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Determination of anandamide and other fatty acyl ethanolamides in human serum by electrospray tandem mass spectrometry

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Abstract

We developed a new selective liquid chromatography–electrospray ionization–tandem mass spectrometry method for the identification and quantification of anandamide (AEA), an endogenous cannabinoid receptor ligand, and other bioactive fatty acid ethanolamides (FAEs) in biological samples. Detection limit (0.025 pmol for AEA and 0.1 pmol for palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)) and quantification limit (0.2 pmol for AEA and 0.4 pmol for OEA and PEA) were in the high fmol to low pmol range for all analytes. Linear correlations ($r^2 = 0.99$) were observed in the calibration curves for standard AEA over the range of 0.025–25 pmol and for standard PEA and OEA over the range of 0.1–500 pmol. This method provides a time-saving and sensitive alternative to existing methods for the analysis of FAEs in biological samples.

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Keywords: Anandamide; Fatty acid ethanolamides; Human; Liquid chromatography; Tandem mass spectrometry

Endogenous cannabinoids, such as anandamide (AEA)¹, and structurally related fatty acid ethanolamides (FAEs) play important roles as physiological modulators of numerous processes in the peripheral and central nervous system [1,2]. Endocannabinoids exert most of their biological actions via activation of cannabinoid receptors. To date two subtypes of G-protein-coupled cannabinoid receptors have been identified: the CB₁ receptor subtype, which is mainly expressed in the brain [3], and the CB₂ receptor subtype,

which is particularly abundant in the immune system [4]. Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are produced together with anandamide when primary neurons in culture are stimulated with membrane-depolarizing agents [5–7]. PEA and OEA are not active on CB₁ receptors. PEA exerts antiinflammatory effects [8,9] and modulates pain initiation [10] while OEA has been shown to elicit satiety and to stimulate lipolysis in rodents by activating the nuclear receptor peroxisome proliferators-activated receptor- α [11,12]. Because of the potential involvement of FAEs in the regulation of different physiological processes, it is important to have sensitive analytical methods for the accurate identification and quantification of these molecules.

Previous analytical studies of FAEs have shown that the levels of these compounds, especially of AEA, are extremely low in biological fluids, such as cerebrospinal fluid [13,14] or

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¹ Abbreviations used: AEA, anandamide; FAE, fatty acid ethanolamide; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; QCs, quality control samples; ESI, electrospray ionization; SRM, selected reaction monitoring.

rat blood [15,16]. In recent years several analytical methods to measure FAEs have been reported; however, the quantification limits of existing methods may hinder the analysis of FAE in human fluids. Existing gas chromatographic and liquid chromatographic methods hardly reach the low quantification limits required for a reliable quantification of FAEs in biological fluids [16,17]. Moreover, gas chromatography (GC) and high performance liquid chromatography (HPLC) methods require derivatization steps during preparation since FAEs are nonvolatile, do not fluoresce, and have weak UV absorption. To overcome these limitations, we established a novel approach for the detection of AEA, PEA, and OEA in human serum: AEA and OEA were analyzed using silver cation coordination and positive electrospray tandem mass spectrometry, whereas, for the quantification of PEA, which lacks a double bond to bind silver cations, we monitored the protonated species. This new sensitive and selective method for determining AEA and its analogs allowed us to achieve higher sensitivity than previously published methods.

Materials and methods

Chemicals

Fatty acyl chlorides (5,8,11,14-eicosatetraenoylchloride, hexadecanoylchloride, and 9-octadecenoylchloride, >99% purity) were from Nu-Chek Prep (Elysian, MN); [$^2\text{H}_4$]-labeled ethanolamine (isotopic atom enrichment = 98%) was from Cambridge Isotope Laboratories (Andover, MA). All solvents were from Fisher Scientific, Schwerte, Germany and all other chemicals were from Sigma–Aldrich, Deishofen, Germany.

