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Identification of aminoethylcysteine ketimine decarboxylated dimer in human plasma

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Abstract

Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) is a natural sulfur-containing tricyclic compound detected, until now, in human urine and bovine cerebellum. Recently, the antioxidant properties of this compound, and particularly its protective effect on the in vitro oxidation of low-density lipoproteins, have been demonstrated. In this paper, the identification of AECK-DD in human plasma by means of gas chromatography, high-performance liquid chromatography and gas chromatography–mass spectrometry, performed after a simple and fast purification procedure, is reported.

Keywords: Aminoethylcysteine ketimine decarboxylated dimer; Low density lipoproteins

1. Introduction

Aminoethylcysteine ketimine decarboxylated dimer (formula in Fig. 3a), simply named AECK-DD hereafter, is a natural sulfur-containing tricyclic compound and a member of a new family of sulfur-containing amino acids (see Ref. [1] for a general review). It was synthesized for the first time by Hermann in 1961 [2] and since then very little progress has been made on the understanding of its chemical and biochemical properties. Only recently the antioxidant properties of this compound have been studied.

A possible biochemical route leading to the formation of AECK-DD could start from the synthesis of aminoethylcysteine (AEC) operated by serine sulfhydrase (EC 4.2.1.22) using serine and cysteamine [3] or serine and pantetheine [4]; a subsequent α-deamination of AEC, operated by L-amino acid oxidase (EC 1.4.3.2) [5,6] or by a transaminase of wide specificity and distribution (e.g. glutamine transaminase, EC 2.6.1.15) [7,8], leads to the production of aminoethylcysteine ketimine (AECK), which spontaneously dimerizes and rapidly loses a carboxyl group yielding the AECK-DD [9]. The biological relevance of this biochemical route is supported by the finding of all these compounds in

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biological tissues. AEC was recently found in human urine [10], AECK was detected in bovine brain and cerebellum [11] and AECK-DD in human urine [12] and in bovine cerebellum [13].

Since previous studies have indicated a protective effect of this compound on the in vitro oxidation of low-density lipoproteins [14,15], the aim of the present work was to ascertain its presence in human plasma and to evaluate its distribution among circulating lipoproteins both in normolipidemic and hypercholesterolemic subjects.

2. Experimental

2.1. Chemicals and materials

AECK-DD was prepared according to [16]. Other reagents were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

2.2. Extraction procedures

Blood from fasting 12 h donors was collected into EDTA (1 mg/ml) and centrifuged at 1000 *g* for 10 min. Plasma was used immediately or stored at −20°C and used within one week after preparation. To 0.5-ml aliquots of each sample 1 ml of methanol and 2 ml of chloroform were added, and the resulting mixture was well shaken. The chloroform fraction was removed and dried, the residue was resuspended in methanol and then directly analyzed by gas chromatography (GC), high-performance liquid chromatography (HPLC) and gas chromatography–mass spectroscopy (GC–MS).

2.3. GC analyses

Gas chromatographic analyses were performed on a Perkin Elmer Sigma 300 chromatograph equipped with a flame photometric detector selective for sulfur-containing compounds. The Supelco glass column (180 cm×2 mm I.D.) was packed with 3% OV-17 on Chromosorb W HP, 100–120 mesh. Temperatures were: 260°C for the column, 270°C for the injector and 300°C for the detector. Flow-rates were: 25 ml/min for N_2 (carrier gas), 65 ml/min for H_2 and 110 ml/min for air.

2.4. GC–MS analyses

GC–MS analyses were performed on a Hewlett–Packard 5970A MSD system. Chromatographic separations were carried out on a 30 m×0.25 mm I.D. fused-silica capillary column coated with cross-linked 5% phenyl-methyl siloxane, film thickness 0.25 μm, as the stationary phase (supplied by Hewlett–Packard). Injection mode: splitless at a temperature of 260°C. Column temperature program: 41°C (3 min), then to 280°C at a rate of 8°C/min and then held for 8 min. The carrier gas was helium at a constant flow of 1.3 ml/min and the average linear velocity was 41 cm/s. The spectra were obtained in the electron impact mode at 70 eV ionization energy; ion source 280°C; ion source vacuum 10^{-5} Torr; mass range from m/z 50 to 240. When the selected ion monitoring acquisition mode was used, the dwell time was 50 ms. Chromatograms and spectra were collected by a Hewlett–Packard 59970 Chem Station.

