Journal of Chromatography B, 895–896 (2012) 186–190

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Short communication

Ultra sensitive measurement of endogenous epinephrine and norepinephrine in human plasma by semi-automated SPE-LC–MS/MS

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ARTICLE INFO

Article history:
Received 7 February 2012
Accepted 17 March 2012
Available online 26 March 2012

Keywords:
Catecholamines
Epinephrine
Norepinephrine
Alumina SPE
Human plasma
Biomarker
LC–MS/MS

ABSTRACT

Measurement of endogenous epinephrine (E) and norepinephrine (NE) in human plasma is very challenging due to lower endogenous concentrations as compared with animal plasma. An LC–MS/MS in combination with alumina-based SPE and derivatization procedure was validated for the measurement of E and NE in human plasma with acceptable intra-day and inter-day accuracy and precision. Sample was extracted with semi-automated alumina 96-well solid phase extraction (SPE) cartridge. The resulting eluent was dried and derivatized using d4-acetaldehyde. The analytes were separated on a monolithic C_{18} column. Extraction efficiencies were >66% for E and NE. The lower limit of quantitation (LLOQ) was 5.00 pg/mL for E and 20.0 pg/mL for NE.

1. Introduction

Catecholamines (epinephrine (E), norepinephrine (NE) and dopamine) play a critical role in physiology, acting as neurotransmitters and hormones. E and NE have been examined as exploratory biomarkers in the study of many disease areas, including diabetes, heart disease, pain, and anxiety [1,2]. The most commonly employed method of E and NE analysis in human plasma in clinical laboratories is reversed-phase LC in conjunction with electrochemical (ECD) or fluorescent detection (FD) [3,4]. Recently, Ji et al. [5] have described an ethylation labeling procedure combined with UPLC–MS/MS assays for determination of rat plasma E and NE with enhanced sensitivity (LLOQ = 50.0 pg/mL).

Because of the low physiological levels of E and NE in human plasma, the procedure for sample extraction and enrichment is critical to develop a more sensitive and selective assay. Methods using phenylboronic acid (PBA), alumina or cation-exchange SPE sorbents have been reported to extract and enrich E and NE from biological samples effectively [6–8]. However these manual approaches were generally time-consuming and inconsistent for sample recovery and assay reproducibility. Here, we describe a new semi-automated alumina-based extraction method for the simultaneous extraction of E and NE in human plasma, followed by reductive ethylation labeling and analysis by LC–MS/MS platform. The LC–MS/MS assay was analytically validated with an LLOQ of 5.00 pg/mL for E and 20.0 pg/mL for NE. The limit of detection (LOD) (S/N = 3.0) for E and NE was 0.500 pg/mL in human plasma. This method with SPE enrichment offers at least 10 fold higher sensitivity than previously reported LC–MS/MS methods.

2. Experimental

2.1. Materials

Formic acid, sodium metabisulfite, L-ascorbic acid, ammonium formate, sodium acetate, cyanoborohydride coupling buffer, epinephrine, and norepinephrine were obtained from Sigma (St. Louis, MO, USA). (±)-Epinephrine-d6 (E-d6) (98.7% D) and (±)-norepinephrine-d6 (NE-d6) HCl (98.7% D) were purchased from C/D/N Isotopes Inc (Quebec, Canada). Acetaldehyde-d4 (D4, 99%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Heptafluorobutyric acid (HFBA) was purchased from Acros Organics (NJ, USA). All other chemicals and reagents were of reagent grade or higher and purchased from Sigma (St. Louis, MO).

2.2. Liquid chromatographic and mass spectrometric conditions

The LC–MS/MS system consisted of a Shimadzu HPLC LC-20 AD binary pump system (Columbia, MD), a CTC Analytics (LEAP) HTS PAL autosampler (Carrboro, NC), and an API 4000 mass

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doi:10.1016/j.jchromb.2012.03.026

spectrometer (AB Sciex, Ontario, Canada) with a TurboIonspray™ source employed for detection. The chromatographic separation was performed on an Onyx Monolith C_{18} column (100 mm × 3.0 mm i.d.) (Phenomenex, Torrance, CA). Mobile phase A (10 mM ammonium formate in water) and mobile phase B (methanol) were operated with gradient elution as (min, % B): (0, 6.0) (0.5, 6.0) (3.50, 15.0) (3.51, 6.0) (3.53, 6.0) (4.50, 60) (4.51, 90) (5.40, 90) (5.41, 6.0) (6.20, 6.0) at the flow rate of 0.700 mL/min. The mass spectrometer was operated in positive-ion, multiple reaction monitoring (MRM) mode. MRM transitions were *m/z* 216.1 → 165.1 for E, 222.1 → 171.3 for E-d6, 234.1 → 183.3 for NE, and 240.1 → 189.4 for NE-d6. Collision energy was 32 eV for both E and E-d6 and 35 eV for both NE and NE-d6.

