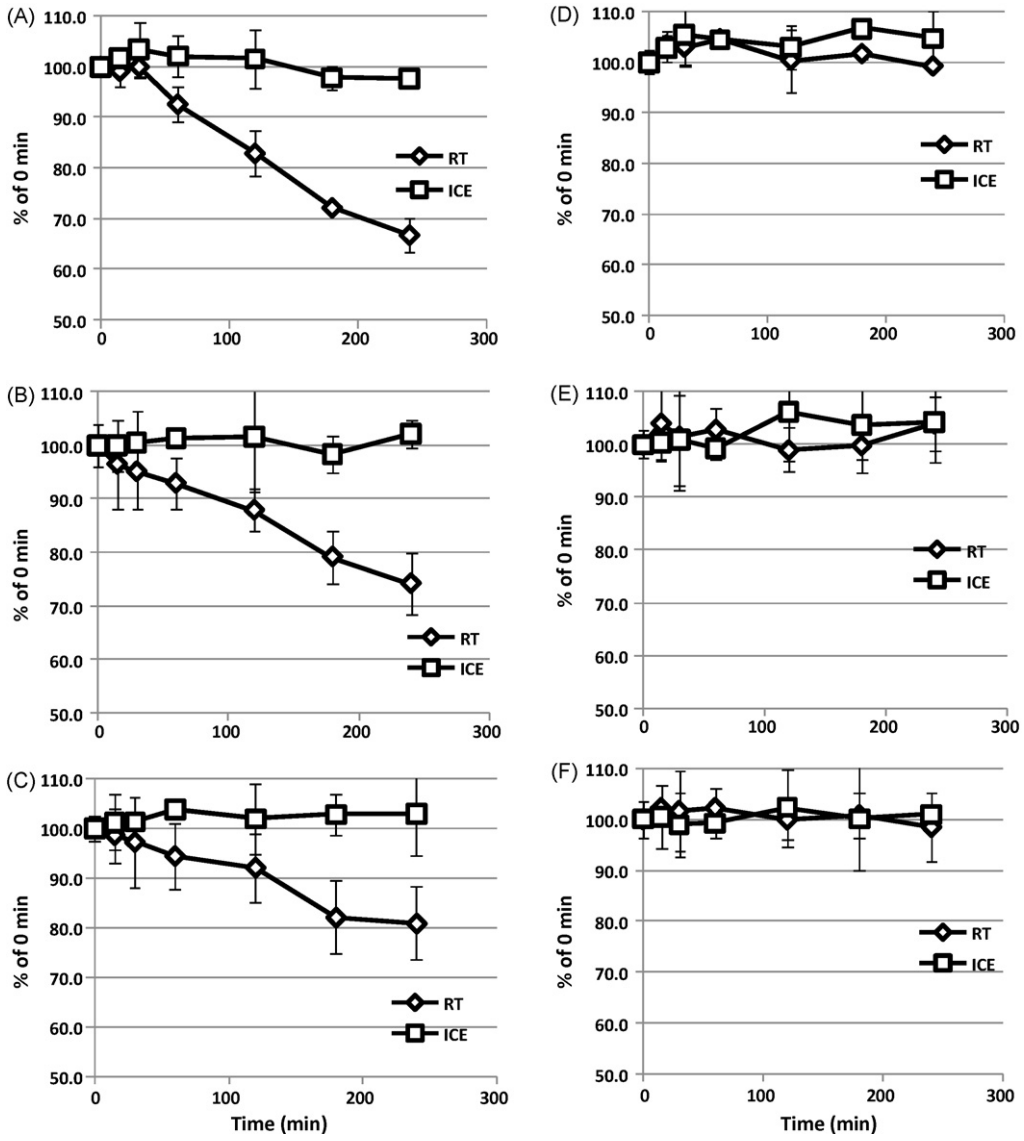


**Fig. 3.** Stability of (A) $D_4$-AEA, (B) $D_2$-OEA, (C) $D_4$-PEA spiked in purchased human whole blood; stability of (D) $D_4$-AEA, (E) $D_2$-OEA, (F) $D_4$-PEA spiked in purchased human whole blood in the presence of PMSF.

