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HPLC study of oxidation products of hydroethidine in chemical and biological systems: ramifications in superoxide measurements

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ABSTRACT

Methods for the detection and quantitation of hydroethidine (HE) and its oxidation products by HPLC analysis are described. Synthetic methods for preparation of authentic standards (2-hydroxyethidium and diethidium) are provided. Potential applications of the HPLC methods to chemical and biological systems are discussed. Specific examples of chromatograms obtained using UV-Vis absorption, fluorescence, electrochemical, and mass spectrometry detectors are provided. The development of a dual electrochemical and fluorescence detection methodology and its applications are described. The HPLC-based method enables analyses of HE and its oxidation products such as ethidium and the dimeric products of HE. The ramifications of HPLC measurement of HE and its oxidation products in the detection and quantitation of 2-hydroxyethidium, the diagnostic marker product of superoxide and HE, in the intracellular milieu are discussed. Similarly, mitochondria-targeted HE conjugated to a triphenylphosphonium group (Mito-HE or Mito-SOX) also forms oxidation products (dimers of Mito-HE and Mito-E⁺) that can affect the detection and quantitation of 2-hydroxy-mito-ethidium, the diagnostic marker product of Mito-HE and superoxide in mitochondria.

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Introduction

Hydroethidine (HE; Fig. 1), also known as dihydroethidium, is a fluorogenic probe that has been used for almost 20 years in the detection of intracellular superoxide radical anion (O₂⁻). The red fluorescent product of HE reaction with superoxide has long been assumed to be the two-electron oxidation product ethidium (E⁺). However, recent publications in well-defined model systems suggest that 2-hydroxyethidium (2-OH-E⁺) is the sole reaction product of HE and O₂⁻ [1–3]. Nonetheless, both fluorescent products (2-OH-E⁺ and E⁺) are typically formed in cells under oxidative conditions in different ratios. In general, E⁺ has been detected at a much higher level than 2-OH-E⁺ [2–4]. Owing to their spectral overlap, it is extremely difficult to distinguish between the two products in biological systems using fluorescence spectroscopy. Attempts to resolve the spectra and quantify selectively the product, 2-OH-E⁺, have been reported [5,6]. The quantitation of 2-OH-E⁺ using fluorescence spectroscopy can also be confounded by its intracellular distribution such as binding to DNA and differential partitioning due to different hydrophobicity and/or

pH, factors that influence the fluorescence quantum yield and positions of the maxima of excitation/emission bands [4]. Increasing evidence suggests that intracellular quantitation of superoxide, based on fluorescence measurements in cellular systems, is inadequate and potentially erroneous [2,4,7,8]. Therefore, the detection and quantitation of 2-OH-E⁺ by HPLC separation of the fluorescent products in cell and tissue extracts has become the method of choice, with over 30 papers published in the past 4 years [1–5,7–33]. There are several modalities with which HE oxidation products can be detected. Although fluorescence detection remains a favorite choice for quantitation of 2-OH-E⁺, UV-Vis absorption, electrochemical, or MS detection methods enable the measurement of less fluorescent and nonfluorescent products of HE at a higher degree of sensitivity. These alternate detection methods have made it possible to detect and quantitate radical-mediated oxidative dimeric products of HE lacking fluorescence under the excitation/emission setup optimized for 2-OH-E⁺ [4]. Recently a mitochondria-targeted analog of HE (Mito-HE, MitoSOX red, Fig. 1) has been synthesized and is being widely used for the quantitation of mitochondria-derived superoxide [5,6]. Despite differences in the intracellular distribution of HE and Mito-HE, their chemical reactivities in redox reactions are very similar (Fig. 2) [5,8]. Thus, in this review we focus on the experiments utilizing HE with the understanding that most of the discussion

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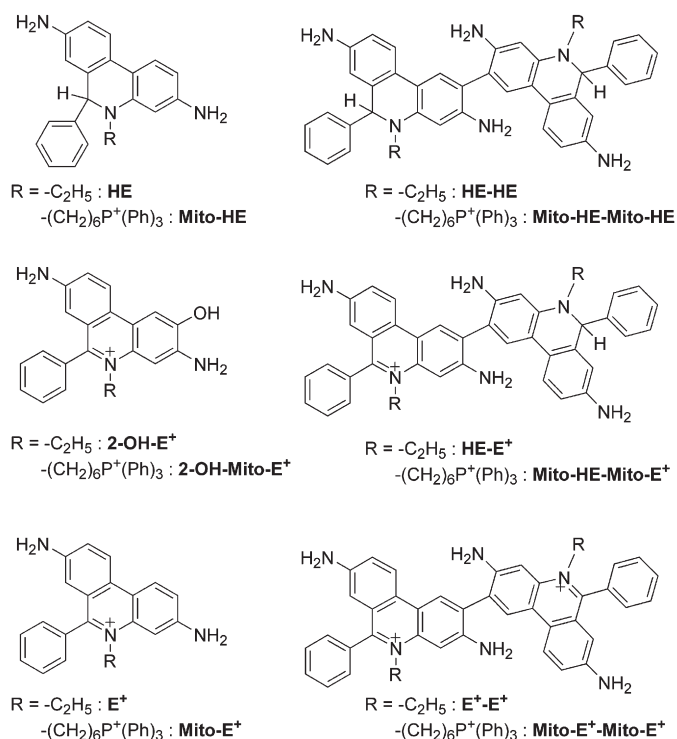


Fig. 1. Structures of HE and Mito-HE and their oxidation products.

concerning the utility and limitation of the HE-based assay is also applicable to Mito-HE.

Principles

As both monomeric (2-OH-E⁺ and E⁺) and dimeric (HE-HE, HE-E⁺ and E⁺-E⁺) products of HE oxidation can be detected in extracts from cells incubated with HE, it is essential to understand their mechanisms of formation, taking into consideration the various oxidant-induced pathways of HE consumption.

The measurement of superoxide radical anion using the HE probe is based on its exclusive conversion to 2-OH-E⁺, which is highly specific for detecting superoxide in biological systems [2,4,34,35]. It was shown that the conversion of HE into 2-OH-E⁺ initially involves a one-electron oxidation of HE to the radical cation. However, this initial reaction step is induced by other one-electron oxidants, as has been shown in the case of hydroxyl radical (OH) and dibromine radical anion (Br₂⁻) and, therefore, is not particularly specific for superoxide (Fig. 2) [10]. What makes this reaction product unique for superoxide is the subsequent step, i.e., the superoxide reaction with the HE radical species (radical cation or neutral radical) or Mito-HE radical, leading to formation of the final product, 2-hydroxyethidium or 2-hydroxy-mito-ethidium [10,20]. In the absence of superoxide, other one-electron oxidants can convert HE and Mito-HE into several dimeric products (e.g., HE-HE, HE-E⁺, and E⁺-E⁺ or their mitochondria-targeted analogs) [8]. As in the case of ferricyanide and ferricytochrome c, both dimeric products and E⁺ have been detected as the products, it is likely that the intermediate HE radical species can dismutate to form ethidium. Ethidium and mito-ethidium can be also formed in a process of direct hydride transfer from HE or Mito-HE to the appropriate hydride acceptor [10] without involving any radical intermediate.

