A Solid Phase Method for the Extraction and Measurement of Anandamide, from Multiple Human Bio-matrices

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Running Title: Extraction of AEA from human bio-matrices

Abbreviations: AEA, *N*-arachidonoyl ethanolamine (anandamide); AEA-d8, deuterated anandamide; LOD, limit of detection; LOQ, limit of quantification; LPE, liquid-phase extraction; SPE, solid-phase extraction; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry.

Keywords: Endocannabinoid, anandamide, solid phase extraction.

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Abstract

N-Arachidonylethanolamine (anandamide, AEA) was the first endocannabinoid to be identified and has since become associated with the mediation of several physiological functions and disease states. AEA has been isolated from numerous tissues and bio-fluids, in the low nM range, using lipid extraction techniques utilising organic solvents. These techniques require the drying down of relatively large volumes of solvents, which make them unsuitable for high-throughput analysis. Here we describe a solid-phase extraction (SPE) method for the investigation of AEA concentrations in human plasma, serum, milk, urine, amniotic fluid, peritoneal fluid, saliva, follicular fluid and fluid from an ovarian cyst. AEA (mean $\pm$ standard deviation) was detected in serum and plasma from blood isolated from 20 adult women (0.68 ± 0.29 and 0.64 ± 0.28 nM, respectively), from pregnant women at term (1.37 ± 0.42 nM) and from umbilical vein (1.26 ± 0.33 nM) and umbilical artery (1.14 ± 0.35 nM), in milk (0.12 ± 0.05 nM) and from amniotic (0.03 ± 0.02 nM), peritoneal (0.93 ± 0.27 nM), follicular (1.17 ± 0.51 nM) and ovarian cyst (0.32 ± 0.01 nM) fluids. AEA was undetectable in saliva and urine. The 60% AEA extraction efficiency achieved with SPE from plasma was superior to the 19% efficiency achieved using the existing organic solvent extraction method. Limits of quantification and detection for AEA were also dramatically improved using SPE (8 fmol/mL and 4 fmol/mL) compared with organic extraction (25 fmol/mL and 18.75 fmol/mL plasma). These improvements allow the use of smaller plasma samples with SPE. Intra- and inter-day variability were comparable and the mean AEA concentrations of pooled plasma samples ($n = 15$, 1.18nM) were identical with the two techniques. Similarly, when 56 plasma

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samples from labouring and non-labouring women were analysed using both techniques no extraction method-dependent differences were observed. Consequently, we provide evidence for a robust SPE technique for the extraction of AEA from bio-matrices to replace the existing liquid extraction methods which is superior in terms of speed, extraction efficiency and sample size required.

Introduction

The endocannabinoid, anandamide (*N*-arachidonylethanolamine; AEA), is an endogenous, unsaturated fatty acid derivative that was first identified in porcine brain [1] and has since been characterised as a ligand for the cannabinoid receptors, CB1 and CB2 [2] and the orphan receptors GPR55 [3] and GPR119 [4]. AEA also binds to the Ca^{2+} -dependent membrane transporter and vanilloid receptor 1 (TRPV1) [5].

Through receptor activation, AEA exhibits pleiotropic physiological effects including hypothermia, anti-nociception, vasodilation and anti-inflammatory responses [2, 6] and has been implicated in several physiological functions and conditions ranging from analgesia, reproduction, vascular tone, obesity, cancer, schizophrenia and multiple sclerosis [7].

AEA has been detected and quantified in several peripheral tissues [8, 9] including human and rodent brain tissues [10, 11]. We have previously employed a methanol: chloroform, liquid-liquid extraction method for isolating AEA from plasma [12, 13]: This limits the number of samples that could be processed at any one time because processing times were lengthy at around 2 hours/sample or longer. Several similarly time-consuming extraction and purification processes have been reported [14-16].

