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Endogenous ethanolamide analysis in human plasma using HPLC tandem MS with electrospray ionization

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ABSTRACT

A sensitive and selective liquid chromatography tandem mass spectrometry (LC\MS\MS) method has been developed for the simultaneous quantification in human plasma of the endocannabinoid anandamide (AEA) and three other related ethanolamides, linoleoyl ethanolamide (LEA), oleoyl ethanolamide (OEA), and palmitoyl ethanolamide (PEA). The analytical methodology requires 50 μL of human plasma which is processed via protein precipitation using a 96-well protein precipitation plate. Chromatographic separation of plasma extract was achieved with a Phenomenex Gemini C6-Phenyl HPLC column (2.1 mm × 50 mm, 5 μm) at a flow rate of 0.30 mL/min using gradient elution and a mobile phase consisting of acetonitrile and 5 mM ammonium formate. All four fatty acid ethanolamides were quantified by positive ion electrospray ionization tandem mass spectrometry, with the detection of ion current signal generated from the selected reaction monitoring (SRM) transition of $[M+H]^+ \rightarrow m/z$ 62. Deuterated anandamide (AEA-d8) was used as an internal standard for all four ethanolamides. The lower limit of quantitation was 0.05 ng/mL for AEA and LEA, 0.5 ng/mL for OEA and 1.0 ng/mL for PEA. Inter-assay precision and accuracy were typically within 12% for the four endogenous analytes and overall extraction recoveries ranged between 40% and 100%.

1. Introduction

The endocannabinoid system, which consists of transmembrane cannabinoid receptors, endogenous ligands (endocannabinoids), the enzymes involved in the synthesis and degradation via metabolism of these ligands, and associated signaling pathways, is known to play an important role in a number of physiological and pathophysiological processes such as drug addiction, obesity, inflammation and cancer [1–4]. Arachidonoyl ethanolamide (anandamide, AEA), an endogenous cannabinoid, was first identified in mammalian brain as a natural ligand for the CB-1 receptor [5]. Other structurally related endogenous ethanolamides such as oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA) and linoleoyl ethanolamide (LEA) have also been identified in biological tissues [6–7]. However, unlike anandamide, these compounds do not bind to cannabinoid receptors with high affinity but may influence endocannabinoid function by competing for catabolic enzymes. Alternatively, OEA, PEA, and/or LEA may be ligands for receptors that have not yet been identified [8]. AEA mimics many pharmacological and behavioral effects of cannabinergic agents, including analgesia and inhibition of locomotor activity. AEA as well as other endocannabinoids accomplish such effects via activation of cannabinoid receptors [9]. It has been shown that activation of both CB-1 and -2 receptors reduces nociceptive processing in animal models of neuropathic pain, with modulation through endocannabinoid signaling interactions. Thus, a possible approach to modulate pain sensation is by increasing the concentration of the endocannibinoid AEA [10–14].

The enzyme fatty acid amide hydrolase (FAAH) catalyzes the hydrolysis of anandamide to produce arachidonic acid and ethanolamine and is known to be the chief enzyme that terminates the activity of anandamide *in vivo*. Consequently, inactivation of FAAH is usually accompanied by elevation in levels of AEA [15–17]. In addition other ethanolamides which may serve as substrates for FAAH play a role in the regulation of different physiological processes. These include PEA, which possess anti-inflammatory properties, and OEA, which is known to regulate feeding and body weight [18–19].

Due to its rapid speed, specificity and sensitivity, high-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has become the bedrock of any modern day bioanalytical laboratory. Given the physiologic relevance of ethanolamides, it is important to have selective and sensitive analytical methods for the accurate identification and quantitation of these molecules in biological matrices, such as human plasma. The primary aim of this work was to validate a bioanalytical

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multiplex assay for four fatty acid amides, arachidonoyl ethanolamide (AEA), palmitoyl ethanolamide (PEA), linoleoyl ethanolamide (LEA), and oleoyl ethanolamide (OEA) for the analysis of human plasma samples. While previous methodology has been reported for measurement of these ethanolamides in biomatrices of preclinical species, this work describes the first validation of analytical methodology for application in human plasma. Though published methods have been reported previously for several ethanolamides [20–22], including some with lower limits of detection, this report presents a novel and simple sample preparation approach to quantitate ethanolamides using 96-well sample extraction technology and simultaneous analysis of a unique multiplex of ethanolamides that include AEA, PEA, LEA, and OEA. The limit of quanitification of the presented methodology is sufficiently below basal endogenous levels for all ethanolamides thereby providing analytical methodology that serves as a tool to enable probing systemically the impact for which FAAH inactivation has on the levels of these endogenous ethanolamides.

2. Experimental

2.1. Chemicals and reagents

AEA, AEA-d8, OEA and PEA were purchased from Cayman Chemicals (Ann Arbor, MI). LEA was obtained from Sigma (St. Louis, MO). HPLC grade acetonitrile, ethanol, methanol, water, and ammonium formate were all purchased from Mallinckrodt (St. Louis, MO). Human stripped charcoal plasma was obtained from Biochemed Pharmacologicals (Winchester, VA) and human EDTA plasma was obtained from Rockland Immunochemicals (Gilbertsville, PA) and Pfizer Laboratories, (St. Louis, MO).

2.2. Liquid chromatography and mass spectrometry

LC/MS/MS analysis was carried out using a high-performance liquid chromatography system consisting of a Shimadzu binary pump model LC-AvP (Shimadzu Scientific Instruments, Columbia, MD) with a CTC PAL autosampler (Leap Technologies, Carrboro, NC). The chromatography system was interfaced to an API 4000 LC/MS/MS quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex Inc., Ontario, Canada) which afforded the positive electrospray ionization (ESI) tandem mass spectrometry detection.