2.3. Preparation of surrogate matrix (human plasma), standards and QC samples

Control human plasma was kept at 37 °C for 48 h to oxidatively degrade the endogenous E and NE below detectable levels (<LOD). Two milliliter of matrix stabilizer solution (317 mg/mL of sodium metabisulfite in water) was added to 98 mL of above heated human plasma and mixed well and further diluted with water (1:1, v/v). This heat treated, stabilized and diluted human plasma was then used as artificial blank (surrogate) matrix to prepare calibration curve standards (STDs) in the concentration range 5.00–500 pg/mL for E and 20.0–2000 pg/mL for NE. Two milliliter of matrix stabilizer solution (317 mg/mL of sodium metabisulfite in water) was also added to 98 mL of pooled human plasma and used for the preparation of QC samples at three concentration levels: LQC (=endogenous baseline (EB)), MQC (=EB + 100 pg/mL for E and EB + 400 pg/mL for NE) and HQC (=EB + 375 pg/mL for E and EB + 1500 pg/mL for NE). Similar to QC samples, all human plasma samples were treated with matrix stabilizer solution (add 0.1 mL of 317 mg/mL sodium metabisulfite to each 4.9 mL of human plasma) before sample analysis.

2.4. Sample preparation

SPE extraction was operated on a high throughput automatic Tomtec Quadra 4 liquid-handling workstation (Tomtec, Hamden, CT, USA). Alumina B 96-well SPE cartridge (Waters, Milford, MA) was conditioned with 1.0 mL of acetonitrile, followed by 1.0 mL of 0.2% ammonia solution (v/v) and 1.0 mL of 0.5 M Tris–EDTA buffer (pH 8.0). Each sample/STD/QC/blank (0.5 mL) was aliquoted to individual wells of a 2 mL 96-well block, followed by the addition of 25 μL of the internal standard (10.0 ng/mL of E-d6 and NE-d6) to each well. The samples were then diluted with 0.8 mL of 0.5 M Tris–EDTA buffer (pH 8.0) and mixed well. The above samples were then loaded onto SPE cartridge, washed with 1× 0.8 mL of 0.5 M Tris–EDTA buffer (pH 8.0) and 3× 0.8 mL of water. Analytes were eluted using 0.55 mL of acetonitrile:water (60:40, v/v) containing 2.5% formic acid. The eluent was evaporated to dryness under a steady stream of nitrogen at 37 °C. Samples were reconstituted in 60 μL of ethylation labeling derivatization solution (pH 5.2), which was prepared freshly just before chemical labeling [5] by mixing 5.25 mL of cyanoborohydride coupling buffer with 1.2 mL of 2 M acetate buffer (pH 5.2), 65 μL of acid solution (containing 500 mg/mL of citric acid and 40 mg/mL of L-ascorbic acid), and 180 μL of acetaldehyde-d4. The resultant 96-well plate was then incubated at 37 °C for 60 min. After derivatization, 8.0 μL of 5% (v/v) HFBA water solution was added to each well of the derivatization plate and mixed well. Each sample (20 μL) was then injected into the LC–MS/MS system for analysis.

2.5. Stability of E and NE in human blood and plasma with or without stabilizers

E and NE are readily oxidized and unstable in whole blood and plasma at room temperature. Sodium metabisulfite (6.34 mg/mL in matrix) or citric acid and L-ascorbic acid solution (25 mg/mL of citric acid and 2 mg/mL of L-ascorbic acid in matrix) were tested as potential stabilizers for E and NE in human whole blood and plasma at 4 °C and room temperature (RT).

