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Reduction of pantethine in rabbit ocular lens homogenate

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Abstract

In several animal models, preliminary studies have indicated that pantethine may inhibit cataract formation. Therefore, preclinical trials need to be conducted to study the pharmacology of pantethine in the ocular lens and to establish its efficacy. Since pantethine, which is a disulfide, can undergo a variety of chemical modifications such as reduction and formation of mixed disulfides, a detailed study was first conducted to determine the stability of pantethine in rabbit lens homogenate. A knowledge of the stability of pantethine in lens homogenate was necessary to establish if pantethine could be metabolized in the time it takes to harvest and homogenize a lens. The results of this study will be used to establish a protocol for harvesting and homogenizing lens samples.

Pantethine (100 μM) is completely reduced to pantetheine in rabbit lens homogenate in about 16 min. About 1.5% of the pantethine added to lens homogenate forms a mixed disulfide with lens proteins, and the remainder is found in the supernatant. The supernatant pantethine concentration decreases exponentially as a function of time, and the terminal half-life for this process is 3.3 min. The free supernatant pantetheine concentration increases in pseudo first order manner as a function of time with a rate constant of 4.3 min. Pantethinase activity is not significant, because the free supernatant pantetheine concentration did not decrease. The exact mechanism of pantethine reduction in rabbit lens homogenate remains to be determined.

Keywords: Electrochemical detection; HPLC; Occular lens homogenate; Pantetheine; Pantethinase; Pantethine; Stability

1. Introduction

Preliminary work by Clark and Benedek [1] indicated that pantethine (Fig. 1) might prevent the formation of cataracts in several animal models. This hypothesis was tested further via preclinical trials at Oculon Corporation. The purpose of the work reported here was to study the stability of pantethine in lens homogenate. The results will indicate if pantethine is chemically modified during the time required to harvest the lens and prepare a lens homogenate. This information will be used to develop a fully validated protocol for ocular lens total pantetheine levels in animals treated with pantethine.

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To conduct the proposed stability study, suitable analytical techniques were required. A review of the literature indicated previously published chromatographic methods for pantetheine, but none for pantethine. Fahey and co-workers derivatized thiols with monobromobianes [2] and applied their HPLC method to the determination of pantetheine in human red blood cells using ion-exchange [3] and reversed-phase chromato-graphy [4]. A fluorescence method for total pantetheine using 4-aminosulphonyl-7-fluoro-2,1,3-benzoxadiazole was also developed at Oculon, but was not published.

Because a method for pantethine had not been reported in the literature, glutathione was selected as a model compound for method selection. Many small, water-soluble thiols and disulfides can be quantitated using HPLC [5]. Thiols such as glutathione can be directly quantitated using HPLC with fluorescence detection (HPLC/FL), but the disulfides cannot be quantitated without a reduction step. Thiols and their corresponding disulfides can be directly quantitated using HPLC with electrochemical [6–11] or mass spectrometric detection [12]. Analysis of reduced and oxidized glutathione in the lens of the eye using HPLC with electrochemical detection (HPLC/EC) has been reported in the literature [13,14]. Therefore, HPLC/EC using a Hg/Au electrode was selected because pantethine could be detected without reduction and derivatization, pantetheine could be detected without derivatization, and an HPLC/EC method for analysis of glutathione in the lens of the eye had been reported [13,14].

Determing the stability of pantethine was important because harvesting the ocular lens and preparing the lens homogenate requires 10 min. During this time period, pantethine could undergo a variety of chemical reactions (Fig. 2). It could be reduced to the free thiol, pantetheine, or it could form a mixed disulfide with protein or nonprotein moieties. Pantethine is rapidly degraded in plasma by pantethinase [15]. However, there are no reports of the stability of pantethine or pantethinase activity in ocular tissues.