On the basis of our current understanding of the chemistry of HE and Mito-HE, we can conclude that 2-OH-E⁺ and 2-OH-Mito-E⁺ formation in cells and tissues incubated with HE or Mito-HE should

arise from the reaction with superoxide. On the other hand, the formation of dimeric products of HE or Mito-HE is indicative of the reaction with one-electron oxidants that oxidize HE or Mito-HE to its radical form. Formation of E⁺ and Mito-E⁺ is less informative, however. Both E⁺ and Mito-E⁺ can be formed during oxidation of HE or Mito-HE enzymatically, by hydride transfer or by a one-electron oxidation mechanism. Although the exact nature of the one-electron oxidant cannot be derived from the structure of the dimeric product, it may include heme proteins (for example peroxidases and cytochrome c), peroxynitrite-derived oxidants, or iron-derived oxidants [8,10,36]. In some cases, however, the exact nature of the oxidant could be established using selective inhibitors of the specific oxidation pathways.

The relatively high chemical reactivity of HE and Mito-HE, while enabling the detection of several oxidants, may be also a limiting factor in the detection of superoxide. High reactivity of the probe together with a high concentration of the co-oxidant generated inside the cells will lead to low steady-state intracellular levels of HE. Under this condition, HE cannot effectively compete with superoxide dismutase (SOD; the rate constants of the reaction with superoxide are ca. $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for SOD [37] and ca. $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for HE [10] and ca. $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for Mito-HE [5]). Although the intracellular HE concentration is determined by the rate of the probe influx and the rate of HE consumption, agents capable of interfering with at least one of those factors may seriously compromise the ability of the probe to compete for superoxide and other oxidants. Therefore, monitoring the intracellular concentration of HE or Mito-HE in each experiment is crucial and changes in HE or Mito-HE concentration should be taken into account for proper interpretation of the experimental results obtained for HE dimer, Mito-HE dimer, or 2-OH-E⁺ and 2-OH-Mito-E⁺.

Materials

The materials to be used are as follows:

- (1) Hydroethidine (Fluka, Cat. No. 37291)
- (2) Mito-HE (MitoSOX red, Invitrogen, Cat. No. M36008)

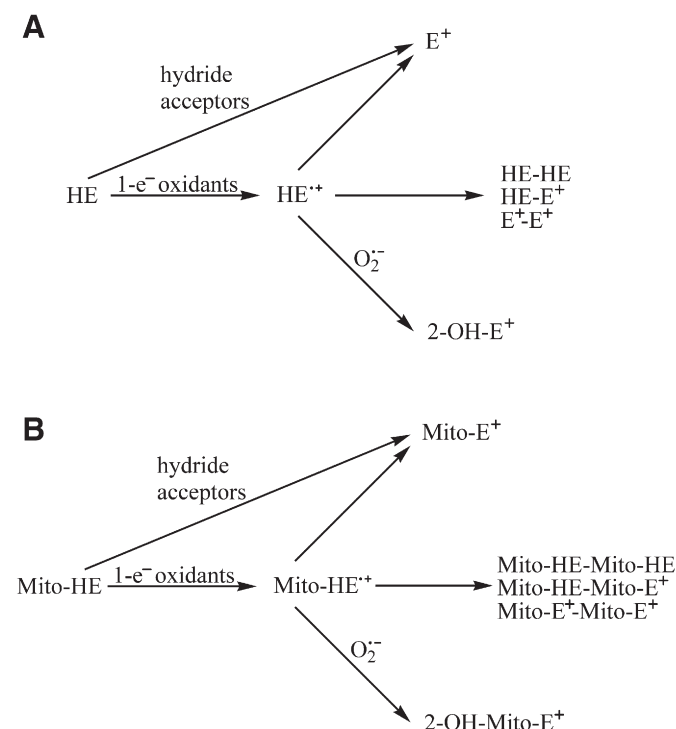


Fig. 2. Oxidation pathways of (A) HE and (B) Mito-HE.

- (3) Dimethyl sulfoxide (DMSO; Sigma, D2650)
- (4) Dulbecco's phosphate-buffered saline (DPBS; Sigma, D8537)
- (5) Triton X-100 (Sigma–Aldrich, Cat. No. T9284)
- (6) Ethidium bromide (Fluka, Cat. No. 46065)
- (7) Perchloric acid, 70% (GFS Chemicals, Item 67)
- (8) Methanol (Sigma–Aldrich, Cat. No. 34860)
- (9) Ethanol (AAPER)
- (10) Phosphoric acid, 85% (J.T. Baker, Cat. No. 0260-02)
- (11) Potassium phosphate, monobasic (Sigma–Aldrich, Cat. No. P0662)
- (12) Potassium phosphate, dibasic (Fluka, Cat. No. 60356)
- (13) Diethylenetriaminepentaacetic acid (DTPA; Fluka, Cat. No. 32319)
- (14) Potassium bromide (Sigma–Aldrich, Cat. No. 243418)
- (15) 1-Butanol (Sigma–Aldrich, Cat. No. 34867)
- (16) Acetonitrile (Sigma–Aldrich, Cat. No. 34851)
- (17) Trifluoroacetic acid (TFA; Thermo Scientific, Cat. No. 28903)
- (18) Formic acid (Riedel-de Haën, Cat. No. 27001)
- (19) Potassium ferricyanide (Sigma–Aldrich, Cat. No. 244023)
- (20) Potassium nitrosodisulfonate (Aldrich, Cat. No. 220930)
- (21) Tetrachloro-1,4-benzoquinone (Fluka, Cat. No. 23280)
- (22) Bradford reagent (Sigma–Aldrich, Cat. No. B6916) or other reagent for determination of protein concentration in cell lysate
- (23) Bovine serum albumin (Sigma, Cat. No. A8806)

Protocol

Synthesis of 2-hydroxyethidium (2–3 days)

2-Hydroxyethidium is synthesized by reacting HE with nitrosodisulfonate (NDS) using two moles of NDS per one mole of HE [20]. For a large-scale synthesis, HE can be synthesized by reduction of E⁺ as described previously [20,38].

- (1) Prepare 0.5 M phosphate buffer, pH 7.4.
- (2) Prepare 1.0 mM DTPA in water.
- (3) Prepare 10 ml of 1 mM NDS in 50 mM phosphate, pH 7.4, 100 μ M DTPA. The concentration of NDS should be determined by spectrophotometry using the extinction coefficient values of 1690 (at 248 nm) and 20.8 M⁻¹ cm⁻¹ (at 545 nm) [20].
- (4) Prepare 20 mM HE in DMSO. The concentration should be determined by spectrophotometry using the extinction coefficients values of 1.8 $\times 10^4$ (at 265 nm) and 9.75 $\times 10^3$ M⁻¹ cm⁻¹ (at 345 nm) [20]. For that purpose, to the quartz cuvette containing 998 μ l aqueous solution of 50 mM phosphate buffer, pH 7.4, and 100 μ M DTPA, add 2.5 μ l of 20 mM HE in DMSO, mix by inversion, and collect the spectrum. Subtract the spectrum of the corresponding mixture with added pure DMSO instead of HE stock solution and determine the absorbance values at 265 and 345 nm for calculating the HE concentration.
- (5) To the glass vial add:
 - (A) 1 ml of 0.5 M phosphate buffer, pH 7.4;
 - (B) 1 ml of 1 mM DTPA;
 - (C) 6 ml of water;
 - (D) 2 ml of 1 mM NDS.
- (6) Mix the solution and add 50 μ l of 20 mM solution of HE in DMSO.
- (7) Incubate the mixture at room temperature for 1 h.