The diverse pathophysiological roles attributed to AEA have generated a requirement for a validated, rapid, reproducible method for the measurement of AEA in several bio-matrices. We recently reported a UPLC-MS/MS method

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for the measurement of AEA in plasma [17]. The major limitation in that method was the liquid-based lipid extraction step. Therefore, a rapid extraction method to replace the time-consuming and labour intensive lipid-extraction technique previously described for the extraction of AEA from bio-matrices was required.

Improvements to liquid-phase extraction (LPE) methods by incorporating a second, silica-based solid phase extraction (SPE), have yielded techniques providing cleaner samples and improved subsequent sample sensitivity and selectivity [11, 14]. However, these published SPE methods were employed only to clean-up the solvent-based lipid extracts and consequently actually increased the sample processing times. The aims of this study were to develop a single-step SPE method without the requirement for prior lipid extraction suitable for the analysis of AEA from several human bio—matrices, to use this SPE method to quantify the levels of AEA in various human bio-matrices, to equate AEA values obtained using SPE and LPE methods and to compare the levels in serum and plasma.

Materials and methods

Chemicals

Anandamide (AEA) and octa-deuterated anandamide (AEA-d8) were obtained from Cayman Chemicals (Ann Arbor, MI). Virodhamine standard was purchased from Tocris Bioscience (Bristol, UK). All solvents and chemicals were of HPLC grade. Acetonitrile, chloroform, formic acid, methanol and ammonium acetate were purchased from Fisher Scientific (Loughborough, UK) and water was obtained using a water purification system (Maxima ELGA, High Wycombe, UK). All mobile phases were filtered through 0.2µm, 47 mm diameter PTFE filters (Waters, Elstree, UK) prior to use. Saline was obtained from Fannin UK Ltd (Reading, UK).

Preparation of standards

AEA, AEA-d8 and virodhamine stock solutions were prepared by drying supplied stocks under nitrogen gas and reconstituting them in acetonitrile (5 mg/ml and 100 µg/ml, respectively). Stocks were stored at -20°C until needed when further dilutions were performed in acetonitrile on ice.

Sample collection

All volunteers gave signed informed consent to take part in the study, which was approved and conducted according to the guidelines of the Leicestershire and Rutland Local Research Ethics Committee.

Whole blood (4 ml) was either collected into EDTA monovettes (Sarstedt Ltd, Leicester, UK) for isolation of plasma or serum gel monovettes (Sarstedt Ltd, Leicester, UK) for collection of serum. All blood samples were transported to the analytical laboratory on ice and were centrifuged within 2 h of collection at 1200 x *g* for 30 min at 4°C. Plasma or serum was transferred to a clean 7 m1Kimble scintillation vial (Kinesis, St Neots, Cambs, UK) and either analysed immediately or stored for no more than 48 h at -80°C.

The bio-matrices studied were adult and fetal plasma and serum, amniotic fluid, milk, urine, saliva, peritoneal fluid, follicular fluid and fluid from an ovarian cyst. Fetal blood was collected from both the vein and artery of a segment of the umbilical cord between two clamps at the time of Caesarean section. To ensure that the samples were from the appropriate vessels, blood gas analyses were performed on the samples using a gas analyzer (make) and samples were accepted only when there was a difference in the pH of the two samples. Amniotic fluid was collected at elective Caesarean section into sterile universal bottles for transport to the laboratory where they were immediately transferred to 7 ml Kimble scintillation vials and kept on ice prior to analysis while milk was collected from breast-feeding mothers 1-4 weeks after birth. Urine was collected from 7 healthy volunteers (2 men, 3 non-pregnant women, 2 labouring women) into polycarbonate containers as midstream captures and aliquots transferred directly into clean Kimble scintillation vials. Saliva was collected from 5 healthy, non-pregnant female volunteers using the Salivette system (Sarstedt Ltd, Leicester, UK). Saliva was then extracted by centrifugation of the Salivettes at 1200 x *g* for 30 min at

