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Comparative determination of purine compounds in carotid plaque by capillary zone electrophoresis and high-performance liquid chromatography

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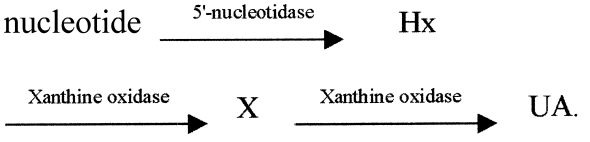
Abstract

Allantoin, uric acid (UA), hypoxanthine (Hx) and xanthine (X) were determined on carotid plaque by capillary zone electrophoresis (CZE) and high-performance liquid chromatography (HPLC). Comparison of the results showed that capillary zone electrophoresis may have similar or even superior analytical performance to HPLC, especially for the determination of allantoin in biological samples.

Keywords: Purine

1. Introduction

Hypoxanthine, xanthine and uric acid are the main products of purine nucleotide catabolism in humans. They are formed in the sequence:



Under normal conditions, they reflect the balance between the synthesis and breakdown of nucleotides. Levels of these products change in various situations; for example they decrease in experimental tumors, when synthesis prevails over catabolism [1–6], and are enhanced during oxidative stress and hypoxia.

Hypoxia resulting from impairment of tissue oxygen action, for example due to ischemia, is a primary cause of cell injury. One of the many biochemical changes occurring during hypoxia, induced cell injury, is a dramatic decrease in adenine nucleotides [7–13], related to enhanced activity of the catabolic enzymes, HPRT (hypoxanthine–guanine–phosphorybosyl-transferase), APRT (adenine–phosphoribosyl-transferase), IMP-5′–nucleotidase and AMP-5′–nucleotidase, due to the presence of oxygen radicals. Nucleotide catabolism involves an accumulation of Hx and X, which are substrates of xanthine oxidase (XO); under such conditions, the activity of XO also increases, due to irreversible conversion of the XO dehydrogenase (D form) to the XO oxidase (O form), accompanied by production of oxygen radi-

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cals. This leads to enhanced production of UA. In most animals, UA is transformed into allantoin by the action of uricase. In humans, no enzyme which oxidizes UA is known, however UA may be transformed into allantoin by chemical oxidation or oxygen radicals. Current analytical methods have revealed the presence of allantoin in serum [14–19], as the first and major product of the oxidation of UA; allantoin has been suggested as a marker of free radical reactions in vivo. The simultaneous determination of allantoin, UA, Hx and X in biological samples is therefore of special interest. Purine compounds are usually determined by HPLC [14–15,20–22] which poses no problems for the separation and determination of Hx, X and UA. On the other hand, methods for the resolution of allantoin by HPLC (which employ either long columns or two columns in series) are often unsatisfactory, unless allantoin is first broken down or derivatized [16,18,19]. Such methods are tedious and not suitable for routine analysis.

Allantoin may also be measured by the colorimetric method of Young and Conway [23], which involves its conversion to glyoxylic acid by sequential hydrolysis under alkaline and acidic conditions. The glyoxylic acid reacts with phenylhydrazine to form a hydrazone which is measured colorimetrically. This method is tedious, time critical and not specific, due to the presence of several keto acids in biological samples.

The main objective of this paper is to develop a methodology for the simultaneous determination of UA, Hx, X and allantoin. Here we compared the performance of two methods for the simultaneous determination of the four catabolites, one based on HPLC and the other on CZE. They were tested on plasma and atherosclerotic plaque. Carotid plaque was chosen because oxidation plays a central role in atherogenesis [24]: hypoxia and oxidative stress occur in the arterial wall, after infiltration of low density lipoprotein (LDL) and its transformation to oxidized-LDL (ox-LDL). The mechanism and the factors responsible for it are unclear, but formation of ox-LDL is widely accepted [25,26]. In carotid plaque, oxidative stress has never been directly demonstrated by other compounds, except ox-LDL. The presence of X, Hx, UA and allantoin in plaque would therefore be the first direct demonstration of oxidative stress.