The reaction mixture contains 2-hydroxyethidium as the major product with a small amount of ethidium as a contaminating by-product (Fig. 3A). The purification of the crude 2-hydroxyethidium can be achieved by silica-gel low-pressure chromatography, using C₁₈ solid-phase extraction cartridges, or by HPLC. The methodology for 2-hydroxyethidium purification has been described previously [4,20]. Below is a description of the HPLC method of purification of

2-hydroxyethidium. The products of HE oxidation are separated on a semipreparative C₁₈ column (Beckmann Ultrasphere, 250 \times 10 mm, 5 μ m), using a gradient of acetonitrile/water containing 0.1% TFA with a flow rate of 2.4 ml/min (Table 1). Under the HPLC conditions shown in Fig. 3A, the 2-hydroxyethidium eluted at approximately 25 min after the injection. The fraction collected should be analyzed by mass spectrometry to confirm the identity of the pure product (see inset in Fig. 3A, mass-to-charge ratio (m/z)=330.16; the intervals between the isotopic peaks should be 1.0 indicating a z value of 1). Please note that the fraction collected is 2-hydroxyethidium dissolved in a mixture of water and acetonitrile containing 0.1% TFA. One can lyophilize the fractions collected to obtain the salt of 2-hydroxyethidium cation and trifluoroacetate anion. The concentration of 2-OH-E⁺ in water at pH 7.4 is determined by spectrophotometry using the absorbance at 470 nm and the extinction coefficient of 1.2 $\times 10^4$ M⁻¹ cm⁻¹ [7].

Synthesis of 2-hydroxy-mito-ethidium (2–3 days)

2-Hydroxy-mito-ethidium can be synthesized by a protocol similar to that described above for 2-hydroxyethidium using NDS [4,5,8] except for the following changes:

- (1) In step 4, owing to the availability of Mito-HE in vials containing 50 μ g of the compound, the concentration of Mito-HE in DMSO should be ca. 5 mM. The concentration should be determined by spectrophotometry in a way similar to that for HE, but using the extinction coefficients values of 1.6 $\times 10^4$ M⁻¹ cm⁻¹ (at 267 nm) and 7.5 $\times 10^3$ M⁻¹ cm⁻¹ (at 355 nm) [4].
- (2) In step 5, instead of 50 μ l of 20 mM HE, use 200 μ l of 5 mM Mito-HE.

As in the case of HE, there is a small contamination of the product by nonhydroxylated cation (Mito-E⁺) and the product should be purified by HPLC. For that purpose the reaction mixture is initially extracted with *n*-butanol (*n*-BuOH) and the organic extract dried as follows:

- (1) Transfer the reaction mixture into two 15-ml tubes (5 ml per tube).
- (2) Add ca. 20 mg of KBr to each tube.
- (3) Add 3 ml of *n*-BuOH to each tube, close the tubes, and vortex for 1 min.
- (4) Leave the tubes for 10 min or centrifuge the tubes (3 min at 1000 g) to obtain separated layers of aqueous (lower) and organic (upper) phases.
- (5) Transfer the upper (organic) layer carefully to a new glass vial (combine the extracts from both tubes) using a glass Pasteur pipette.
- (6) Repeat steps 3 and 4 two more times and combine all the extracts together.
- (7) Evaporate the *n*-BuOH under air or nitrogen flux or using a rotary evaporator with the temperature set at 40 °C.

Redissolve the dry residue in 1 ml of ethanol and dilute with 9 ml of water containing 0.1% TFA. 2-Hydroxy-mito-ethidium is separated from mito-ethidium using the same HPLC conditions as described for 2-hydroxyethidium purification, but using a different gradient (Table 1). The fraction collected should be analyzed by mass spectrometry to confirm the identity of the pure product (m/z =326.66; the intervals between the isotopic peaks should be 0.5 indicating a z value of 2; for the deprotonated form m/z =646.30; the intervals between the isotopic peaks should be 1.0 indicating a z value of 1). The final product can be lyophilized and stored at 4 °C as a trifluoroacetate salt. The concentration of 2-OH-Mito-E⁺ after dissolving in water can be determined at pH 7.4 by spectrophotometry using the absorbance at 478 nm and the extinction coefficient of 9.4 $\times 10^3$ M⁻¹ cm⁻¹ [5].

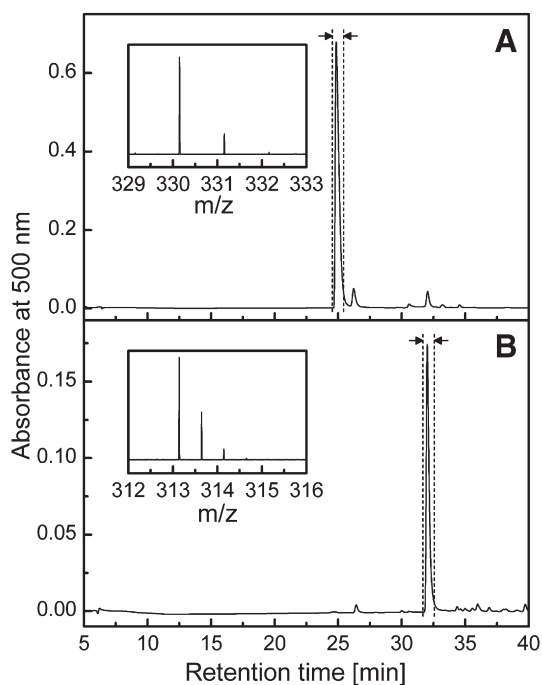


Fig. 3. HPLC chromatograms collected during purification of (A) 2-OH-E⁺ and (B) E⁺-E⁺ on a Beckmann Ultrasphere semipreparative column (250 × 10 mm) using the gradient described in Table 1 and a flow rate of 2.4 ml/min. The vertical dashed lines and arrows mark the beginning and end of the fraction collection. Insets show the MS spectra of the collected fractions.

Synthesis of mito-ethidium (2–3 days)

Mito-ethidium can be synthesized by reacting Mito-HE with chloranil (tetrachloro-1,4-benzoquinone) [4,8] as follows:

- (1) Prepare 10 ml of a 2 mM solution of chloranil in methanol.
- (2) Prepare a 5 mM solution of Mito-HE in DMSO.
- (3) Prepare a 0.5 M solution of phosphate buffer, pH 7.4.
- (4) Prepare a 1.0 mM solution of DTPA in water.
- (5) To the glass vial add:
 - (A) 1.3 ml of 0.5 M phosphate buffer, pH 7.4;
 - (B) 1.3 ml of 1 mM DTPA;
 - (C) 10 ml of water;
 - (D) 33 μ l of 2 mM chloranil in MeOH.
- (6) Mix the solution and add 131.6 μ l of 5 mM HE in DMSO.
- (7) Incubate the mixture at room temperature for 4 h.