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4°C and the supernatant transferred to a clean 7 ml Kimble scintillation vial. Peritoneal fluid was collected from 10 female subjects at laparoscopy and transported to the analytical laboratory on ice and centrifuged at 1200 x *g* for 30 min at 4°C to remove any contaminating blood components. Supernatant was transferred to a clean 7 ml Kimble scintillation vial. Follicular fluid was obtained by trans-vaginal aspiration of follicles during egg collection in *in-vitro* fertilisation procedures from 8 women at the Assisted Conception Unit, Leicester Royal Infirmary. The single ovarian cyst was obtained from an oophorectomy specimen. This was a benign cyst confirmed histologically to be a mucinous cystadenoma.

Liquid-phase extraction of AEA from human biomatrices

Liquid-phase extractions (LPE) were performed according to Habayeb *et al.*, (2004) with minor modifications [11, 17]. To determine extraction efficiencies from plasma, serum or saline (2 ml), 1.25 pmol/ml AEA-d8 internal standard was added prior to thorough mixing on a desktop vortex. Protein was precipitated by the addition of 2 ml ice-cold acetone followed by centrifugation at 1200 x *g* for 10 min at 4°C. The supernatant was transferred to a fresh Kimble scintillation vial and the acetone evaporated under a gentle stream of nitrogen gas. Lipid extraction was then performed on the remaining mixture by the addition of 2 ml of methanol: chloroform (1:2 v/v) followed by gentle mixing by repeated inversion. The sample was centrifuged at 1200 x *g* for 10 min at 4°C and the lower chloroform layer recovered into a fresh Kimble scintillation vial and then dried under a stream of nitrogen before reconstitution in acetonitrile (80 µl). To estimate the efficiency of the lipid

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extraction procedure, peak areas obtained for 2.5 pmol AEA-d8 extracted from plasma were compared with 2.5 pmol AEA-d8 standard in 80 µl acetonitrile.

Solid Phase Extraction of AEA from human bio-matrices

Plasma (0.5 ml), serum (0.5 ml), umbilical cord plasma (0.25-0.5 ml), amniotic fluid (2 ml), milk (2 ml), urine (2 ml) peritoneal fluid (0.5-1 ml), follicular fluid (0.5-1.0 ml) and saliva (1 ml) were spiked with 2.5 pmol/ml AEA-d8 internal standard and diluted, where necessary, to 1 ml with deionised water. All samples were vortexed thoroughly and centrifuged at 16000 x g for 5 min at 4°C. Oasis HLB 1cc cartridges (Waters, Elstree, UK) were pre-conditioned and equilibrated using 1 ml methanol and 1 ml H₂O via a vacuum manifold (Waters, Elstree, UK). Samples were introduced onto the cartridges and drawn under gentle vacuum at a flow rate of about 1 ml/min. The cartridges were washed with 1 ml 40% aqueous methanol and AEA was eluted in 1 ml acetonitrile. The eluate was dried under a constant stream of nitrogen and reconstituted in 80 µl acetonitrile and transferred to a HPLC sample vial ready for UPLC-MS/MS analysis as described previously [17].

Validation of SPE analysis of AEA

The SPE method was validated using pooled, depleted human plasma frozen at -20°C supplied by the Blood Transfusion Unit, Leicester Royal Infirmary (Leicester, UK). To determine extraction efficiency and inter- and intra-day assay variability, liquid extraction and SPE were performed over 3 days, n=5. Each sample was analysed in triplicate by UPLC-MS/MS. To overcome the

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contribution of endogenous AEA, limits of detection and quantification were determined for these extraction methods using plasma spiked with AEA-d8 and saline spiked with both AEA-d8 and AEA. Similarly, peak areas of AEA-d8 extracted from plasma were compared with those from non-extracted standards to calculate extraction efficiencies for the two extraction techniques. Finally, the AEA levels in plasma samples from 56 pregnant volunteers were determined using both extraction methods to investigate inter-assay variability. These 56 samples came from both labouring and non-labouring women at term.