Synthesis of unlabeled and [$^2\text{H}_4$]-labeled standard FAEs

Standard unlabeled and [$^2\text{H}_4$]-labeled FAEs were synthesized by the reaction of the corresponding fatty acyl chlorides with unlabeled or [$^2\text{H}_4$]-labeled ethanolamine, as described previously [16]. Briefly, fatty acylchlorides were dissolved in dichloromethane (10 mg/ml) and allowed to react with an excess of unlabeled or [$^2\text{H}_4$]-labeled ethanolamine for 15 min at 0–4 °C. The reaction was stopped by adding water. After stirring and phase separation, the upper aqueous phase was discarded and the organic phase was washed several times to remove remaining ethanolamine. This reaction results in the quantitative formation of FAEs, which were concentrated to dryness under stream of N_2 and reconstituted in chloroform at a concentration of 20 mM. FAE solutions were stored at –20 °C until used. The identity and chemical purity (>98%) of the synthesized FAEs were determined by HPLC/MS, MS ion trap, and proton nuclear magnetic resonance (^1H NMR). ^1H NMR spectra were recorded on a Bruker GN 500 MHz spectrometer; chemical shifts are reported in parts per million (ppm), using TMS as internal standard in CDCl_3 : OEA, ^1H NMR (CDCl_3) δ : 5.84 (br s, 1H), 5.31 (m, 2H), 3.57 (m, 2H), 3.27 (m, 2H), 2.51 (br s, 1H), 2.17 (t, 2H), 2.01 (m, 4H), 1.59

(m, 2H), 1.31 (br d, 20H), 0.88 (t, 3H); PEA, ^1H NMR (CDCl_3) δ : 5.87 (br s, 1H), 3.73 (m, 2H), 3.47 (m, 2H), 2.51 (br s, 1H) 2.20 (t, 2H), 1.70 (m, 2H), 1.29 (br d, 24H), 0.88 (t, 3H); AEA, ^1H NMR (CDCl_3) δ : 6.12 (br s, 1H), 5.35 (m, 8H), 3.70 (m, 2H), 3.41 (m, 2H), 3.11 (br s, 1H), 2.81 (m, 6H), 2.23 (m, 2H), 2.18 (m, 4H), 1.74 (m, 2H), 1.36 (m, 6H), 0.89 (t, 3H).

Human serum samples

The human serum samples were taken from eight subjects, four males and four females, who were part of a control group of healthy volunteers in a clinical trial aimed at evaluating the role of endocannabinoids in psychiatric disorders. No relevant use of medical or illicit drugs or alcohol was allowed in these subjects. Consumption of alcohol was not allowed for 1 week prior to participation in the study. In addition, all subjects passed a routine examination of blood and plasma without any pathological findings. All volunteers were informed and gave written consent that their samples would be stored and used for future research. All subjects were free of any relevant health problems and of diagnosable psychopathology according to DSM-IV criteria. The Ethical committee of the Medical Faculty of the University of Cologne reviewed and approved the protocol of this study and procedures for sample collection, storage, and analysis.

Sample collection and extraction

Venous blood samples were obtained from human subjects with Sarstedt serum monovettes and immediately centrifuged at 4000 rpm for 5 min. Serum (S) was aliquoted into 1 ml fractions, transferred into 2 ml glass vials, and immediately stored at –80 °C for further analysis. Additionally, venous blood was drawn into BD Vacutainer CPT tubes (Becton–Dickinson, NJ, USA). The Vacutainer CPT Tube combines a citrate anticoagulant with a Ficoll Hypaque density fluid and a polyester gel barrier, which separates the two liquids. These tubes allow the collection of whole blood and the separation of mononuclear cells. The tubes were immediately centrifuged at 2750 rpm for 25 min at room temperature in a horizontal rotor (Eppendorf, Hamburg, Germany). After centrifugation, the mononuclear cells were resuspended into the remaining serum by inverting the unopened Vacutainer CPT Tube gently 5 to 10 times. Serum with mononuclear cells (SC) was also stored at –80 °C for further analysis.

Construction of calibration curves

Standard calibration curves were constructed by adding a constant amount of deuterium-containing standards (25 pmol for [$^2\text{H}_4$]AEA and 500 pmol for [$^2\text{H}_4$]OEA and [$^2\text{H}_4$]PEA) to increasing amounts of the corresponding unlabeled FAEs. To determine the accuracy and precision of our method (see below), quality control samples (QCs)

were prepared by diluting independently prepared standard solutions at four concentration levels of the analytes (20, 10, 0.4, and 0.2 pmol/ml for AEA (QCs 1–4); 400, 20, 2, and 0.4 pmol/ml for PEA and OEA (QCs 1–4)) and a constant amount of deuterium-containing standards (25 pmol for [$^2\text{H}_4$]AEA and 500 pmol for [$^2\text{H}_4$]OEA and [$^2\text{H}_4$]PEA). All standard and QC solutions were stored at -20°C until used.