This reaction mixture contains mito-ethidium as a major product. Mito-E⁺ can be extracted from the reaction mixture using *n*-BuOH and purified by HPLC using the same method as described above for 2-OH-Mito-E⁺. The identity of the pure product should be confirmed by mass spectrometry ($m/z=315.66$; the intervals between the isotopic peaks should be 0.5 indicating a z value of 2; for the deprotonated form $m/z=630.31$; the intervals between the isotopic peaks should be 1.0 indicating a z value of 1). The obtained product can be lyophilized and stored at 4 °C as the trifluoroacetate salt. The concentration of Mito-E⁺ after dissolving in water can be determined at pH 7.4 by spectrophotometry using the absorbance at 488 nm and the extinction coefficient of $5.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [5].

Synthesis of diethidium (2–3 days)

The synthesis of diethidium is based on the oxidation of hydroethidine by potassium ferricyanide [8] as follows:

- (1) Prepare 10 ml of a 1 M solution of K₃Fe(CN)₆ in water.
- (2) Prepare a 20 mM solution of HE in DMSO.

- (3) Prepare a 0.5 M solution of phosphate buffer, pH 7.4.
- (4) Prepare a 1.0 mM solution of DTPA in water.
- (5) To the glass vial add:
 - (A) 1 ml of 0.5 M phosphate buffer, pH 7.4;
 - (B) 1 ml of 1 mM DTPA;
 - (C) 8 ml of 1 M K₃Fe(CN)₆.
- (6) Mix the solution and add 50 μ l of 20 mM HE in DMSO.
- (7) Incubate the mixture at room temperature for 1 h.

The reaction mixture thus obtained contains diethidium as a major product along with other oxidation products. The pure form of diethidium can be isolated using an HPLC apparatus equipped with a fraction collector and a semipreparative column. However, owing to the high concentration of K₃Fe(CN)₆ in the reaction mixture, it is desirable to extract the product from the reaction mixture containing the ferricyanide so as to avoid precipitation of ferricyanide inside the HPLC column. The extraction can be carried out using *n*-BuOH as described above for the preparation of 2-hydroxy-mito-ethidium.

The procedure for purification of diethidium by HPLC is similar to the one described above for 2-hydroxyethidium (Table 1), except for its retention time (Fig. 3B). The identity of the collected compound should be confirmed by MS analysis (see the inset in Fig. 3B, $m/z=313.15$; the intervals between the isotopic peaks should be 0.5 indicating a z value of 2). As with 2-OH-E⁺, the collected fractions can be lyophilized to obtain the solid salt of diethidium trifluoroacetate. After being dissolved in the aqueous solution of phosphate buffer (pH 7.4), the concentration of diethidium can be determined by spectrophotometry using an extinction coefficient of $1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 497 nm.

Synthesis of di-mito-ethidium (2–3 days)

The synthesis of di-mito-ethidium (Mito-E⁺-Mito-E⁺) is based on the oxidation of Mito-HE by potassium ferricyanide [8] as follows:

- (1) Prepare a 10 mM solution of K₃Fe(CN)₆ in water.
- (2) Prepare a 5 mM solution of Mito-HE in DMSO.
- (3) Prepare a 0.5 M solution of phosphate buffer, pH 7.4.
- (4) Prepare a 1.0 mM solution of DTPA in water.
- (5) To the glass vial add:
 - (A) 1 ml of 0.5 M phosphate buffer, pH 7.4;
 - (B) 1 ml of 1 mM DTPA;
 - (C) 8 ml of water;
 - (D) 150 μ l of 10 mM K₃Fe(CN)₆.
- (6) Mix the solution and add 100 μ l of 5 mM Mito-HE in DMSO.
- (7) Incubate the mixture at room temperature for 15 min.

The reaction mixture thus obtained contains di-mito-ethidium as a major product along with other oxidation products. Mito-E⁺-Mito-E⁺ can be extracted from the reaction mixture using *n*-BuOH and purified by HPLC by the same method as described above for 2-OH-Mito-E⁺.

Table 1

The gradient elution conditions used for purification of the oxidation products of HE and Mito-HE by semipreparative HPLC

	Time (min)	0.1% TFA in water	0.1% TFA in MeCN
HE	0	90%	10%
	46	30%	70%
	50	0%	100%
	55	0%	100%
	60	90%	10%
Mito-HE	0	60%	40%
	30	45%	55%
	40	0%	100%
	50	0%	100%
	55	60%	40%

The identity of the pure product should be confirmed by mass spectrometry analysis ($m/z=315.15$; the intervals between the isotopic peaks should be 0.25 indicating a z value of 4; for the deprotonated form $m/z=419.87$, and the intervals between the isotopic peaks should be 0.33 indicating a z value of 3). The product can be lyophilized and stored at 4 °C as the trifluoroacetate salt.

Cell experiment (2–4 h)

- (1) Incubate bovine aortic endothelial cells (BAEC) or other cells of interest in medium containing 10 μM HE for 20–60 min.
- (2) Stop the incubation by replacing the medium with equivalent volume of ice-cold DPBS.
- (3) Remove DPBS and scrape the cells in 1 ml of ice-cold DPBS.
- (4) Transfer the cell suspension into an Eppendorf tube (1.5–2 ml) and place the tube on ice.
- (5) Centrifuge the cell suspension to pellet the cells: 5 min \times 1000 g at 4 °C.
- (6) Remove the supernatant and freeze the cell pellets and store at or below –80 °C or immediately process the samples for HPLC analysis.

Preparation of the sample for HPLC analysis (4–5 h)

- (1) Add 150–200 μl of 0.1% Triton X-100 in DPBS to the tube with the cell pellet.
- (2) Lyse the cells by aspirating and dispensing back the suspension using the insulin syringe with a needle. Repeat the step 10 times.
- (3) Transfer 2–10 μl (depending on the expected protein concentration and protein assay used) of the cell lysate into an empty tube for the determination of the protein concentration in the cell lysate. For each sample the protein concentration should be determined at least twice to check the experimental reproducibility.
- (4) Transfer 100 μl of the cell lysate to a tube containing 100 μl of 0.2 M solution of HClO_4 in MeOH, vortex the mixture, and place on ice. Leave the mixture on ice for 1–2 h to allow protein precipitation.
- (5) After incubating on ice centrifuge the samples for 30 min at 20,000 g at 4 °C.
- (6) Transfer 100 μl of the supernatant into a tube containing 100 μl of 1 M solution of potassium phosphate buffer, pH 2.6, and vortex.
- (7) Centrifuge the samples for 15 min at 20,000 g at 4 °C.
- (8) Transfer the supernatant into an HPLC vial equipped with a 200- μl conical insert, seal the vial, and place the sample back on ice.
- (9) Place the samples in the refrigerated autosampler and run the analyses.

HPLC analysis (60–95 min per sample)

The separation of HE and its oxidation products by HPLC is based on the consecutive elution of the analytes using a gradient of acidified acetonitrile/water from low (mobile phase A) to high (mobile phase B) concentration of acetonitrile. The conditions of the HPLC analyses, as routinely used in the authors' laboratory, are shown in Table 2 for HE and Table 3 for Mito-HE.