2. Experimental

2.1. Chemicals

Allantoin, uric acid, hypoxanthine and xanthine were purchased from Sigma (St. Louis MO, USA). Methanol (HPLC grade) was obtained from Baker (Phillipsburg, NJ, USA). Potassium dihydrogen phosphate, potassium monohydrogen phosphate and disodium tetraborate were obtained from Merck (Darmstadt, Germany).

2.2. Standard solutions

Stock solutions of all compounds (1 mg/ml) were prepared by dissolving pure standards in MilliRo water. Fresh working solutions were prepared by diluting the stock solutions with water.

2.3. Sample preparation

Carotid plaque and plasma were obtained from eleven patients (eight males and three females, age 68–80 years) undergoing carotid endarterectomy at the Institute Vascular Surgery, Siena University. Seven of these subjects were symptomatic (five males and two females) and four were asymptomatic (three males and one female). Stenosis was $>60\%$.

Each specimen was rinsed twice in cold phosphate buffered saline (PBS) solution to minimize blood residue. The samples were at the laboratory within 2 h, where they were stored at $-20^{\circ}C$ until analysis. The plaque was frozen under nitrogen and pulverized (three cycles for 1 min) in a micro-dismembrator (Braun, Melsungen, Germany). The powder was washed with 1 ml water and centrifuged three times for 10 min at 12 000 *g*.

A 500 μl plasma (or serum) volume was extracted with 100 μl 4 *M* perchloric acid (PCA). The extracts were centrifuged for 10 min at 12 000 *g*. The supernatant was neutralized with 5 *M* potassium hydroxide (KOH) (about 35 μl), cooled for 1 h and

centrifuged (12 000 *g* for 10 min). Supernatants plaque and plasma were analyzed immediately.

2.4. Equipment

For HPLC separation, a Varian Vista 5500 high-performance liquid chromatograph equipped with a variable-wavelength UV detector (Varian, model 2550) and electronic integrator (Varian, Model 4290) was used. A ready-to-use prepacked Supelcosil LC-18 column (Supelco, 250×4.6 mm I.D., 5 μm), with precolumn (20×4.6 mm I.D.) packed with the same material (Supelguard,Supelco) completed the analytical system.

Two solvents, 0.01 *M* potassium phosphate buffer at pH 5.5 (A) and methanol (B) were used. The mobile phase was run it the following gradient: B: 0% at 0 min, 10% at 10 min, 20% at 20 min, 0% at 30 min. The next sample was injected after a further 10 min. The flow-rate was 1 ml/min and the detection wavelength 220 nm. Allantoin can be detected at 220 nm. The detection of UA at 220 nm has a higher sensitivity than at 292 nm.

For electrophoretic separation a Waters Quanta 4000 instrument was used. Analyses were performed in an uncoated silica capillary (57 cm×75 μm I.D.) with the window at a distance of 50 cm. UV detection was at 214 nm. Hydrostatic injection was used. The capillary was cleaned by flushing sequentially with 100 m*M* sodium hydroxide (NaOH), MilliRo water and separation buffer for 20 min each. Between analyses, the capillary was flushed for 1 min with the separation buffer prior to injection of the next sample.

For the separation of allantoin, UA, Hx and X, a 20 m*M* borate buffer was employed, with the current stable around 30–35 μA. The final optimized conditions were pH 10.1, 16 KV and 9 s load.

3. Results

For the HPLC separation of allantoin, UA, Hx and X, we first checked the effects of different pH values of the mobile phase which are reported in Fig. 1. The pH chosen for the chromatography was 5.5. The standard peaks of allantoin, UA, HX and X were eluted at 3.3, 6.6, 11.8 and 13.8 min respectively. Good linearity was obtained for all amounts of allantoin, Hx (0.2–500 μ*M*), UA and X (0.1–500 μ*M*) used, injected with a 20 μl loop. The equations obtained were $y=66.2x$ for allantoin, $y=332.5x$ for UA, $y=159.1x$ for Hx, and $y=292.9x$ for X (y= peak area, x=concentration). The correlation coefficients were always greater than 0.99. The overall run-to-run and day-to-day precision of the retention times are shown in Table 1.