Quantification of AEA

AEA and AEA-d8 quantification was achieved using an UPLC-MS/MS system comprising an Acquity UPLC in line with a Quattro Premier tandem mass spectrometer (Waters, Elstree, UK) as described previously [17]. In brief, separation was accomplished with an Acquity UPLC BEH C₁₈ (2.1 x 50 mm) column maintained at 40°C. The two mobile phases comprised A (2 mM ammonium acetate containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). Gradient conditions were as follows: 0-0.5 min, 80% A; 1.5 min, 0% A; 2.5 min, 80% A then re-equilibrated at 80% A until 3.5 min. Samples were kept at 4°C throughout. Quantification of analytes was achieved using tandem electro-spray mass spectrometry in positive ion mode (ES⁺). Product ions were monitored in multiple reaction monitoring mode. Transitions employed for AEA and virodhamine and for AEA-d8 were m/z 348.3>62.3 and m/z 356.3>63.3, respectively. Injection volumes for samples

and standards were 7 μ l. Eight-point calibration curves in triplicate were performed.

Virodhamine, a related endocannabinoid, has an identical molecular weight to AEA and also fragments to a daughter ion with the same mass as the daughter ion of AEA; consequently the method described here will detect both AEA and virodhamine. Virodhamine and AEA elute at 1.52 and 1.68 min, respectively [17].

Statistical Analysis

All AEA concentrations are presented as the mean $\pm$ standard deviation. Pairwise comparison between volunteer groups was performed using two-tailed Students' *t*-test.

Results

Solid-phase method development

During development of the SPE method of AEA extraction employed here extensive optimisation was undertaken with respect to choice of wash and elution solutions and sample pre-treatment using pooled, stock plasma from multiple donors spiked with 2.5 pmol/mL AEA-d8. Acetonitrile, methanol, isopropanol, ethyl acetate, chloroform, acetone and mixtures of the above with or without acidification (2% v/v acetic acid) were investigated with respect to their capacity to elute AEA from the SPE cartridges. Elution with acetonitrile gave the highest extraction efficiency with acidification presenting no advantage over non-acidified eluants (data not shown). Wash buffers comprising different concentrations of aqueous methanol were investigated and 40% aqueous methanol represented the highest methanol concentration that did not elute AEA from the SPE cartridge (data not shown). Finally, dilution of plasma 1:1 with either 5% (v/v) aqueous phosphoric acid or 5% (v/v) ammonium hydroxide prior to SPE did not increase the extraction efficiency for AEA (data not shown) suggesting that changes in pH do not improve extraction efficiency of AEA from un-modified plasma.

Validation of a solid-phase method of AEA extraction from human plasma and comparison with liquid-phase extraction.

To compare the robustness and accuracy of the SPE extraction technique in relation to the LPE technique, previously employed for the analysis of AEA in plasma, the AEA levels in pooled, depleted plasma were assayed using each

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extraction technique over 3 days. No significant difference in pooled plasma AEA concentration was observed between the two methods, over 3 days (n = 15). The mean value observed using either extraction technique was 1.18nM (Table 1). Inter- and intra-day variability for extraction of AEA using both techniques was excellent with an intra-day variation of less than 5% and an inter-day variability of approximately 12% (Table 1). Relative standard deviations were 12.7% and 11.9% for LPE and SPE methods, respectively. Consequently, in these respects the two methods are highly comparable. The greatest difference between the two methods is in their extraction efficiencies. SPE demonstrated a 3-fold improvement (60%) over LPE (19%) (Table 1, Figure 2). This improved extraction efficiency with SPE contributed to the significantly smaller plasma samples (0.5 ml) required to quantify AEA compared with LPE (2 ml).

The limits of detection (LOD) and quantification (LOQ) were greatly improved using the SPE extraction method. LOQ (signal-to-noise ratio > 10) values for SPE and LPE were 8 and 25 fmol/ml plasma, respectively. LOD (signal-to-noise ratio > 3) values were 4 and 18.75 fmol/ml.