Extraction of AEA, PEA, and OEA from serum

To quantify FAEs, aliquots (1 ml) from S and SC (total volume, 3–5 ml) were spiked with 25 pmol of [$^2\text{H}_4$]anandamide (d_4 -AEA) and 500 pmol of [$^2\text{H}_4$]palmitoylethanolamide (d_4 -PEA) and [$^2\text{H}_4$]oleoylethanolamide (d_4 -OEA) as internal standards and subjected to acetone precipitation of protein. Since FAEs are unstable (unpublished observations) and subject to degradation by fatty acid amide hydrolase in body fluids at room temperature [1], all samples were kept on ice (0 – 4°C) during processing. Supernatants were collected and their volumes were reduced under a stream of N_2 . Lipids were extracted with chloroform/methanol (2:1, vol/vol), and chloroform phases were evaporated to dryness under N_2 , reconstituted in chloroform and methanol (2:3, 80 μl total), and transferred to 2.0-ml screw topped vials with 0.1-ml glass inserts (Thermo Electron, Dreieich, Germany). The extracts were stored at -20°C until analyzed. The lipophilic FAEs are stable in methanol/chloroform (unpublished data) but even if a minimal degradation of FAEs should occur, an equal amount of internal standards would be lost so that the ratio between the two (which is used to quantify FAEs) remains unchanged.

LC-ESI-MS-MS conditions

LC-ESI-MS-MS analysis was performed with a Surveyor HPLC system (Thermo Electron) coupled to a TSQ Quantum triple-quadrupole mass spectrometer (Thermo Electron) operating in positive electrospray ionization (ESI^+) mode with selected reaction monitoring (SRM). Samples 20 μl from extracted serum samples were injected into a 3- μm Hypersil BDS C_{18} column (100×2.1 mm; Thermo Electron) and eluted using a linear gradient of 70 μM aqueous silver acetate solution (Eluent A) and a 70 μM methanolic silver acetate solution (Eluent B) (30% A:70% B to 100% B in 5 min followed by a 5 min hold at 100% B). The column was reequilibrated at 30% A:70% B for 2 min. The flow rate was 200 $\mu\text{l}/\text{min}$. Under these conditions FAEs eluted from the column at retention times between 6.7 and 7.7 min. Including the time necessary to reequilibrate the column, the total time required for one sample analysis was 12 min. For FAEs detection and quantification, the SRM transitions were m/z 456.2 $\rightarrow$ 438.2 (AEA), 460.3 $\rightarrow$ 442.3 (d_4 -AEA), 438.2 $\rightarrow$ 420.2 (OEA), 434.2 $\rightarrow$ 416.2 (d_4 -OEA), 300.3 $\rightarrow$ 62.3 (PEA), and 304.3 $\rightarrow$ 66.3 (d_4 -PEA). For ESI^+ the following parameters were employed: spray voltage 4500 V, capillary temperature

350°C , sheath gas pressure 60 (arbitrary units), and auxiliary gas pressure 2 (arbitrary units).

Statistical analysis

AEA, OEA, and PEA values in S and SC samples were expressed in pmol/ml of blood. Means $\pm$ SE ($n=8$) were compared by using the paired Student's t test (significant if $\alpha < 0.05$).

Results and discussion

Ionization and fragmentation

For AEA and OEA, we exploited the ability of silver cations to complex with the double bonds of these molecules to form charged species $[\text{M} + \text{Ag}]^+$, which in turn undergo conversion to the gas phase via the ESI source. This process has long been known to occur in unsaturated lipids [18] and has been successfully used for their analyses [19,20]. In the case of PEA, which does not have any double bond, the mass spectrum was dominated by the protonated molecular ion $m/z = 303.3$ $[\text{M} + \text{H}]^+$ [Fig. 1A, PEA mass spec], whereas full-scan analysis of AEA and OEA in the absence of Ag^+ showed protonated molecular ions of considerably less intensity than the corresponding silver adducts [Fig. 1B, AEA mass spec and Fig. 1C OEA mass spec]. Direct infusion of AEA and OEA in $\text{MeOH}:\text{H}_2\text{O}$ with 70 μM Ag^+ gave base peaks corresponding to $m/z = 456.2$ $[\text{M} + \text{Ag}]^+$ (for AEA) and $m/z = 438.2$ $[\text{M} + \text{Ag}]^+$ (for OEA). Silver ion coordination resulted in the observation of two $[\text{M} + \text{Ag}]^+$ ions—separated by two mass units—due to the parent isotopic ions, ^{107}Ag (52%) and ^{109}Ag (48%).

