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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DIRECT QUANTITATION OF OXY RADICALS IN MYOCARDIUM AND BLOOD BY MEANS OF 1,3-DIMETHYLTHIOUREA AND DIMETHYL SULFOXIDE

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SUMMARY

A direct, sensitive (50–200 ng), simple and specific high-performance liquid chromatographic (HPLC) method is described for the quantitation of oxy radicals by means of the consumption of dimethyl sulfoxide (DMSO) by the hydroxy radical and dimethylthiourea (DMTU) by hydrogen peroxide. The specific scavengers catalase and L-methionine were used to quantitate hydrogen peroxide and OH, respectively. The DMSO and DMTU peaks were separated and identified by HPLC on a Waters C_{18} Resolve 10-μm Radial-Pak column with an isocratic mobile phase (5% aqueous methanol) at 2 ml/min with UV detection (DMSO, 214 nm; DMTU, 240 nm). The OH concentrations were extrapolated by a luminol chemiluminescence technique. A linear relationship was obtained for DMTU consumption by hydrogen peroxide in the range 0.25–0.40 mM with a coefficient of variation (C.V.) of 8.8 ± 2.1% and for DMSO consumption by hydroxy radicals in the range 0.1–3.2 μM OH, with a C.V. of 9.6 ± 3.6%. The limits of detection for this method were 50 ng of hydrogen peroxide for DMTU and 200 ng of OH for DMSO. Hydrogen peroxide averaged 10.5 ± 3.6 nmol/ml in blood and 56.4 ± 5.3 μmol/g wet weight in left ventricular (LV) tissue. The hydroxy radical concentration was 0.1 μM in blood and 0.3 μM in LV tissue.

INTRODUCTION

Based on indirect evidence from scavenging experiments, the oxygen metabolites superoxide (O_2^-), hydroxy (OH) and peroxy radicals (OOH) and hydrogen peroxide are present in variable concentrations in both normal and disease states[1–4]. Scavenging enzymes, such as superoxide dismutase (SOD) and catalase, are large, highly charged particles, which probably do not reach key intracellular locations when given exogenously. On the other hand, dimethylthiourea (DMTU) and dimethyl sulfoxide (DMSO), specific scavengers of hydrogen peroxide and OH, respectively, are small and highly mobile across cell membranes, affecting both intra- and extracellular compartments. As they are both relatively non-toxic, they may be suitable for use in biological systems as *in vivo* oxygen metabolite scavengers. DMTU at low concentra-

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tions (1 m*M*) consumes hydrogen peroxide without affecting OH. However, at 100 m*M* concentration it consumes approximately 23% of the OH available. DMSO, on the other hand, specifically scavenges OH but not hydrogen peroxide at 1 m*M*.

The use of DMTU and DMSO consumption as a marker for oxy radicals in biological systems was previously limited because the small amounts of hydrogen peroxide and OH that occur in these systems could not be measured[5,6]. We have developed a direct, simple and specific high-performance liquid chromatographic (HPLC) method with UV detection for the quantitation of the micromolar concentrations of OH and hydrogen peroxide produced in biological systems. This technique is based on their consumption by DMSO and DMTU. The concentrations of DMSO and DMTU selected (1 m*M*) produced no cross-scavenging of OH and hydrogen peroxide. Quantitative data were obtained by measuring the DMSO and DMTU consumption in the absence and presence of L-methionine and catalase, which are specific scavengers of OH and hydrogen peroxide, respectively.

EXPERIMENTAL

Materials

HPLC-grade methanol (Fisher, Springfield, NJ, U.S.A.) was used. Analytical-reagent grade chemicals were obtained from the following sources: DMTU (Lancaster Synthesis, Windham, NH, U.S.A.), DMSO (Aldrich, Milwaukee, WI, U.S.A.), hydrogen peroxide (Fisher), iron(II) sulfate, EDTA, catalase and L-methionine (Sigma, St. Louis, MO, U.S.A.). Water was purified with a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.).