either immediately processed to generate plasma (within 5 min of collection) or incubated at room temperature or on ice for different periods of time. This experiment was designed to mimic the conditions in a clinical study. PMSF was not used in order to avoid complicating the clinical study sample collection procedure, which will be developed based on the results of this experiment. The concentrations of AEA, OEA, and PEA were measured and plotted to a concentration–time relationship (Fig. 5). In contrast to the results obtained from purchased blood, where the concentrations remained largely unchanged, the endogenous ethanolamides elevated quite rapidly in fresh blood. The initial mean concentration of AEA in plasma separated from fresh whole blood was $0.242 \pm 0.086$ ng/mL. It increased ~3-fold to $0.627 \pm 0.107$ ng/mL at room temperature by the end of the 2 h incubation, and ~2-fold to $0.496 \pm 0.081$ ng/mL on ice (Fig. 5A). The initial plasma concentrations of OEA and PEA were $1.587 \pm 0.481$ and $2.673 \pm 0.447$ ng/mL, respectively. They also elevated at room temperature (~1.8-fold for OEA and ~1.5-fold for PEA) and to a less extent on ice (~1.5-fold for OEA and ~1.3-fold for PEA) (Fig. 5B and C). This set of observations suggests that biosynthesis/release of ethanolamides from blood cells is more predominant than hydrolysis in determining the *ex vivo* change of ethanolamide concentrations in fresh blood. If the plasma is not separated from blood cells promptly, the concentrations of ethanolamides could be significantly overestimated. Based the observations made in this set of experiments, it is critical to minimize the time and standardize the blood collection and processing procedure for clinical studies.

### *3.4. Phase 0 study—diurnal variations/food effects of ethanolamide levels*

In order to use plasma ethanolamides as biomarkers of FAAH activity, it is important to understand the biological variability of plasma ethanolamide levels. Naturally occurring variability such as those caused by diurnal changes and food intake may impact plasma ethanolamide concentrations and complicate the interpretation of the biomarker data obtained in clinical trials. It has been reported that AEA, OEA, and PEA in rat brain exhibit diurnal variations [26,27]. However, to our knowledge, the diurnal variations in human have never been characterized. To better understand the variability of plasma ethanolamides in humans, a Phase 0 study was conducted. The Phase 0 study did not involve administration

of any novel or marketed pharmaceutical agent. It was a single-center study that simulated typical single-ascending dose and multiple-ascending dose studies in population, diet, and activity restrictions. Plasma samples were collected for the measurement of basal ethanolamide levels. To control the *ex vivo* change in plasma ethanolamide concentrations, a standard procedure was used which included: (1) the maximal time allowed from blood collection to start of centrifugation for plasma separation would be less than 3 min; (2) because low temperature slows the elevation process, the blood would be collected into pre-chilled vacutainers and all the samples would be kept on ice; (3) the overall time between blood collection and freezing the plasma would be less than 15 min; (4) all samples would be processed in a consistent manner. By taking these precautions, the *ex vivo* change of ethanolamide levels was expected to be kept to the minimum.

The results of the Phase 0 study for AEA are demonstrated in Fig. 6. It was found that the mean plasma AEA concentration from the 8 subjects around 8 a.m. on Day 1 was $0.257 \pm 0.066$ ng/mL ($n = 8$). It fluctuated around ~0.20–0.25 ng/mL before 12 p.m. After the standardized meal was given at noon, mean AEA concentration declined substantially to $0.154 \pm 0.036$ ng/mL at 14 p.m., and continued to drop to $0.126 \pm 0.022$ ng/mL at 16 p.m., although it was not clear whether this was a food induced effect or a diurnal effect. The concentration flattened at 18 p.m. at $0.130 \pm 0.011$ ng/mL before another standardized meal was given. After the meal, the concentration then further declined slightly to $0.108 \pm 0.007$ ng/mL. During the evening and night, AEA concentration gradually recovered, increasing to $0.172 \pm 0.059$ ng/mL at 8 a.m. on Day 2, and to $0.257 \pm 0.098$ ng/mL at noon, which was almost the same level as on Day 1. After the meal was given, a decline to the same extent as Day 1 was again observed. Shown in Fig. 7, the basal plasma concentration of (A) OEA and (B) PEA was $1.845 \pm 0.501$ and $1.819 \pm 0.439$ ng/mL, respectively. Nearly identical changes to that of AEA were observed during the two-day study, as depicted in the figure. Overall, plasma AEA, OEA, and PEA exhibited large intra-day variations, which may be caused by food intake and circadian rhythm. Especially, the first meal after an extended fast seemed to correlate with a significant decline in plasma concentrations of AEA, OEA, and PEA, although additional studies with varying times of meal administration would be needed to confirm this. Also significant, it was found there was large inter-subject variability in plasma ethanolamide levels. Evident in Figs. 6 and 7, the inter-subject variability appeared more significant during the waking hours than at night in most cases.