HPLC with absorbance/fluorescence detection (HPLC–Abs/Fl)

The chromatogram of the mixture of HE, 2-OH- E^+ , E^+ , and E^+-E^+ obtained using the HPLC system equipped with both UV–Vis absorption and fluorescence detectors is shown in Fig. 4 and for Mito-HE and its oxidation products in Fig. 5A. The setup of HPLC with

UV–Vis absorption and fluorescence detection has been published elsewhere [2,8,20]. The detectors are configured in a series such that the eluent flows initially through the absorption detector and then through the fluorescence detector. Here we show that by taking advantage of the wavelength programming capability of modern fluorescence detectors, one can detect HE as well as the 2-OH- E^+ and E^+ with a high degree of sensitivity. Under the conditions used (Fig. 4), the signal intensity of HE is nearly 2 orders of magnitude higher than those of 2-OH- E^+ and E^+ (Fig. 4). The fluorescence parameters for 2-hydroxyethidium were modified slightly to improve the sensitivity of detection.

The mobile phases for the HPLC system with UV–Vis absorption and fluorescence detectors are prepared as follows:

- Mobile phase A: Mix 1 L of water with 1 ml of TFA.
- Mobile phase B: Mix 1 L of acetonitrile (MeCN) with 1 ml of TFA.

HPLC with electrochemical detection (HPLC–EC)

Fig. 6 shows the chromatogram of an equimolar mixture of HE, 2-OH- E^+ , E^+ , and E^+-E^+ obtained using the HPLC system equipped with electrochemical (coulometric) and fluorescence detectors. The HPLC–EC chromatogram of an equimolar mixture of Mito-HE, 2-OH-Mito- E^+ , and Mito- E^+ is shown in Fig. 5B and that of the reaction mixture of Mito-HE with potassium ferricyanide in Fig. 5C. The HPLC–EC instrument setup is shown in Table 2 for single electrochemical [7,8,10] as well as for dual electrochemical/fluorescence options. Because of the destructive nature of the coulometric detection (i.e., 100% of the analyte undergoes oxidation), the fluorescence detector cannot be placed in a series with electrochemical detectors. Instead, the flow leaving the column is split into two detectors. As discussed above, the fluorescence detector can be programmed so that both HE and 2-OH- E^+/E^+ can be detected with high sensitivity. One of the advantages of dual detection is that even with coeluting materials, there is a high likelihood that their electrochemical and fluorescence properties are different. By the same token, obtaining the same values of the concentration of analytes using both modes of detection is a very good indicator of the purity of the eluting compound. In addition, dual detection enables the quantitation of several analytes in a single HPLC run. For example, we were able to monitor doxorubicin uptake using the fluorescence detector from cells treated with HE and doxorubicin (unpublished data).

With regard to the HPLC with electrochemical detection technique, the water used for preparing the mobile phase should first be passed through a C_{18} cartridge (for example, an Alltech Prevail SPE cartridge) to remove the traces of organic contaminants that can be present even after purification using a typical Millipore or an equivalent system. In all the procedures described below for HPLC–EC, the same purification method should be employed for water preparation. Instead of TFA the acidic phosphate buffer is used in the mobile phase for HPLC–EC. Described below is the procedure for preparation of the mobile phase for HPLC–EC system.

Phosphate buffer, 0.2 M, pH 2.6. To a 1-L volumetric flask add:

- 19.05 g KH_2PO_4 ;
- 4.1 ml H_3PO_4 (85%);
- water (prepared as described above) up to 1 L.

Filter the solution using a vacuum filtering system and 0.2- μm filter. Check the pH of the solution after dilution to obtain 50 mM phosphate buffer. The pH value should be within the range 2.5–2.7.

Mobile phase A. To a 1-L volumetric flask add:

- 250 ml 0.2 M phosphate buffer, pH 2.6;
- 500 ml water;
- 100 ml acetonitrile;
- water up to 1 L.

Table 2
The instrumental setup for the analysis of HE and its oxidation products by HPLC with different detectors

	UV-Vis absorption and fluorescence detection			Electrochemical detection			MS detection		
Mobile phase A	0.1% TFA in water			50 mM potassium phosphate buffer, pH 2.6; 10% MeCN; water			0.1% formic acid in water		
Mobile phase B	0.1% TFA in MeCN			50 mM potassium phosphate buffer, pH 2.6; 60% MeCN; water			0.1% formic acid in MeCN		
Column	Kromasil C ₁₈ 250×4.6 mm; 5 μm, 100 Å			Synergi Polar RP 250×4.6 mm; 4 μm, 80 Å			Synergi Polar RP 250×2.0 mm; 4 μm, 80 Å		
Flow rate	0.5 ml/min			0.5 ml/min			0.1 ml/min		
Typical inj. vol.	50 μl			50 μl			20 μl		
Gradient	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
	0	90	10	0	60	40	0	80	20
	46	30	70	30	0	100	60	0	100
	47	0	100	40	0	100	70	0	100
	55	0	100	45	60	40	75	80	20
	60	90	10	60	60	40	95	80	20
Detector settings	UV-Vis absorption (diode array) detector: 220, 242, 290, 370, 500 nm			Potentials on coulometric electrodes: 0, 200, 280, 365, 400, 450, 500, 600 mV. In the case of dual detection the potentials on the electrodes are 0, 220, 320, 450, 500, 600 mV and the fluorescence parameters are 0–17 min, λ _{ex} =358 nm, λ _{em} =440 nm; 17–45 min, λ _{ex} =490 nm, λ _{em} =565 nm.			Total ionic current collected over the range of m/z values of 100–1000. Positive ions detection mode.		
	Fluorescence detector								
	Time (min)	λ _{ex} (nm)	λ _{em} (1) (nm)	λ _{em} (2) (nm)					
0–25	358	440	450						
25–60	490	567	596						

Filter the solution using a vacuum filtering system and 0.2-μm filter. Keep the mobile phase refrigerated until use.

Mobile phase B. To a 1-L volumetric flask add:

- 250 ml 0.2 M phosphate buffer, pH 2.6;
- 140 ml water;
- 600 ml acetonitrile;
- water up to 1 L.

Filter the solution using a vacuum filtering system and 0.2-μm filter. Keep the mobile phase refrigerated until use.

