Journal of Chromatography B, 878 (2010) 3231–3237

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantitative analysis of anandamide and related acylethanolamides in human seminal plasma by ultra performance liquid chromatography tandem mass spectrometry

Akwasi A. Amoako[a], Timothy H. Marczylo[a], Patricia M.W. Lam[a], Jonathon M. Willets[a], Amanda Derry[b], Janine Elson[b], Justin C. Konje[a,*]

[a] Endocannabinoid Research Group, Reproductive Science Section, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester LE2 7LX, United Kingdom
[b] Assisted Conception Unit, University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary, Leicester LE1 5WW, United Kingdom

ARTICLE INFO

Article history:
Received 22 May 2010
Accepted 24 September 2010
Available online 1 October 2010

Keywords:
Endocannabinoids
Anandamide
Oleoylethanolamide
Palmitoylethanolamide
Seminal plasma
Mass spectrometry

ABSTRACT

The endocannabinoids anandamide, palmitoylethanolamide and oleoylethanolamide have been detected in human seminal plasma and are bioactive lipids implicated in regulation of sperm motility, capacitation and acrosome reaction. Several methods exist for endocannabinoid quantification but none have been validated for measurement in human seminal plasma. We describe sensitive, robust, reproducible solid phase and isotope-dilution UHPLC-ESI-MS/MS methods for the extraction and quantification of anandamide, palmitoylethanolamide and oleoylethanolamide in human seminal plasma. Precision and accuracy were evaluated using pooled seminal plasma over a 4 day period. For all analytes, the inter- and intraday precision (CV%) was between 6.6–17.7% and 6.3–12.5%, respectively. Analyses were linear over the range 0.237–19 nM for anandamide and oleoylethanolamide and 0.9–76 nM for PEA. Limits of detection (signal-to-noise >3) were 50, 100 and 100 fmol/mL and limits of quantification (signal-to-noise >10) were 100, 200 and 200 fmol/mL, respectively for anandamide, palmitoylethanolamide and oleoylethanolamide. Anandamide and oleoylethanolamide were stable at −80 °C for up to 4 weeks, but palmitoylethanolamide declined significantly. We assessed seminal plasma from 40 human donors with normozoospermia and found mean (inter-quartile range) concentrations of 0.21 nM (0.09–0.27), 1.785 nM (0.48–2.32) and 15.54 nM (7.05–16.31) for anandamide, oleoylethanolamide and palmitoylethanolamide, respectively. Consequently, this UHPLC-ESI-MS/MS method represents a rapid, reliable and reproducible technique for the analysis of these endocannabinoids in fresh seminal plasma.

1. Introduction

Cannabis, is known to affect semen quality. Its active component Delta-9-tetrahydrocannabinol (Δ-THC) binds to cannabinoid receptors. The endocannabinoids are an important group of bioactive lipid signalling molecules. Anandamide (*N*-arachidonylethanolamide, AEA) is the most extensively studied endocannabinoid and is known to activate cannabinoid receptors (CB1 and CB2) which are widely distributed in most central and peripheral tissues including the reproductive system [1–3]. Recent evidence suggests that AEA also activates non-CB1 and non-CB2 receptors including the ion-channel transient receptor potential vanilloid (TRPV1) receptor [4]. AEA is purported to be co-synthesized with palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) from cell membrane phospholipid precursors in response to depolarizing agents, neurotransmitters, and hormones [5,6]. PEA and OEA are not agonists for the CB1 and CB2 receptors. Their exact biological effect and molecular mechanism by which these occur remain elusive but they are purported to act as entourage compounds enhancing the biological activity of AEA by inhibiting its degradation by fatty acid amide hydrolase (FAAH). Significant levels of AEA, PEA and OEA have been detected in human follicular fluid and seminal plasma at nanomolar concentrations [7–9]. Recent studies have also revealed that OEA and PEA have antioxidant, antimicrobial and anti-inflammatory properties and play a significant role by protecting the sperm cell membrane from peroxidative damage [10–12]. Spermatozoa are sequentially exposed to declining levels of AEA as they swim from the ejaculate deposited in the vagina to the fertilization site in the oviductal ampulla [9]. Changes in the concentrations of these endocannabinoids may have significant adverse effects on sperm quality. To be able to investigate these potential changes, it is essential to have a reliable robust method of quantifying endocannabinoids in seminal plasma. Although AEA, PEA and OEA have previously been detected in seminal plasma [8,9] the methodology utilised in these publications had not been validated.

* Corresponding author. Tel.: +44 116 252 5826; fax: +44 116 252 5846.
E-mail address: jck4@le.ac.uk (J.C. Konje).

1570-0232/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2010.09.024

Furthermore, AEA extraction from biofluids using silica chromatography, as used in these published studies, but not with C18 solid phase, greatly overestimate concentrations due to deuterium loss from internal standards [13]. Ex vivo processing times have a significant effect upon levels of AEA in some biofluids and tissues [14,15]. The aims of this study were therefore to develop and validate methods for sample handling, AEA extraction using C18 solid-phase extraction and quantification of *N*-acylethanolamides (NAEs) by UHPLC-ESI-MS/MS in human seminal plasma.

2. Materials and methods

2.1. Materials

Solvents and ammonium acetate were of HPLC grade. AEA and formic acid were from Sigma Aldrich (Poole, Dorset, UK), PEA, OEA, and the deuterated NAEs (AEA-d8, PEA-d4 and OEA-d2) each of >98% purity (and >99% deuterated content) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetonitrile, chloroform, methanol, and ammonium acetate were purchased from Fisher Scientific (Loughborough, UK) and HPLC grade water was obtained using a water purification system (Maxima ELGA, ELGA, High Wycombe, UK). Mobile phases were filtered through 0.2 μm, 47 mm diameter PTFE filters (Waters UK Ltd., Elstree, UK) prior to use. Oasis HLB solid phase extraction (SPE) cartridges (1cc, 30 mg) were purchased from Waters UK Ltd.