To demonstrate further that the plasma AEA concentrations are comparable irrespective of the extraction technique employed, we measured AEA concentrations in plasma obtained from pregnant, female volunteers at term. Figure 3 demonstrates that for all samples, the AEA concentrations were not altered significantly by changes in the extraction method used. The AEA

concentrations obtained following SPE relative to concentrations observed following LPE were 100.75 ± 8.67 % (mean $\pm$ standard deviation, n = 56).

Measurement of AEA in other human blood samples.

AEA was clearly detected and easily quantified in human plasma, serum and in plasma collected from umbilical vein and umbilical artery (Figure 4, Table 2). In the 56 plasma samples analysed, the AEA concentration ranged from 0.51 to 3.99 nM with a mean value of 1.38 nM. AEA was also extracted from and measured in serum (n = 20) where the mean AEA concentration was 0.68 ± 0.29 nM (Table 2). Plasma collected from the same volunteers at the same time as the serum samples yielded a comparable mean AEA concentration of 0.64 ± 0.28 nM. The concentration of AEA in serum relative to plasma in these samples was 106 ± 8 %.

When plasma was collected from 10 women undergoing elective Caesarean section, the AEA concentration (1.37 ± 0.42 nM) closely resembled that obtained in the larger cohort of women (Table 2). AEA was also detected in plasma obtained from umbilical artery and from umbilical vein at levels comparable to those present in maternal plasma (Table 2). In this cohort of women no significant differences in AEA concentrations were observed between umbilical artery and umbilical vein or between umbilical samples and maternal plasma (Table 2).

Measurement of AEA in other human bio-matrices

The SPE technique was employed successfully to isolate AEA from human milk, follicular fluid, peritoneal fluid and fluid drained from an ovarian cyst at levels that could be clearly measured using UPLC-MS/MS (Figure 5). Comparatively low, but measurable levels of AEA were extracted from amniotic fluid using SPE but AEA was not detected in saliva and though spectrograms from urine suggested the presence of AEA, especially in the women in active labour, the levels were below the LOQ (8 fmol/mL) for this analytical method (Figure 5). Extraction efficiencies of d8-AEA from the different bio-matrices were comparable, indicating that the lack of AEA retrieval from urine and saliva in normal circumstances was not due to technical problems associated with these fluids (data not shown). AEA concentrations vary greatly between the different bio-matrices that were analysed with the following concentration gradient: follicular fluid>peritoneal fluid>ovarian cyst fluid>milk>amniotic fluid (Table 2).

Virodhamine, which has a retention time of 1.54 min using this system [17], and is a possible confounding contaminant of AEA measurement was not detected in any of the bio-matrices.

Discussion

The myriad of studies reporting a central role of endocannabinoids (such as AEA) in several physiological processes including reproduction and anti-nociception [18-20] and several pathological conditions such as cancer, obesity, schizophrenia and multiple sclerosis [7] has made the quantification

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of these endocannabinoids an absolute necessity if their precise roles in these physiological and pathological states are to be further unravelled. Unfortunately the existing liquid-based, organic solvent methods for the extraction of AEA do not lend themselves well to the routine investigation of a large number of samples because of the lengthy processing time involved. By contrast, the SPE format is easily adaptable to scaling up and high throughput automation. The method benefits from the additional advantages of not utilising volatile solvents such as chloroform and improved sample cleanup which diminishes matrix effects on subsequent MS analysis. Consequently, we have assessed a method of extraction of the endocannabinoid, AEA, from bio-matrices using Oasis HLB solid phase cartridges and compared it with our existing LPE method that uses methanol: chloroform (1:2 v/v) as the primary solvent. Overall, the SPE method out-performed the LPE method (Table 1) with marked improvements in extraction efficiency, limits of detection and quantification, speed of analysis and the amount of sample required. The extraction efficiency observed using the LPE method was only 19% (Table 1). There are, to the best of our knowledge, no values for recovery of AEA from plasma using LPE methods but recoveries for extraction of AEA from brain tissues are 71-97 % [10, 11, 21]. Two of these studies in rodent brain have employed silica-based SPE [10, 11] the other C18 reverse phase SPE [21] for the clean-up of LPE tissue extracts. Silica-based SPE methods are not suitable for extraction of AEA from bio-fluids because they require that AEA be in an organic solvent and we have already shown that AEA does not partition efficiently into organic solvents from bio-fluids. Furthermore, it has also been demonstrated that use of silica SPE, but not C18 SPE, can lead to