2.6. Method validation

The method was validated for linearity, matrix effects, accuracy, precision and extraction efficiency. Peak area ratios were calculated by dividing the area of each analyte by that of its internal standard. Calibration curves were constructed using these peak area ratios with a weighted ($1/x^2$) least-squares linear regression analysis. Precision (expressed as percent relative standard deviation, % RSD) and accuracy (expressed as relative percent error) were calculated at three levels of QC samples. Six replicates of each QC level were analyzed with each validation run to determine the intra-day accuracy and precision. This process was repeated three times in order to determine the inter-day accuracy and precision. Extraction efficiencies were determined using E and NE-spiked plasma samples ($n = 5$) at the concentrations of 100 and 375 pg/mL for E or 400 and 1500 pg/mL for NE. Extraction efficiency was calculated by dividing the peak area of analytes spiked before extraction by the peak area of analytes sample at equal concentration in the same matrix spiked after extraction. Matrix effects for quantitation of E and NE were determined using 3 different lots of plasma samples by comparing the differences between peak area ratios of spiked standards in human plasma samples and control (artificial plasma). Analyte stability through freeze–thaw (3 cycles), at room temperature (24 h) with sodium metabisulfite stabilizer, long-term stability at −80 °C, and extract stability in an autosampler (72 h) ($n = 5$) were also assessed.

3. Results and discussion

3.1. MRM and chromatographic conditions

Chromatographic retention and separation play key roles for the bioanalysis of derivatized E and NE. Derivatized NE generated the cross-talk response in derivatized E MRM channel. Therefore, baseline chromatographic separation between derivatized E and derivatized NE is necessary to prevent this crosstalk from impacting the analytical results. Different common reversed phase columns such as C_{18}, C_8, phenyl, phenyl-hexyl, and C_{18} monolith columns were screened and the Onyx Monolith C_{18} column (100 mm × 3.0 mm i.d.) provided the best chromatographic separation and peak shapes.

3.2. Strategies for extracting E and NE from plasma

Optimizing sample preparation conditions is critical in the achievement of acceptable recovery and reproducibility for E and NE. Protein precipitation using acetonitrile has been shown to work well for rat plasma [5], but the sensitivity with this method is insufficient to quantify endogenous levels of E in human plasma. It is necessary to enrich E from human plasma to obtain a LLOQ in the low pg/mL range. However, it is quite challenging to extract E and NE from biological samples using traditional silica based solid-phase extraction (SPE) due to their high polarity, low molecular weight and chemical instability under basic conditions [9]. Alumina and boric acid isolation SPE procedures for the enrichment of E and NE from plasma have been frequently