2. Experimental

2.1. Chemicals

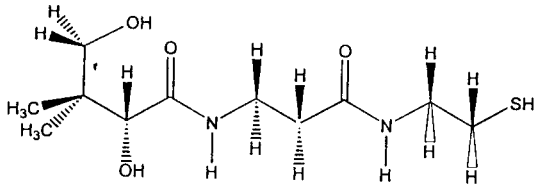
Optima grade HPLC solvents were obtained from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide (99.999 + %, electronic grade), chloroacetic acid (99 + %), redistilled 70% perchloric acid (99.999%), electronic grade mercury, redistilled nitric acid (99.999 + %), and disodium ethylenediaminetetraacetic acid (EDTA, 99 + %) were supplied by Aldrich Chemical Company (Milwaukee, WI). Dithiothreitol (DTT, 99%) was supplied by Fluka Chemical (Ronkonkoma, NY). Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP, 98.8%) was purchased from Pierce Chemical (Rockford, IL).

Pantethine (99.9%, Fig. 1) and bovine lens proteins were obtained from Sigma (St. Louis, MO). Pantetheine (Fig. 1) was synthesized, purified and characterized by Dr. Sham Patil at Oculon Corporation. The estimated purity of pantetheine using reversed-phase HPLC with UV detection at 230 nm is 92%, and the water content of the product is 7.7%. Pantetheine and pantethine were stored at −20°C.

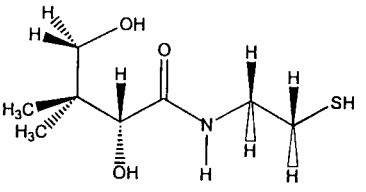
Bis-(pantoyl-β-aminoethyl) disulfide (Fig. 1), the internal standard for total pantetheine and pantethine determinations, was purified and characterized by Dr. Shashank Otiv at Oculon Corporation and was stored at −20°C. Pantoylcysteamine (Fig. 1), the internal standard for supernatant-free pantetheine analysis, was synthesized by reducing bis-(pantoyl-β-aminoethyl) disulfide with TCEP in acetate buffer. Purification was accomplished as described in Section 2.5.3. Pantoylcysteamine was stored at 4°C.

2.2. HPLC/EC equipment for pantetheine and pantethine assays

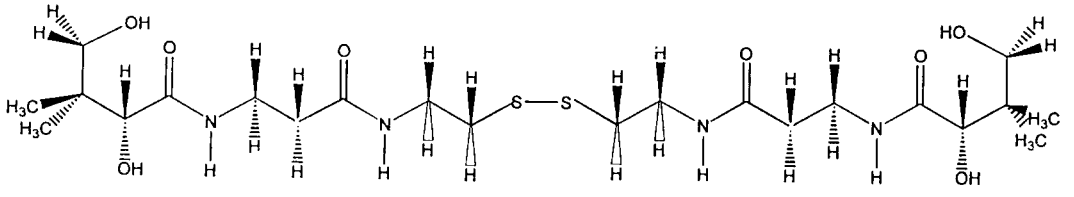
The chromatographic system consisted of a model 626 HPLC pump with associated column heater (Waters, Milford, MA), a model LC-44C electrochemical detector and flow cell (Bioanalytical Systems, West Lafayette, IN) and a model 9125 injector (Rheodyne, Cotati, CA). A 3.0 mm × 150 mm stainless-steel column packed with



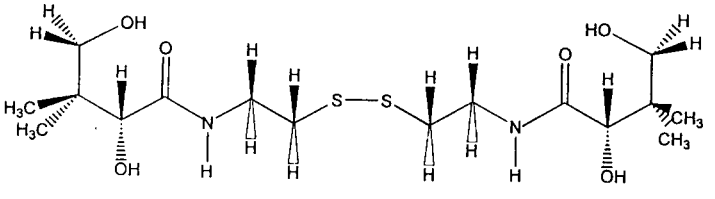
pantetheine



pantoylcysteamine



pantethine



bis-(pantoyl-β-aminoethyl) disulfide

Fig. 1. Structures of pantetheine, pantethine, pantolycysteamine and bis-(pantoyl-β-aminoethyl) disulfide.