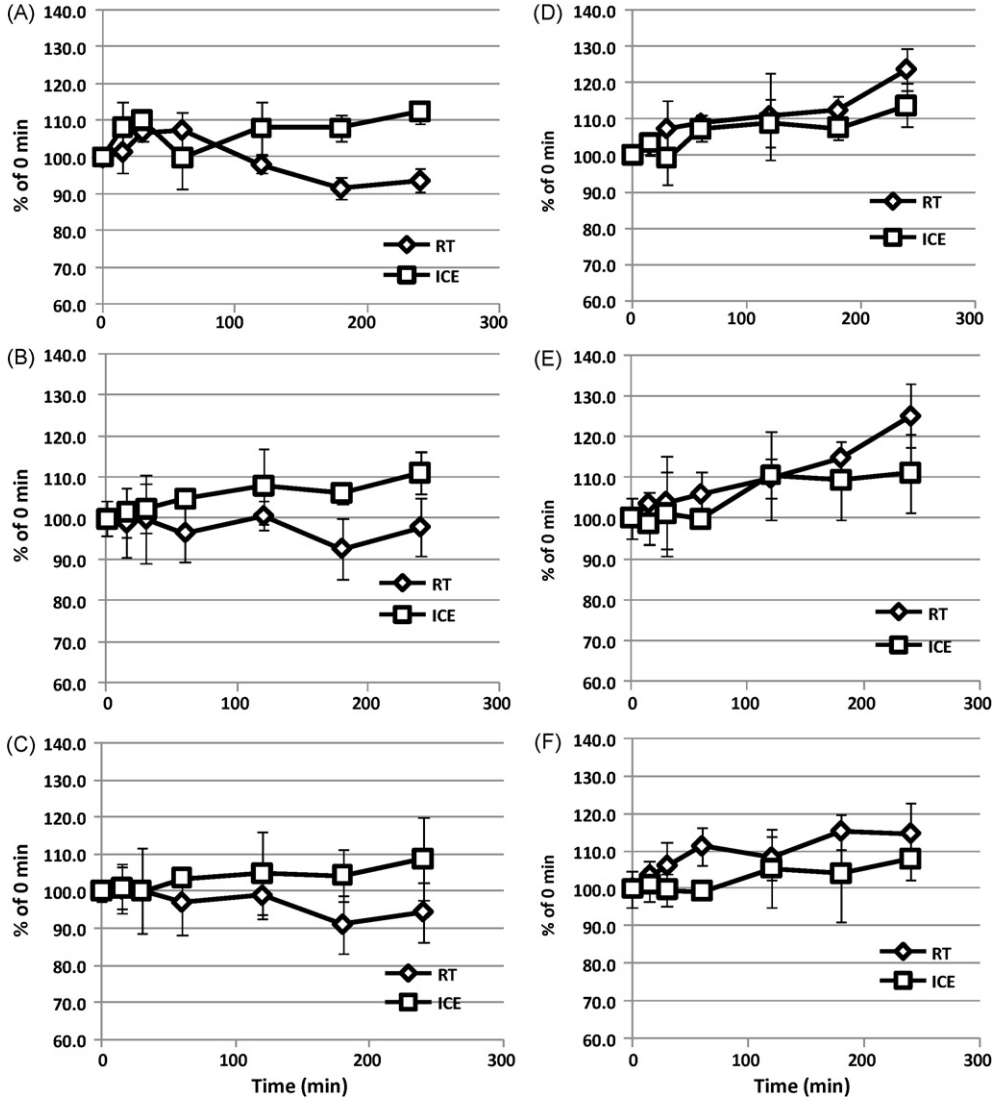


**Fig. 4.** Stability of endogenous (A) AEA, (B) OEA, (C) PEA in purchased human whole blood; stability of endogenous (D) AEA, (E) OEA, (F) PEA in purchased human whole blood in the presence of PMSF.