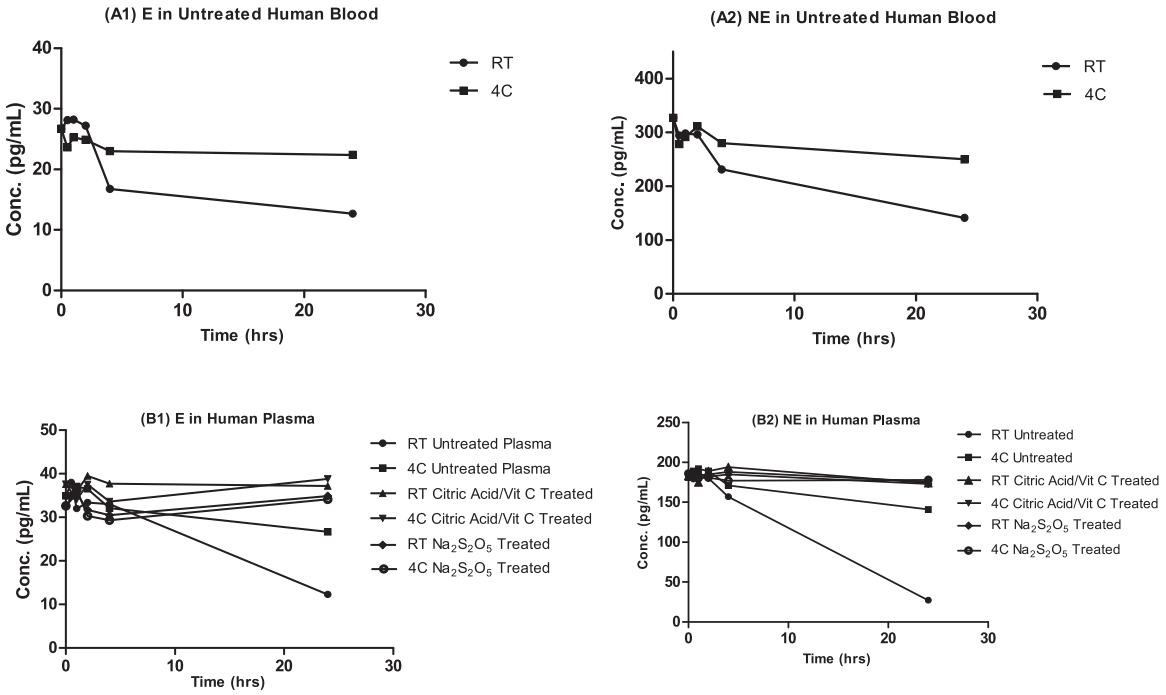

Fig. 1. Matrix stability assessment of E and NE in human blood and plasma (A1) E in human blood without stabilizers at room temperature and 4 °C, (A2) NE in human blood without stabilizers at room temperature and 4 °C, (B1) E in human plasma with or without stabilizers at room temperature and 4 °C, and (B2) NE in human plasma with or without stabilizers at room temperature and 4 °C.

applied in LC-ECD based assays, however, the accuracy of calculated E and NE concentrations might be compromised due to unexpected interferences and oxidation of E and NE under high pH conditions [10,11].

Attempts in using ion-pair reversed phase SPE or using mixed cation exchange (MCX) SPE were conducted. Unfortunately, extraction efficiencies of both approaches were less than 15% (data not shown). Next, Waters Alumina B 96-Well cartridges were evaluated and significantly enhanced the extraction efficiencies of analytes with extraction efficiencies above 66.0% for E and above 68.1% for NE.

3.3. Surrogate matrix selection for human plasma

A common challenge in biomarker analysis is to select an appropriate surrogate matrix for the preparation of a standard calibration curve. An ideal surrogate matrix should be free of target analytes and is identical or similar to unknown sample matrices with regard to ionization effects and extraction efficiency. To this end, human plasma was kept at 37 °C for 48 h to thermally degrade the endogenous E and NE. Matrix stabilizer solution (317 mg/mL of sodium metabisulfite in water) was then added to the above heat treated human plasma to stabilize the E and NE spiked in to create

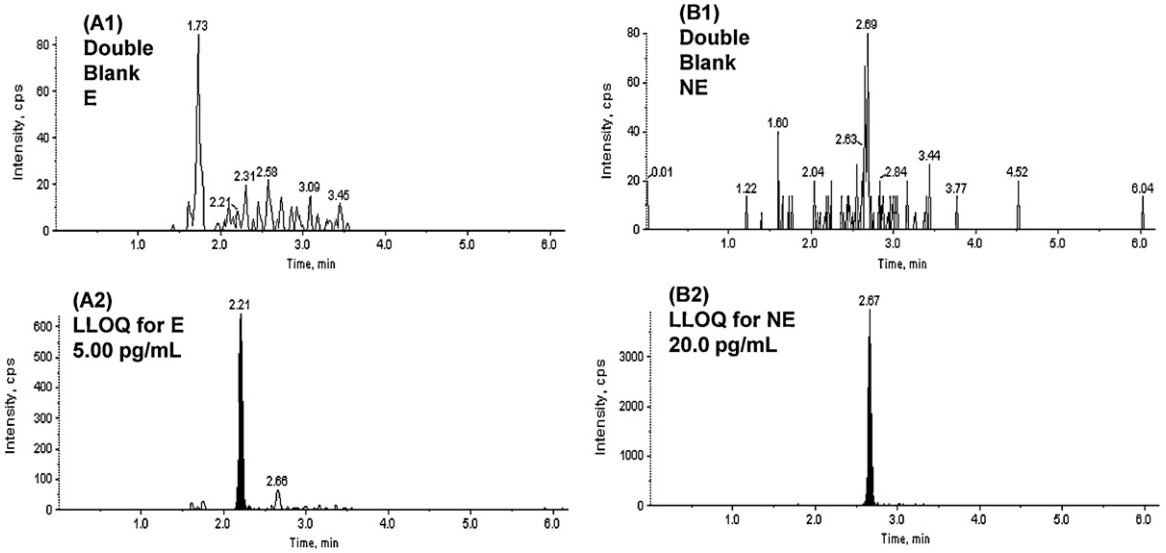

Fig. 2. Representative LC–MS/MS chromatograms of LLOQ (5.00 pg/mL for E and 20.0 pg/mL for NE) in artificial plasma (A1) blank for E, (B1) blank for NE, (A2) 5.00 pg/mL of E spiked in artificial plasma, and (B2) 20.0 pg/mL of NE spiked in artificial plasma.