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loss of deuterium from internal standard [21] causing inaccuracy in the determination of AEA. In addition, these methods were developed for brain tissue using concentrations of AEA ranging from 20-2000 pmol/g [10, 11, 21] which are not representative of the low pmol/ml AEA concentrations observed in plasma [12, 13, 16, 17, 24]. An additional problem when modifying AEA extraction methods for tissues for use with bio-fluids is that extraction media are added to tissues in large quantities (40-500 fold w/v) [10, 11, 21] which is possible because of the small size of the tissue samples being extracted. Using such fold-excesses of solvents to extract AEA from plasma is impractical (i.e., for a 2 ml plasma sample we would require 80-1000 ml extraction solvent). The lower sample: solvent ratio may explain, in part, the limited extraction efficiency observed here. Increasing solvent volume may increase the extraction efficiency but processing time will be increased accordingly because of the increased solvent volumes to be evaporated.

The levels of circulating AEA we obtained were comparable whether from serum (0.68 ± 0.29 nM) or plasma (0.64 ± 0.28 nM). Consequently, determination of AEA levels from blood appears to be unaffected by the processing method. AEA concentrations were not changed significantly whether SPE or LPE techniques were employed (Figure 3). When plasma AEA concentrations obtained following SPE were related to LPE the difference was negligible (Figure 3).

There is considerable variation in the published levels of plasma AEA. The levels presented herein compare favourably with those previously published in non-pregnant volunteers (0.37 nM), by Fernandez-Rodriguez et al [22] and

with those previously published by our group [13,17] for labouring women in two separate reports at term (2.5 nM, and 2.3 nM, respectively). The levels of AEA measured by both LPE and SPE methods in plasma from non-pregnant woman was 0.64 nM and 1.38 nM (range 0.51-3.99 nM) for term pregnant women. These data differ from those reported by other groups, 2.58 and 3.73 nM for non-pregnant women [23, 24]. The difference could be related to the physiological state or the timing of processing of their biological samples. For example, we have standardised the collection procedure for our blood samples and have observed variations in the levels of plasma AEA if the blood is stored for more than 2 h prior to processing (unpublished observations).

We demonstrate here, for the first time, that AEA is present in plasma from umbilical arteries and umbilical vein at term at levels comparable to circulating, maternal AEA levels (Table 2). Park et al have previously reported the presence of fatty acid amide hydrolase, the enzyme responsible for degradation of AEA, in placenta at term and hypothesised that this enzyme regulates exposure of the fetus to AEA [25]. Consequently, our discovery of AEA in umbilical blood at levels comparable to those of the maternal circulation is unexpected and suggests that the placenta is not a barrier to AEA in term non-labouring women and that the levels in the umbilicus are representative of the levels of AEA produced by the fetus at term prior to labour. The role of AEA in the fetus at term requires further investigation.

Unlike the related exocannabinoid, Δ^9 -tetrahydrocannabinol, which is normally determined in these two bio-matrices in forensic laboratories, no measurable AEA was found in either urine or saliva (Table 2, Figure 5) though some AEA was found in urine from labouring women at concentrations below the limit of quantification (8 pM). There is also clear evidence that in the mouse, AEA is synthesized in the kidney vasculature and acts both in the kidney and salivary gland to alter respective functioning [26]. However, in women AEA is not present in urine, in detectable enough quantities except during labour.