For CZE, the separation of compounds was investigated in the pH range 8.9–10.1 (Fig. 1). The best results, in term of efficiency of separation, were achieved at pH 10.1. The four compounds were resolved in 20 min. The migration times were: 13.1 for Hx, 13.7 for allantoin, 14.3 for X and 15.7 for UA. A linear calibration was obtained with concentrations of standard solutions between 1 and 50 μM. The equations obtained were $y=1085.1x$ for allantoin, $y=2032.4x$ for UA, $y=3150.5x$ for Hx and $y=1855.6x$ for X (y=peak area, x= concentration). The correlation coefficients were always greater than 0.93.

The reproducibility of migration times and areas of standard peaks, under the optimized conditions, are reported in Table 2. When our procedure was used to analyze carotid plaque and plasma, the day-to-day precision of the retention time was not reproducible (data not published), presumably because the presence of PCA, also if neutralized, deteriorated the compounds, so we decided to analyze them on the same day. A typical HPLC chromatogram and a typical CZE electropherogram are reported in Fig. 2 for plasma and Fig. 3 for plaque. The concentrations of allantoin obtained by HPLC were unsatisfactory, because this compound falls in a region with many interfering peaks, and we did not derivatize it to improve its resolution. The peaks of Hx, X and UA were well separated after the HPLC run. All four peaks were clearly distinguished after CZE.

The levels of the various metabolites in plaque and plasma are reported in Table 3. Similar values of Hx, X and UA were by HPLC and CZE. UA concentrations in plasma were similar to those reported in the literature [14,19,27]; those of allantoin, Hx and X were slightly different. Plasma levels of