2.3. Individual standard solutions and calibration standards

Ethanol stock solutions were prepared individually for AEA, LEA, OEA, and PEA at 1 mg/mL. From these stock solutions, secondary stock solutions and working standard (WS) solutions were prepared with these solutions containing all four analytes. The secondary standard solution was prepared by the addition of the individual stock solutions to a volumetric flask to achieve concentrations of 1, 1, 10 and 20 μg/mL for AEA, LEA, OEA and PEA, respectively upon dilution with 50:50 methanol:H_2O. This secondary standard solution was stored at −20 °C. The working standard solution, for which the plasma calibration standards were generated, was freshly prepared daily with 5-fold dilution of the secondary standard solution with 30:70 acetonitrile:water to yield concentrations of 0.2, 0.2, 2, and 4 μg/mL of the ethanolamide analytes. The high plasma calibration standard (STD8) was prepared by the addition of 10 μL of working standard to 190 μL of charcoal stripped human plasma. The charcoal stripped plasma is simply plasma processed with activated charcoal for global removal of free, low molecular weight hormones. This source of plasma matrix was determined to contain lower endogenous levels of the tested ethanolamides when compared against other human plasma sources. The remaining calibration standards were then prepared via serial dilutions of high calibration standards to achieve the levels as shown in Table 1. The internal standard solution (AEA-d8) was prepared at a concentration of 100 ng/mL in acetonitrile.

Table 1
Calibration standards concentrations.

STD ID	Final plasma concentration (ng/mL)			
	AEA	LEA	OEA	PEA
STD8	10	10	100	200
STD7	5	5	50	100
STD6	2.5	2.5	25	50
STD5	1	1	10	20
STD4	0.5	0.5	5	10
STD3	0.25	0.25	2.5	3
STD2	0.1	0.1	1	2
STD1	0.05	0.05	0.5	1

2.4. Quality control and stability samples

Quality control samples (QC) were prepared from human control plasma fortified with EDTA at three analyte concentration levels representing the high-, mid- and low-end of the calibration standard curve (HQC, MQC, and LQC). Charcoal stripped plasma was not utilized for the preparation of the QC's therefore the total concentration of each analyte in the individual QC samples includes the fortified amount plus any basal endogenous levels (EL) present in the blank matrix. The LQC and HQC quality control concentrations served as the stability samples tested throughout the validation.

2.5. Sample preparation procedure

The sample preparation procedure for the analysis of AEA, LEA, OEA, and PEA in plasma is as follows. A 50 μL aliquot of plasma was precipitated with 250 μL of acetonitrile containing the internal standard (AEA-d8) using 2 mL capacity Strata Protein Precipitation plates (Phenomenex, Torrance CA). The Strata plates represent a solvent first extraction methodology in which the acetonitrile containing internal standard (IS) is added in the wells prior to plasma. An oleophobic membrane placed onto the filter stack prevents premature leaking of the organic solvent for up to 30 min. The plasma sample preparation procedure is a simplified three-step procedure involving the loading of the acetonitrile containing internal standard, addition of the plasma sample to the plate, and after gentle agitation, the elution of the supernatant into a clean 96-well collection plate. An adhesive cover was placed on the plate before the plate was shaken for approximately 3 min and then placed on a vacuum box over a collection plate for elution. The vacuum was set at 10 mm Hg and turned on for 10–15 s to allow for complete elution. The collection plate is then dried under N_2 and reconstituted using 100 μL of 30% acetonitrile/water solution. The plate is shaken for 3 min and placed onto an autosampler where 10 μL of the reconstituted extract was injected onto the LC/MS/MS system.

Table 2
Quality control sample concentrations.

QC ID	Final plasma concentration (ng/mL)			
	AEA	LEA	OEA	PEA
LQC	0.15 + el	0.15 + el	1.5 + el	3.5 + el
MQC	1.5 + el	1.5 + el	15 + el	35 + el
HQC	7.5 + el	7.5 + el	75 + el	175 + el
LQC stab[1]	0.15 + el	0.15 + el	1.5 + el	3.5 + el
HQC stab[1]	7.5 + el	7.5 + el	75 + el	175 + el

el: endogenous level present in plasma.

Table 3
Linearity assessment determined from three analytical batch runs.

Conc (ng/mL)	STD1 0.05	STD2 0.10	STD3 0.25	STD4 0.5	STD5 1	STD6 2.5	STD7 5	STD8 10	R^2
(a) Linearity assesment of AEA determined in three batch runs									
Mean	0.05	0.10	0.25	0.50	0.95	2.53	5.04	10.1	0.9987
S.D.	0.0015	0.0058	0.020	0.017	0.025	0.091	0.243	0.310	0.0004
%CV	3.1	5.6	8.0	3.5	2.67	3.6	4.8	3.1	0.04
%RV	1.3	3.3	0.0	0.0	−5.3	1.3	0.8	0.7	NA
(b) Linearity assesment of LEA determined in three batch runs									
Mean	0.050	0.10	0.26	0.53	0.99	2.50	4.80	9.90	0.9985
S.D.	0.0012	0.0059	0.026	0.025	0.021	0.091	0.159	0.075	0.0008
%CV	2.3	6.1	10.2	4.8	2.1	3.9	3.3	0.8	0.08
%RE	1.3	4.3	4.0	5.3	−0.7	−0.1	−3.9	−0.9	NA
Conc (ng/mL)	STD1 0.05	STD2 0.10	STD3 0.25	STD4 0.5	STD5 10	STD6 25	STD7 50	STD6 100	R^2
(c) Linearity assesment of OEA determined in three batch runs									
Mean	0.49	1.03	2.54	5.03	8.69	25.1	49.2	102	0.9988
S.D.	0.0058	0.020	0.136	0.081	0.305	0.100	0.608	5.56	0.0016
%CV	1.2	1.9	5.4	1.6	3.5	0.4	1.2	5.5	0.2
%RV	−1.3	3.0	1.5	0.7	−13	0.4	−1.6	2.0	NA
(d) Linearity assesment of PEA determined in three batch runs									
Mean	0.98	2.03	5.28	10.2	17.6	52.1	102	196	0.9980
S.D.	0.035	0.162	0.188	0.393	0.700	0.961	2.00	1.00	0.0002
%CV	3.6	8.0	3.6	3.8	4.0	1.8	2.0	3.6	0.02
RE	−2.5	1.7	5.5	2.4	−12.0	4.1	2.0	−2.0	NA