Data on AEA levels in other biological fluids is scarce. Schuel et al [27] measured AEA in follicular fluid (2.9 nM), amniotic fluid (7.9 nM), milk (5.1 nM) and in ovarian cyst fluid (2.7 nM). Here we also determined levels of AEA in these matrices but AEA concentrations were consistently lower (Table 2). The limited number of samples analysed and the relatively poor LOQ in the method utilised in the former study may explain the considerable divergence in AEA concentrations between the two studies but confirm their existence in these bio-matrices.

AEA in ovarian cyst fluid was only found to be 0.32 nM in this study but this was only from one sample and may be a consequence of the pathology of this sample. Follicular fluid contained AEA at 1.17 nM which is significantly lower than that described by Schuel et al [27]. This divergence is not a consequence of the extraction method employed as we found that AEA concentrations were not changed significantly when follicular fluid AEA was extracted using the LPE method (1.25 ± 0.57 nM) and thus this discrepancy may be related to

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pathology. In Schuel's study [27], AEA levels were also considerably higher in amniotic fluid suggesting that the reduced levels reported here may be a consequence of the stage at which amniotic fluid was collected. In the previous study, amniotic fluid was collected from women undergoing termination of pregnancy at an average gestation of 16.2 weeks whereas in this study amniotic fluid was collected during elective Caesarean section at term (>37 weeks of completed gestation). The fetus constantly cycles amniotic fluid by ingesting and then urinating such that at term, amniotic fluid is essentially fetal urine. The absence of quantifiable levels of AEA in urine suggests that any locally produced AEA is markedly diluted by fetal urine, or is removed following ingestion.

AEA levels in milk (0.12 ± 0.05 nM) were 80% lower than plasma levels (0.62 ± 0.28 nM). The levels reported herein are a fraction of the levels reported by Schuel (5.1 ± 2.1 nM) [27] but closely resemble those of Fride et al [28] where AEA was found at 0.156 nM. AEA concentration in milk varies dependent upon when it is collected with no AEA found previously in early human milk [28]. There are no data demonstrating the presence of anandamide synthesising enzymes in the normal human breast and so a reliable conclusion as to why the levels we report differ from those of Schuel cannot be made. Accurate measurement of AEA in milk and whether levels are modulated by environmental factors such as diet require further investigation.

Di Marzo et al [29] have previously demonstrated that mouse peritoneal macrophages produce AEA in small quantities. Here we demonstrate for the

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first time that human peritoneal fluid, from women with endometriosis, contains AEA (Table 2) at concentrations (0.57-1.24 nM) comparable with those found in plasma. Whether AEA levels correlate with severity of disease or could act as a diagnostic indicator, requires further examination.

Conclusions

The SPE technique described above offers a viable option for the extraction of AEA from biological matrices with advantages over LPE techniques in terms of extraction efficiency, amount of sample required, limits of quantification, sample processing times, suitability for high-throughput screening, simplicity and safety (no requirement for volatile solvents). Improved extraction efficiency has meant that AEA can be measured in biological fluids with low AEA concentrations and in fluids where often only small volumes of samples may be available (e.g., umbilical plasma, follicular fluid, peritoneal fluid). AEA was measured for the first time in peritoneal fluid and plasma from both umbilical vein and artery. Both the fetus, via the umbilical cord, and neonate via breast milk, receive AEA from the mother. The role of AEA in these circumstances and relevance to perinatal health require further investigation. Furthermore, detection of AEA in peritoneal fluid of women with endometriosis at levels comparable to those found in the circulation suggests that the role of AEA in this pathology requires further investigation.

Acknowledgements

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Figure Legends

Figure 1

Structure of AEA showing the proposed site of fragmentation during UPLC-MS/MS and sites of deuteration in AEA-d8 (*).