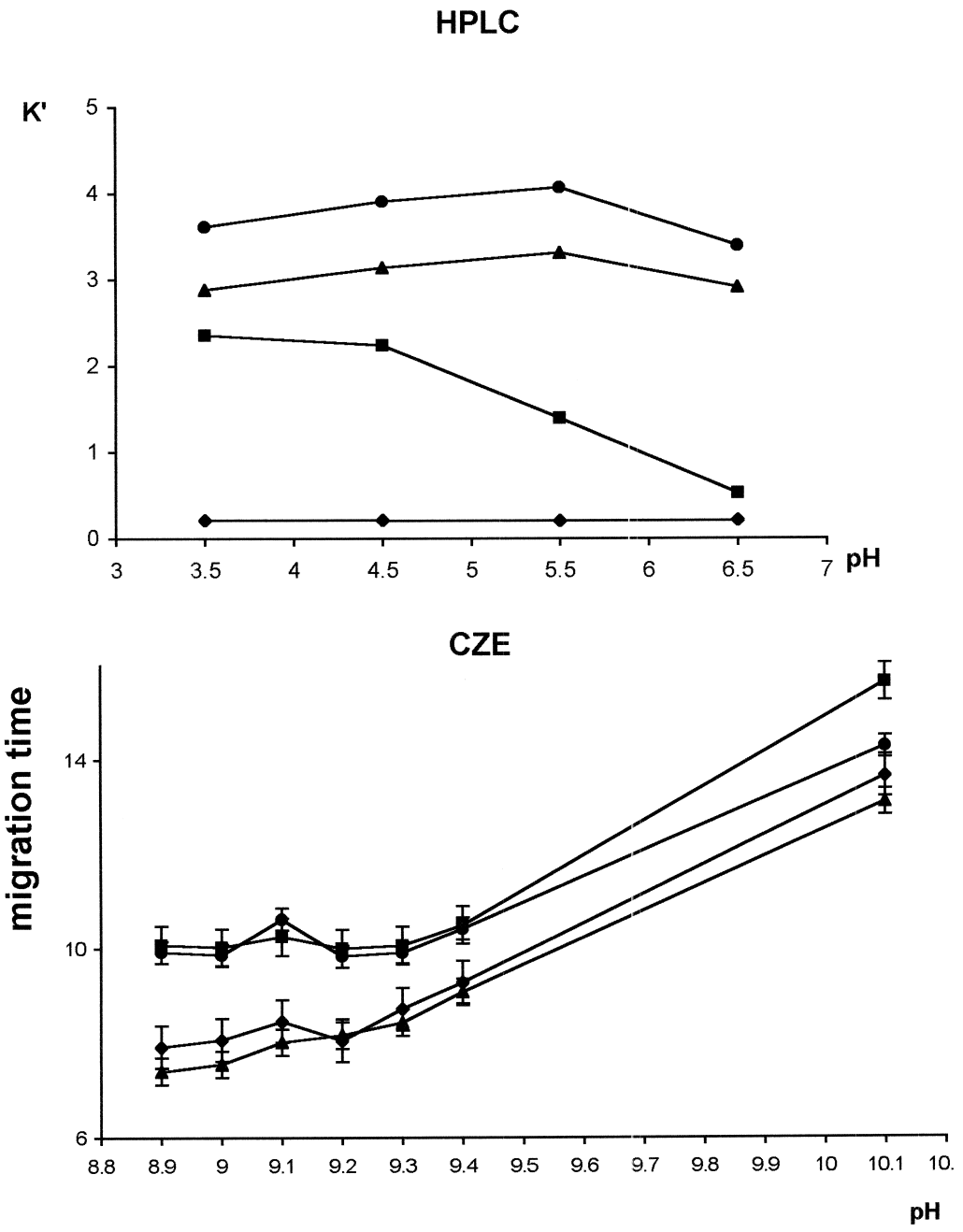

Fig. 1. Effect of pH on HPLC capacity factor (K′) values and CZE migration time of allantoin (♦), UA (■), Hx (▲) and X (●).

allantoin, Hx and X have not yet been standardized in humans.

No data on the concentrations of the same compounds in plaque is available in the literature. Concentrations of Hx, X and allantoin determined by us were remarkably higher in plaque than in plasma,

Table 1
Precision of retention times and peak areas of allantoin, uric acid, hypoxanthine and xanthine standards determined by HPLC

Parameter	Compound	Retention time (min)	SD (5)	C.V. (%)	Peak area (arbitrary units)	SD (5)	C.V. (%)
Between-run precision (same day)	Allantoin	3.3	0.0	0.2	131.1	3.9	2.9
	UA	6.6	0.0	0.3	764.6	2.0	0.3
	Hx	11.8	0.0	0.2	320.5	9.1	2.9
	X	13.8	0.1	0.5	603.5	16.0	2.7
Between-day precision (7 days)	Allantoin	3.3	0.1	1.4	134.9	2.8	2.1
	UA	6.6	0.1	1.4	736.8	12.2	1.7
	Hx	11.7	0.2	1.8	322.8	5.1	1.6
	X	13.7	0.3	2.5	624.7	11.7	1.9

only UA concentrations were lower in plaque. The plaque/plasma ratio was 2.8 for allantoin, 0.1 for UA, 81.5 for Hx and 30.6 for X. Most of data found show high standard deviation due to heterogeneity within and between plaque samples.

The data obtained by HPLC and CZE was processed by standard linear regression analysis and regression analysis of Passing and Bablok. The following equations were:

Allantoin	$y=1.6+0.5x$	$p=0.01$
Hypoxanthine	$y=1.1+x$	$p<1\times10^{-3}$
Uric acid	$y=0.2+1x$	$p<1\times10^{-3}$
Xanthine	$y=0.8+1.0x$	$p<1\times10^{-3}$

The correlation coefficients were highly significant, indicating that the two methods give similar results.