2.2. Instrument and chromatographic conditions

Quantitative analysis of AEA, PEA and OEA was performed with a UHPLC-ESI-MS/MS system comprising an Acquity UHPLC coupled to a Quattro Premier tandem mass spectrometer (Waters UK Ltd.). Separation was achieved using an Acquity UHPLC BEH C_{18} (2.1 mm × 50 mm, 1.7 μm) column maintained at 40 °C. Mobile phases were A (2 mM ammonium acetate containing 0.1% formic acid, 5% acetonitrile) and B (acetonitrile containing 0.1% formic acid). LC gradient conditions were: 0–0.5 min, 20% B; 2.5 min, 100% B; 3.5 min, 20% B then re-equilibrated at 20% B until 4.0 min. The flow rate was 0.7 mL/min. Samples were maintained at 4 °C throughout. Analytes were quantified using electrospray ionization tandem mass spectrometry in positive ion mode (ES^+). Source parameters included capillary voltage of 1 kV, cone voltage 21 V, source temperature 120 °C, desolvation temperature 440 °C, cone gas flow 50 L/h and desolvation gas flow 800 L/h. Subsequently, MS/MS conditions for monitoring each precursor $[M+H]^+$ ion comprised entry, collision and exit energies of 6, 16 and 2 eV, respectively. Product ions were monitored in selected reaction monitoring (SRM) mode. Mass transitions were as follows: AEA (*m*/*z* 348.3 > 61.9), AEA-d8 (*m*/*z* 356.4 > 63.0), OEA (*m*/*z* 326.5 > 61.9), OEA-d2 (*m*/*z* 328.4 > 61.9), PEA (*m*/*z* 300.4 > 61.9) and PEA-d4 (*m*/*z* 304.4 > 61.9) (Fig. 1). Injection volumes for samples and standards were 7 μL. Seven-point combined AEA, OEA and PEA calibration curves in triplicate spiked with internal standards were performed. Peaks from standards and analytes were integrated using Masslynx software version 4.1. Quanlynx software calculated the concentration of each NAE using calibration curves of concentration against relative response calculated as follows:

$$\text{Relative response}\,(y) = \frac{\text{Peak area}}{(\text{IS area}/[\text{IS}])}$$

2.3. Standard solutions

AEA was diluted in acetonitrile to make a stock solution of 5 mg/mL. AEA-d8 stock solutions were prepared by drying the supplied stock under nitrogen and reconstituting in acetonitrile to a concentration of 100 μg/mL. OEA and PEA were dissolved in ethanol at 10 mg/mL and 2.5 mg/mL, respectively. OEA-d2 (1 μg/μL) and PEA-d4 (1 μg/μL) were supplied as ethanol stocks. All stock solutions were stored at −20 °C.

2.4. Sample preparation and analysis

This study was approved and conducted according to the guidelines of the Leicestershire and Rutland local research ethics committee. All participants signed informed consent to take part in the study. Seminal fluid was obtained from men who attended the Andrology Unit of the Leicester Royal Infirmary for routine semen analysis. Seminal fluid was collected by masturbation into a sterile plastic container after 2–5 days of sexual abstinence. Samples were allowed to liquefy at room temperature for 1 h. From an aliquot of the seminal fluid, the standard seminal parameters were examined according to the World Health Organization (WHO) criteria. Semen parameters were characterized based on WHO reference normal values (World Health Organization, 1999) (semen volume >2 mL, sperm concentration $>20 \times 10^6$/mL, sperm number per ejaculum $>40 \times 10^6$ and sperm motility >50% motile sperm). Excluded from this study were donors with previous or current use of recreational drugs, current use of any medication known to affect semen quality (e.g. sulphasalazine), presence of any systemic disease like diabetes or hypertension or a history of vasectomy.

Samples were transported to the analytical laboratory on ice and processed within 2 h of production. Seminal fluid was transferred into a clean 7 mL Kimble scintillation vial (Kinesis, St. Neots, Cambs, UK) and centrifuged at 1200 × *g* for 30 min at 4 °C to separate seminal plasma from spermatozoa and cellular debris. The supernatant was transferred into a clean 7 mL Kimble scintillation vial. Endocannabinoid extraction was either performed immediately or the seminal plasma stored at −80 °C for later analysis.