Table 3
The instrumental setup for the analysis of Mito-HE and its oxidation products by HPLC with various detectors

	UV-Vis absorption and fluorescence detection			Electrochemical detection		
Mobile phase A	0.1% TFA in water			50 mM potassium phosphate buffer, pH 2.6; 10% MeCN; water		
Mobile phase B	0.1% TFA in MeCN			50 mM potassium phosphate buffer, pH 2.6; 60% MeCN; water		
Column	Kromasil C ₁₈ 250×4.6 mm; 5 μm, 100 Å			Synergi Polar RP 250×4.6 mm; 4 μm, 80 Å		
Flow rate	0.5 ml/min			0.5 ml/min		
Typical injection volume	50 μl			50 μl		
Gradient	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
	0	80	20	0	35	65
	10	55	45	30	0	100
	30	45	55	50	0	100
	40	0	100	55	35	65
	46	0	100	70	35	65
	50	80	20			
	65	80	20			
Detector settings	UV-Vis absorption (diode array) detector: 220, 270, 290, 365, 500 nm			Potentials on coulometric electrodes: 0, 200, 280, 365, 400, 450, 500, 600 mV		
	Fluorescence detector: λ _{ex} =510 nm, λ _{em} =595 nm					

HPLC with MS detection (HPLC-MS)

The HPLC-MS chromatogram of the mixture of HE, 2-OH-E⁺, E⁺, and E⁺-E⁺ is shown in Fig. 7. As mass spectrometry detection is based on the mass-to-charge ratio, the HPLC-MS system offers a high

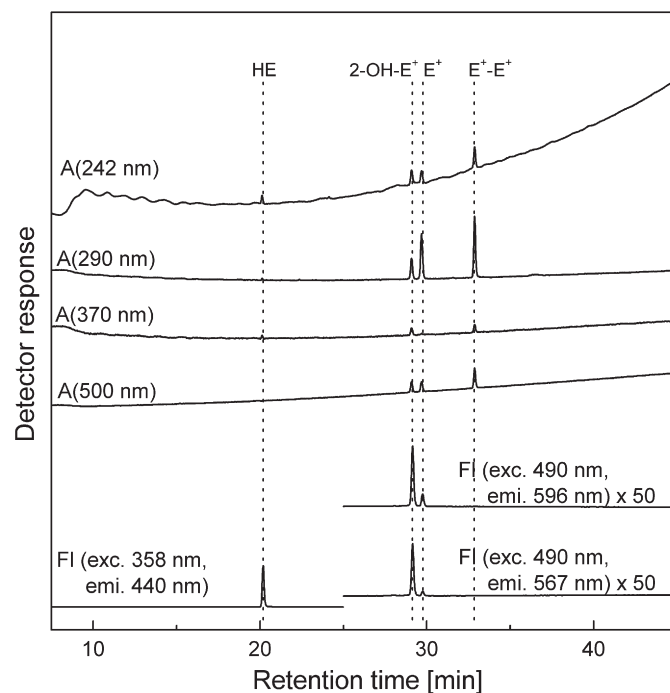


Fig. 4. Chromatogram (HPLC-Abs/FI) of the mixture of HE, 2-OH-E⁺, E⁺, and E⁺-E⁺ (1 μM each) in an aqueous solution of 0.1 M potassium phosphate, pH 2.6, containing 25% MeOH. All conditions are as shown in Table 2 except the injection volume was decreased to 5 μl to avoid saturation of the fluorescence detector due to HE signal. The intensity of the fluorescence signal of 2-OH-E⁺ and E⁺ (25–45 min) has been multiplied by 50 to appear on the same intensity scale as the HE fluorescence signal.

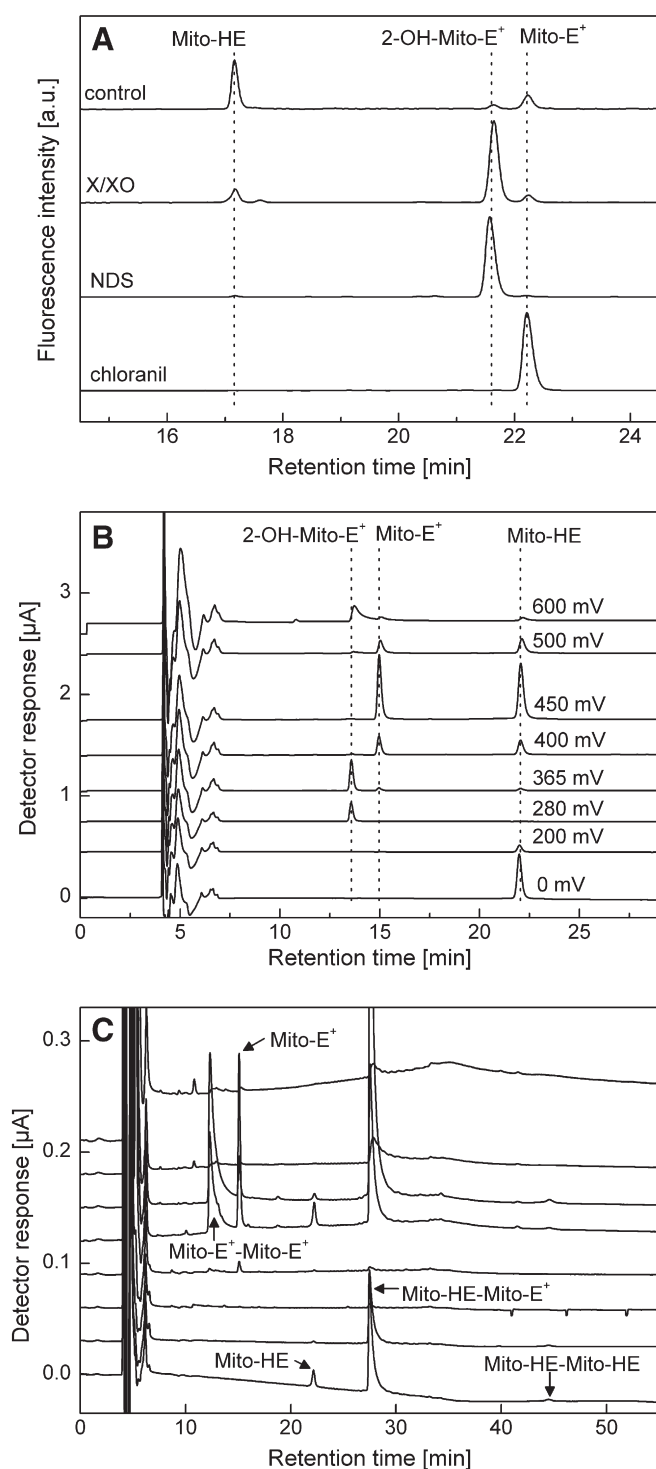


Fig. 5. HPLC analyses of Mito-HE and its oxidation products. (A) HPLC absorbance/fluorescence analysis (as described in Table 3) of products formed from Mito-HE (50 μ M) incubated without any additive (control) or with xanthine (1 mM)/xanthine oxidase (2 mU/ml) (X/XO) or nitrosodisulfonate (NDS; 100 μ M) or chloranil (25 μ M) for 1 h in phosphate buffer (50 mM, pH 7.4) containing DTPA (0.1 mM). Samples were analyzed after dilution (1:1) with ice-cold phosphate buffer (1 M, pH 2.6). (B) HPLC-EC chromatogram of a mixture of Mito-HE, 2-OH-Mito-E⁺, and Mito-E⁺ (ca. 1 μ M each) in a phosphate buffer, pH 2.6 (saturated solution), containing 25% (by volume) methanol. (C) HPLC-EC chromatogram of a mixture of Mito-HE (50 μ M) and potassium ferricyanide (100 μ M) incubated for 15 min in phosphate buffer (50 mM, pH 7.4) containing DTPA (0.1 mM). Before analysis, the sample was diluted (1:9) with ice-cold phosphate buffer (1 M, pH 2.6). (Adapted from [8].)

