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Determination of aminoethylcysteine ketimine decarboxylated dimer in human plasma and cultured cells by high-performance liquid chromatography with electrochemical detection

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Abstract

Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) is a natural compound with antioxidant properties of a new family of sulfur-containing amino acids. It has been detected in human urine and plasma, in mammalian cerebellum and, more recently, in dietary vegetables. In the present study, a simple, highly sensitive method using a high-performance liquid chromatography system with electrochemical detection (ECD) has been developed. The method showed excellent precision and accuracy. It has been found to be about 100-fold more sensitive than gas chromatographic method and 2000-fold more sensitive in respect to the liquid chromatography method with UV detection. The method showed the required features of specificity and sensitivity to detect aminoethylcysteine ketimine decarboxylated dimer in human plasma and in cultured cells after in vitro supplementation.

Keywords: Aminoethylcysteine ketimine decarboxylated dimer

1. Introduction

Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) (Fig. 1) is a natural sulfur-containing tricyclic member of a new family of sulfur-containing amino acids (see [1] for a general review). Ketimines and their derivatives have been detected in mammalian tissues and fluids [2–6]. Some of them have been found in the central nervous system, and for one of them the binding to bovine brain cortex membranes has been demonstrated [7], suggesting a possible neurochemical role.

AECK-DD is the product of spontaneous dimerization and decarboxylation of aminoethylcysteine ketimine. AECK-DD has been found in human urine [8] and plasma [9], in mammalian cerebellum [10] and, more recently, in dietary vegetables [11]. AECK-DD

Abbreviations: AECK-DD, aminoethylcysteine ketimine decarboxylated dimer; HPLC, high-pressure liquid chromatography; ECD, electrochemical detection; PBS, phosphate buffered saline

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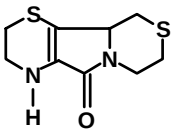

Fig. 1. Aminoethylcysteine ketimine decarboxylated dimer.

possesses a strong antioxidant activity. It displays a protective effect on copper-induced oxidation of low-density lipoprotein [12], at concentrations comparable to those found in human plasma. A scavenging activity on hydroxyl radicals, peroxynitrite and its derivatives, comparable to that of alpha-tocopherol and more potent than ascorbic acid and glutathione has been described [13–16].

Gas chromatography has been used up till now to detect aminoethylcysteine ketimine decarboxylated dimer. An high-pressure liquid chromatography (HPLC) procedure coupled with UV detection has been occasionally reported to support the identification of AECK-DD in human plasma [9] and food [11]. Besides this, no methods have been reported in the literature for the qualitative and quantitative determination of AECK-DD by high-performance liquid chromatography techniques.

In this study, we developed a specific and sensitive method for the detection of AECK-DD by HPLC with electrochemical detection (ECD). The method has been applied to determine the level of AECK-DD in human plasma and the intracellular levels of AECK-DD in cultured cells after in vitro supplementation.

2. Materials and methods

2.1. Chemicals and reagents

AECK-DD was synthesized according to Fontana and co-workers [15]. All chemicals and reagents were of analytical grade or HPLC grade. Distilled water was purified using a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Cell culture

Human monocytic cells (U937) were maintained in continuous cell suspension at 37 °C under 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 40 μg/ml gentalin. AECK-DD was dissolved in ethanol and further diluted in culture medium. U937 cells were cultured at 0.3×10^6 cells/ml for 24 h in the presence or in the absence (control) of AECK-DD (50 and 250 μM) (ethanol final concentration ≤0.12%).

2.3. Samples preparation

2.3.1. Plasma samples

To obtain plasma, blood from 12 h-fasting donors was collected into EDTA (1 mg/ml) and centrifuged at 1000 × *g* for 10 min. Plasma was used immediately or stored at −80 °C and used within 1 week after preparation. Plasma samples were treated for AECK-DD extraction essentially as previously described [9]. Methanol (1 ml) and chloroform (2 ml) were added to 0.5 ml plasma. The resulting mixture was vortexed vigorously for 5 min, then centrifuged at 2000 × *g* for 5 min. The chloroform fraction was removed and the sample was extracted with additional 2 ml chloroform, vortexed for 5 min and centrifuged at 2000 × *g* for 5 min. The two organic layers were combined and evaporated and dried under nitrogen flow. The residue was dissolved in 0.1 ml absolute methanol and vortexed for 3 min, then added with 0.2 ml sample buffer (1.25% glacial acetic acid, 10% methanol in water), vortexed for 3 min, and filtered prior to HPLC–ECD analysis. Appropriate dilutions, when required, were performed with sample buffer.