Previously described liquid phase (LPE) and solid phase (SPE) techniques for the extraction of AEA from bio-fluids were employed with slight modifications for the extraction of endocannabinoids from the seminal plasma [16]. For the SPE method seminal plasma (0.5 mL) was diluted to 1 mL by adding 5% phosphoric acid and spiked with 2.5 pmol/mL AEA-d8, 2.5 pmol/mL OEA-d2 and 5 pmol/mL PEA-d4. Samples were thoroughly mixed by vortexing for 10 s and were then centrifuged at 1200 × *g* for 2 min at 4 °C. They were next loaded onto Oasis HLB 1cc cartridges which had been preconditioned and equilibrated with 1 mL of methanol and 1 mL of distilled H_2O. The samples were drawn through the cartridges under gentle vacuum at a flow rate of approximately 1 mL/min using a Vacmaster vacuum manifold (Biotage, Uppsala, Sweden). The cartridges were washed with 1 mL of 40% aqueous methanol, and AEA, PEA and OEA were eluted in 1 mL of acetonitrile. The eluant was evaporated to dryness under a gentle stream of nitrogen. The dried extract was reconstituted in 80 μL of acetonitrile, transferred into a clean HPLC vial and loaded onto the UHPLC–MS/MS system for quantification of these NAEs [16,17]. Liquid phase extractions were performed using the technique described previously for plasma [17,18]. Samples of seminal plasma (2 mL) were transferred into clean 7 mL Kimble scintillation vials and spiked with 1.25 pmol/mL of AEA-d8, 1.25 pmol/mL of OEA-d2 and 2.5 pmol/mL of PEA-d4 internal standard before thorough mixing with a desktop vortexer. To precipitate protein, 2 mL of ice-cold acetone was added to the sample and centrifuged at 1200 × *g* for 10 min at 4 °C. The supernatant was transferred to a clean Kimble scintillation vial and was evaporated under a gentle stream of nitrogen to remove the acetone. Methanol: chloroform (2 mL, 1:2 (v/v)) was added to the remaining sample and gently mixed by repeated inversion followed by centrifugation at 1200 × *g* for 10 min at 4 °C. The

lower chloroform layer was aspirated and transferred into a fresh Kimble vial. The extract was evaporated to dryness under a gentle stream of nitrogen and then reconstituted in 80 μL of acetonitrile, vortexed for 30 s, transferred into a clean HPLC vial and loaded onto the UHPLC–MS/MS system for quantification of NAEs [16,17]. To determine if any discrepancies in endocannabinoid measurements might be a consequence of the extraction procedure, pooled seminal plasma was divided into two aliquots of 0.5 mL and 2 mL for SPE and LPE, respectively ($n = 6$). AEA, OEA and PEA were quantified and the extraction efficiencies and calculated concentrations compared. To estimate the efficiency of the extraction procedures, peak areas obtained for 2.5 pmol AEA-d8 and OEA-d2 and 5 pmol PEA-d4 extracted from seminal plasma using each extraction technique were compared with 2.5 pmol AEA-d8 and OEA-d2 and 5 pmol PEA-d4 standards in 80 μL acetonitrile.

2.5. Calibration and linearity

Seven-point calibration curves were prepared in triplicate from stock solutions in acetonitrile on ice prior to each experiment at concentrations of 0.2375–19 pmol/mL for AEA and OEA and 0.9–76 pmol/mL for PEA spiked with deuterated internal standards (IS, 12.5 pmol/mL of AEA-d8, PEA-d4 and OEA-d2). Concentration ranges were chosen based upon the expected concentrations of NAEs in seminal plasma from previously published work [8] allowing for overestimation due to deuterium transfer as discussed in Section 1 [13]. Calibration standards were analysed by UHPLC-MS/MS. The slope, intercept, and coefficient of correlation of these calibrations were determined by linear regression analysis. To ascertain the consistency of calibration curves, calibrations were performed on three separate days for comparison.

2.6. Limits of detection and quantification

All seminal fluid samples investigated contain endogenous levels of NAEs consequently, limits of detection (signal-to-noise ratio >3) and quantification (signal-to-noise ratio >10) were determined for the deuterated internal standards. Seminal fluid samples (0.5 mL) were spiked with decreasing concentrations of deuterated NAE standards ($n = 3$) and extracted by SPE. Peaks corresponding to the deuterated standards in the spectrograms were analysed for signal-to-noise ratio using Masslynx and LOD and LOQ ascribed to concentrations consistently giving ratios of 3 or more and 10 or more, respectively.

2.7. Recovery and matrix effects

Recovery for each extraction method was determined using the deuterated standards ($n = 20$): Plasma samples were spiked with deuterated internal standards as described above and extractions performed. The same amounts of these standards were then spiked into acetonitrile (final volume 80 μL) to make a non-extracted control. Recovery was determined by the relative peak areas of standards in sample compared with non-extracted control. Matrix effects may confound these recovery values so were also investigated using deuterated standards. For this purpose seminal plasma (0.5 mL, $n = 3$) was extracted using SPE without the addition of deuterated standards. Dried sample was re-suspended in acetonitrile containing deuterated standards and matrix effects determined by the peak areas of these samples relative to a non-extracted control.

2.8. Precision and accuracy

Precision of the UHPLC-ESI-MS/MS method was assessed using QC samples at high, medium and low concentrations of AEA (19, 0.95 and 0.48 nM), OEA (19, 4.75 and 0.95 nM) and PEA (38, 19, and 7.6 nM). Precision was determined following 16 injections of these QCs. Accuracy was calculated following nine injections of three concentrations of AEA (3.33, 6.65 and 133 fmoles on column), OEA (6.65, 33.3 and 133 fmoles on column) and PEA (33.3, 66.5 and 133 fmoles on column).

Inter-day and intra-day variability of measured NAE concentrations in seminal plasma were determined in aliquots of pooled seminal plasma (0.5 mL) frozen at −80 °C. NAE levels were determined in batches of five over a 4 day period using SPE and variability expressed as the relative standard deviations (RSD = (SD/Mean) × 100%). As NAEs are present in all seminal plasma samples, inter- and intra-day variability was determined only at one concentration i.e., the actual NAE content of human seminal plasma.

