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Short communication

Determination of lipoic acid by precolumn derivatization with monobromobimane and reversed-phase high-performance liquid chromatography

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Abstract

A new method for the quantitation of lipoic acid in plasma and tissues based on the selective precolumn derivatization of thiols with the fluorescent label monobromobimane is described. After extraction with diethyl ether, the dithiolane ring of lipoic acid is opened by reduction with $NaBH_4$ before the free thiols can react with the label. Separation and quantitation was achieved by reversed-phase HPLC and fluorescence detection. The concentration–response curve was linear from 20 to 3000 n*M* in plasma. The recovery as determined with [^{3}H]lipoic acid was 60.9% from plasma and 61.4% from rat heart tissue. The pretreatment of samples with *N*-ethylmaleimide makes it possible to differentiate between reduced and oxidized forms of lipoic acid. © 1998 Elsevier Science B.V.

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1. Introduction

The metabolic role of lipoic acid (1,2-dithiolane-3-pentanoic acid, LP) as cofactor of the enzymes of oxidative decarboxylation is well known [1]. In addition, non-protein-bound LP attracted attention as a multifunctional antioxidant. Especially the reduced form, dihydrolipoic acid (DHLP), showed protective activity against oxidative damage in many in vitro tests and biological systems [2]. Beneficial effects of exogenously administered lipoic acid were observed in numerous disorders ranging from diabetes to AIDS [2]. The use of LP for treatment of diabetic neuropathy is approved in Germany. The compound also found an application as a food additive.

The detection and quantitation of LP is usually achieved by extraction from biological materials in solvents and separation by GC [3,4] or by HPLC with electrochemical detection [5] or, more selectively, with a dual gold–mercury electrode [6]. This procedure is highly sensitive, but the concentration–response curve is only linear in a narrow concentration range, and laborious reconstitution of the Hg-electrode is necessary. Recently, a procedure to separate the *R*- and *S*-enantiomers for pharmacokinetic studies by precolumn formation of diastereomeric derivatives with *o*-phthalaldehyde and D-phenylalanine was presented, but differentiation between LP and DHLP was not reported [7].

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Another approach would be to use thiol-reactive compounds for precolumn derivatization. The bimanes, especially monobromobimane (mBBr, 1*H*,7*H* - pyrazolo-[1,2 - *a*] - pyrazole - 1,7 - dione,3 - (bromomethyl)-2,5,6-trimethyl), were originally introduced by Kosower [8] with the aim of analyzing SH-functions in proteins and of quantifying other naturally occurring thiols like glutathione and cysteine. The broad applications of these reagents have recently been reviewed [9,10]. The bimanes readily and quantitatively form relatively stable fluorescent adducts with all naturally occurring sulfohydrils in aqueous media at room temperature and at a slightly alkaline pH. In addition, the selective determination of reduced and oxidized forms as well as free and protein-bound forms has been achieved by the concomitant application of SH-blocking reagents like *N*-ethylmaleimide (NEM), of reductants like $NaBH_4$ or dithiothreitol, and of protein precipitation [9].

The objective of the present communication is to evaluate, for the first time, the use of mBBr for the detection and quantitation of LP in biological materials. A HPLC-separation procedure with fluorescence detection was developed, and methods to distinguish between LP and DHLP were evaluated. The procedure shows excellent sensitivity with a linear concentration–response curve over a broad concentration range. It is therefore well suited for physiologic and pharmacokinetic studies of LP.

2. Experimental

2.1. Materials

The monobromobimane label (THIOLYTE) was obtained from Calbiochem (Bad Soden, Germany) and racemic LP was from Sigma (Deisenhofen, Germany). *R*-α-Lipoic acid-[^{3}H] carrying ^{3}H at C_8 to a specific activity of 3920 kBq/μmol was custom-synthesized by Amersham, UK, in cooperation with BASF, Ludwigshafen, Germany. The HPLC-grade solvents were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade, and were purchased from Merck or Sigma. Human plasma with Li-heparinate as anticoagulant from numerous patients was kindly provided by the central analytical laboratories of the hospital of Humboldt-University, Berlin, Germany.