2.5. HPLC analyses

HPLC analyses were carried out with a Waters chromatograph equipped with a model 626 pump, a 6005 controller and a model 996 photodiode array detector linked to a Millennium 2010 Data Station.

The column (250×4 mm I.D.) was packed with ODS, 5 μm (supplied by Hypersil). The mobile phases were: (A) water and (B) acetonitrile–water (80:20, v/v). A linear gradient from 100% A to 100% B was developed in 30 min. The flow rate was 1 ml/min at room temperature and the spectra were recorded at 308 nm.

2.6. Plasma fractioning

Blood from healthy, normolipidemic donors fasting 12 h was collected into EDTA (1 mg/ml). EDTA–plasma was used immediately to prepare the following fractions: the first one containing VLDL and IDL (fraction at density<1.019 g/ml); the second one containing LDL and HDL (1.019<d<1.210 g/ml) and the last one, the fraction at density>1.210 g/ml, containing essentially albumin. These fractions were isolated at plasma density and with a 2.973 molal NaBr solution, respectively, by

ultracentrifugation at 100 000 *g* at 14°C in a 40.3 Ti rotor for 18 and 22 h, respectively.

2.7. LDL preparation

Blood from healthy, normolipidemic donors fasting 12 h was collected into EDTA (1 mg/ml). EDTA–plasma was used immediately to prepare low-density lipoproteins (LDL) by sequential ultracentrifugation on a KBr gradient [14]. The LDL band (density=1.019–1.063 g/ml) was collected by aspiration and dialyzed in the dark for 2 h against 200 volumes of 150 m*M* NaCl and 10 μ*M* EDTA, then for 60 h against 150 m*M* NaCl, which was changed at least two times. LDL was stored in a vial under nitrogen at 4°C in the dark and used within one week after preparation.

The protein content of LDL solution was determined by the method of Lowry et al. [17], using bovine serum albumin as the protein standard.

2.8. Capacity of LDL to bind AECK-DD

The capacity of AECK-DD to bind to LDL was examined. AECK-DD (50 μ*M* final concentration) was incubated overnight with human LDL (1 mg protein/ml) at 37°C. As controls, two additional mixtures were incubated overnight: AECK-DD in PBS at the same concentration and LDL without AECK-DD. All mixtures were dialyzed for 24 h against 200 volumes of PBS (at least two changes). At the end of dialysis 0.5 ml aliquots were extracted with a mixture of methanol–chloroform (1:2, v/v), then desiccated, resuspended in methanol and analyzed by GC.

3. Results

In Fig. 1, is reported a typical GC profile of a plasma sample extracted as described in the previous section, resuspended in 100 μl of methanol and directly injected into the gas chromatograph. It shows the presence of a sulfur-containing compound having the same retention time as AECK-DD and co-eluting with the authentic compound.

Plasma samples were analyzed also by HPLC and

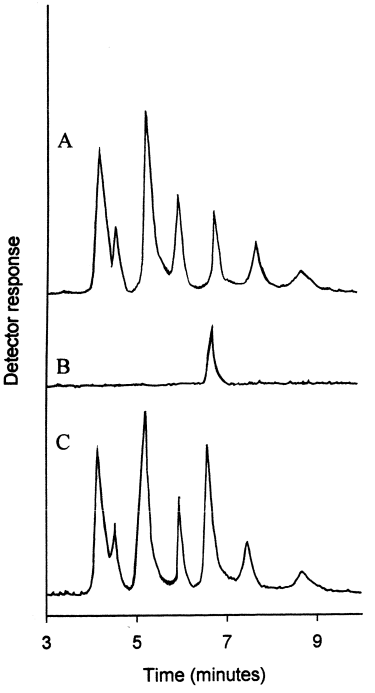

Fig. 1. GC profile, as obtained using a flame photometric detector selected for sulfur-containing compounds, of (A) human plasma extracted as reported in Section 2; (B) 12.5 ng of pure AECK-DD; (C) the same extract added with 12.5 ng of pure AECK-DD.

a typical chromatogram is reported in Fig. 2. The AECK-DD peak eluted from HPLC was collected, dried, solubilized in methanol and submitted to GC