2.9. Stability

To investigate the effect of processing time on the concentrations of the endocannabinoids, samples ($n = 5$) were divided into aliquots. One aliquot was processed within 2 h of sample production whilst the second aliquot was processed 4 h after sample production. Processing times include the 1 h required for liquefaction. Times were based upon a reasonable time for sample handling by the Andrology Unit and transfer to our laboratories (2 h) and that significant ex vivo increases in AEA concentrations have been observed by 4 h in previously investigated tissues (blood, placenta, and foetal membranes) [15]. Initially the stability of NAEs in seminal plasma samples during storage at −80 °C was investigated in matched samples after a single freeze-and-thaw cycle. Samples with previously quantified NAE levels on the day of collection ($n = 5$), were thawed on ice after storage at −80 °C for 1 and 4 weeks, extracted and then analysed. Extraction was performed by the SPE method. Values obtained were compared with those obtained on the day of sample collection and stability expressed as the percentage change in concentration. In addition, pooled seminal plasma was assessed for the effect of multiple (1–3) freeze–thaw cycles upon NAE concentrations ($n = 4$ per cycle). For each cycle, sample was flash frozen in liquid nitrogen for at least 15 min then thawed on ice prior to sample extraction.

2.10. Method application

Men attending the Andrology Unit gave written informed consent and were recruited onto this study. Only seminal plasma from those men with normal seminal parameters ($n = 40$) according to the WHO guidelines was used for these investigations. Seminal plasma was obtained and NAE's extracted by SPE as described above and levels of NAEs determined by UHPLC-ESI-MS/MS.

2.11. Statistical analysis

Data analysis was performed within GraphPad Instat version 3, Graphpad Software, San Diego, CA. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Comparison of SPE and LPE methods for the extraction of AEA, PEA and OEA

No significant differences were observed for NAE concentrations in matched seminal plasma samples ($n = 8$) whether SPE or LPE extraction was used. The mean ± SEM were as follows: AEA 0.19 ± 0.07 and 0.20 ± 0.07; OEA 2.23 ± 0.46 and 1.98 ± 0.43 and PEA 21.83 ± 6.7 and 21.63 ± 6.7 for SPE and LPE, respectively.

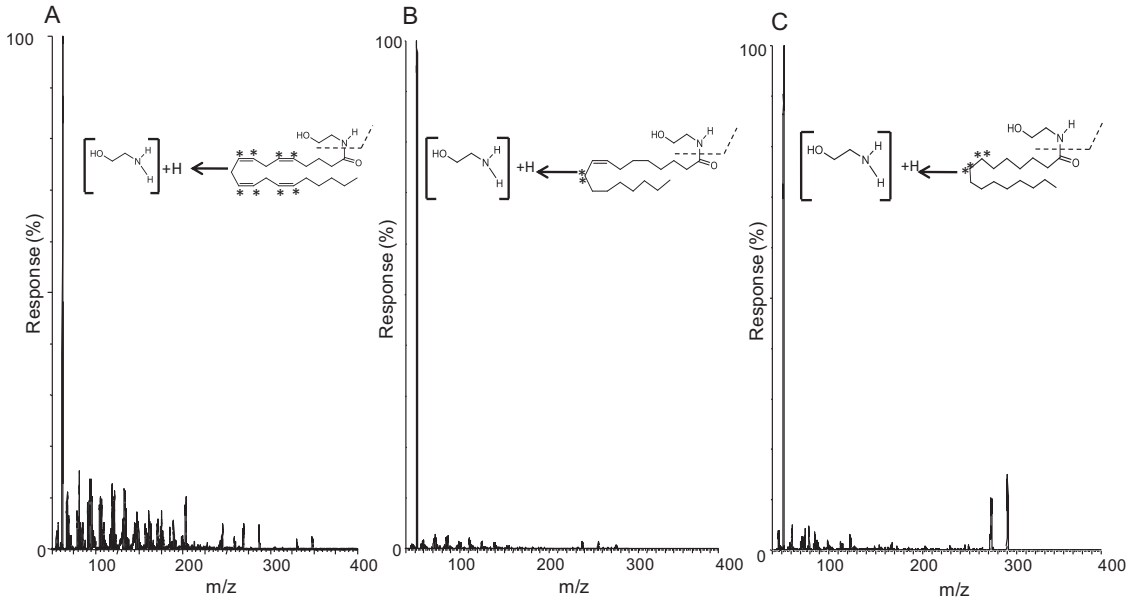

Fig. 1. SRM daughter ion spectrograms for AEA (A), OEA (B) and PEA (C) showing predominant daughter ions at 62 amu for each NAE. Structures of the NAEs are presented with proposed fragmentation (—) and sites of deuteration (*).

Regression analysis of a plot of SPE against LPE for each analyte showed a linear response with R^2 values of 0.998 for AEA, 0.995 for OEA and 0.996 for PEA (Fig. 2). Bland–Altmann analysis of individual samples analysed using both extraction techniques shows a percentage variation within the preset limit of 20% (Fig. 2). Although no significant differences in seminal plasma NAE concentrations were observed using the two extraction methods, the extraction efficiencies for NAEs from seminal plasma using SPE were double those achieved with LPE (Table 1). Consequently, the SPE technique required a smaller sample volume than LPE (0.5 mL versus 2 mL, see Section 2). This is particularly advantageous as ejaculate volumes may be less than 2 mL in many of the samples obtained (range: 0.5–6.0 mL). Extracts obtained following SPE were assessed for matrix effects using spiked deuterated standards. Ion suppres-

