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Note

Determination of ergothioneine in red blood cells by high-performance liquid chromatography

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Ergothioneine (ESH) is a widely distributed thiol [1], whose biological role has not yet been established. Potential roles are linked to its capacity to bind certain metal ions [2–4], to the metabolism of essential metal (copper and zinc), to the inhibition of metalloenzymes [5] or in general to maintenance of the cellular redox potential. Several methods have been developed for the determination of ESH. The earliest colorimetric methods [6–9] lacked specificity and sensitivity. Recently, several high-performance liquid chromatographic (HPLC) methods have been proposed for biological thiols [10–13]. These methods are very sensitive for glutathione and cysteine, but they require precolumn derivatization and show poor sensitivity toward ESH [10–13]. This is probably related to the equilibrium with the thionic form [1].

The only HPLC method described in the literature for ESH without derivatization [14] was tested in our laboratory but separation was not satisfactory. We therefore set out to develop a specific method to measure physiological levels of ESH.

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EXPERIMENTAL

Chemicals

L-Ergothioneine dihydrate was purchased from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate (SDS, electrophoretic grade), ion-exchange resins AG2X8 (200–400 mesh, Cl^- form) and AG50X8 (100–200 mesh, H^+ form) were purchased from Bio-Rad (Richmond, CA, U.S.A.). Bidistilled water was prepared in our laboratory and filtered through an all-Pyrex Millipore apparatus; all other HPLC solvents were HPLC grade. Acetonitrile was from Omnia Res. (Milan, Italy) and methanol from Carlo Erba (Milan, Italy).

Trifluoroacetic acid (HPLC grade) was purchased from Pierce (Rockford, IL, U.S.A.), sodium chloride (analytical grade) from Merck (Darmstadt, F.R.G.) and heptane and pentane sulphonate (sodium salts, HPLC grade) and ammonium formate (analytical grade) from Carlo Erba.

Chromatography

A Beckman (Fullerton, CA, U.S.A.) System Gold liquid chromatograph (266 pump module) was used in combination with a variable-wavelength detector (166 module). The detector was set at 260 nm. A reversed-phase column (250 mm × 4 mm I.D.) packed with Nucleosil 5C-18 (5 μm) (Macherey-Nagel, Düren, F.R.G.) was used. The mobile phase was water (containing 0.5 g/l trifluoroacetic acid, 2 m*M* SDS and 2 m*M* sodium chloride)–acetonitrile–methanol (200:50:25, v/v). The column was at room temperature and was protected by a 4 mm × 4 mm guard column (LiChrocart, Merck).

Standard solutions of ESH in bidistilled water were kept frozen until use and were then diluted with the eluent or with water.

Sample preparation

Male CD-Cobs rats (Charles River, Calco, Italy) (400 ± 50 g body weight) were anaesthetized with diethyl ether and decapitated. Blood was collected in heparinized tubes and centrifuged (20 min at 400 *g*). Samples of 0.5 ml of the packed red blood cells (RBCs) were mixed with 4 ml of bidistilled water for haemolysis and vortexed. The mixture was kept for 10 min in a boiling water-bath. After centrifugation (20 min at 2000 *g*), 2 ml of the clear supernatant were passed through an ion-exchange resin column, formed by a mixed bed of AG2X8 and AG50X8. The AG2X8 Cl^- form was converted into the OH^- form following Bio-Rad instructions, washed with water and equilibrated with 20 bed volumes of a 1 *M* solution of ammonium formate. The AG50X8 H^+ form was converted into the NH_4^+ form in the same manner. A 2.5-ml plastic disposable syringe was filled with 0.5 ml of each resin. After packing, columns were washed with 4 ml of bidistilled water. The samples were eluted with 1.5 ml of bidistilled water. The eluate was dried on a Rotavapor and redissolved in 1 ml of the HPLC solvent system.

RESULTS

Chromatography

Several solvent mixtures were tested (water–methanol and water–acetonitrile in different proportions) and several reversed-phase columns (C_{18}, C_8 and C_2). None of these mixtures and columns gave a retention volume different from V_0. Water–methanol and water–acetonitrile containing phosphate buffer with a pH ranging from 3 to 2 were tested. Addition to these mixtures of several ion-pairing reagents (pentane sulphonate, heptane sulphonate, SDS) did not significantly prolong ESH retention times.