Figure 2.

UPLC-MS/MS spectrograms for the measurement of AEA and AEA-d8 in the same sample of human plasma following (A) liquid-phase extraction and (B) solid-phase extraction

Figure 3.

AEA concentrations determined following extraction from plasma obtained from pregnant women at term using LPE and SPE techniques. AEA was extracted from plasma collected from 56 labouring or non-labouring women at term as described in materials and methods using SPE (■) and LPE (◇). Results are representative of the mean AEA concentration following three replicate injections onto the UPLC-MS/MS.

Figure 4.

Representative UPLC-MS/MS spectrograms of AEA (m/z 348.3>62.3) in human blood samples following SPE. (A) Plasma, (B) Serum, (C) Plasma obtained blood collected from umbilical artery and (D) Plasma obtained from blood collected from umbilical vein. Variable intensities between spectrograms are accounted for by the vertical scale bars.

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Figure 5.

Representative UPLC-MS/MS spectrograms of AEA (m/z 348.3>62.3) in human bio-matrices following SPE. **(A)** Milk, **(B)** Amniotic fluid, **(C)** Peritoneal fluid, **(D)** urine, **(E)** Saliva, **(F)** Fluid drained from an ovarian cyst, **(G)** Follicular Fluid. Variable intensities between spectrograms are accounted for by the vertical scale bars.

Table 1. Comparison of the solid-phase (SPE) and liquid-phase (LPE) AEA extraction methods using pooled human plasma.

PARAMETER	LPE	SPE
Extraction efficiency (%)	19	60
Intra-day variability (%)	2.9	4.0
Inter-day variability 3 days (%)	12.6	11.6
Limit of Detection (pM)	18.75	4
Limit of Quantification (pM)	25	8
[AEA] (mean±SD, n=15) nM	1.18±0.15	1.18±0.14
Processing time for 10 samples	~3.5 h	~0.4 h
Amount of plasma required/test	2 mL	0.5 mL

Intra-day variability was determined for the measurement of 5 plasma samples. Inter-day variability is representative of 5 plasma samples measured on 3 separate days. Limits of detection and quantification are presented as the spiked plasma AEA-d8 concentrations which provided peaks with signal to noise values greater than 3 and 7, respectively. The extraction efficiencies determined over the 3 days (n = 15) are significantly different ($P < 0.0001$) using students' paired t test.

Table 2. AEA concentrations in human biomatrices.

BIOMATRIX (n = number of samples)	AEA CONCENTRATION (nM)
Plasma (n = 20)	0.64 ± 0.28 (0.19-1.32)
Serum (n = 20)	0.68 ± 0.29 (0.16-1.31)
Maternal plasma (at term, n = 10)	1.37 ± 0.42 (0.81-1.91)
Umbilical artery plasma (n = 10)	1.14 ± 0.35 (0.75-1.53)
Umbilical vein plasma (n = 10)	1.26 ± 0.33 (0.91-1.84)
Amniotic fluid (n = 5)	0.03 ± 0.02 (0.01-0.05)
Milk (n = 3)	0.12 ± 0.05 (0.09-0.18)
Follicular Fluid (n = 8)	1.17 ± 0.51 (0.51-1.90)
Peritoneal fluid (n = 10)	0.93 ± 0.27 (0.57-1.24)
Urine (n = 7)	<0.004
Saliva (n = 5)	0.000
Ovarian Cyst Fluid (n = 1)	0.32 ± 0.01 (0.30-0.34)

Results are presented as mean ± SD with the range of concentrations given in parentheses. Ovarian cyst fluid, data is presented as the mean ± standard deviation of 2 separate extractions of the same sample on two separate days each analysed three times. Plasma and serum were collected from non-pregnant, female volunteers. Maternal plasma represents samples obtained from pregnant volunteers at term which also provided umbilical plasma samples.

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Figure 1

Figure 2

Figure 3

Figure 4

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