4. Discussion

As demonstrated by our results, CZE opens a new possibility for the quantitative determination of allantoin, Hx, UA and X in biological samples. CZE elution is as simple to perform as HPLC gradient elution. CZE is equally fast, but requires much smaller volumes than HPLC, which makes it possible to work with biological materials available in very limited amounts. Both optimized systems show high reproducibility of retention times and peak areas. The CZE results were similar to those found by HPLC for UA, Hx and X. The CZE gave a more precise determination for allantoin, even when the concentration was low. This was due to the capacity of CZE, respect to HPLC, to improve separation of allantoin from the contaminants present in serum and plaque extracts. Our study shows that CZE can be

Table 2
Precision of migration times and peak areas of allantoin, uric acid, hypoxanthine and xanthine standards determined by CZE

Parameter	Compound	Migration time (min)	SD (5)	C.V. (%)	Peak area (arbitrary units)	SD (5)	C.V. (%)
Between-run precision (same day)	Hx	13.1	0.2	1.6	312	14.3	4.6
	Allantoin	13.7	0.2	1.6	107	6.1	5.7
	X	14.3	0.3	2.4	203	12.2	6.0
	UA	15.7	0.3	2.5	167	6.2	3.7
Between-day precision (7 days)	Hx	13.0	0.2	2.1	320	35.0	5.5
	Allantoin	13.7	0.2	2.5	109	9.8	4.5
	X	14.3	0.3	2.4	190	14.6	4.1
	UA	15.8	0.3	2.5	169	6.5	3.8