The mixture that increased the retention time contained 2 m*M* SDS and 2 m*M* sodium chloride in water, brought to pH 2.3 with trifluoroacetic acid (0.5 g/l). Sodium chloride was added to prevent peak tailing. The aqueous solution was filtered through a 0.45-μm Millipore cellulose ester filter (Bedford, MA, U.S.A.), before being mixed with methanol and acetonitrile: water–methanol–acetonitrile, 200:25:50, v/v. The retention time of ESH with this solvent system

TABLE I

RECOVERIES OF ERGOTHIONEINE ADDED TO PACKED RBCs

Each result is the average of three determinations.

Rat No.	Endogenous level (μg per 0.5 ml)	Amount added (μg per 0.5 ml)	Measured level (μg per 0.5 ml)	Recovery (mean ± S.D.) (%)
1	2.03	1	2.88	95 ± 5
1	2.03	2	4.05	101 ± 2
2	2.55	1	3.35	94 ± 2
2	2.55	2	4.27	94 ± 3
3	4.80	2	6.45	94 ± 4

TABLE II

ERGOTHIONEINE CONCENTRATION IN PACKED RBCs

Rat No.	Concentration (mean ± S.D., $n=3$) (μg/ml)	C.V. (%)
1	4.06 ± 0.10	2.5
2	5.11 ± 0.13	2.5
3	9.61 ± 0.54	5.6
4	9.32 ± 0.23	2.5
5	4.32 ± 0.09	2.1
6	7.89 ± 0.48	6.0
7	8.73 ± 0.13	1.4
8	4.60 ± 0.05	1.1
Mean ± S.D.	6.70 ± 1.58	

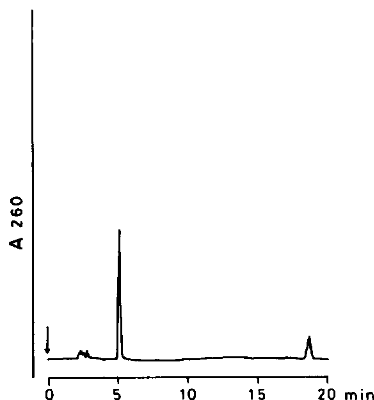

Fig. 1. Typical chromatogram after heat precipitation and ion-exchange purification of RBC extract. The absorbance detector was operated at 260 nm and the full scale of the ordinate is 0.05 absorbance units.

was ca. 5 min. The minimum detectable amount was 250 pg. The calibration curve was linear in the range 12.5–100 ng of ESH.

Addition of 1 or 2 μg of ESH to 0.5 ml of packed RBCs gave recoveries that always exceeded 90% (Table I). The mean $\pm$S.D. level of ESH in RBCs measured in eight rats was 6.7 ± 1.58 μg/ml of packed cells (Table II). This is comparable with data previously reported by Melville and Lubschez [7]. The coefficient of variation (C.V.) ranged from 1 to 6%.

The specificity of the method was tested by treating the samples with an excess of copper(I) oxide, which complexes and precipitates ESH [1]. The undissolved copper(I) oxide was filtered off through a 0.45-μm HV Millipore filter before the samples were chromatographed. This led to the disappearance of the ESH peak.

The method is sensitive enough to detect ESH, the second major thiol present in animal tissues, even with only a small amount of biological material (less than 0.5 ml of packed RBCs) and at an instrumental sensitivity range that avoids baseline problems (Fig. 1).

Under our chromatographic conditions no other peak interfered with ESH determination. The retention time could be increased to ca. 6.5 min by using slightly higher concentrations of trifluoroacetic acid (data not shown).

DISCUSSION

In our hands the results with the method described in ref. 14 were not reliable. The retention volume of ESH always corresponded to the V_0 of the column, with no clear separation from other peaks present in the RBC extracts. ESH, being an inner salt, insoluble in organic solvents, does not interact effectively with a reversed phase, unless the eluent is highly acidic and contains a small percentage of an organic modifier [10].

The only method reported hitherto for the detection of ESH in biological materials is that of Fahley et al. [11]. This method, however, requires pre-column

derivatization with monobromobimane, the use of a modified amino acid analyser and a long analysis time; it also has a low fluorescence yield. The modification of this method [12] does not appear to have the sensitivity and reliability of our method. Our method appears to be the least time-consuming to date and enables us to measure ESH in RBCs in significant amounts. The purification step seems specific, reproducible and rapid enough for the study of the physiological role of ESH. The fact that ESH is found in so many living organisms [1] may have general physiological implications that need to be clarified.

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