2.6. LC/MS/MS methodology

AEA, LEA, OEA, and PEA, and the IS AEA-d8 were separated chromatographically with a Phenomenex Gemini C6-Phenyl column (2.1 mm × 50 mm, 5 μm) by gradient elution. The mobile phase consisted of solvent A (5 mM ammonium formate in water) and solvent B (acetonitrile, acn). The gradient was as follows: solvent B was held at 45% for 0.2 min, linearly ramped from 45% to 90% in 2.4 min, held at 90% for 0.8 min and then immediately brought back to initial conditions of 45% ACN for re-equilibration. Total run time was 3.5 min with a flow rate of 0.30 mL/min. The mass spectrometer was operated in positive ion ESI mode for the detection of AEA, LEA, OEA, and PEA along with the internal standard (IS). Multiple reaction monitoring was performed with the $[M+H]^+$ selected for each analyte and monitoring the transitions m/z 348 → 62 for AEA, m/z 324 → 62 for LEA, m/z 326 → 62 for OEA, m/z 300 → 62 for PEA, and m/z 356 → 63 for AEA-d8 (Figs. 1–5). Mass spectrometer parameters were optimized for detection of the analyte transitions

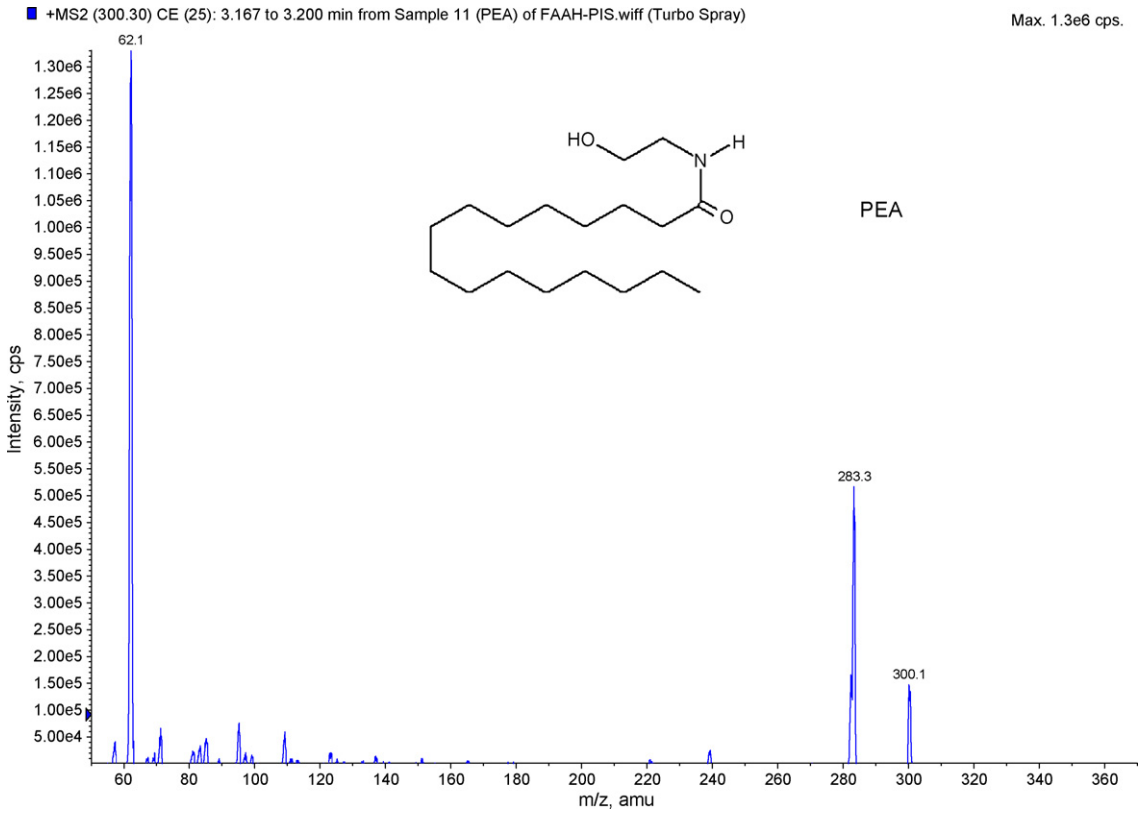

Fig. 1. Structure and product ion mass spectrum for PEA following dissociation of [M + H] + @ m/z 300.