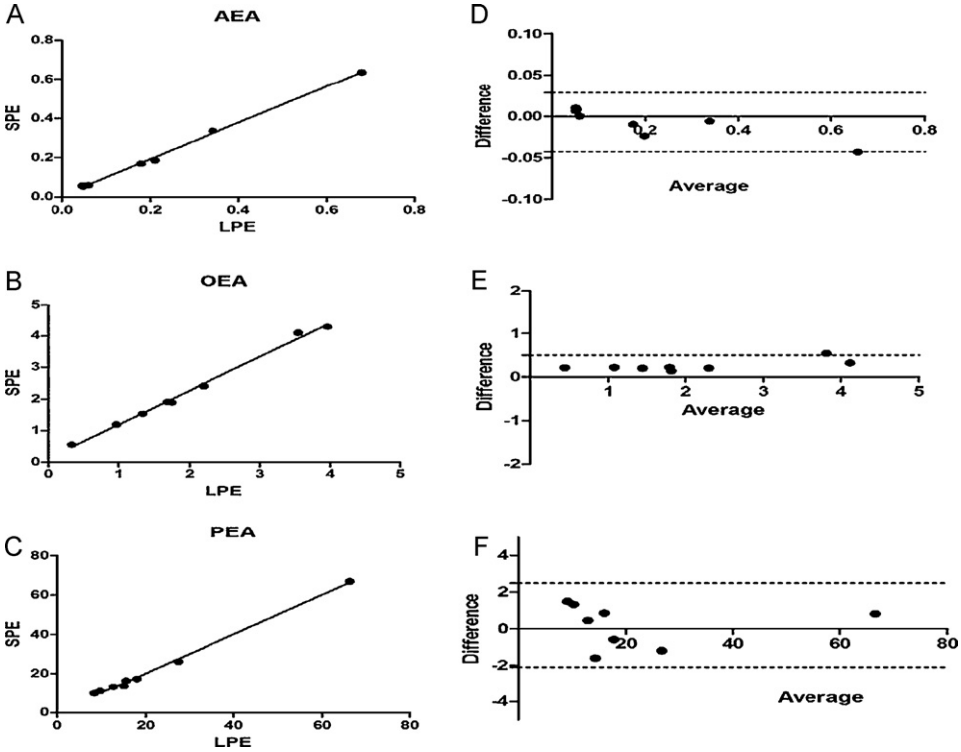

Fig. 2. Comparison of SPE and LPE extraction techniques ($n = 8$) for the quantification of AEA, PEA and OEA in human seminal plasma. Linear regression analysis shows a near perfect correlation between SPE and LPE for (A) AEA, $R^2 = 0.998$; $p < 0.0001$ (B) OEA, $R^2 = 0.995$; $p < 0.0001$ and (C) PEA, $R^2 = 0.996$; $p < 0.0001$. Bland–Altmann plot of difference versus average shows good 95% limits of agreement between the two extraction techniques for (D) AEA, (E) OEA and (F) PEA. The deviations between samples are all within 20%.

Table 1
Comparison of extraction efficiencies between SPE and LPE.

	Mean extraction efficiency (%)	SEM	95% CI
SPE ($n = 14$)			
AEA	54	3.1	46.8–60.3
OEA	49	3.9	40.5–57.4
PEA	30	2.3	24.6–34.6
LPE ($n = 10$)			
AEA	21	2.2	15.5–26.1
OEA	25	2.9	18.6–31.9
PEA	14	1.7	10.0–17.5

sion was observed at 14%, 6% and 24% for AEA, OEA and PEA, respectively.

3.2. Validation of UHPLC-MS/MS analysis of AEA, PEA and OEA

AEA/AEA-d8, OEA/OEA-d2 and PEA/PEA-d4 were eluted from the UHPLC and detected by MS/MS at the following retention times; 2.27 ± 0.003 min (%RSD of 0.13%), 2.47 ± 0.004 min (%RSD of 0.16%) and 2.42 ± 0.004 min (%RSD of 0.17%), respectively (Fig. 3). In all seminal fluid samples a second peak with SRM of m/z 328.2 > 61.9 was observed at a retention time of 2.67 min (Fig. 3B and C). This peak had good baseline separation from the peak representing OEA-d2. Co-elution studies with authentic standard identified this peak to be representative of stearoylethanolamide (data not shown). This NAE was not quantified in these samples because of the lack of any available labelled standard. The inter-day ($n = 20$) and intra-day ($n = 5$) variability for AEA, PEA and OEA were as follows: 6.3% and 6.6% for AEA, 10.2% and 11.2% for OEA and 12.5% and 17.7% for PEA. For all analytes, the inter-day variability was within a CV% of 6.6–17.7% and that of intraday was 6.3–12.5%, all were within acceptable limits (Table 2). Regression analysis indicated that the plots for AEA, PEA and OEA standards were linear over the range of 0.237–19 nM for AEA and OEA and 0.9–76 nM for PEA and the correlation coefficient values (R^2) of the regression lines were greater than 0.99 (Table 2). Precision data for 133 fmol (19 nM) injections of AEA, OEA and PEA on column were calculated to have mean concentrations of 19.00 ± 0.58 nM, 19.00 ± 0.54 nM and 19.00 ± 1.03 nM, respectively. Precision for 3.33 fmol (0.48 nM), 6.65 fmol (0.95 nM) AEA; 6.65 fmol (0.95 nM), 33.25 fmol (4.75 nM) OEA; 53.2 fmol (7.6 nM) and 266 fmol (38 nM) PEA on column had means of 0.47 ± 0.03 nM, 0.93 ± 0.08 nM; 0.97 ± 0.04 nM, 4.91 ± 0.26 nM; 7.08 ± 0.22 nM and 38.82 ± 2.61 nM, respectively. RSD values were acceptable for all of these concentrations as they were below 10% (Table 2).