2.3.2. Cell extracts

After 24 h culture in the presence or in the absence (control) of AECK-DD, U937 cells ($(20–40) \times 10^6$ cells) were pelleted by centrifugation, washed twice with 50 ml Dulbecco's phosphate buffered saline (PBS), and resuspended in 0.5 ml 0.5% Triton in PBS. After sonication (15 s) to facilitate lysis, cell suspensions were extracted with chloroform (2 ml × 2 ml) by 5 min vortexing, and centrifuged at 2000 × *g* for 5 min. The two organic layers were combined and evaporated under nitrogen flow. The residue was dissolved in 0.1 ml absolute methanol, vortexed for 3 min, then added with 0.3 ml sample buffer, vortexed for 3 min,

centrifuged 5 min at 14,000 × *g* and filtered prior to HPLC–ECD analysis.

2.4. HPLC instrumentation

The HPLC consists of a Perkin-Elmer Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT) with a gradient pump, column thermoregulator and autosampling injector (Gilson, Beltline, Middleton, WI) equipped with an electrochemical coulometric detector (Coulochem II, ESA, Bedford, MA). An high sensitivity coulometric analytical cell (ESA, Bedford, MA, code no. 5011) was used, containing two chambers (1 and 2), each chamber includes a porous graphite analytical electrode, platinum/palladium reference electrode and counter electrode. Electrode 1 was kept at 0 mV throughout the study, while electrode 2 was set at +400 mV or at different potential as specified. Turbochrom chromatography workstation software was used for data processing. Operating conditions were as follows: column temperature, 30 °C; flow rate, 1 ml/min; injection volume, 50 μl; sensitivity range, 100 nA; filter, 2 s. Chromatographic separations were performed on a Supelcosil LC-18 C_{18} column (5 μm particle size, 250 mm × 4.6 mm i.d.) including a guard column (C_{18}, 5.0 μm particle size, 20 mm × 4.0 mm i.d.; both Supelco, Bellefonte, PA). Mobile phases A and B were employed. Solution A contained 1.25% glacial acetic acid in water (pH 2.8); solution B was absolute methanol. Isocratic elution was used with 65% A, 35% B at 1 ml/min flow (retention time 20.2 min).

To further confirm the identification of AECK-DD, in a separate set of experiments, gradient elution was applied to plasma and cell extracts samples. The following gradient was used: 0–40 min, from 90% a, 10% b to 70% a, 30% b, linear gradient; 41–60 min, 70% a, 30% b; 61–75 min, from 70% a, 30% b to 90% a, 10% b (retention time of AECK-DD 50.9 min).

Prior to HPLC analysis, all samples were filtered using millex-HV filters (Millipore, Bedford, MA) with 0.45 μm pore size.

2.5. Voltammetric study

Voltammetric characterization of AECK-DD was achieved by hydrodynamic voltammogram of 200 ng/ml solution of AECK-DD in sample buffer. Hydrodynamic voltammogram was obtained during a series of experiments in which the detector potential (electrode 2) was raised by 50 mV increments (range 100–600 mV).

2.6. Calibration and quantitative analysis

The method of external standards was used for calibration. A standard stock solution of AECK-DD (1 mg/ml in methanol) was stored at −80 °C and used within 1 week. For the calibration curve, progressive dilutions in sample buffer of the stock solution were prepared (300, 150, 100, 50, 20, 10 ng/ml) and three replicates of each standard dilution were analyzed. The calibration curve for peak area versus the amount of AECK-DD was obtained. The calibration curve was determined on each day of analysis. For quantitative determination, peak areas in the sample chromatograms were correlated with the concentrations according to the calibration curve.