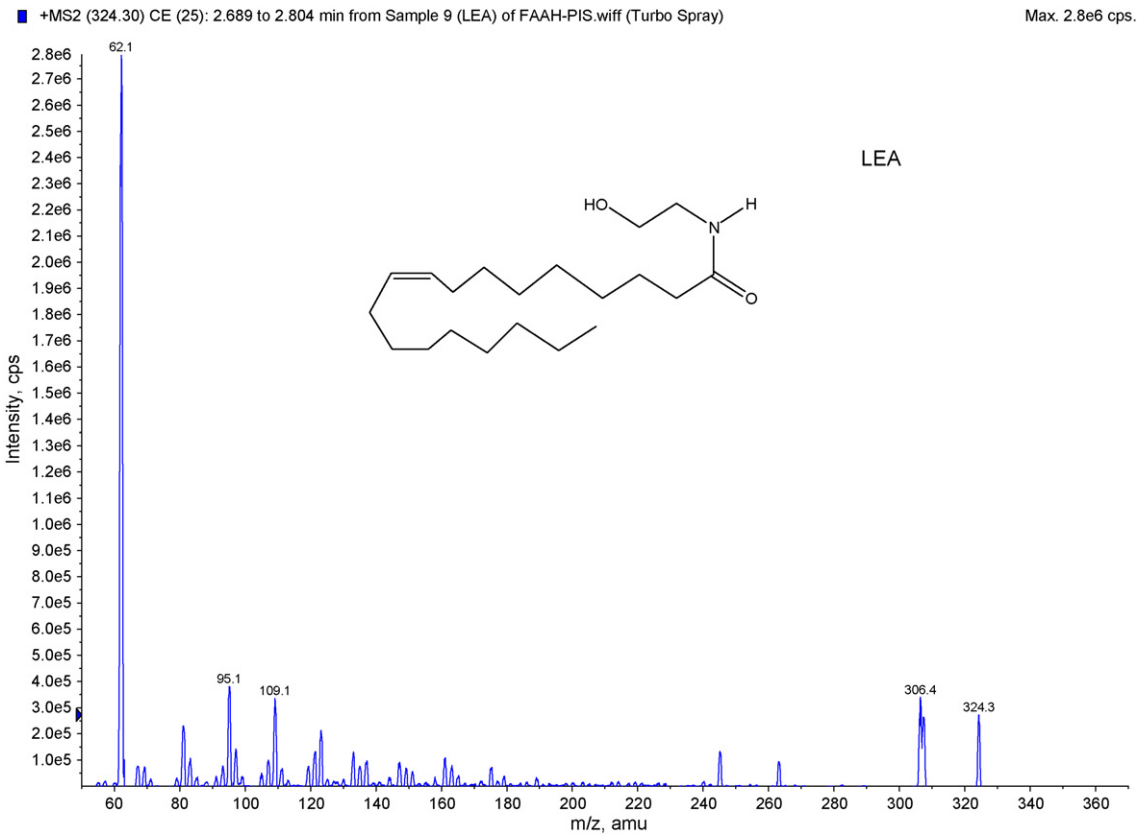

Fig. 2. Structure and product ion mass spectrum for LEA following dissociation of [M + H] + @ *m*/*z* 324.

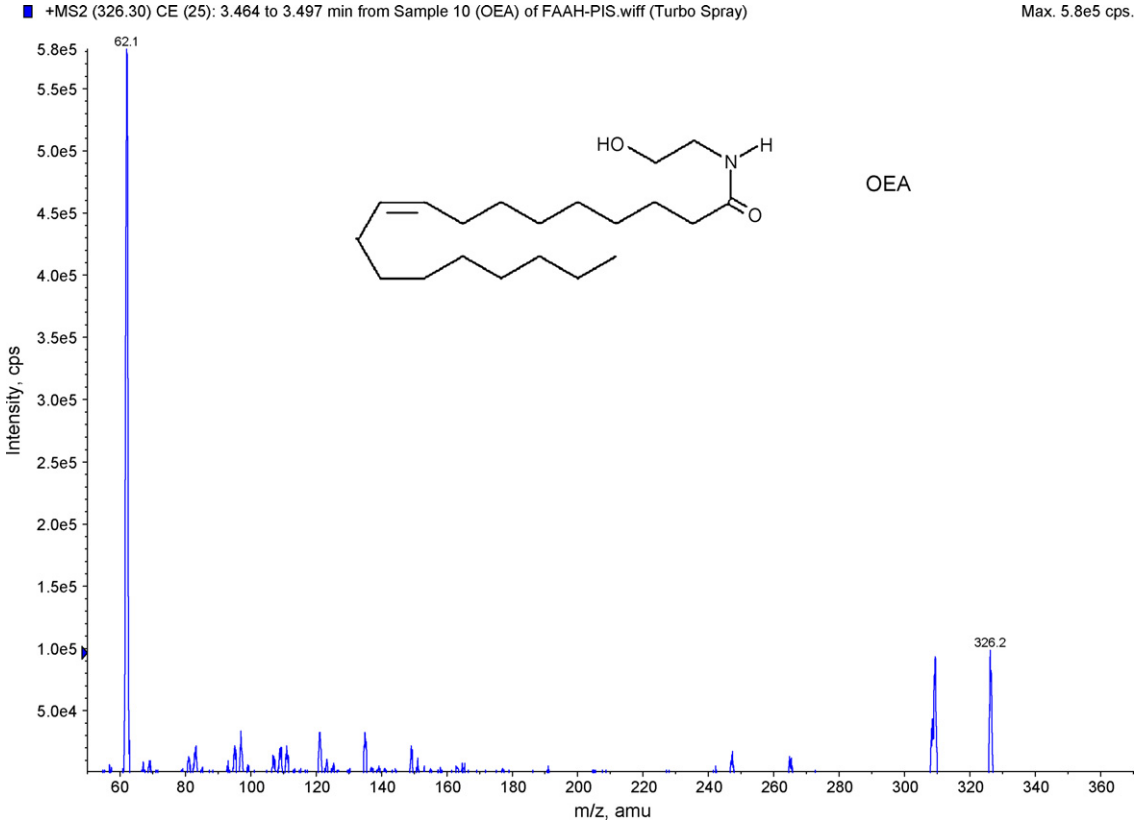

Fig. 3. Structure and product ion mass spectrum for OEA following dissociation of [M + H] + @ *m*/*z* 326.