Zorbax Stable Bond™, 3.5 μm, octyldecylsiloxane silica (Mac-Mod Analytical, Chadds Ford, PA) was prepared with titanium frits.

2.3. Preparation of the working electrode

The following materials were purchased from Buehler (Lake Bluff, IL) for electrode polishing: 2.875 in, 15 μm silicon carbide fibrpol PSA discs; 2.875 in, 9 μm aluminum oxide fibrpol PSA discs; 2.875 in nylon polishing cloth PSA discs, 3 μm Metadi II diamond polishing compound; 2.875 in microcloth polishing PSA discs; and Masterpolish.

Spent amalgam was removed with 7.9 N nitric acid. The electrode was polished with a 15 μm disc, 9 μm disc, 3 μm polish on a nylon disc and alumina on a microcloth. The electrode was rinsed with water and methanol after the acid wash and polishing step. A drop of mercury ($\approx$5

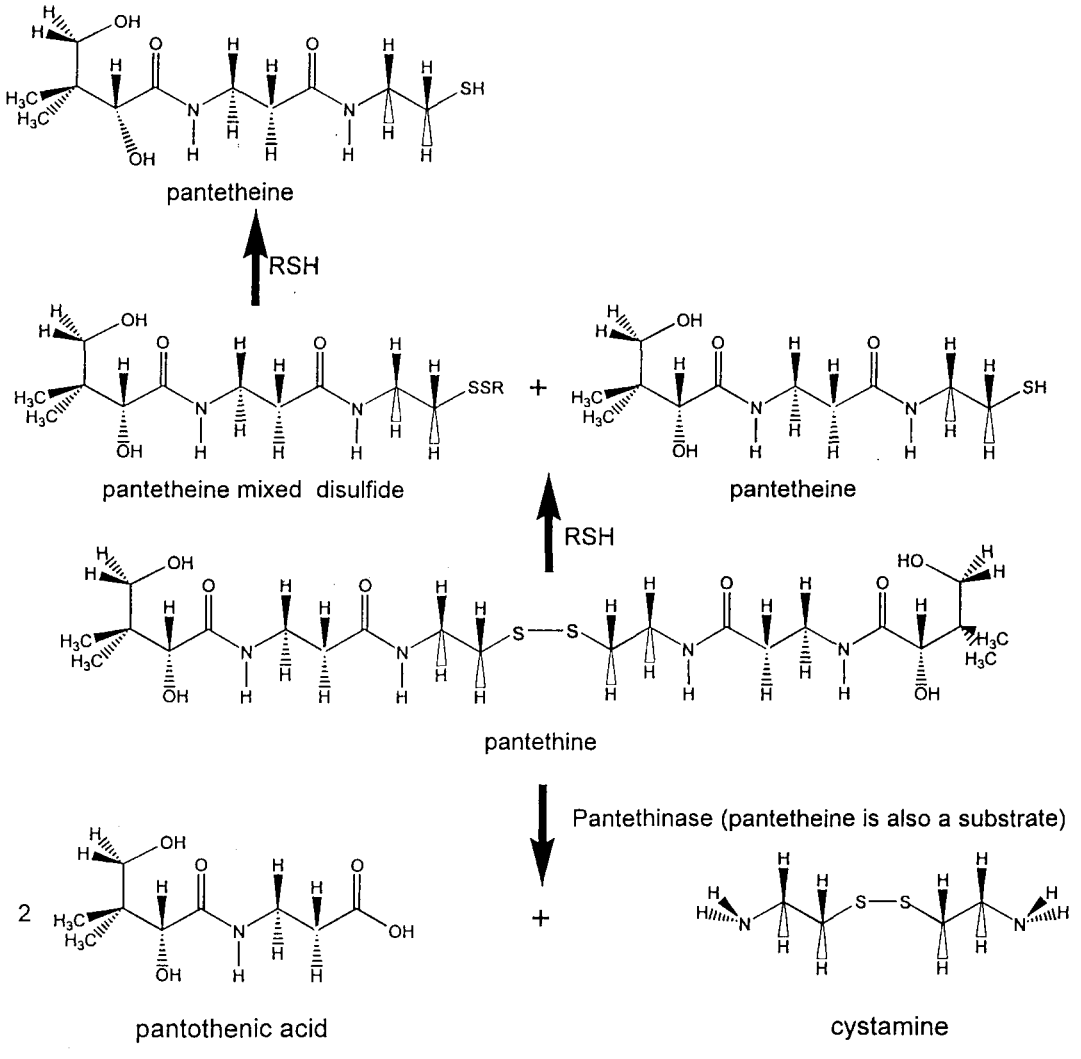


Fig. 2. Some possible degradation pathways of pantethine in lens homogenates.

μl) was applied to the electrode. After $\approx$30 min, most of the mercury was removed from the surface with an index card. The electrode was conditioned in air overnight. The working electrode surface was renewed weekly.

2.4. Lens harvesting and homogenizing

A rabbit eye was enucleated, frozen on dry ice and stored at −70°C until ready for dissection. The lens was dissected while the eye was still frozen. The lens ($\approx$200–300 mg) was weighed, 0.9% saline (1:3 w/v) was added, and the lens was homogenized by sonication. Aliquots of the lens homogenate were used for the assays described below.