2.7. Method validation: repeatability, precision and recovery

To evaluate the repeatability (intra-assay precision), aliquots of cell extracts (control) were spiked with pure AECK-DD (range 20–300 ng/ml) and analyzed three consecutive times under the conditions described previously. Inter-assay precision (intermediate precision) was evaluated analyzing the same samples over a 5-day span. Repeatability and intermediate precision were also evaluated on human plasma by measuring the same plasma sample five consecutively times or five successive days. Repeatability and intermediate precision were estimated as the coefficient of variation (CV%) on the replicate measurements, intra-day and inter-day, respectively.

The recovery of AECK-DD was tested on plasma samples by the spiked method in three independent experiments. In each experiment, known amounts (25, 50, 100, 250 ng) of pure AECK-DD were added to 0.5 ml of the same plasma sample before extraction.

The recovery of AECK-DD on cell extracts was tested by the spiked method in three independent experiments, by adding known amounts (25, 50, 75, 150 ng) of pure AECK-DD to 0.5 ml AECK-DD-free

cell extracts in Triton/PBS and extracted as previously described. The absolute recovery (%) was expressed as [17]:

$$\frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of pure standard analyte (unprocessed)}}$$

2.8. Statistical analyses

Data resented are means ± standard deviation ($n \geq 3$). Statistical analysis was performed using a one-factor analysis of variance (ANOVA, Scheffe's method) for multiple comparison.

3. Results and discussion

3.1. Voltammetric characterization of AECK-DD

Electrochemical detection of AECK-DD has never been accomplished in the past, therefore the voltammetric behavior of AECK-DD was evaluated. Hydrodynamic voltammogram of AECK-DD is reported in Fig. 2. The $E_{1/2}$ has been calculated at +260 mV. To enhance sensitivity, we selected +400 mV as working potential for our experiments.

3.2. AECK-DD identification in human plasma and cell extracts

Fig. 3A shows the liquid chromatogram of standard AECK-DD, eluting at 20.2 min, obtained with a detector potential of +400 mV. In Fig. 3B, a typical chromatogram obtained from human plasma sample is reported. A peak was identified as AECK-DD on the basis of both retention time and coelution with standard AECK-DD (Fig. 3C), indicating the presence of endogeneous AECK-DD in human plasma. The same sample analyzed at +600 mV (Fig. 3D) showed a more complex elution pattern compared to that obtained at +400 mV (Fig. 3B). The amount of AECK-DD detected in plasma samples from ten healthy subjects (Table 1) has been found in agreement with values reported previously by gas chromatographic technique [9].

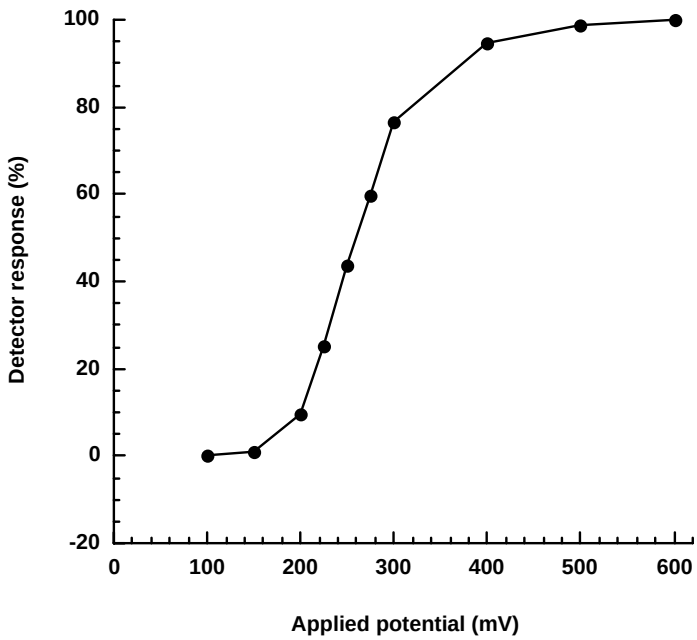

Fig. 2. Hydrodynamic voltammogram of standard AECK-DD. A solution of standard AECK-DD (200 ng/ml) was run on the HPLC at different potential settings of electrode 2 on the electrochemical detector. The current obtained at +600 mV was considered as 100% response.