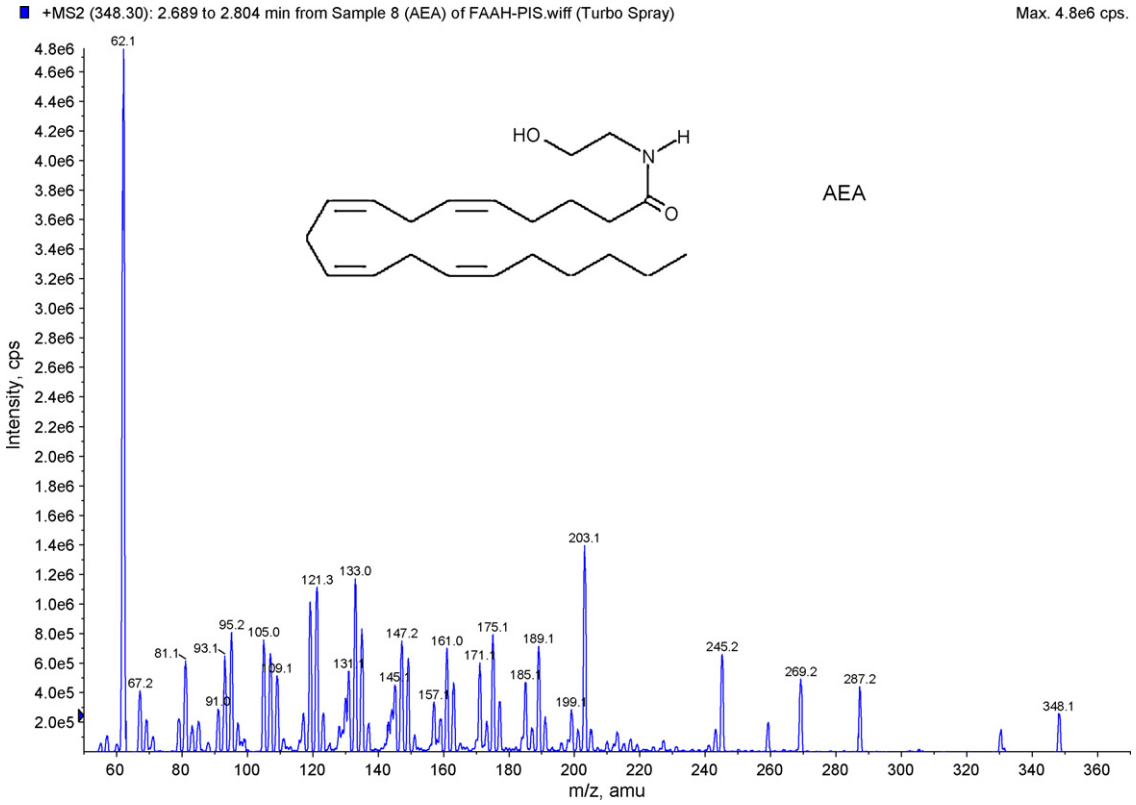

Fig. 4. Structure and product ion mass spectrum for AEA following dissociation of [M + H] + @ m/z 348.

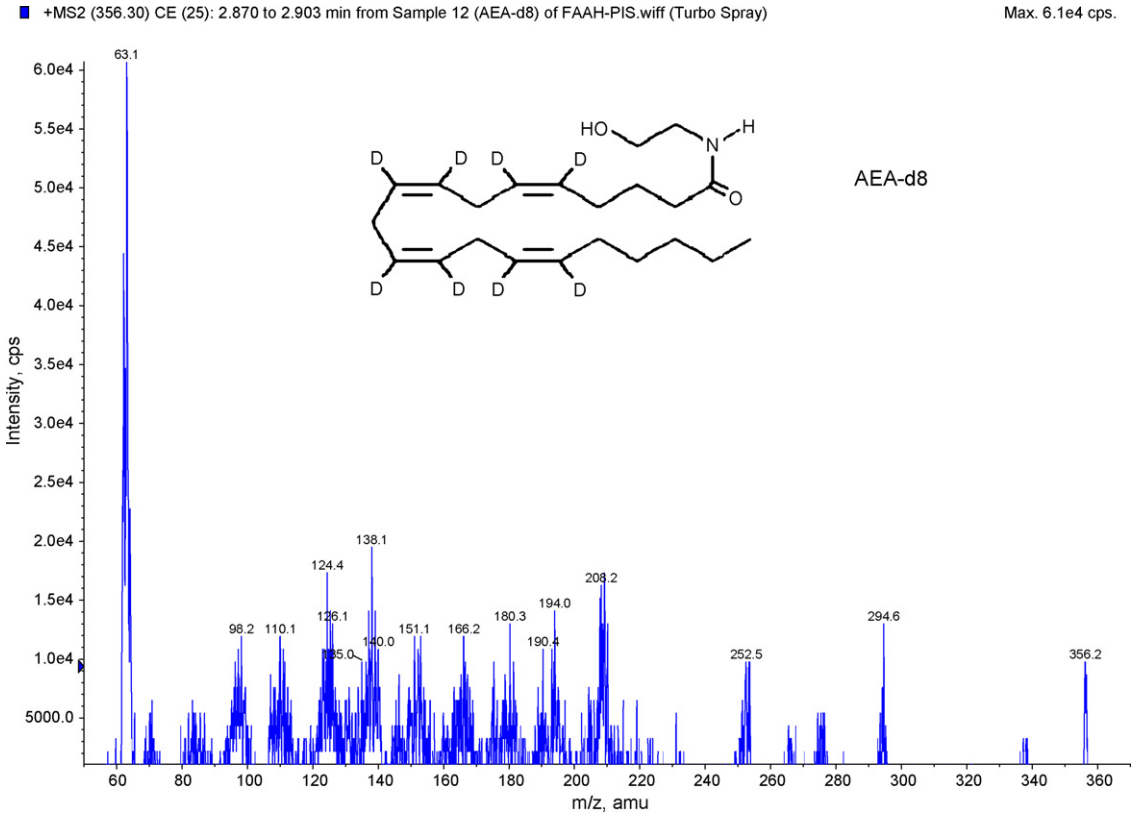

Fig. 5. Structure and product ion mass spectrum for AEA-d8 following dissociation of [M + H] + @ m/z 356.

Table 4
Precision and accuracy for AEA, LEA, OEA and PEA in plasma QC samples.

Sample ID	Mean conc (ng/mL)	Intra-day[a]						Inter-day	
		Day 1		Day 2		Day 3		%CV	%RE
		%CV	%RE	%CV	%RE	%CV	%RE		
AEA									
LQC	0.16	7.4	0.5	4.6	−0.1	5.5	−1.0	11.6	5.6
MQC	1.54	2.9	−3.9	5.7	4.6	5.2	4.7	6.8	2.7
HQC	7.75	2.6	−3.3	3.5	6.2	5.2	6.3	6.0	3.3
Average		4.3	−2.2	4.6	3.6	5.3	3.3	8.1	3.9
LEA									
LQC	0.14	9.9	1.6	5.6	−1.4	2.5	−1.9	18.3	−5.2
MQC	1.43	3.4	−2.9	3.2	−6.6	6.6	−5.0	9.4	−4.7
HQC	7.32	2.6	−1.1	3.5	−3.4	5.6	−3.8	4.8	−2.5
Average		5.3	−0.8	4.1	−3.8	4.9	−3.6	10.3	−4.1
OEA									
LQC	1.37	7.0	−8.5	4.6	−3.8	4.7	−1.0	11.2	−8.7
MQC	13.7	6.3	−15.0	4.4	−7.3	6.0	−2.2	8.7	−8.9
HQC	69.4	4.3	−14.5	5.5	−6.7	6.6	−0.9	8.3	−7.5
Average		5.9	−12.7	4.8	−5.9	5.8	−1.4	9.4	−8.4
PEA									
LQC	3.89	5.1	15.0	3.8	5.9	5.5	1.4	10.4	11.2
MQC	36.2	6.9	7.4	4.9	0.0	4.9	2.4	6.5	3.5
HQC	170	3.1	2.1	4.3	−7.3	3.9	−3.3	5.5	−2.8
Average		5.0	8.2	4.3	−0.5	4.8	0.2	7.5	3.9