2.2. Preparation of DHLP

Stock solutions of *R,S*-LP in acetonitrile (100 m*M*) were 50-fold diluted with solution A (5 m*M* EDTA/NaOH, pH 7.85, 0.9% NaCl). Aliquots of 50 μl were mixed with the same volume of a freshly prepared solution (1 *M*) of $NaBH_4$ in water. After 10 min at room temperature, the reaction was stopped by freezing in liquid N_2 or at −80°C. Twenty microliters of 2 *M* HCl were added before the solution was allowed to thaw at room temperature. The pH of the solution was then adjusted to about 7.5 by addition of 400 μl of 100 m*M* NH_4HCO_3. The concentration of thiols was immediately determined by Ellman's procedure [11].

2.3. Preparation of samples

Homogenates from rat heart muscle were prepared by disrupting the tissue with an Ultra-Turrax at full speed in ice-cold phosphate buffer (10 m*M* Na-phosphate, pH 7.4, 2 m*M* EDTA, 0.9% NaCl) at a concentration of 10 ml/g wet weight. Aliquots (0.25–2.0 ml) of plasma or of freshly prepared heart-homogenate were spiked with 5–50 μl of LP or DHLP in concentrations of 1–10 μ*M* in solution A to obtain concentrations of LP/DHLP in the samples ranging from 5 to 5000 n*M*. In some cases, [^{3}H]lipoic acid (0.67–3.36 kBq) in toluene solution was added to the samples directly before they were mixed with ether for extraction as described in Section 2.4. The concentration of LP in solution A was controlled by the Ellman procedure [11] after reduction with $NaBH_4$ (Section 2.2).

2.4. Extraction and labeling with mBBr

The following procedures were partly adopted from several recently described methods for the derivatization of natural thiols or metal-complexing drugs [9,10,12,13]. Briefly, LP was extracted from acidified samples with diethyl ether and reduced to DHLP by $NaBH_4$ to render both thiols susceptible to the derivatization with mBBr.

Samples (250 μl) of plasma or rat heart homoge-

nate were mixed with 15 μl EDTA solution (100 m*M*, pH 7.8 with NaOH) and 50 μl of 5 m*M* 1,4-dithioerythritol (DTE) in 10-ml vials before they were incubated at room temperature for 5 min. Lipoic acid was extracted by adding 1.5 ml diethyl ether followed by 10 μl 1 *M* phosphoric acid and thorough vortexing. Phase separation was achieved by centrifugation at 2500 *g* for 3 min. The ether phases were transferred into 2-ml reaction vials and concentrated to dryness under a stream of nitrogen. The extraction with ether, but without further addition of phosphoric acid, was repeated twice. The combined dry ether extracts were resuspended in a mixture of 70 μl SDS/EDTA solution (0.11% SDS, 15 m*M* EDTA, 0.9% NaCl), 5 μl 1 m*M* DTE and 50 μl freshly prepared 1 *M* $NaBH_4$. The reduction proceeded at room temperature for 15 min before the reaction was stopped by freezing in liquid nitrogen or at −80°C. The $NaBH_4$ was destroyed as in Section 2.2, and the acid was neutralized by adding 400 μl 3% (w/v) $NaHCO_3$. Thiols were then labeled by adding 5 μl of a 100 m*M* solution of mBBr in acetonitrile and by incubating the vials in the dark for 90 min. The major parts of the free reagent and of the DTE derivative were removed by extraction with 1 ml dichloromethane. Phases were separated by centrifugation for 3 min in a caps centrifuge. The aqueous phases were removed and directly subjected to HPLC.

2.5. Chromatography

The mBBr derivative of DHLP was separated from the non-reacted reagent and other nonspecific reaction products by reversed-phase HPLC using a Hypersil MOS (C_8) 5-μm column (100 mm×4.6 mm I.D.) from Hewlett–Packard (Palo Alto, CA, USA). The mBBr-labeled compounds were detected and quantified by measuring the fluorescence at 385 nm (excitation) and an emission wavelength of 470 nm. For the determination of radioactivity in the effluent, fractions of 0.5 or 1.0 ml were sampled and mixed with 8 ml of liquid scintillation fluid (Optiphase ''HiSafe'', Fisons Chemicals, Loughborough, UK). Aliquots of 50 μl of the aqueous phase after dichloromethane extraction were usually injected. The separation was achieved by isocratic elution at a flow-rate of 1 ml/min and at a column temperature of 40°C with 25% acetonitrile in 1% acetic acid adjusted to pH 3.95 with NH_3 as mobile phase. The chromatographic system consisted of a LC-6A pump, a SIL-6B autoinjector, a SCL-6B system controller, and a RF-535 fluorescence monitor from Shimadzu Europa GmbH, Duisburg, Germany. Peak integration and other calculations were carried out by means of the Shimadzu C-RA4 integrator.