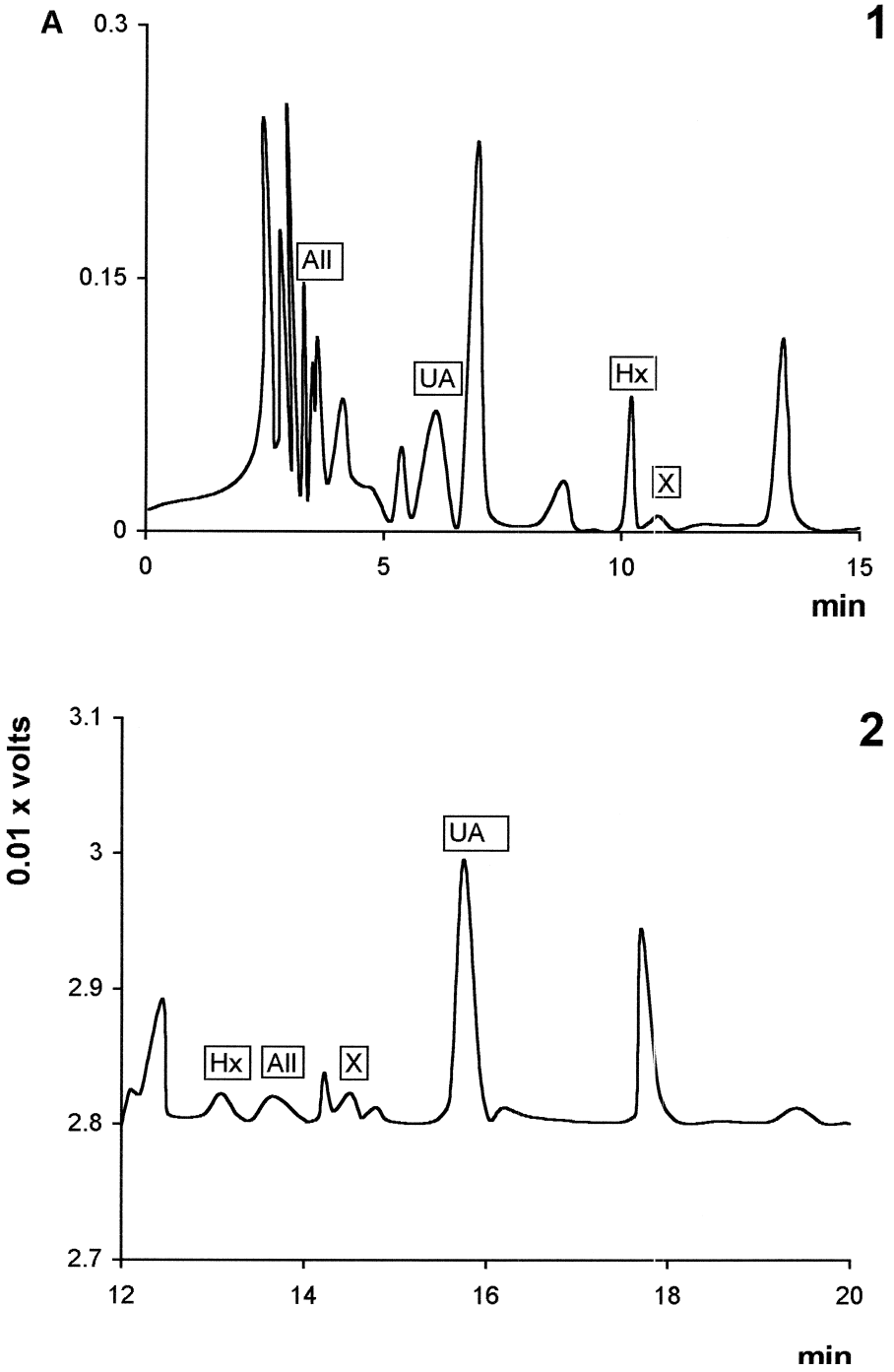

Fig. 2. Typical HPLC chromatogram (1) and a typical CZE electrophoregram (2) of perchloric extract of plasma.

used to determine purine compounds, especially allantoin, in biological samples, as recently reported by Alfazema et al. [28] for urine.

Although no conclusion can be drawn from this preliminary data, the presence of Hx, X and UA in carotid plaque does not seem to be due to mere