[a] Not replicates for each QC.

indicated and included the declustering potential (DP) of 80 V, the entrance potential (EP) of 10 V and a collision energy (CE) of 25 V. All raw data was processed using Analyst Software ver. 1.4.1 (Applied Biosystems/MDS Sciex Inc., Ontario, Canada).

2.7. Validation procedure

To ensure that this methodology demonstrated sufficient rigor such that the data could be confidently utilized to make biomarker based decisions within the context of drug development programs, the following analytical assessments were made: linearity, precision and accuracy, analyte recovery from plasma, ion suppression, specificity and analyte stability.

2.7.1. Linearity, precision and accuracy

The linearity of the response of each analyte was assessed over their respective calibration range for three analytical batch runs. The precision and accuracy of the assay was determined for each analyte at three QC concentration levels in human control plasma over the three batch runs. These QC concentrations included the known fortified level added to the plasma plus the unknown endogenous concentration of analyte. Assessments of the endogenous levels found in the blank plasma used in the preparation of QC samples were made within each analytical batch run. The fortified level measurement was then calculated by subtracting the mean background level value from the entire concentration determined in the QC sample. Precision was denoted by a percent coefficient of variance (%CV) calculated by dividing the standard deviation by the mean and then multiplied by 100; (S.D./Mean × 100). The accuracy was denoted by a percent relative error (%RE), calculated by subtracting the theoretical level from the mean amount divided by the theoretical amount and then multiplied by 100; [((Mean − Theoretical)/(Theoretical)) × 100].

2.7.2. Stability

2.7.2.1. Long term stability. The long term storage stability of each analyte in human plasma was tested up to 8 weeks. Spiked quality control samples were prepared at two different concentrations, LQC and HQC levels ($n = 6$) to assess stability upon storage at −20 °C. A standard calibration curve was freshly prepared on the day of analysis when determining the stability of the analytes in the matrix.

2.7.2.2. Freeze/thaw stability. Freeze/thaw stability was tested at concentration levels, LQC and HQC ($n = 6$), by freezing the samples overnight then allowing them to thaw the next day at room temperature and then repeating the process again until three full cycles had been completed. Stability samples were quantitated against a freshly prepared standard calibration curve.

2.7.2.3. Benchtop plasma stability. Benchtop stability samples were prepared at the LQC and HQC levels and kept on a lab bench at room temperature for 4 h before analysis. Replicates of six were analyzed for each concentration against a freshly prepared calibration curve.

2.7.2.4. Whole blood stability. Whole blood stability was evaluated to assess any changes in analyte concentration that may occur following the draw of blood samples. Whole blood stability was tested for endogenous AEA levels only at room temperature using blood collected from four volunteers, two males and two females. Blood samples were collected in K2-EDTA tubes and then sat at room temperature for up to 2 h (0, 15, 30, 45, 60, 90 and 120 min) until the

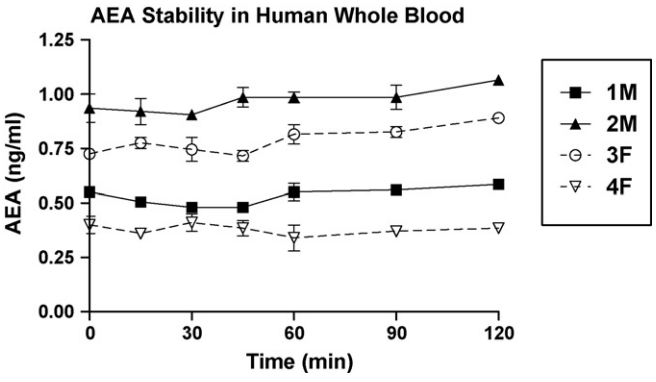

Fig. 6. Fresh whole blood stability of AEA at room temperature.

Table 5
Long term stability data at −20 °C.

Stability sample	Week 1			Week 8		
	Mean conc. (ng/mL)	%CV	%RE	Mean conc. (ng/mL)	%CV	%RE
AEA	Wk 1, el = 0.084			Wk 1, el = 0.066		
LOC	0.24	5.3	5.4	0.21	7.3	−3.7
HOC	8.23	2.4	8.5	6.95	12.0	−8.3
LEA	Wk 1, el = 0.58			Wk 1, el = 0.45		
LOC	0.66	6.7	−10.5	0.63	10.4	4.8
HOC	8.21	4.1	1.4	6.90	12.2	−13.2
OEA	Wk 1, el = 0.98			Wk 1, el = 1.35		
LOC	2.21	7.0	−11.0	2.89	10.1	1.4
HOC	71.0	3.2	−6.6	79.0	9.9	3.4
PEA	Wk 1, el = 2.37			Wk 1, el = 1.40		
LOC	6.86	6.3	16.9	5.40	11.4	7.1
HOC	199	4.2	12.5	154	14.7	−12.7

samples were centrifuged and the plasma layer was removed and processed for quantitative analysis.

2.7.2.5. Autosampler stability. Autosampler stability is defined as the stability of the analytes in the processed sample extract which defines the time interval that may be tolerated prior to injection of the sample extract onto the LC/MS/MS system following the plasma sample preparation. This stability was tested for over 18 h by re-injecting the same LQC and HQC plasma extracts repetitively ($n = 6$), and evaluating the concentration against a freshly prepared standard curve.