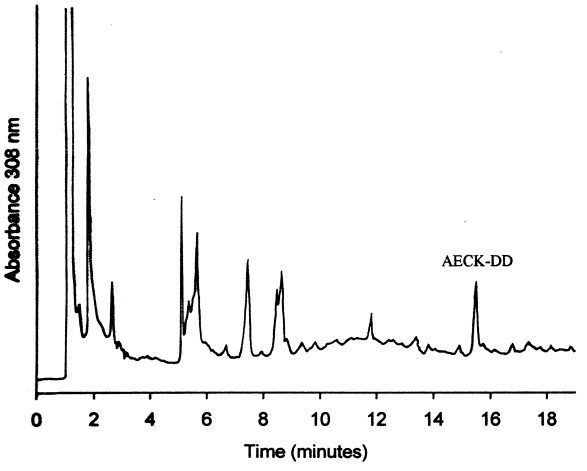

Fig. 2. HPLC elution pattern of human plasma extracted as reported in Section 2. The effluent was monitored at 308 nm.

showing the same chromatographic pattern as the authentic compound.

These data were confirmed by GC−MS, where the mass spectrometer was used both in the scan and in the selected-ion monitoring mode. In Fig. 3a, is reported the mass spectrum of a pure sample of synthesized AECK-DD: the mass spectrum shows a fragmentation pattern where the molecular ion m/z 228 and three other ions, m/z 200, 154 and 126 with a relative abundance of 29.7, 48.5 and 16.8%, respectively, were the most abundant. The mass spectrum of an extracted plasma sample (Fig. 3b) shows the typical fragmentation pattern of the authentic AECK-DD and similar relative intensities of the more abundant ions (30.6% for m/z 200, 49.5% for m/z 154 and 16.6% for m/z 126) and minor ions from unknown compounds. As previously reported [13], the fragmentation patterns seems to imply loss of CO ($m=28$) and/or loss of radical species as CH_2S ($m=46$) through different routes. e.g., the loss of CO from the molecular ion likely could lead to the fragment m/z 200 and the following loss of a CH_2S radical may explain the formation of the fragment m/z 154. The fragment m/z 182 may be produced by loss of the radical CH_2S from the molecular ion; following loss of CO may result again in the formation of the fragment m/z 154. The loss from this fragment of acetylene C_2H_4 may lead to the formation of the fragment m/z 126.

Fig. 4 shows selected ion chromatograms of authentic synthesized AECK-DD (A) and of that extracted from human plasma (B). In both is present

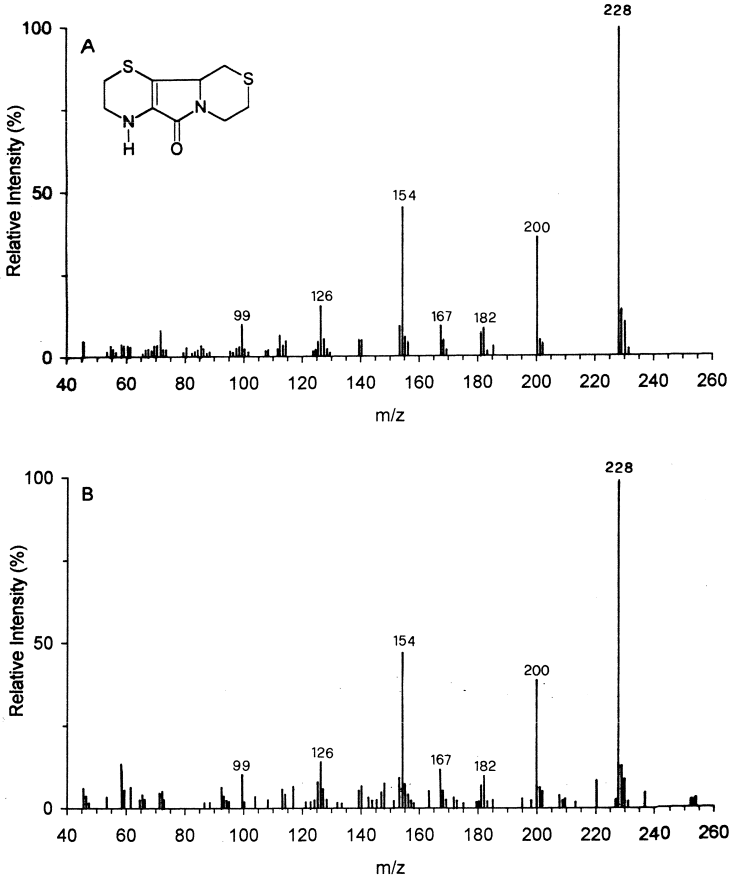

Fig. 3. Mass spectrum of (A) pure AECK-DD and (B) a methanolic extract of human plasma, as obtained by electron impact at 70 eV. In A the formula structure of AECK-DD is shown.