The collision-induced dissociation of the Ag^+ adducts (AEA and OEA) and the protonated species of PEA resulted in specific product-ion spectra; the most intense fragments were chosen for quantification. Thus the m/z 456.2 $\rightarrow$ 438.2 was used for SRM analysis of AEA and m/z 438.2 $\rightarrow$ 420.2 for OEA. Tetra-deuterated internal standards were also dissolved in a $\text{MeOH}/70 \mu\text{M}$ Ag^+ solution in water (1:1 vol/vol). The m/z 460.3 $\rightarrow$ 442.3 and 434.2 $\rightarrow$ 416.2 were used for SRM analysis of d_4 -AEA and d_4 -OEA, respectively.

Calibration and recovery

Standard calibration curves were constructed by adding a constant amount of deuterium-containing standards (25 pmol for d_4 -AEA and 500 pmol for d_4 -OEA and d_4 -PEA) to increasing amounts of the corresponding unlabeled FAEs, followed by analysis of $[\text{M} + \text{Ag}]^+$ or $[\text{M} + \text{H}]^+$ ions in SRM. The ratios of unlabeled and labeled ions were plotted against the amount of spiked FAEs and the calibration curves were constructed using linear regression. R^2 was 0.9987 for AEA, 0.9972 for OEA, and 0.9975 for PEA, respectively, indicating a linear response for all the analytes.

For recovery studies, serum samples from the same volunteer were spiked with 2, 5, 10, and 25 pmol of AEA, PEA,

Fig. 1. Fragmentation spectra from the collisionally induced dissociation of $[\text{PEA}+\text{H}]^+$ (A), $[\text{AEA}+\text{Ag}]^+$ (B), and $[\text{OEA}+\text{Ag}]^+$ (C) in the presence of silver ion in positive-ion ESI mode. Inset details fragmentation site of the each analyte.

and OEA, 25 pmol of d_4 -AEA, and 500 pmol of d_4 -PEA and d_4 -OEA ($n=3$) before and after their processing. The recovery was determined by comparing the peak areas of spiked samples with those of unextracted standard solutions containing the same concentrations of FAE. The naturally occurring amount of FAEs in the serum was considered a blank value of the serum samples which has been subtracted from each value. The resulting percentage of recovery, as displayed in Table 1, was higher than those

reported previously. In particular, Kingsley and Marnett [21] reported a recovery of AEA ranging between 51 and 82%, whereas in the study of Koga et al. [17], AEA recovery was between 67 and 73%.

Accuracy and precision

We assessed the accuracy and precision of our method by measuring the recovery of known amounts of FAEs in the presence of deuterium-labeled standards. Precision is expressed as percentage coefficient of variation (CV), by dividing the standard deviation by the sample mean and multiplying the resulting value by 100.

Accuracy is expressed as the ratio between the actual and the nominal values observed. For the evaluation of the intraday and interday precision and accuracy, four different QC samples—high (QC1), mid-range (QC2), and low range (QC3 and QC4)—were processed and analyzed in replicates of four on 5 consecutive days. The predefined limits for precision and accuracy were set at a maximum of 15% or rather 20% (at lowest limit of quantification) variation or

Table 1
Average percentage recovery for AEA, PEA, and OEA

	2.5 pmol/ml (low level)	10 pmol/ml (intermediate level)	25 pmol/ml (high level)
Recovery AEA (%)	92	93	99
Recovery PEA (%)	76	82	99
Recovery OEA (%)	98	91	98

For recovery studies, serum samples from the same volunteer were spiked with FAEs (concentrations shown in the table) and 25 pmol d_4 -AEA and 500 pmol d_4 -PEA and d_4 -OEA ($n=3$) before and after their processing. The recovery was determined by comparing the peak areas of spiked samples with those of unextracted standard solutions containing the same concentrations of FAE.

Table 2
Accuracy and precision from QCs

Compound	QC-Level	Intraday		Interday	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
AEA	QC1	1.3	12.2	4.0	10.6
	QC2	2.8	0.8	2.2	0.9
	QC3	7.7	8.7	7.0	6.7
	QC4	3.4	3.3	12.9	3.1
OEA	QC1	0.4	3.9	1.1	3.6
	QC2	0.6	−4.0	1.7	−3.2
	QC3	6.2	2.6	13.3	8.6
	QC4	3.1	0.9	4.5	0.4
PEA	QC1	0.7	5.6	1.5	5.0
	QC2	2.3	2.2	3.3	1.8
	QC3	5.3	14.1	13.7	15.8
	QC4	15.2	−0.3	15.6	0.7

Precision is expressed as percentage coefficient of variation (CV). Accuracy is expressed as the ratio between the actual and the nominal values observed. For evaluation of the intraday and interday precision and accuracy, four different QC samples (high (QC1), midrange (QC2), and low range (QC3 and QC4)) were processed and analyzed in replicates of four on 5 consecutive days.

mean deviation, respectively. Table 2 summarizes the results obtained in five independent experiments and shows that precision and accuracy were always within the predefined limits.