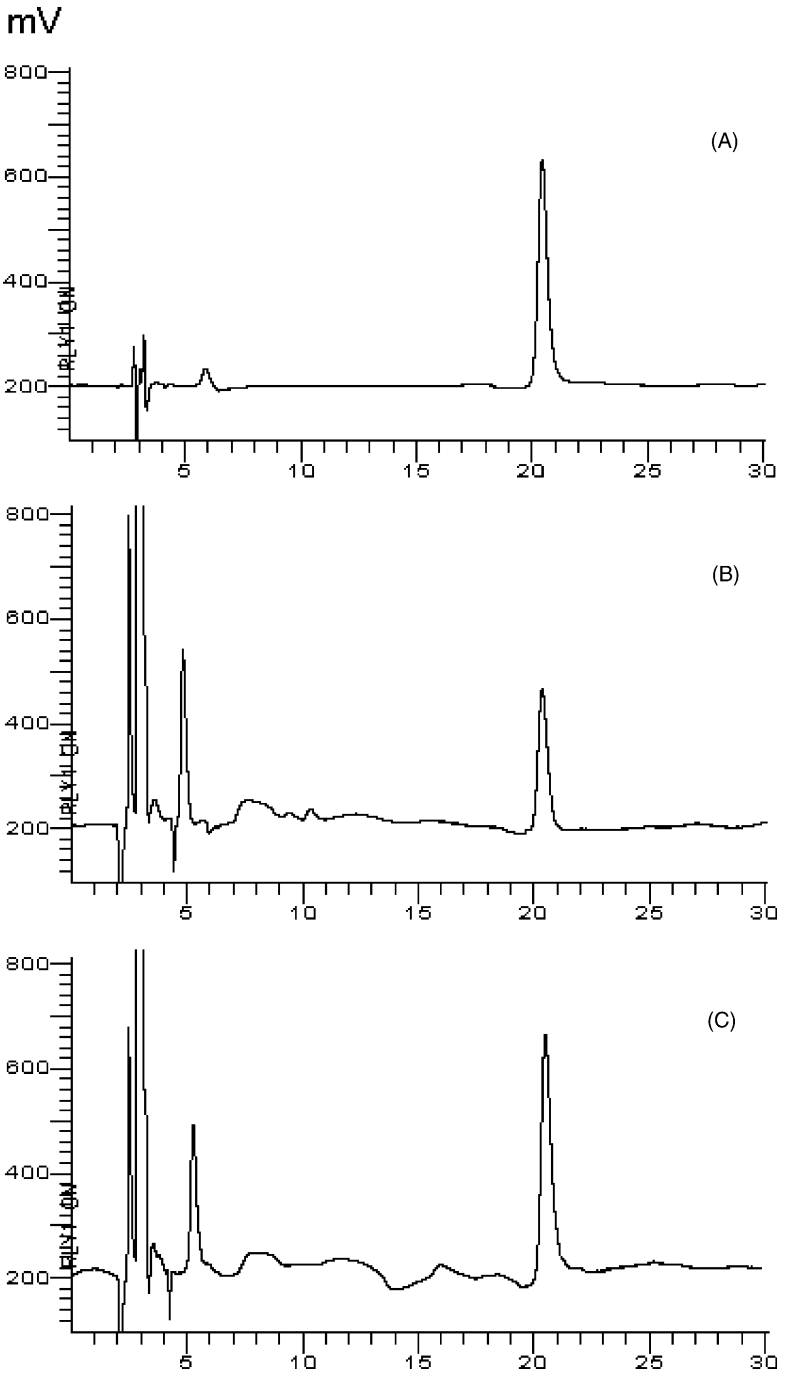

Fig. 3. High-performance liquid chromatography of: (A) standard AECK-DD, (B) plasma sample, (C) plasma sample + standard AECK-DD, with detector potential at +400 mV (electrode 2), (D) plasma sample as in (B), analyzed at +600 mV (electrode 2). Plasma extraction procedure, HPLC conditions and isocratic elution were as reported in Section 2.