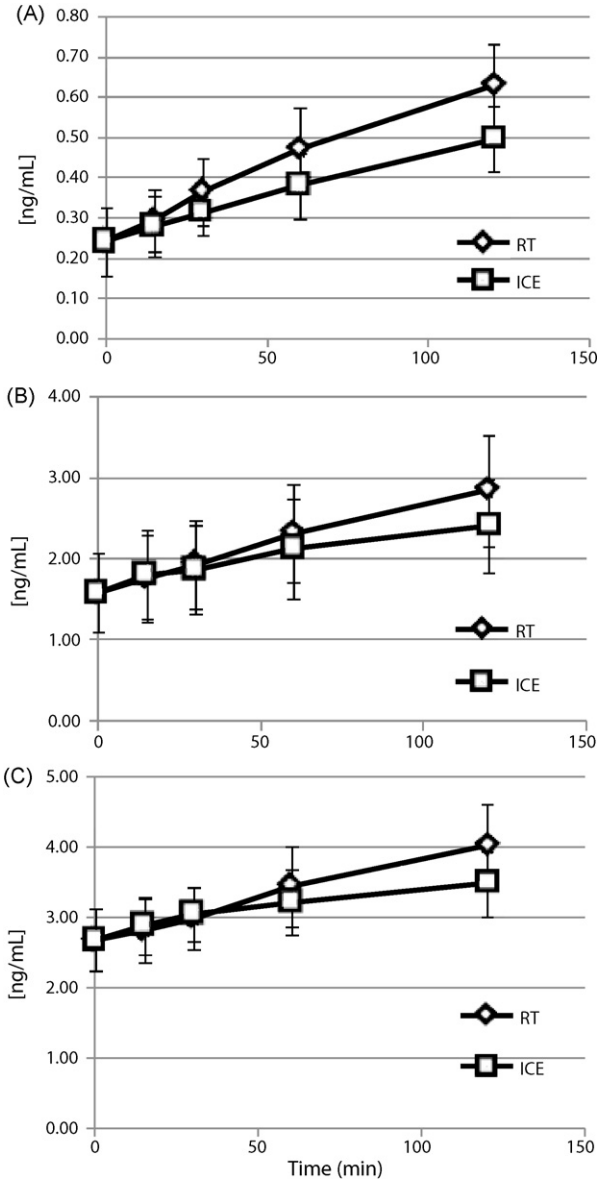


**Fig. 5.** Mean concentrations of endogenous (A) AEA, (B) OEA, (C) PEA in plasma generated from human whole blood incubated at different time intervals immediately after collection from healthy blood donors ($n = 6$). PMSF was not used in this experiment.