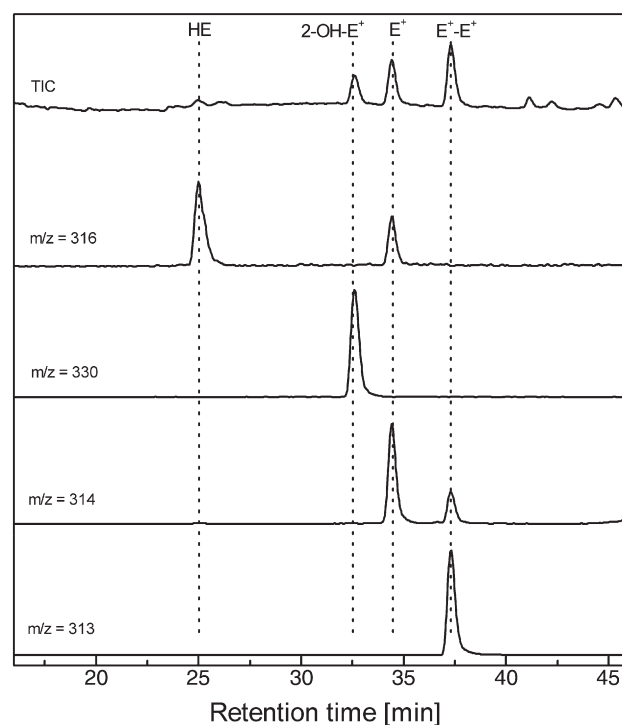


Fig. 6. HPLC-EC chromatogram of the mixture of standards (as described for Fig. 4). The chromatographic conditions are the same as described in Table 2 for HPLC with dual electrochemical/fluorescence detection. (A) The fluorescence signal. The arrow indicates the time at which the fluorescence detector settings were changed. The intensity of the fluorescence signal of 2-OH-E⁺ and E⁺ (17–30 min) has been multiplied by 40 to appear on the same intensity scale as the HE fluorescence signal. (B) The signals detected on a CoulArray electrochemical detector.

selectivity compared to other detection methods. The mobile phase for HPLC-MS should be prepared as follows:

- Mobile phase A: Mix 1 L of water with 1 ml of formic acid.
- Mobile phase B: Mix 1 L of MeCN with 1 ml of formic acid.

Calculations and expected results

HPLC-EC analysis of the BAEC lysate

The HPLC-EC chromatogram obtained from BAEC incubated with HE is shown in Fig. 8. The major peaks in the chromatogram are due to ethidium and the dimeric products, whereas the peak of 2-OH-E⁺ is the smallest one. This indicates that most of the HE is oxidized via multiple pathways intracellularly.

Calculations

The steps used for quantitation of the analytes in the cell lysates are described in Table 4 using the data shown in Fig. 8. For quantitation purposes, the area of the peaks (column 4) corresponding to the analytes of interest (column 1) were measured using the software provided with the HPLC system. Based on the known dependence of the peak areas on the concentration of the analytes, the concentration of the analytes in the sample taken for HPLC analysis is calculated (column 5). The concentration of the analytes in the cell lysates is calculated by multiplying the concentration shown in column 5 by 4, which takes into account the dilutions made during sample processing. As the numbers of cells in different samples may vary, the results are normalized to the protein concentration in the cell lysates (column 7). The amount of the sample per milligram of protein is shown in column 8. Because of the lack of availability of pure standards of HE-HE and HE-E⁺,

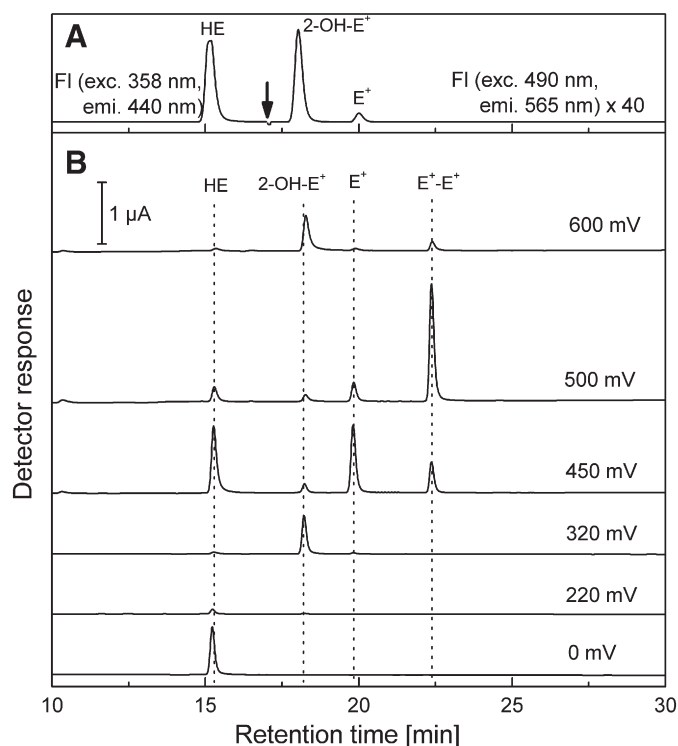


Fig. 7. HPLC–MS chromatogram of the mixture of standards (as described for Fig. 4). The chromatographic conditions are the same as described in Table 2. The traces at specific m/z values have been extracted from total ionic current (TIC) trace using the software provided with HPLC–MS system.

these peak areas can be directly normalized to the protein concentration in cell lysates and compared between different treatments.

Caveats

Changes in intracellular HE concentration

Due to the competition between HE or Mito-HE and other intracellular scavengers of superoxide (mainly, SOD), the yield of 2-hydroxyethidium or 2-hydroxy-mito-ethidium is dependent, not only on the rate of superoxide production, but also on the ratio of the concentrations of HE or Mito-HE and other scavengers. Thus, changes in HE or Mito-HE and/or other scavengers will alter the yield of 2-OH-E⁺ or 2-OH-Mito-E⁺ even at a constant rate of superoxide production. The same logic is applicable to the formation of other oxidative products of HE or Mito-HE in cells as well. Therefore, any measurements of HE or Mito-HE products (2-OH-E⁺, E⁺, or HE dimers and 2-OH-Mito-E⁺, Mito-E⁺, or Mito-HE dimers) in response to a specific treatment should always be accompanied by simultaneous monitoring of HE or Mito-HE uptake into cells.

Extraction methods

To recover HE and its oxidation products from cells and tissues, various extraction methods have been proposed [2–4,11,19]. Whereas the authors previously used the extraction method based on mixing (1:1) of the cell lysate with acidic methanol, it is now apparent that there exist other methods, using pure methanol or acetonitrile, for improved yield, depending on the cell or tissue type. Thus, it is preferred to use several extraction methods and ultimately choose the one that gives the best yield of the analytes without causing HE degradation during sample processing. Due to the different physicochemical properties of mitochondria-targeted analogs of HE, the experiment with Mito-HE may require an extraction method that is different from that for HE to obtain a similar recovery of the analytes. *Note:* The authors previously observed that the extraction with *n*-BuOH causes a partial oxidation of HE,

lowering the yield of HE and artifactually increasing the concentration of the oxidation products determined in cell lysates, compared to extraction with methanol or acetonitrile.