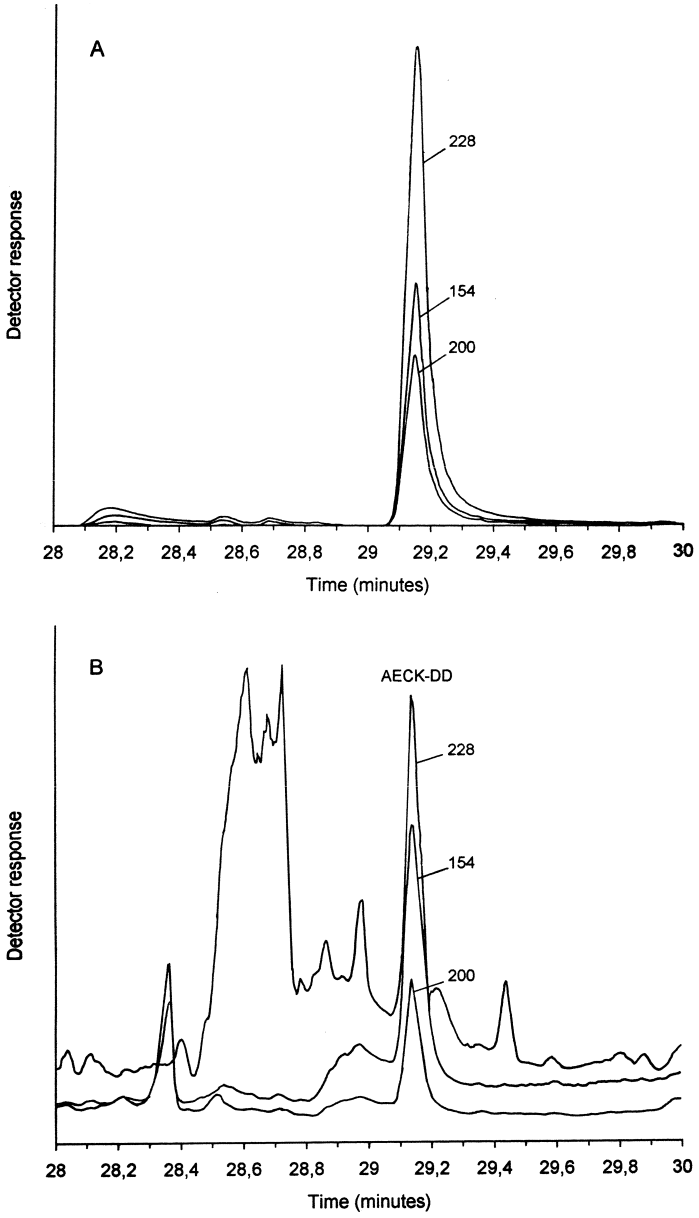

Fig. 4. Selected-ion chromatograms of (A) pure AECK-DD and (B) the compound extracted from human plasma, obtained by monitoring the three more abundant ions, m/z 228, 200 and 154 present in the mass spectrum of the authentic synthesized compound.

a peak eluted at the same retention time of AECK-DD for each of the three m/z values.

Therefore, by means of the three techniques used, GC, HPLC and GC–MS, the human plasma extracted compound is undoubtedly identified as the decarboxylated dimer of aminoethylcysteine ketimine.

Quantitative analyses were performed by GC, while qualitative identification was confirmed by GC–MS. Quantitation of AECK-DD was calculated

Table 1
Summary of linear regression data for AECK-DD[a]

Parameters	Day				
	1	2	3	4	5
Slope	1313	1280	1289	1290	1325
y-Intercept	−7698	−7789	−7653	−7378	−9327
Correlation coefficient (*r*)	>0.99	>0.99	>0.99	>0.99	>0.99

[a] Standard curves were calculated each day for five days using the average of four peak ratios for concentration.

from the area of the GC peak compared to the five-point standard curve. The calibration curve was linear from 5 to 100 ng. The correlation coefficients (*r*) for all calibration curves and the regression equations, calculated in five different days, are summarized in Table 1. The lower limit of detection was 5 ng (absolute quantity), at a S/N ratio of 3. The recovery of authentic AECK-DD was tested by the spiked sample method in four different experiments. In each experiment three different quantities of this compound (400, 800 and 1200 ng) were added to 0.5 ml of the same plasma. At the end of the extraction procedure the residue was dissolved in methanol and analyzed by GC. The recovery averaged 99±13%.