The apparatus consisted of a Waters Model 510 liquid chromatograph, coupled to a Waters U6K injector and a 490 programmable multi-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.), A Digital 350 computer/controller and printer (LA 50) completed the system (Digital, Merrimack, NH, U.S.A.). The column used was a Waters Resolve C_{18} Radial-Pak 10-μm cartridge measuring 8 mm × 10 cm. Injections were made with a 25-μl Hamilton syringe (Waters Assoc.).

Methods

The mobile phase contained 5% methanol in Millipore water, routinely prepared, filtered through the Duropore filter (0.22 μm) (Waters Assoc.) and degassed the day of use. The flow-rate was 2 ml/min and the sample volume injected was 20 μl. Absorption spectra were recorded at 214 nm for DMSO and 240 nm for DMTU.

Standards of DMTU (0.25–4.0 m*M*), DMSO (0.5–3.5 m*M*), hydrogen peroxide (100–700 μM), catalase (200 μg/ml) and L-methionine (10 m*M*) were used on the day of preparation. Hydroxy radical (1.0 μM) was produced with Fenton's reagent[7] [1 m*M* iron(II) sulfate–0.4 m*M* hydrogen peroxide–1 m*M* EDTA] and quantitated by the luminal chemiluminescence technique[8].

Blood samples consisted of 100 μl of fresh heparinized blood, which was mixed with 50 μl of DMTU or DMSO (1 m*M*) and 50 μl of phosphate buffer (pH 7.4) within 1–2 s of collection. The samples were mixed in a Vortex mixer, centrifuged for 5 min (1100 *g*, 4°C) and a 20-μl sample was injected on to the column.

Left ventricular tissue (50 mg) was quickly frozen (1–2 s after harvest) in liquid nitrogen, powdered under dry nitrogen and mixed with 50 μl of DMTU or DMSO (1

m*M*) and 100 μl of phosphate buffer (pH 7.4). The mixture was again mixed for 3 min, centrifuged for 5 min (1100 *g*, 4°C) and a 20-μl sample was injected on to the column.

Scavenger data with L-methionine and catalase (50 μl of each) were obtained by incubation with the specimens for 1 min at room temperature. The scavenger solution volume replaced the phosphate buffer in these experiments.

RESULTS AN DISCUSSION

The calibration graphs for DMTU absorbance at 240 nm (0.25–4.0 m*M*) and for DMSO absorbance at 214 nm (0.5–3.5 m*M*) were linear. Linear regression analysis indicated the following characteristics for these curves: DMTU, slope = 192, r = 0.999, intercept = 1.6; DMSO, slope = 64, r = 1.000, intercept = 4. The actual chromatograms for 1 m*M* concentrations DMTU and DMSO are shown in Fig. 1.

The calibration graph for hydrogen peroxide concentration by means of DMTU (1 m*M*) consumption was also linear. Addition of catalase (200 μg/ml) completely inhibited DMTU consumption, confirming that hydrogen peroxide was responsible for the DMTU in both tissue and blood. As the DMTU consumption graph was linear, the peak-height difference represented the concentration of hydrogen peroxide in both standard and sample. Spiking the blood sample with 500 μM hydrogen peroxide yielded quantitative results with this method. The coefficient of variation for this technique was 8.8 ± 2.1% (n = 10) in the range 50–60 m*M* with recoveries of 99 ± 0.6% and a sensitivity of 50 ng.

In human blood, the hydrogen peroxide concentration was 10.5 ± 3.6 nmol/ml and in swine LV tissue 56.4 ± 5.3 μmol/g wet weight (Table I). The amount of hydrogen peroxide measured in human blood and tissue represents the concentration that exceeds the rate of turnover of native complex of the catalase.