Photo-oxidation of HE

Exposure of HE to light can induce modification of HE, forming oxidation products (e.g., 2-hydroxyethidium and ethidium) [7,11]. Light-induced oxidation of HE to E⁺ may also be catalyzed by 2-OH-E⁺ [7]. Therefore, exposure of samples containing HE to light should be minimized. If samples containing HE and 2-OH-E⁺ are exposed to light, E⁺ formation that is proportional to the concentration of 2-OH-E⁺ will occur. Thus, although E⁺ is not a direct product of the reaction of HE with superoxide, it can be increased under conditions generating superoxide (e.g., xanthine/xanthine oxidase) in the presence of HE and light [7]. It is likely that Mito-HE will undergo a similar type of photochemical oxidation.

Interaction of HE with SOD mimetics

The propensity of HE to undergo oxidation may limit the range of agents used to stimulate or inhibit production of superoxide or other ROS because these agents may be able to directly react with HE, leading to misinterpretations. It has been shown that manganese porphyrin, Mn(III)TBAP (manganese(III) tetrakis(4-benzoic acid)porphyrin chloride), a widely used SOD mimetic, can directly oxidize HE, leading to decreased formation of 2-hydroxyethidium, a superoxide-specific product [7]. Thus, the expected decrease in 2-hydroxyethidium after incubation of the cells with Mn(III)TBAP could arise from HE depletion in cells in a process unrelated to superoxide formation. Indeed, it has been recently reported that Mn(III)TBAP does not possess an SOD-like activity owing to its unfavorable redox properties [39]. The possibility of a direct reaction between HE and the antioxidant reagents should be taken into consideration for proper interpretation of results.

Interaction of HE and Mito-HE with heme proteins

HE and Mito-HE have also been reported to react with various heme proteins, including cytochrome *c*, horseradish peroxidase,

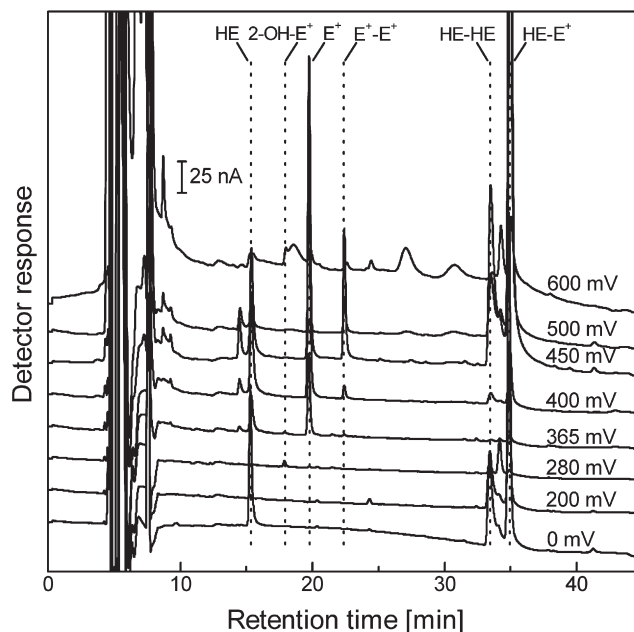


Fig. 8. HPLC–EC chromatogram of the extract from BAEC incubated at 37 °C for 20 min with 10 μM HE in DMEM containing 2% FBS. Before HPLC analysis the sample was processed as described in the text.

Table 4

Example of the calculations of the amount of HE and its oxidation products in the BAEC lysate, based on the chromatogram shown in Fig. 8

(1) Analyte	(2) Retention time (min)	(3) Potentials used for quantitation (mV)	(4) Sum of peak areas (μC)	(5) Conc. in HPLC vial (μM)	(6) Conc. in cell lysate (μM)	(7) Conc. of protein in cell lysate (mg/ml)	(8) Amount of the analyte per mg protein (nmol/mg)
HE	15.3	0, 200	1.65	0.20	0.81	3.6	0.22
2-OH-E ⁺	17.9	200, 280, 365	0.089	0.0089	0.036	3.6	0.010
E ⁺	19.8	280, 365, 400, 450, 500	7.09	0.43	1.72	3.6	0.48
E ⁺ -E ⁺	22.4	365, 400, 450, 500	1.98	0.066	0.26	3.6	0.073
HE-HE	33.4	0, 200, 280	3.77			3.6	1.05 ^a
HE-E ⁺	34.9	0, 200, 280	6.60			3.6	1.83 ^a

^a For HE-HE and HE-E⁺, instead of concentrations in the cell lysate, the peak areas (column 4) have been normalized to the protein concentration and expressed in the units of $\mu\text{C}\cdot\text{ml}\cdot\text{mg}^{-1}$.

myoglobin, hemoglobin, and mitochondrial respiratory complex IV [8,11,36,40–43]. In most cases the heme proteins can catalyze the oxidation of HE in the presence of H₂O₂. H₂O₂ alone does not oxidize HE or Mito-HE. The major products of those reactions catalyzed by heme/H₂O₂ reactions are dimeric products of HE oxidation (HE-HE, HE-E⁺, and E⁺-E⁺) and ethidium cation [8]. Similar reactivity has been also observed in the case of Mito-HE and several dimeric products have been detected. As the rate constant of the reaction of HE and Mito-HE with cytochrome *c* is relatively high (10^4 – 10^5 M⁻¹ s⁻¹ [8]), the consumption of HE and Mito-HE by heme proteins may significantly affect the availability of the probes for other oxidants, especially in mitochondria, owing to the high concentration of cytochrome *c* within the mitochondrial intermembrane compartment. These reactions will seriously hamper the use of HE and Mito-HE in the detection of superoxide. Thus, monitoring intracellular HE and Mito-HE along with their oxidation products, including E⁺ or Mito-E⁺ and the dimers, is essential for proper interpretation of results.

Binding of HE and Mito-HE to the vial walls

An additional confounding factor in the HE-based assay of superoxide and oxidants is the binding of HE and its oxidation products to the vial walls [4]. This can significantly affect the quantification of 2-hydroxyethidium and HE dimers. Because of their physicochemical properties the binding extent can be an even bigger problem in the case of Mito-HE and its oxidation products [4]. The binding process can be inhibited by acidification of the solution and by using a suitable organic solvent. However, a higher content of organic solvent in the samples may affect the retention of HE and its products on the reversed-phase HPLC column. Thus, the amount of organic solvent should be optimized to protect the analytes from binding to the vial walls while at the same time not affecting their retention on the HPLC column. In our laboratory the extent of binding is usually minimized by keeping the samples for HPLC analysis under acidic conditions (pH 2.6) and in the presence of 25% methanol.

Recently, new fluorescent probes for detecting intracellular hydrogen peroxide have been developed [44–47]. It is critical to develop an HPLC-based experimental approach similar to that described in this study for rigorous interpretation of cellular detection and quantitation of hydrogen peroxide using these probes.

The knowledge of the chemical reactivity (including the rate constants) of the probes along with their intracellular concentration is essential for any quantitative analyses of products derived from such probes.

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