In Table 2, the AECK-DD plasma values of 14 healthy normolipidemic subjects are reported. The values range from 513 to 768 ng/ml plasma (mean 658±70). In Table 3, the values of the plasma AECK-DD concentration of two kinds of dislipidemic patients are reported: for the three homozygous hypercolesterolemic subjects the values were 569, 585 and 660 ng/ml plasma (mean 605±48) and then there were six subjects with polygenic hypercolesterolemia, whose values' ranged from 552 to 925 ng/ml plasma (mean 691±130). One-way analysis of variance plus the Newman Keuls multiple comparison test shows that no significative differences of AECK-DD concentration were present between the three groups analyzed.

The capacity of AECK-DD to bind to LDL was also examined. Supplementation of LDL (1 mg protein/ml) with 50 μ*M* AECK-DD for 20 h at 37°C, followed by extensive dialysis in PBS to remove unbound material, resulted in the binding of about 5% of the compound to LDL.

To verify whether AECK-DD is associated to a particular plasma fraction, three different fractions isolated by sequential ultracentrifugation were analyzed: one containing IDL and VLDL ($d<1.019$ g/ml), another one containing LDL and HDL (1.019 g/ml$<d<$1.210 g/ml) and the last one containing essentially albumin ($d>1.210$ g/ml). Each fraction

Table 2
AECK-DD plasma levels in healthy, normolipidemic subjects[a]

Normolipidemic subject	AECK-DD concentration (ng/ml plasma)
1	701±39
2	644±27
3	559±42
4	708±19
5	715±15
6	656±27
7	665±24
8	732±46
9	591±40
10	513±30
11	768±40
12	631±34
13	706±25
14	629±37

[a] All values are mean±SD of three measurements.

Table 3
AECK-DD plasma levels in familial hypercolesterolemic (FH) and polygenic hypercolesterolemic (PH) subjects[a]

Subject	AECK-DD concentration (ng/ml plasma)
FH	
1	569±9
2	585±10
3	660±10
PH	
1	925±42
2	682±44
3	738±25
4	614±61
5	552±55
6	633±13

[a] All values are mean±SD of three measurements.

was extracted as reported in the previous section and analyzed by GC. The results show that AECK-DD is evenly distributed in the three fractions.

4. Discussion

The results reported above clearly demonstrate the presence of AECK-DD in human plasma. Because of its low solubility in water, it can reasonably be assumed to be bound to hydrophobic regions of macromolecular constituents, but the results indicate that it binds to LDL by only 5% and is present in the three plasma fractions analyzed. It can, therefore. be concluded that this compound is evenly distributed in human plasma, even if its binding to plasma proteins needs further investigation.

The presence of AECK-DD in plasma may be of importance due to the antioxidant properties of this molecule, which has been found to interact with reactive oxygen species [16], to protect membranes and lipidic structures from oxidative damage [18], and to be a scavenger of hydroxyl radicals [19], peroxinitrite and its derivatives [15]. Particularly, the concentration values of AECK-DD in human plasma are similar to those that exert a protective effect against LDL oxidation in vitro, as reported in previous papers [14,15]. In fact, starting from a 25 nM concentration, AECK-DD exerts its protective effect against the oxidative damages occurring in LDL both at the protein and lipid moiety levels. At higher (μM) concentrations it has a protective effect also against $ONOO^-$-induced tyrosine nitration and α_1-antiproteinase inactivation [15].

Due to the antioxidant properties of this compound against LDL oxidation [14,15], which are involved in the formation of the early atherosclerotic lesions [20–25], two kind of hypercolesterolemic subjects were also analyzed. The first group was composed, due to the very low impact of the pathology, of only three homozygous FH patients (FH, familial hypercholesterolemia, a rare inherited genetic disorder due to defects in the low density lipoprotein receptor), and the second group was composed of six patients with PH (polygenic hypercholesterolemia, a form with moderately high plasma levels of cholesterol due both to several genes and environmental factors).

Neither the lack of a preferential distribution of AECK-DD among the various fractions of circulating lipoproteins, nor the absence of increased plasma levels in hypercholesterolemic subjects invalidate the hypothesis of a protective effect of AECK-DD, also in vivo, on LDL oxidation.

Further studies are needed to evaluate whether AECK-DD levels can contribute significantly to the anti-oxidant potential of human plasma.

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