Accuracies for 3.33 fmol, 6.65 fmol and 133 fmol of AEA on column were 98.4 ± 8.4%, 95.1 ± 8.1% and 99.5 ± 4.3%, respectively. Accuracies for 6.65 fmol, 33.3 fmol and 133 fmol of OEA on column were 98.9 ± 5.3%, 104.9 ± 5.8 and 98.2 ± 4.1%, respectively. The accuracy for 33.3 fmol of PEA was 103.7 ± 13.0%, 100.5 ± 5.3% for 66.5 fmol and 98.2 ± 5.1% for 133 fmol (Table 2).

The limits of detection (LOD) and quantification (LOQ) were determined by spiking the human seminal plasma with decreasing amounts of deuterated AEA, OEA and PEA until concentrations at which the signal-to-noise ratio of >3 and >10 were achieved for LOD and LOQ, respectively. LODs were 50 fmol/mL, 100 fmol/mL and 100 fmol/mL for AEA, PEA and OEA, respectively and LOQs were 100 fmol/mL, 200 fmol/mL and 200 fmol/mL for AEA, PEA and OEA, respectively (Table 2). Limits were obtained for deuterated NAEs because all samples contained endogenous NAE that would confound determination of LOD and LOQ for non-deuterated NAEs.

3.3. Stability and sample handling

The levels of PEA remained relatively stable at 2 and 4 h ex vivo (9.273 ± 2.12 versus 9.730 ± 2.22; $p = 0.37$). There was an increase in the levels of AEA and OEA between 2 and 4 h, although this did not reach statistical significance (0.089 ± 0.014 versus 0.1435 ± 0.061; $p = 0.35$ for AEA and 1.060 ± 0.46 versus 1.501 ± 0.59; $p = 0.09$ for OEA). For AEA and OEA, a single freeze–thaw cycle did not significantly alter concentration over 4 week storage at −80 °C. However, there was significant variation in the concentration of PEA after a single freeze–thaw cycle. PEA levels declined significantly after 1 week ($p = 0.03$) and at 4 weeks ($p = 0.01$) when stored at −80 °C. These observations for repeated freeze–thaw cycles were consistent with the observed inter-day variability (Table 2). Concentrations of AEA, OEA and PEA were 0.15, 2.25 and 18.5 nM, respectively in fresh, pooled seminal plasma. AEA concentrations changed modestly with 13.3, 6.6 and 13.3% increases observed following 1, 2 and 3 freeze–thaw cycles. OEA likewise showed modestly higher concentrations with 13.7, 5.7 and 3.5% increases in concentration, respectively. Following 1 freeze–thaw cycle, PEA concentration increased by 0.6%, but 8.5% and 3.4% decreases in concentration were observed after 2 and 3 freeze–thaw cycles. Over the 4 measurements (fresh and three freeze–thaw cycles) the coefficients of variability for AEA, OEA and PEA showed very good consistency at 5.89, 5.52 and 4.34%, respectively.

3.4. Biological application

The validated method was applied to quantify the levels of AEA, OEA and PEA in the seminal plasma of 40 men with nor-

Table 2
Linearity ($n = 3$), intra- and inter-day coefficient of variation, LOQ and LOD of the UHPLC-ESI-MS/MS method for the quantification of AEA, OEA and PEA in human seminal plasma.

Parameter	AEA	OEA	PEA
Intra-day variability (CV%) $n = 5$	6.3	10.2	12.5
Inter-day variability (CV%) $n = 20$	6.6	11.2	17.7
LOD (fmol/mL)	50	100	100
LOQ (fmol/mL)	100	200	200
R^2 (±SD, $n = 3$)	0.998 ± 0.001	0.993 ± 0.003	0.990 ± 0.009
Slope (±SD, $n = 3$)	3.65 ± 0.19	2.63 ± 0.07	3.12 ± 0.85
Intercept (±SD, $n = 3$)	0.12 ± 0.06	0.60 ± 0.22	3.86 ± 1.30
Precision (RSD)			
High	3.1% (133 fmol)	2.9% (133 fmol)	5.4% (266 fmol)
Medium	8.8% (6.65 fmol)	5.4% (33.3 fmol)	5.4% (133 fmol)
Low	5.8% (3.33 fmol)	4.0% (6.65 fmol)	3.1% (53.2 fmol)
Accuracy			
High	99.5 ± 4.3% (133 fmol)	98.2 ± 4.1% (133 fmol)	98.2 ± 5.1% (133 fmol)
Medium	95.1 ± 8.1% (6.65 fmol)	104.9 ± 5.8 (33.3 fmol)	100.5 ± 5.3% (66.5 fmol)
Low	98.4 ± 8.4% (3.33 fmol)	98.9 ± 5.3% (6.65 fmol)	103.7 ± 13.0% (33.3 fmol)