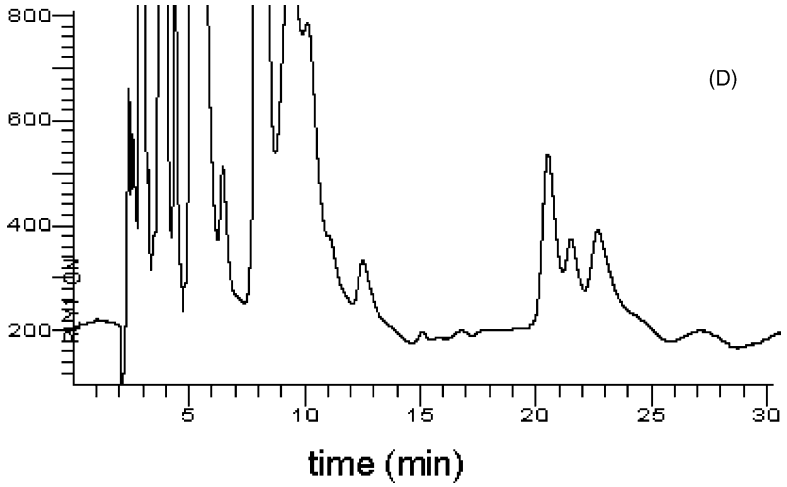

Fig. 3. (*Continued*).

Our electrochemical method was further applied to monitor the uptake of AECK-DD in cultured U937 cells. Fig. 4B shows a typical chromatogram obtained at +400 mV from cells cultured for 24 h in medium containing 50 μm AECK-DD. A peak was identified as AECK-DD on the basis of both retention time and coelution with standard AECK-DD (Fig. 4C), indicating the occurrence of endogenous AECK-DD in cell extracts after AECK-DD supplementation. No peaks corresponding to AECK-DD were detected in cells cultured in the absence of AECK-DD (Fig. 4D). In Fig. 4E, the same cell extract showed in Fig. 4B was analyzed at +600 mV. The content of AECK-DD measured with our method was 0.10 ± 0.01 and 0.47 ± 0.08 ng/10^6 cells for cells grown in the presence of 50 and 250 μm AECK-DD, respectively. The identification of AECK-DD in plasma and cell extracts samples was further confirmed by gradient elution analysis, in which AECK-DD was eluted at 50.9 min (data not shown).

Table 1
AECK-DD concentration in human plasma[a]

Subject	AECK-DD (ng/ml)
1	125.0 ± 3.1
2	519.8 ± 6.6
3	469.2 ± 5.7
4	114.1 ± 4.8
5	304.0 ± 2.6
6	447.9 ± 4.3
7	105.7 ± 3.6
8	385.3 ± 5.4
9	500.4 ± 3.2
10	498.1 ± 4.6

[a] Values are means ± S.D. ($n = 3$).

3.3. Calibration and validation of the proposed method

The response of AECK-DD was linear in the tested concentration range of 10–300 ng/ml (Fig. 5). The regression coefficient for peak area versus concentration (ng/ml) was $R = 0.998$.

Repeatability and intermediate precision, measured by injection of spiked cell extracts, were found to be ≤5.5 and ≤3.2% for intra-assay and inter-assay,

Table 2
Repeatability and intermediate precision

Nominal value (ng AECK-DD/ml)	Repeatability CV (%) $n = 3$	Intermediate precision CV (%) $n = 5$
20	0.1	3.2
50	4.6	0.1
100	4.9	0.8
150	2.9	2.4
200	3.9	2.1
300	5.5	2.1