Table 1
QC sample results showing the intra-run ($n = 6$) and inter-run precision ($n = 18$) (% RSD) and accuracy (% RE) of the SPE-LC–MS/MS assay used to quantitate E and NE in human plasma.

Analytes	Theoretical concentration (pg/mL)	Intra-day			Inter-day		
		Measured concentration (pg/mL)	RSD (%)	RE (%)	Measured concentration (pg/mL)	RSD (%)	RE (%)
E	26.2 (EB)	26.0 ± 0.843	3.2	−0.8	26.2 ± 0.997	3.8	0.0
	126 (MQC)	131 ± 5.28	4.0	4.0	132 ± 4.51	3.4	4.8
	401 (HQC)	434 ± 8.80	2.0	8.2	432 ± 11.7	2.7	7.7
NE	80.8 (EB)	80.0 ± 2.46	3.1	−1.0	80.8 ± 1.36	1.7	0.0
	481 (MQC)	493 ± 10.5	2.1	2.5	480 ± 14.3	3.0	−0.2
	1580 (HQC)	1680 ± 65.2	3.9	6.3	1650 ± 85.9	5.2	4.4

standards, and the matrix was further diluted with water (1:1, v/v). To this end, this heat treated, stabilized, and diluted human plasma was used as blank matrix (artificial plasma) to prepare calibration curve standards (STDs).

3.4. Stability of E and NE in human plasma with or without stabilizers

Analyte stability is critical for establishing proper sample handling conditions for clinical research sites. Our results indicated that E and NE were not stable at room temperature within 4 h in human blood without stabilizers (37.0% and 29.4% of nominal, respectively) (Fig. 1(A1) and (A2)). However, E and NE in untreated human blood were much more stable at 4 °C (up to 2 h). Plasma E and NE levels reduced to 64.8% and 85.0% of nominal, respectively, at room temperature for 24 h without stabilizers while remained stable for at least 24 h when treated with sodium metabisulfite (6.34 mg/mL in matrix) or citric acid and ascorbic acid solution (25 mg/mL of citric acid and 2 mg/mL of ascorbic acid in matrix) (Fig. 1(B1) and (B2)). From our stability assessment, it was determined that it is not necessary to add the stabilizers to blood and plasma immediately following collection or processing. It is recommended that upon collection, whole blood samples be kept at 4 °C and centrifuged within 60 min (also at 4 °C). The separated plasma should then be frozen immediately at −80 °C and stored until sample analysis.