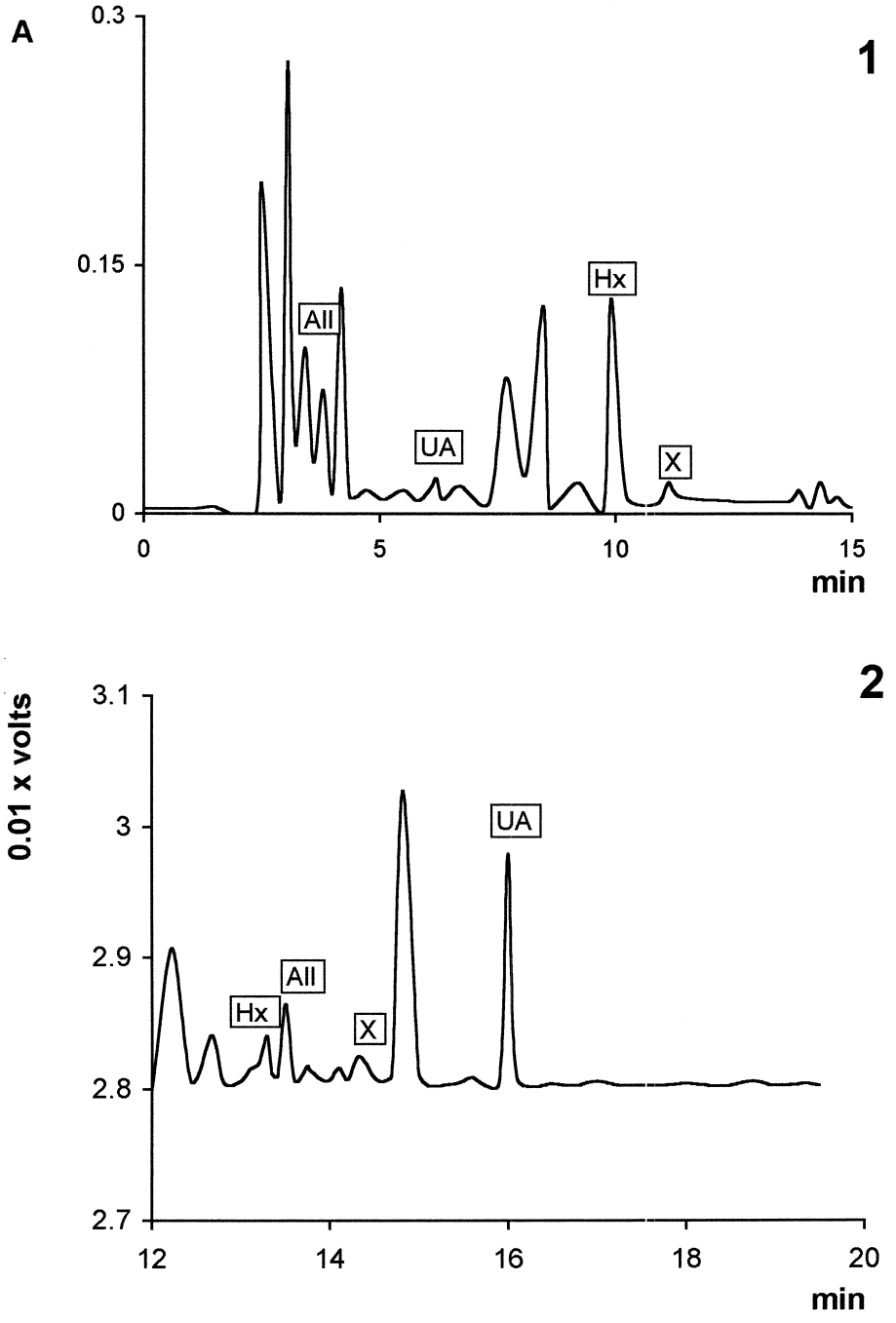

Fig. 3. Typical HPLC chromatogram (1) and a typical CZE electrophoregram (2) of carotid plaque.

accumulation from plasma, but to a local intrinsic phenomenon, in which the oxidative degradation of nucleotides plays a specific role. In fact, when the results from plaque were compared to those from plasma, the concentration of UA was significantly lower, while those allantoin, Hx and X were much

Table 3
Mean concentrations (±standard deviation) of allantoin, UA, Hx and X in plaque and plasma samples determined by HPLC and CZE

Plaque	Allantoin (nmol/g)	Uric acid (nmol/g)	Hypoxanthine (nmol/g)	Xanthine (nmol/g)
HPLC	145.4±48.1	40.0± 39.0	170.3±110.4	20.5±19.5
CZE	90.6±33.4	50.7±40.4	171.3±90.1	22.4±20.1
Plasma	(nmol/ml)	(nmol/ml)	(nmol/ml)	(nmol/ml)
HPLC	59.8±30.7	295.0±40.6	2.1±2.0	1.7±0.6
CZE	31.2±12.5	284.1±30.6	2.5±2.4	1.7±0.7

higher. Such differences are not well compatible with passive accumulation from plasma to plaque, but suggest endogenous production from nucleotide catabolism, with UA rapidly oxidized to allantoin. Free radical reactions has been implicated as an important factor in the pathogenesis of atherosclerotic vascular diseases and the high levels of allantoin for human plasma could be represent a possible indicator of free radical damage in vivo. In fact, UA may be oxidized to allantoin by various reactive species and it is postulated that UA functions as an antioxidant in plasma.

A much larger patient population with histological and clinical data is required for the investigation of this interesting finding, and the determination of mean values and range and their role in the pathology of atheroma. However, this is the first time these products have been determined in carotid plaque. They are the first direct demonstration of oxidative stress in plaque. Knowledge of their biochemical composition could improve our understanding of atherosclerosis and its pathogenesis.

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