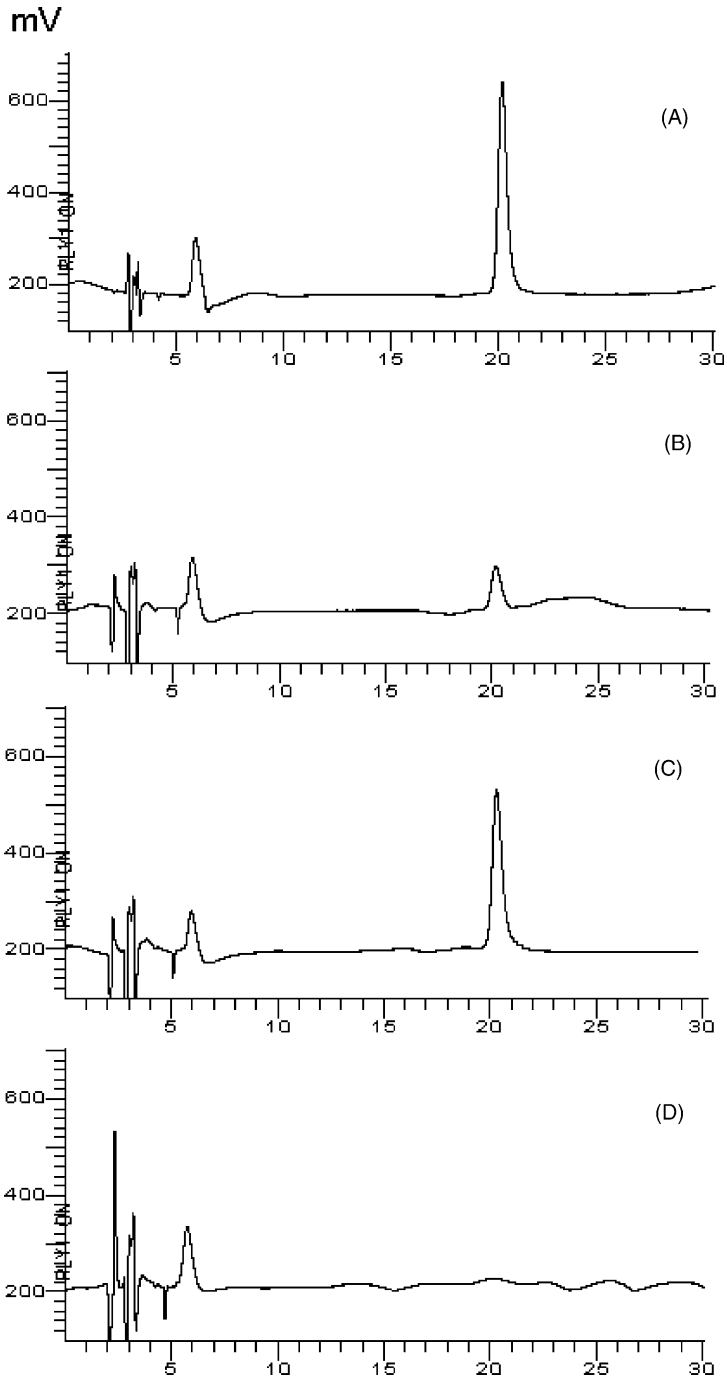

Fig. 4. High-performance liquid chromatography of: (A) standard AECK-DD, (B) cell extract from cells supplemented with 250 μm AECK-DD for 24 h, (C) cell extract as in (B) +standard AECK-DD, (D) cell extract from control, non-supplemented cells, with detector potential at +400 mV (electrode 2), (E) cell extract as in (B), analyzed at +600 mV (electrode 2). Cells extraction procedure, HPLC conditions and isocratic elution were as described in Section 2.

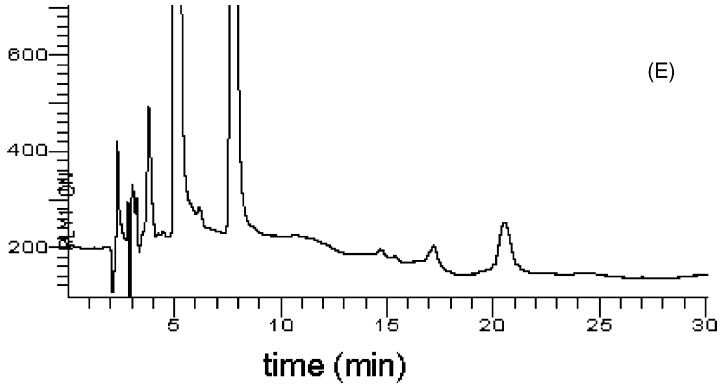

Fig. 4. (*Continued*).

respectively (Table 2). Repeatability and intermediate precision values obtained measuring the same plasma sample five consecutively times or five successive days were 1.8 and 2.8%, respectively. The limit of detection, on the basis of a signal-to-noise ratio of 3:1, has been found to be 0.05 ng (absolute amount).