2.7.3. Recovery and ion suppression

The overall apparent recovery, representing recovery (extraction efficiency) of the analyte in the extraction procedure as well as analyte signal that could be masked in the detection procedure by ion suppression, was calculated for the four analytes. This was achieved by comparing the peak area responses generated from the SRM chromatograms of extracted plasma samples versus samples derived from neat solvent at the same concentration. Ion suppression was evaluated by continually measuring the analyte response under conditions where the effluent entering the LC/MS/MS system consisted of a concurrent infusion of a neat standard solution containing the four analytes which was added to the post-column HPLC effluent upon injection of an extracted plasma blank. This approach was conducted for seven sources of plasma.

3. Results and discussion

3.1. Linearity, precision and accuracy

During methods development and prior to commencement of the validation, the extraction procedure was tested for well to well contamination when using the Strata precipitation plates. This was accomplished by placing a spiked neat HQC sample adjacent to neat blank solution throughout the plate. The analytical procedure outlined in Section 2.5 was followed and the wells containing the neat blank solution were subsequently injected onto the LC/MS/MS system. The results confirmed that no cross well contamination occurred with this procedure.

The linearity of the calibration curves was determined over the specified ranges shown in Table 1. Challenges exist, as often in the case of developing methodology for endogenous soluble biomarkers, when the analyte itself is present in the blank matrix used to prepare the calibration standards. In developing the present assay, all human control matrix available had detectable concentrations of the four ethanolamides and analytical sensitivity was required below the endogenous amounts in order to accurately quantitate those values. Therefore the calibration standards for the assay were prepared in charcoal stripped human plasma which contained levels estimated to be 2–5-fold lower than the lower limit of quanititation of each analyte. For each analyte, the calibration curve showed a coefficient of determination greater than 0.998. Concentrations were back-calculated from the calibration curves for each standard using linear regression with a $1/\text{conc}^2$ weighting (Tables 2 and 3).

Table 6
Freeze thaw, benchtop and autosampler stability.

Stability sample	Autosampler stability			Benchtop stability			Freeze/thaw stability		
	Mean conc. (ng/mL)	%CV	%RE	Mean conc. (ng/mL)	%CV	%RE	Mean conc. (ng/mL)	%CV	%RE
AEA	el = 0.0082			el = 0.19			el = 0.19		
LOC	0.24	5.8	0.3	0.28	4.3	0.8	0.29	4.7	3.6
HOC	8.59	2.5	13.2	7.92	2.5	3.8	8.59	4.1	12.6
LEA	el = 0.47			el = 1.56			el = 1.56		
LOC	0.62	3.9	−0.6	1.57	1.9	−8.1	1.67	9.2	−2.6
HOC	8.05	4.2	1.0	8.49	2.7	−6.3	9.05	3.8	−0.1
OEA	el = 0.78			el = 2.17			el = 2.17		
LOC	2.35	8.5	2.4	3.81	4.1	3.7	3.99	11.9	8.6
HOC	82.5	5.9	8.8	74.8	3.0	−3.2	80.1	5.0	3.7
PEA	el = 1.17			el = 3.54			el = 3.54		
LOC	5.30	3.9	13.4	7.59	6.0	7.8	7.51	9.0	6.6
HOC	189	2.9	7.5	1.49	1.9	−16.3	158	5.1	−11.1

el = endogenous levels.