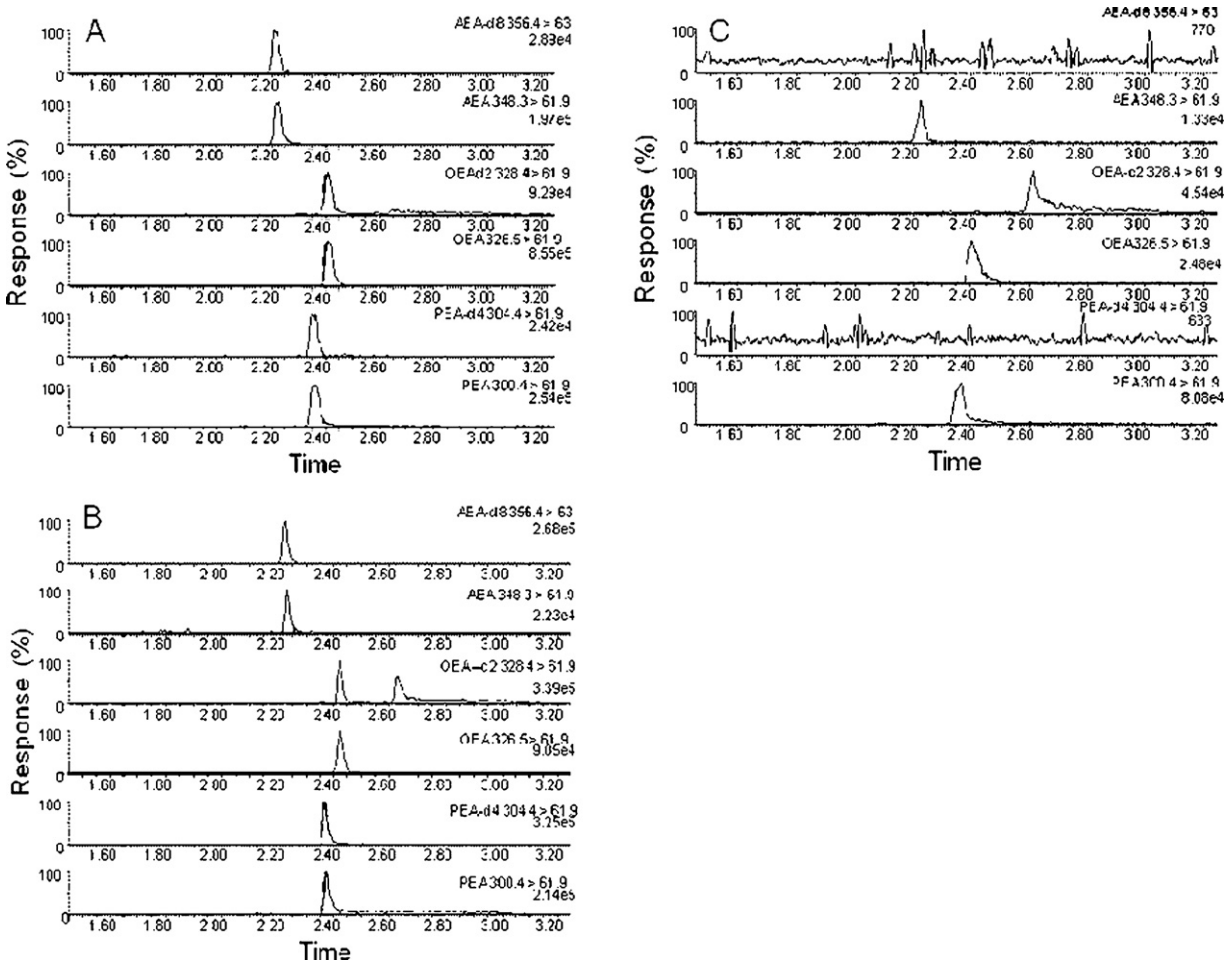

Fig. 3. Typical spectrograms for detection of *N*-acylethanolamides in a standard (A), a typical seminal plasma sample (B) and in seminal plasma without deuterated internal standards (C).

mal semen parameters. All samples were processed within 2 h of production. The mean (IQR) concentrations of 0.21 nM (0.09–0.27), 1.785 nM (0.48–2.32) and 15.54 nM (7.05–16.31) for AEA, OEA and PEA, respectively (Table 3).

4. Discussion

Since the presence of AEA, OEA and PEA in human seminal plasma was first reported [8] there has been growing interest in the exact role these compounds play in sperm function and there have been suggestions that at physiological levels they may influence key processes controlling sperm function and gamete interaction. Despite this, quantification of AEA in human seminal plasma has only been conducted in a very small number of volunteers with unknown fertility status [8]. To date no attempt has been made to establish the normal physiological levels of these compounds and whether there is any relationship between sperm functions and levels of the NAEs. This may in part be due to methodological difficulties in quantitation of these compounds. Several analytical methods have been described to quantify these NAEs in several biofluids (excluding seminal plasma) and discrepancies in the levels quantified have been reported [13]. In the limited studies of AEA in seminal fluid, no validation of the methodology has been described [8].

Table 3
Concentrations of the endocannabinoids AEA, OEA and PEA in human seminal plasma in normozoospermic subjects.

Analyte (nM)	Mean (nM)	IQR	95% CI
AEA	0.21	0.09–0.27	0.13–0.27
OEA	1.79	0.48–2.32	1.21–2.36
PEA	15.54	7.05–16.31	9.91–21.17

All samples ($n = 40$) were processed in duplicate.

The above data demonstrate that the UHPLC-ESI-MS/MS method provides good linearity and has sufficient sensitivity, precision, and accuracy for the simultaneous identification and quantification of low nanomolar concentrations of AEA, PEA and OEA in human seminal plasma (Table 2). Our results show that low nanomolar concentrations of the NAEs can be readily measured in human seminal plasma and thus the method may be applied in future studies aimed at investigating the effect of these NAEs on human sperm function. Consistent with previous reports, the levels of PEA and OEA were significantly higher than AEA in human seminal plasma ($p < 0.0001$; one way ANOVA) [8].