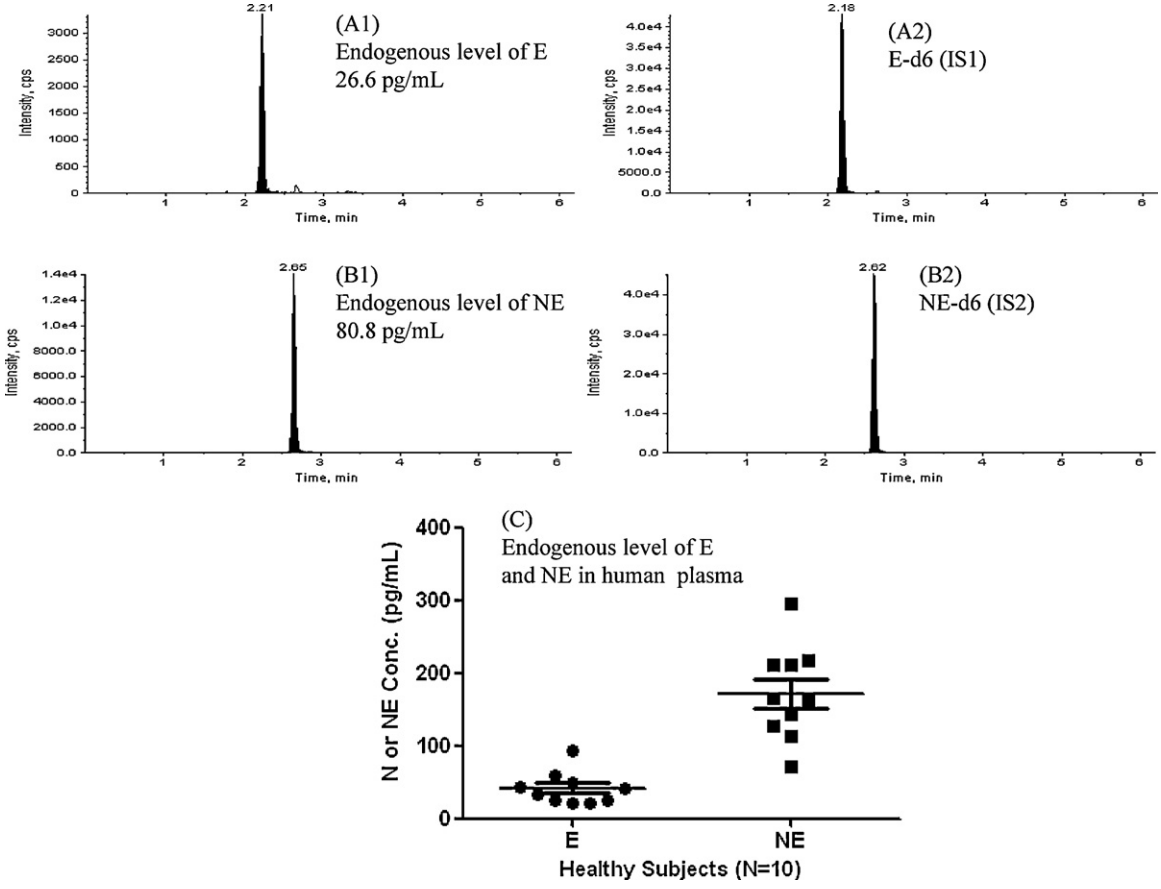

Fig. 3. Representative LC–MS/MS chromatograms of monitoring endogenous E and NE in the commercial healthy human plasma, (A1) 26.6 pg/mL of basal level of E in an individual human plasma, (A2) E-d6 (IS1), (B1) 80.8 pg/mL of basal level of NE in an individual human plasma, (B2) NE-d6 (IS2), and (C) the endogenous level of E and NE in 10 individual healthy human plasma.

3.5. Assay validation results

The calibration curves showed excellent linear response ($R^2 > 0.9971$) over the concentration range of 5.00–500 pg/mL for E and 20.0–2000 pg/mL for NE. The limit of detection (LOD) (S/N = 3.0) for both E and NE was 0.500 pg/mL in human plasma. Representative chromatograms obtained from a blank sample in artificial plasma and artificial plasma spiked with the LLOQ standard (5.00 pg/mL for E and 20.0 pg/mL for NE) are shown in Fig. 2. Matrix effects in 3 plasma samples for E and NE ranged from 0.8 to 3.4% and 2.4 to 4.9%, respectively. These results suggest that no significant matrix effect is present during analysis of the processed samples. Acceptable results for intra- and inter-day precision and accuracy for E and NE in human plasma were obtained and shown with % RSD ≤ 5.2% and % RE ≤ 8.2% (Table 1) based on 2001 US FDA Bioanalytical Method Validation (BMV) guidance [12]. The basal levels of E and NE in QC plasma samples were 26.6 pg/mL for E and 80.0 pg/mL for NE. Representative chromatograms are presented in Fig. 3.

Stock solution stability (30 days), freeze–thaw (3 cycles) or room temperature (24 h) stability with stabilizer, extract stability (72 h), and long-term stability for at least 32 days at −80 °C with or without stabilizer were also evaluated. Our results indicated that E and NE were stable under all these conditions.

3.6. Endogenous human plasma levels of E and NE in healthy subjects

The plasma of ten individual healthy subjects was analyzed to assess the range of endogenous levels that were to be expected in a clinical study. The endogenous levels ranged from 21.7 pg/mL to 92.8 pg/mL for E and 71.0 pg/mL to 296 pg/mL for NE in human plasma (Fig. 3(C)).

4. Conclusions

It has been a huge challenge for clinical laboratories to develop a robust assay to measure basal levels of E and NE in human plasma. In this work, we present a high-throughput SPE-LC–MS/MS assay for E and NE in human plasma. Acceptable assay performance for E and NE measurement was obtained with an LLOQ of 5.00 pg/mL for E and 20.0 pg/mL for NE in human plasma. Matrix stability assessment suggested that it is not necessary to add stabilizers to blood and plasma at clinical sites.

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