3. Results and discussion

The mBBr derivative of LP in extracts from plasma and heart tissue samples eluted at the same retention time of about 12.3 min as pure LP which was directly reacted with the fluorescent label (Fig. 1). This peak was sufficiently separated from the main contaminants, non-reacted mBBr and the mBBr derivative of DTE. These compounds eluted between about 1 and 5 min. The addition of DTE to the media for extraction and derivatization markedly increased the recovery of labeled LP, in accordance with the

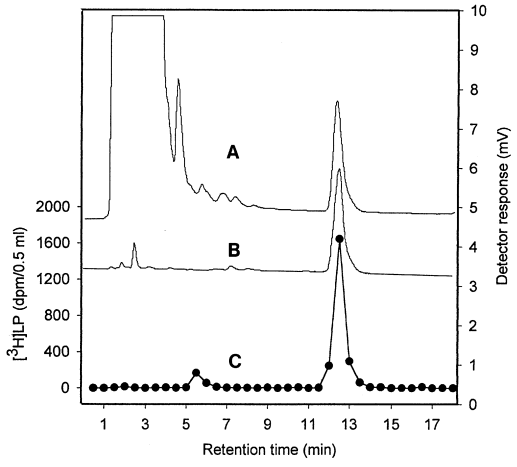

Fig. 1. (A) Detection of mBBr-LP by reversed-phase HPLC. LP was spiked into human plasma, extracted, reduced by $NaBH_4$, derivatized with mBBr, and chromatographed as described in Section 2; (B) another sample of LP was directly reduced and derivatized with the label; (C) [^{3}H]LP was spiked into plasma and prepared for HPLC by extraction, reduction, and derivatization. Peaks were detected by measuring fluorescence in the effluent (A and B), while fractions of 0.5 ml (30 s) were collected in C, and the radioactivity of each fraction was determined (●). The concentrations of LP in the samples were 300 n*M* (A) and 250 n*M* (B).

observation of Mansoor et al. [13]. The presence of other natural sulfohydrils like glutathione and cysteine did not interfere with the detection of lipoic acid (results not shown).

Several minor peaks were consistently detectable in the chromatograms (Fig. 1A). They probably derived from traces of endogenous thiols or from unspecific reaction products of mBBr with non-thiol compounds such as SDS or buffer substances [14]. No attempts were made to identify these peaks because they were base-line separated from the mBBr derivative of LP.

The elution pattern of [^{3}H]LP-spiked plasma samples showed a second, minor peak at about 6 min comprising 8–10% of the total radioactivity, in addition to the main peak of the mBBr derivative of LP (Fig. 1C). This second peak coeluted with non-reacted reduced [^{3}H]LP, and it was increased in relation to the main peak when the incubation time with mBBr was reduced. This peak therefore represents DHLP that escaped reaction with the fluorescent label. The extent of derivatization was not increased after extended incubation with mBBr or other variations of the derivatization procedure, but it was highly constant under the applied conditions.

The calibration curves (Fig. 2) as measured with varying concentrations of external LP in plasma were linear with a coefficient of correlation of more than 0.999. The concentration–response curve demonstrated linearity at least over another order of magnitude (not shown in Fig. 2) resulting in a useful concentration range of quantitation from 20 to 3000 n*M*. The lower limit of quantitation (20 n*M*) compares favorably with other recently reported [5,7] values which were obtained by electrochemical detection (48 n*M*) or derivatization with *o*-phthalaldehyde (72 n*M*).