The absolute recovery, ranging from 98.7 to 110.5% for plasma samples and from 92.2 to 98.6% for cell extracts within the concentration interval, demonstrated the efficiency of the method (Table 3). Regression analysis between the calculated and nominal concentrations from the assayed spiked samples demonstrated the absence of matrix and blank effects, showing a linear relationship ($R = 0.999$ and 1 for plasma and cell extracts, respectively), with slope and intercept not significantly different, respectively, from 1 to 0 [18] for both plasma and cell extracts.

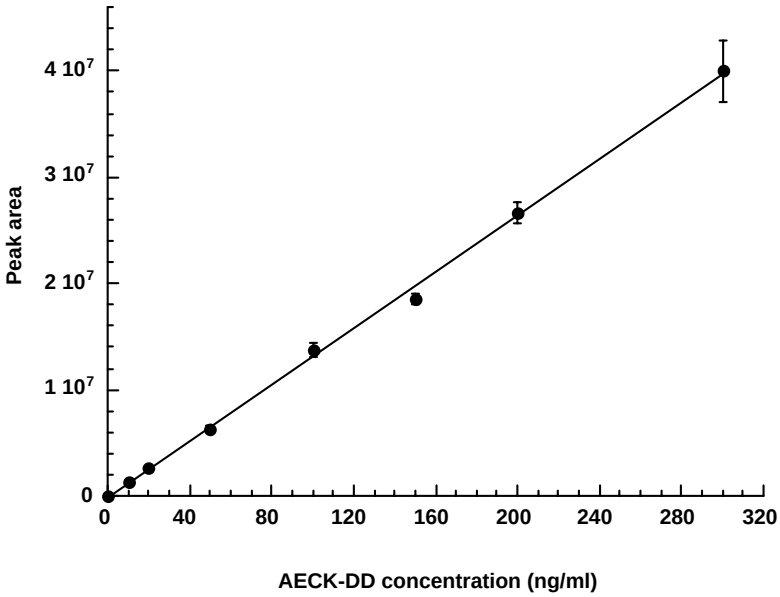

Fig. 5. Calibration curve of AECK-DD at concentration ranging from 10 to 300 ng/ml. Value are means ± S.D. ($n = 3$). HPLC conditions and isocratic elution were as reported in Section 2, with detector potential at +400 mV (electrode 2).

Table 3
Recovery on plasma samples and cell extracts

AECK-DD added (ng)	Plasma samples (percent recovery)[a,b]	Cell extracts (percent recovery)[a]
25	98.7 ± 2.5	92.2 ± 4.0
50	97.9 ± 12.1	98.6 ± 4.7
75	–	97.4 ± 5.3
100	110.5 ± 7.1	–
150	–	96.3 ± 2.5
250	104.5 ± 1.1	–

[a] Values are means ± S.D. of three independent experiments. AECK-DD was added to 0.5 ml sample.
[b] For plasma samples, the endogenous content of AECK-DD was subtracted from calculations.

4. Conclusion

The assay developed in this paper showed excellent precision and accuracy. The detector potential used in our experiments (+400 mV) to detect AECK-DD in human plasma and cell extracts showed a high selectivity. However, due to the high sensitivity of electrochemical detection, it is possible to work at potential lower than +400 mV to obtain further increase in selectivity, retaining a good sensitivity at the same time.

The proposed method has been found to be about 100-fold more sensitive (lower detection limit 0.05 ng, absolute amount) than gas chromatographic method (lower detection limit 5 ng, absolute amount) and 2000-fold more sensitive in respect to the liquid chromatography method with UV detection (lower detection limit 100 ng, absolute amount) [9]. The proposed method is sensitive enough to allow the quantitative determination of AECK-DD not only in human plasma, but also in cells.

Despite the increasing evidences of an antioxidant role for AECK-DD and its presence in biological fluids and tissues, there are no studies describing AECK-DD levels in cells. This is partially due to the lack of sensitive method for measuring AECK-DD. Our results show, for the first time, that AECK-DD can be measured in cells following an in vitro supplementation. The proposed assay might represent a useful tool for studying absorption, cellular transport, tissue storage and cellular localization of AECK-DD.

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