Stability of plasma samples

The stability of serum samples during storage at -80°C was investigated after a single freeze-and-thaw cycle. Serum samples were thawed at room temperature, extracted, and analyzed. The samples were kept on ice while they were being processed. The remaining serum was refrozen at -80°C and thawed and prepared after 2 months. For comparison, QC1 and QC4 samples were thawed, analyzed, refrozen, and analyzed after 3 months. Storage at -80°C guarantees the stability of the samples for at least 3 months [22]. Stability was calculated by comparing the analyzed data with recently prepared reference solutions.

Stability is expressed as a percentage coefficient and is calculated by dividing the deviation between sample and reference values by the reference mean and multiplying the resulting value by 100. The predefined limits for stability were set at 20% for variation and mean deviation. QC stability was in the predefined ranges of variation (Table 3A) but the results for the freeze-thaw-stability of the serum samples showed a variation from 18 to 88% (Table 3B).

Limit of detection and quantification

The limits of detection, defined as the lowest quantity that can be detected, were 0.025 pmol for AEA and 0.1 pmol for both OEA and PEA. The higher detection limits obtained for OEA and PEA were due to the use of 500 pmol of deuterated standards, which per se contain

Table 3
Stability of standard solutions (A) and serum samples (B) after a freeze-and-thaw cycle

Freeze–thaw–stability of QCs (%)	QC Level				
	QC1	QC4			
A					
AEA	−7.0	−9.1			
PEA	−2.8	−12.7			
OEA	−0.5	3.1			
Freeze–thaw–stability of serum samples (%)	Serum sample number				
	1	2	3	4	5
B					
AEA	−44.3	−50.5	−77.2	−27.2	−53.2
PEA	−37.8	−56.9	−28.8	−18.6	−88.0
OEA	−62.3	−67.6	−61.4	−30.1	−70.7

Serum samples were thawed at room temperature, extracted, and analyzed. The remaining serum was refrozen and thawed and prepared after 2 months again. For comparison, QC1 and QC4 samples were thawed, analyzed, refrozen, and analyzed after 3 months again. Stability was calculated by comparing the analyzed data with recently prepared reference solutions.

some amount of unlabeled PEA or OEA. Since endogenous OEA and PEA are more abundant than AEA and therefore their quantification can be achieved at higher detection limits, we used this concentration of d_4 -OEA and d_4 -PEA to improve the recovery of these analytes. The limits of quantification, defined as the lowest quantity that can be measured with acceptable accuracy ($\text{CV} < 15$ or rather 20%), were 0.2 d_4 pmol for AEA ($\text{CV} = 15.0\%$, $n = 4$) and 0.4 pmol for OEA ($\text{CV} = 6.0\%$, $n = 4$) and PEA ($\text{CV} = 13.9\%$, $n = 4$).

Human plasma samples

The analysis of AEA, PEA, and OEA levels in human blood samples collected from eight healthy volunteers shows that there is no statistically significant difference in FAE levels in S and SC samples (Table 4).

Fig. 2 shows a representative chromatogram of serum FAEs. The chromatogram of extracted serum resulting from SRM detection shows AEA, PEA, and OEA in addition to their tetra-deuterated internal standards d_4 -AEA, d_4 -PEA, and d_4 -OEA. The analyte retention times (in minutes) were as follows: AEA (7.88), d_4 -AEA (7.89), PEA (8.50), d_4 -PEA (8.51), OEA (8.57), and d_4 -OEA (8.62). The chromatograms of extracted SC samples had identical profiles.