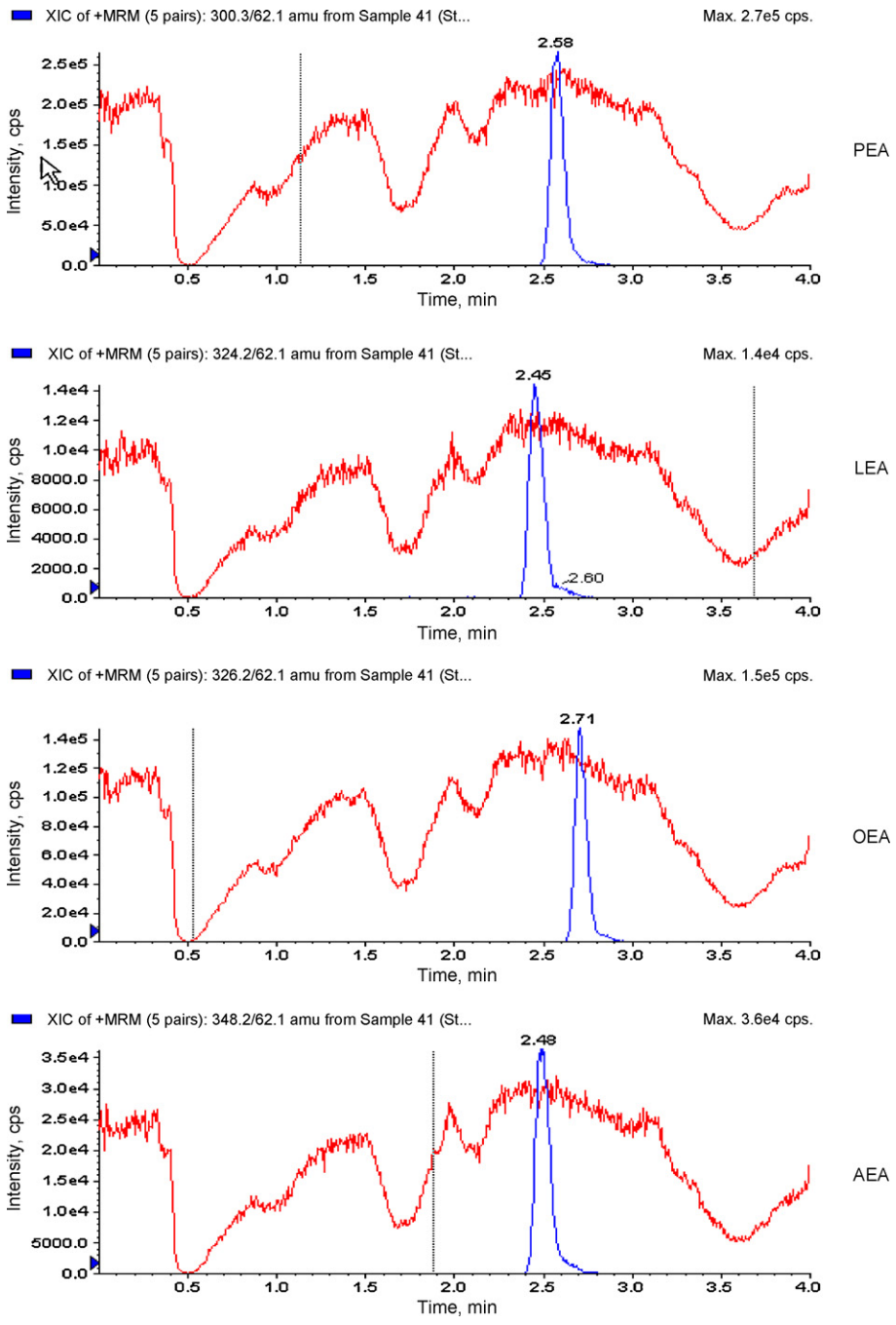

Fig. 7. Reconstructed ion chromatograms obtained with the simultaneous infusion of a neat ethanolamide solution and the HPLC effluent following injection of a blank plasma extract. Overlayed with the ion chromatograms is a representative HPLC trace of an extracted sample which indicates the retention time for each analyte.

The applicability of using charcoal stripped plasma for accurately and precisely quantify ethanolamides in human plasma was established by using quality control (QC) samples. These QC samples were prepared by spiking blank human plasma with known amounts of ethanolamides at three different levels across the assay calibration range. Human plasma was pooled to prepare the QC matrix containing endogenous levels (EL) of ethanolamides. The endogenous levels were calculated on a daily basis for each analytical run by taking the mean amount of six replicate samples. Intraday precision and accuracy was assessed over three separate days by preparing one standard calibration curve along with six replicates of LQC, MQC and HQC samples (Table 4). Inter-day precision and accuracy, including mean concentration values, is also shown in the table (mean endogenous levels have been back subtracted from the mean concentrations).

The limit of detection (LOD) for each analyte was defined as the lowest on column signal obtainable with a signal to noise of 3 or higher. Based on these criteria, the LOD for each ethanolamide was approximately 100 fg on column. In comparison the limit of quantitation (LOQ) is defined as the lowest quantifiable standard and is represented as STD1 in Table 1. The on column LOQ was 250 fg for AEA and LEA, 2.5 pg for OEA and 5 pg for PEA. While typically an LOQ for most analytes is set at or near the LOD, the assay requirements for each endogenous analyte given the baseline plasma levels of ethanolamides did not require such stringent LOQ's. The concentration of the ethanolamides would increase upon enzyme inhibition,

and then return back to near baseline levels hence the LOQ requirements were simply defined by basal plasma levels of the individual ethanolamides and not driven by what the analytical methodology could provide.

3.2. Stability

The sample long term stability at −20 °C was evaluated to establish acceptable storage conditions for plasma study samples. Stability data are shown in Tables 5 and 6. Endogenous levels, which are calculated by taking the mean of six replicate control blanks, are denoted as *el.* and have been incorporated into the tables. A stability time-course was generated for AEA in human whole blood. The data presented in Fig. 6 indicates that AEA concentration determined in plasma is stable over the course of 2 h for which human whole blood was at room temperature.

3.3. Recovery and ion suppression

A neat solution of the medium QC (MQC) concentration was infused at a flow rate of 15 μL/min during chromatographic analysis of a plasma blank extract. The matrix effect was examined qualitatively by the simultaneous introduction into the MS/MS detector of a post-column infusion of the four ethanolamides with the effluent emerging from the HPLC system following chromatographic separation of a blank plasma extract [23]. Representative data from this experiment which was conducted with seven sources of blank plasma is shown in Fig. 7. Overlaying the MS/MS response is a representative reconstructed ion chromatogram of a MQC extracted plasma sample which indicates the retention time for each of the analytes. Of note is the consistent response generated by each analyte in the proximity of the retention time for each respective ethanolamide. In addition, the response within this temporal region is comparable to that observed when the neat analyte solution is infused with mobile phase emerging from the HPLC in the absence of endogenous constituents from an injected sample extract. In our assay methodology, elution of each analyte occurs within a region of the chromatogram characterized by minimal ionization suppression of signal due to constituents within the plasma matrix extract. These results thus lend additional confidence to the validity of data generated when employing this bioanalytical assay.

The absolute recovery of AEA, LEA, OEA and PEA in plasma was determined by comparing the analytical response associated with plasma extracts from an HQC sample with the response generated from a neat sample in solvent at an identical concentration ($n = 6$). Utilizing this approach, it was determined that the overall recovery ranged from 41% (OEA), 61% (AEA and LEA) to 99% (PEA). The lower recovery for OEA, AEA and LEA may be due to non-specific binding from the Strata plates. Given that the accuracy and precision data for the analytes is typically within 12% suggests that the recovery is consistent throughout the calibration range and therefore the lower recovery (<61%) observed would not be expected to compromise data quality in the analysis of unknown samples.

4. Conclusions

We describe a sensitive and selective LC/MS/MS assay procedure using 96-well protein precipitation plates for the specific and quantitative analysis of multiplexed AEA, LEA, OEA and PEA in human plasma. The assay uses only one deuterated internal standard, AEA-d8, unlike some previously reported methods, which reduces assay complexity while maintaining the analytical rigor required. The results presented support the validation of the assay with respect to accuracy and precision, linearity, and stability, for use in measuring these four ethanolamides in human plasma. The analysis requires 50 μL of human plasma and improves the sample processing time through elimination of the multiple steps required for classic protein precipitation techniques, liquid/liquid and solid phase extractions. In addition to reduced cycle time, transferability of the assay to others is greatly enhanced because of the simplified three-step sample preparation technique utilizing a 96-well plate. The use of 96-well precipitation plates and 96-well technology allows for the possibility to fully leverage automated laboratory sample handling systems such as the Hamilton, Tecan or Biomek automated workstations, which can further enhance productivity while reducing sample processing times [24,25]. While the assay presented here is for application in human plasma, the methodology has also been used in the analysis of plasma generated from pre-clinical species.

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