The concentrations obtained for NAEs from seminal plasma in the 40 normozoospermic men contrast markedly with the previously reported levels of the NAEs using a different HPLC-MS/MS method following lipid extraction with methanol: chloroform (2:1) and sample clean-up with silica stationary phase [8,9]. We observed significantly lower mean NAE concentrations for AEA and OEA (0.21 and 1.79 nM, respectively) compared with previously reported levels (12.1 and 32.9 nM, respectively) but relatively comparable levels of PEA (15.54 nM compared with the previously observed 31.5 nM [8]. Possible explanations for these discrepancies include

differences in sample preparation, extraction and fractionation. For example, the high levels of the analytes reported previously may in part be due to the use of silica based columns which have been associated with a loss of deuterium from the labelled AEA internal standard which leads to a marked over-estimation of AEA content [13]. Furthermore, we have shown that NAE concentrations increase the longer the samples are allowed to undergo liquefaction and this time was not standardized in these earlier studies. Extraction efficiencies presented here for AEA using LPE (21%) and SPE (54%) are comparable with those described using these methods to extract AEA from plasma (19% and 60%, respectively) [16].

Recovery of NAEs from seminal plasma using SPE was approximately twice that obtained using LPE (Table 1); the SPE technique requires smaller sample volumes of seminal plasma than LPE and consequently allows for the majority of samples to be processed in duplicate and for even the smallest sample volume (0.5 mL) to be processed accurately. The use of SPE achieved greater extraction efficiencies for all analytes compared to the LPE extraction technique (Table 1). The mean seminal plasma concentrations of each NAE obtained using the SPE technique ($n = 8$) differs slightly from the mean concentrations from the 40 normozoospermic samples and this likely to be due to the length of time between sample collection and processing, pooling of samples to obtain adequate volumes as well as the effects of storage. Investigations into the effects of relatively short-term storage at −80 °C and repeated freeze–thaw cycles upon seminal plasma NAE concentration showed a decrease in PEA over time and multiple freeze–thaw cycles. It is therefore recommended that where analysis of PEA is required, storage of seminal plasma samples at −80 °C should be avoided. Given the prolonged stability of endocannabinoid standards at −80 °C, if storage of samples is required due to the unavailability of UHPLC-ESI-MS/MS for immediate analysis then NAEs should be extracted from fresh samples and the dried extracts stored at −80 °C until such time that they can be analysed. Alternatively, storage in liquid nitrogen may be an option. However, the stability of NAEs under these storage conditions has not been reported.

Acknowledgements

This work was funded in part by generous grants from the University Hospitals of Leicester National Health Services Trust and by Perkin Elmer through a grant to support the Endocannabinoid Research Laboratory of University of Leicester.

The sponsors had no role in the design and conduct of the study; analysis and interpretation of the data; and preparation of this manuscript.

References

[1] M. Maccarrone, B. Barboni, A. Paradisi, N. Bernabo, V. Gasperi, M.G. Pistilli, F. Fezza, P. Lucidi, M. Mattioli, J. Cell Sci. 118 (2005) 4393.
[2] M. Rossato, F. Ion Popa, M. Ferigo, G. Clari, C. Foresta, J. Clin. Endocrinol. Metab. 90 (2005) 984.
[3] H. Schuel, L.J. Burkman, Biol. Reprod. 73 (2005) 1078.
[4] L. De Petrocellis, T. Bisogno, M. Maccarrone, J.B. Davis, A. Finazzi-Agro, V. Di Marzo, J. Biol. Chem. 276 (2001) 12856.
[5] T. Bisogno, K. Katayama, D. Melck, N. Ueda, L. De Petrocellis, S. Yamamoto, V. Di Marzo, Eur. J. Biochem. 254 (1998) 634.
[6] D. Piomelli, M. Beltramo, A. Giuffrida, N. Stella, Neurobiol. Dis. 5 (1998) 462.
[7] M.R. El-Talatini, A.H. Taylor, J.C. Elson, L. Brown, A.C. Davidson, J.C. Konje, PLoS One 4 (2009) e4579.
[8] H. Schuel, L.J. Burkman, J. Lippes, K. Crickard, E. Forester, D. Piomelli, A. Giuffrida, Chem. Phys. Lipids 121 (2002) 211.
[9] H. Schuel, L.J. Burkman, J. Lippes, K. Crickard, M.C. Mahony, A. Giuffrida, R.P. Picone, A. Makriyannis, Mol. Reprod. Dev. 63 (2002) 376.
[10] A. Ambrosini, G. Zolese, S. Ambrosi, L. Ragni, L. Tiano, G. Littarru, E. Bertoli, F. Mantero, M. Boscaro, G. Balercia, Biol. Reprod. 74 (2006) 659.
[11] A. Ambrosini, G. Zolese, S. Ambrosi, E. Bertoli, F. Mantero, M. Boscaro, G. Balercia, J. Androl. 26 (2005) 429.
[12] A. Ambrosini, G. Zolese, M. Wozniak, D. Genga, M. Boscaro, F. Mantero, G. Balercia, Mol. Hum. Reprod. 9 (2003) 381.
[13] S. Hardison, S.T. Weintraub, A. Giuffrida, Prostag. Other Lipid Mediat. 81 (2006) 106.
[14] M. Vorgeser, D. Hauer, S.C. Azad, E. Huber, M. Storr, G. Schelling, Clin. Chem. Lab. Med. 44 (2006) 488.
[15] T.H. Marczylo, P.M.W. Lam, A.A. Amoako, J.C. Konje, Anal. Biochem. 400 (2010) 155.
[16] T.H. Marczylo, P.M. Lam, V. Nallendran, A.H. Taylor, J.C. Konje, Anal. Biochem. 384 (2009) 106.
[17] P.M. Lam, T.H. Marczylo, M. El-Talatini, M. Finney, V. Nallendran, A.H. Taylor, J.C. Konje, Anal. Biochem. 380 (2008) 195.
[18] O.M. Habayeb, A.H. Taylor, M.D. Evans, M.S. Cooke, D.J. Taylor, S.C. Bell, J.C. Konje, J. Clin. Endocrinol. Metab. 89 (2004) 5482.