Sample analyses

The international guidelines and requirements for the validation of a method and the quantitative evaluation of the compounds were followed in all samples [23–26]. Although our stability tests for the QCs did not show a significant decrease in any analyte concentration, we found a massive loss of all FAEs in serum samples after one freeze-thaw cycle. These findings have a tremendous influence on the interpretation of prior research results since they show

Table 4

Concentrations of the fatty acid ethanolamides AEA, PEA, and OEA in serum (S) and serum with cells (SC) of eight healthy volunteers

Sample number	AEA (pmol/ml)		PEA (pmol/ml)		OEA (pmol/ml)	
	S	SC	S	SC	P	SC
1	0.63	0.77	26.63	28.40	2.52	5.19
2	0.58	0.61	11.68	33.66	2.73	2.80
3	0.86	0.75	26.49	26.49	4.06	4.24
4	0.44	0.46	10.17	25.42	2.79	2.72
5	0.49	0.67	25.75	22.59	2.68	4.15
6	1.29	1.28	26.32	34.81	2.75	3.11
7	2.06	1.56	63.89	27.30	8.21	8.14
8	1.10	1.60	36.70	33.93	3.51	5.88
Mean $\pm$ SE	0.93 $\pm$ 0.19	0.96 $\pm$ 0.16	28.44 $\pm$ 5.91	29.07 $\pm$ 1.60	3.66 $\pm$ 0.68	4.53 $\pm$ 0.65
<i>p</i> value	0.91		0.92		0.37	

Results are expressed in pmol/ml of blood. Means $\pm$ SE ($n = 8$) were compared by using the paired Student's *t* test (significant if $\alpha < 0.05$).

that the stability of FAEs in serum samples are labile to freeze–thaw cycles. In our study we have directly aliquoted the serum samples after centrifugation into 1 ml fractions and stored them immediately at -80°C for further analysis. This procedure guaranteed just one freeze–thaw cycle per sample. So we were able to exclude any loss of FAEs by the influence of several freeze–thaw cycles. There were no interfering signals observed during the process of validation and no carry over evidence was found.

Conclusions

We established a sensitive and selective method for the quantitative analysis of AEA, PEA, and OEA in human

serum based on LC-ESI-MS-MS. Our method is appropriate for the analysis and quantification of FAEs in the concentration range found in human serum as it is concordant with international regulatory guidelines for the validation of quantitative methods. Compared to previous reports on FAE analysis [16,17], the use of the tandem mass filter technique and the silver ion coordination offers several advantages: it provides faster sample processing and higher sensitivity and allows for improved detection of AEA, OEA and PEA compared to previously described methods for AEA, OEA, and PEA [16,17,21]. Using tandem mass spectrometry, we were also able to unambiguously identify each specific analyte and avoid erroneous determinations from contaminants displaying retention times identical to those of the FAEs analyzed. However, the AEA, OEA and PEA values in plasma obtained with our new method were in the same range as those reported in the literature for endocannabinoid measurements in plasma using other MS methods [13,27]. The new method is tremendously time-saving since one run lasts only 12 min compared to 30–40 min with existing methods [16,21]. Our simple and cost-saving extraction method with chloroform/methanol provides a rapid and inexpensive approach to extract serum FAEs from a variety of contaminants. Gas chromatographic–mass spectrometry is commonly used for lipid analysis. However, since the FAEs are very lipophilic and nonvolatile agents, derivatizations, such as trimethylsilylation, are needed. The use of GC–MS would thus make the sample preparation more complicated. Furthermore, GC–MS methods need another purification step, which is generally required by solid-phase extraction or HPLC. In conclusion, the use of LC-MS/MS makes our new method optimal for FAE analysis and allows one to prepare and analyze a large number of samples in a relatively short period of time.

Comparing FAEs concentration in serum to that of serum with mononuclear cells was an important question to address since mononuclear cells have the biosynthetic machinery to produce endocannabinoids [28–30]. Furthermore, they proliferate during inflammatory states, a condition that has been associated with elevated fatty acid ethanolamide levels [31,32]. The selection process of our healthy volunteers excluded other potentially influencing

Fig. 2. Chromatogram of extracted human serum resulting from SRM detection showing PEA, d₄-PEA, OEA, d₄-OEA, AEA, and d₄-AEA. Analytes were eluted from a C18 column with a MeOH:H₂O gradient containing 70 μM silver acetate (70–100% in 5 min followed by 3-min hold at 100% MeOH).

factors such as alcohol use. We could not find any difference of the concentrations of AEA, PEA and OEA in serum and serum with mononuclear cells. These results may be ascribed to the fact that FAEs are produced on demand and are not stored in cells. Therefore, an increase of FAE levels may be observed only after activation of mononuclear cells. On the other hand, we found a significant loss of FAEs after repeating freeze–thaw cycles of the same samples, suggesting that storage techniques should be taken into account when analyzing FAEs in blood samples.

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