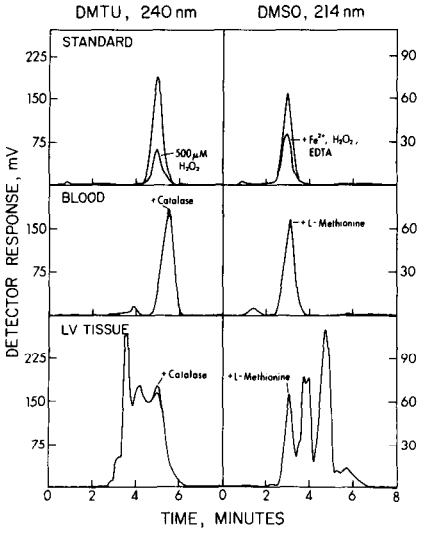

Fig. 1. Chromatogram of standards, human blood and swine LV tissue in the presence of DMTU (1 m*M*) and DMSO (1 m*M*).

TABLE I

HYDROGEN PEROXIDE CONCENTRATION IN STANDARDS, HUMAN BLOOD AND SWINE LV TISSUE DETERMINED BY HPLC FROM THE DMTU CONSUMPTION AND SCAVENGING BY CATALASE

Results are mean values (n = 10).

Sample	*Peak height (mV)*		*Hydrogen peroxide concentration (μM)*
	DMTU	*DMTU + catalase*	
DMTU (1m*M*)	185	185	0
Hydrogen peroxide (500 μ*M*)	182	60	496
Human blood + hydrogen peroxide (500 μ*M*)	181	55	512
Human blood	181	186	10.5
Swine LV tissue	173	185	56.4

TABLE II

PRECISION OF METHODOLOGY

Method	*Detects*	*Range (μM)*	*C.V. (%)*	*Sensitivity (ng)*
DMTU	H_2O_2 concentration	50–600	8.8 ± 2.1	50
DMSO	OH concentration	50–800	9.6 ± 3.6	200

TABLE III

HYDROXY RADICAL CONCENTRATION IN STANDARDS, HUMAN BLOOD AND SWINE LV TISSUE BY HPLC FROM THE DMSO CONSUMPTION AND SCAVENGING BY L-METHIONINE

Results are mean values (n = 10).

Sample	*Peak height (mV)*		*OH (hydrogen peroxide) concentration (μM)*
	DMSO	*DMSO + L-methionine*	
DMSO (1 m*M*)	62	62	0
OH (1 μ*M*)*	60	45	0.95
Human blood + OH (1 μ*M*)	60	44	1.07
Human blood	62	63	0.10
Swine LV tissue	60	63	0.30

* Fe^{2+} (1 m*M*)–hydrogen peroxide (0.4 m*M*)–EDTA (1 m*M*) with the luminal chemiluminescence technique yielded 1.0 μ*M* of OH radical equivalents.

DMSO (1 m*M*) consumption by OH radicals (produced with Fenton's reagent, and quantitated by luminol chemiluminescence) was directly proportional to the concentration of hydrogen peroxide in the reagent in the range 0.2–1.4 m*M*. L-Methionine (10 m*M*) quantitatively scavenged the OH radicals. The peak height difference in the chromatograms *vs.* OH concentration yielded a calibration graph for hydroxy radical concentration in a manner identical with the DMTU calibration. Spiking the blood sample with Fenton's reagent (1 μM OH) yielded quantitative data. The coefficient of variation (C.V.) for the OH determination was 9.6 ± 3.6% (n = 10) in the range 50–800 μM hydrogen peroxide (0.1–3.2 μM OH). The recovery was 95 ± 2.2% and the sensitivity was 20 ng (Table II). Neither blood nor LV tissue showed significant concentration of OH [0.011 ± 0.004 μM and 0.30 ± 0.08 μmol/g wet weight, respectively (Table III)]. The concentration of hydroxy radicals in blood (0.01 ± 0.004 μM) and in LV tissue (0.30 ± 0.08 μmol/g wet weight) were also negligible.

This HPLC method provides a direct, highly specific, sensitive and reproducible quantitation of hydroxy radicals and hydrogen peroxide in biological systems. The ability of the scavengers, DMSO and DMTU, to cross cell membranes and their relative non-toxicity indicates their value when used alone or in combination, at appropriate millimolar concentrations, to detect specific oxy-derived radicals and potentially protect a biological system *in vivo* from their damaging effects.

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