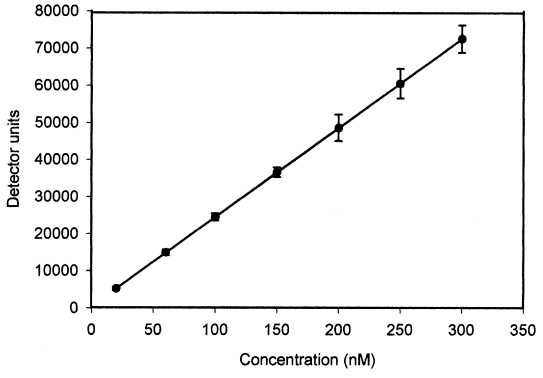

Fig. 2. Concentration–response curve of LP detection. Aliquots of LP stock solutions were spiked into human plasma to obtain the indicated concentrations in the sample. The symbols and bars represent the means and S.D. of four independent determinations. The regression line (correlation coefficient 0.99999) of the means is also shown.

The recovery of [^{3}H]LP was 60.9±1.7% (mean±S.D., n=6) from plasma and 61.4±4.0% (mean±S.D., n=4) from rat heart tissue. These values are lower than those in some other procedures for determination of LP [5,7]. The higher loss of LP is probably due to the additional steps of reduction of LP with $NaBH_4$ and of the subsequent acidification and neutralization before the derivatization with mBBr is feasible. The extraction with dichloromethane also contributed to some losses (8.4±3.8%, mean±S.D., n=4) of the mBBr derivative. But this result does not reduce the usability of the mBBr procedure in any way because only minor variations of the recovery were observed during eight weeks (61.6±0.9%, mean±S.E., n=12). The recovery as determined with [^{3}H]LP amounted to 99.4% (mean of two experiments) of the value which was obtained by evaluation of the signal from the fluorescence detector. This result shows that the [^{3}H]LP was not contaminated by other radioactive compounds.

The observation with many animal systems that exogenously applied LP is readily reduced in some tissues to DHLP is of major interest for pharmacological investigations because both forms exert different antioxidant properties [2]. We therefore tried to use the mBBr procedure to differentiate between oxidized and reduced LP. Initial experiments showed that the direct derivatization of DHLP in plasma led to inconsistent results due to low recovery. In another approach, the samples were pretreated with NEM to mask free sulfohydrils before reduction with $NaBH_4$. The content of DHLP is then calculated as the difference between total LP as measured by the standard procedure and the content after incubation with NEM. Fig. 3 shows that a masking of more than 90% was achieved at concentrations of the reagent from 0.6 to 1.0 m*M*. Consequently, the masking by NEM allows the determination of the redox status of LP, but the recovery of DHLP (53.0±3.0%,

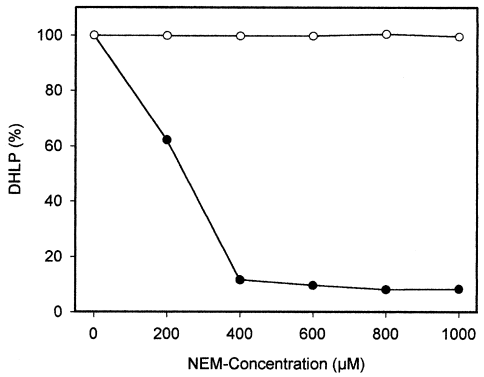

Fig. 3. Effect of NEM on the derivatization of DHLP with mBBr. Freshly reduced LP (●) or the same amount of non-treated LP (○) was spiked into human plasma to a concentration of 250 n*M*. The samples were then incubated with NEM at concentrations of 0.2–1.0 m*M* to block free sulfohydrils. LP was subsequently detected by the standard mBBr procedure. The results are presented as percentage of blanks without NEM treatment. The data are the mean of three (●) or two (○) independent experiments.

mean±S.D., $n=4$) is lower due to the noncomplete reaction with NEM.

Taken together, labeling with mBBr is a procedure which is well suited to determine LP in tissues and body fluids, especially for pharmacokinetic investigations. The recently reported levels of LP after administration of therapeutic doses [7] are clearly within the range of quantitation. It has to be noted that the basic level of unbound LP in plasma and tissues was neither detectable by other similar methods [6,7] nor by our procedure. A small part of LP within cells is covalently bound to protein via lysyl groups. The reduction with $NaBH_4$ does not release LP from these bonds, but this fraction of LP serves as cofactor for enzymes of the ketoacid metabolism [1] and it can therefore be neglected in studies of the antioxidant properties for therapeutic purposes. Furthermore, the mBBr method requires only standard HPLC equipment, while an electrochemical detector with Hg/Au electrodes which needs laborious reconstitution is used in the other available HPLC procedure for the SH-selective detection of LP [6].

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