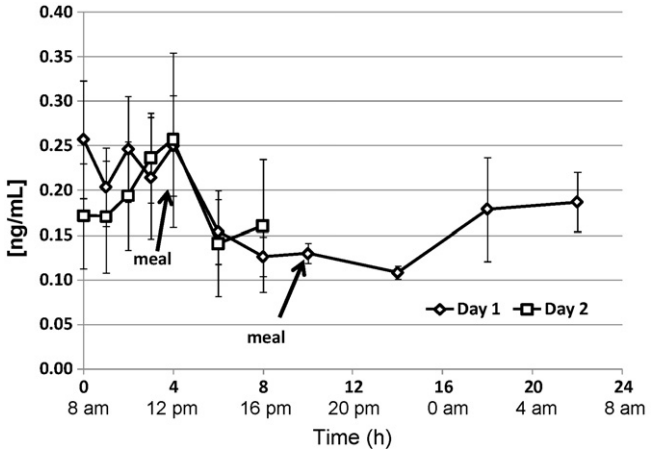


**Fig. 6.** Mean concentration–time profile of AEA obtained from healthy subjects ($n = 8$) in the Phase 0 study.

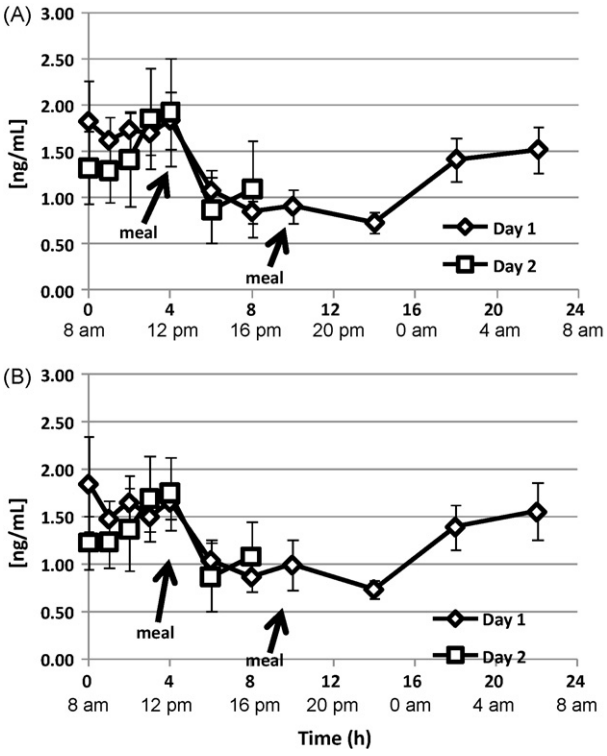


**Fig. 7.** Mean concentration–time profile of (A) OEA and (B) PEA obtained from healthy subjects ($n = 8$) in the Phase 0 study.

The above observations on variations of plasma ethanolamide levels provide valuable information that can facilitate the design of a Phase I study for elucidation of any changes in ethanolamide levels caused by drug treatment. First of all, due to the large inter-subject variability, the Phase I study should conduct intra-subject comparisons rather than a comparison between placebo-treated versus drug-treated subjects. Secondly, the changes of ethanolamide concentrations during the day preclude a comparison between 0 h (pre-dose) and the following time points. Rather, the ethanolamide levels on the day before drug treatment (Day −1) should be used as the baseline and compared to those obtained on the day of drug treatment (Day 1). Finally, because the time-frame and magnitude of changes in ethanolamide levels that would be caused by drug treatment may not be large, the probability to detect the changes reliably could be increased by serial blood sampling for ethanolamides as was used in this Phase 0 study. Although logistically challenging and expensive, serial sampling would allow an area under the curve (AUC) approach to compare the concentrations on Day −1 to those of Day 1, as typically done in pharmacokinetic studies. After trends are elucidated, it may be possible to reduce the blood sampling to fewer points taken at key times post-dose.

## 4. Conclusions

We described a highly selective, sensitive, and high-throughput LC–MS/MS assay for simultaneous quantitation of ethanolamides, including AEA, OEA, and PEA in human plasma. Unlike the previously reported methods, this assay used a “surrogate analyte” strategy which reduced the issue of potential interference from the endogenous analytes. The assay demonstrated excellent accuracy, precision, linearity, specificity for the intended purpose of using plasma AEA, OEA, and PEA as biomarkers in clinical trials. The lower limit of quantitation was sufficient to capture the basal levels of all the analytes in human plasma. Stability of the analytes was also

thoroughly investigated in current study, and it was found that plasma AEA, OEA, and PEA demonstrated good bench-top stability, freeze–thaw stability, and long-term frozen stability. In whole blood, it was confirmed that AEA, OEA, and PEA are subjected to two processes, one being *de novo* synthesis and release from blood cells, the other being the enzymatic hydrolysis by FAAH. It was found that AEA, OEA, and PEA concentrations elevated significantly in freshly collected blood, indicating that *de novo* synthesis and release from blood cells were the predominant processes affecting ethanolamide levels. Accordingly, appropriate sample collection and processing procedures for clinical studies were established to minimize any *ex vivo* changes in concentration. Most importantly, the assay was applied to a Phase 0 study which enrolled healthy volunteers. Large inter-subject and intra-subject variations in plasma ethanolamides were observed, attributable in part to diurnal variations and the ingestion of food. Variability in ethanolamide concentrations and factors which may contribute to this variability should be taken into consideration during the design of clinical studies and interpretation of data produced. The results of the analytical and clinical studies described in this paper indicate plasma ethanolamide levels are a potentially useful pharmacodynamic marker for determining FAAH target engagement.

## Acknowledgments

The authors gratefully acknowledge the technical support and valuable input from Rhys Salter, Charlene Abrams, Mark J. Karbarz, Wende Wu, Nenad Sarapa, and Gary Romano. We also thank Prof. Gerard Hopfgartner, University of Geneva, for helpful advice regarding the specificity experiments with the API 4000 QTrap mass spectrometer.

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