2.5. Total pellet, total supernatant and free supernatant pantetheine assays (Figs. 3A–3C)

2.5.1. Chromatographic conditions

The mobile phase was a solution of a citric acid

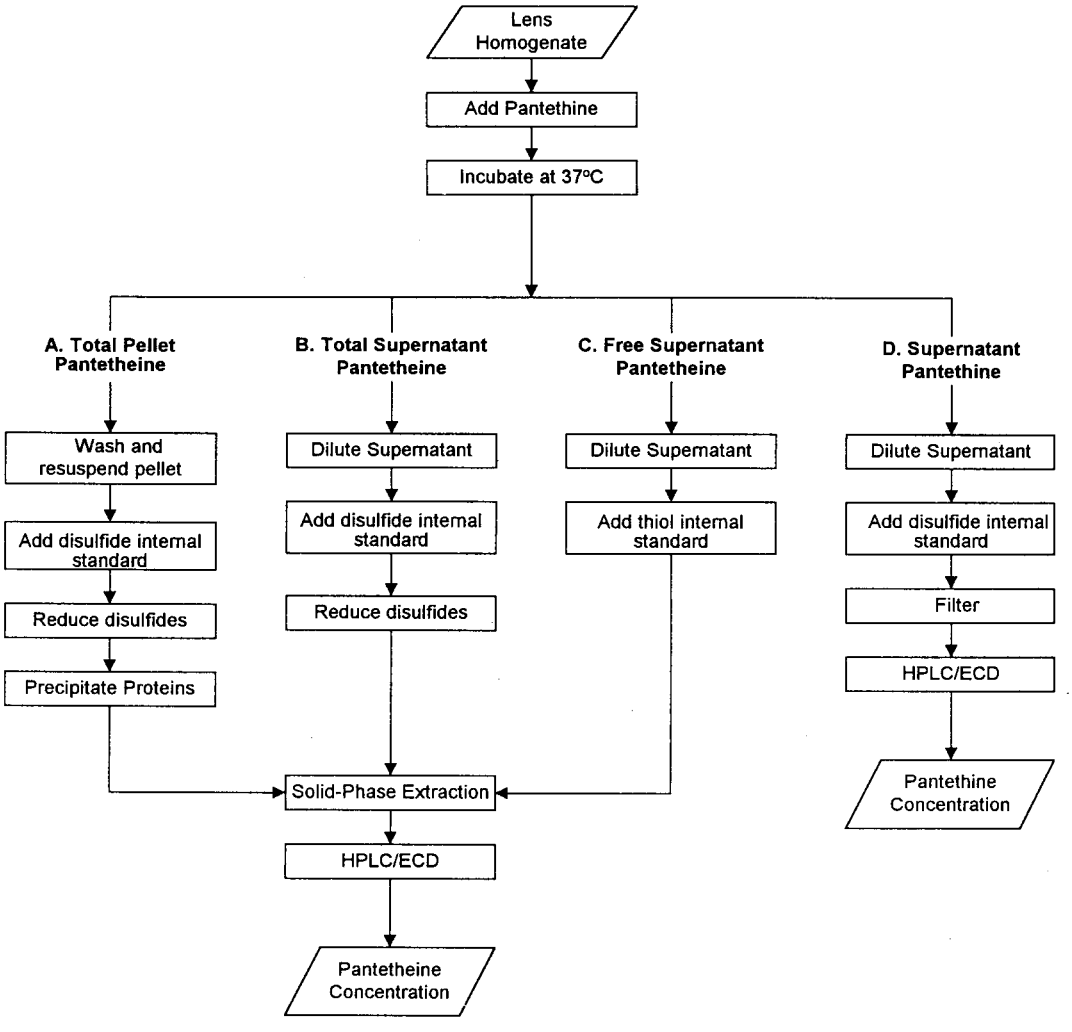


Fig. 3. Pantetheine and pantethine assay flow chart.

buffer (50 mM, pH 2.6 with 0.2 mM EDTA) and acetonitrile (93%:7%, v/v). A single Hg/Au amalgam electrode (Waters Corporation, Milford, MA) was prepared as described in Section 2.3. The flow was set to 1.0 ml min^{-1} and the column was heated to 40°C. The detector was set to +0.150 V, 10 or 20 nA full scale and a time constant of 0.10 Hz.

2.5.2. Standards and quality control

Pantethine stock standard and stock quality control solutions were prepared independently in water. A 20 μl aliquot of each stock solution was diluted 1:10 with a 10% (w/v) solution of bovine lens protein in physiological saline. A 10 μl aliquot of 5.0 μM bis-(pantoyl-β-aminoethyl) disulfide was added to each standard or quality control sample followed by 400 μl of reducing reagent (1.0 mM TCEP, 20 mM acetate, pH 4.8). For the free supernatant pantetheine assay, pantoyl cysteamine was used for the internal stan dard. The samples were heated at 37°C for 1 h. At the end of the reduction, 400 μl of precipitating reagent (0.6 M chloroacetic acid, 0.1 M perchloric

acid and 1.0 mM EDTA, pH 2.8) was added and the samples were vortexed.

2.5.3. Solid-phase extraction

The supernatants were filtered using a centrifugal filter at 10°C. Solid-phase extraction (SPE) tubes (ENVI-18, 100 mg, Supelco, Bellefonte, PA) were conditioned with 1.0 ml of methanol followed by 2.0 ml of a phosphate buffer (100 mM, pH 2.8 with 0.2 mM EDTA, conditioning solvent). The filtered supernatants were extracted under a vacuum of 5 in Hg at a rate of 2–3 drops per minute. The columns were dried by gradually increasing the vacuum to 15 in Hg and holding for 30 s. The compounds were eluted with 0.2 ml of acetonitrile. Residual solvent was reduced to about 10 μl in a centrifugal vacuum concentrator (Labconco, Kansas City, MO). The sample was reconstituted in 200 μl of conditioning solvent.

2.5.4. Total pellet pantetheine assay (Fig. 3A)

Lens homogenates were first brought to 37°C. Duplicate samples of 100 μM pantethine in lens homogenate were incubated at 37°C. A 20 μl aliquot was removed from each tube of lens homogenate at 5, 10, 15, 20, 25 and 30 min, added to 180 μl of precipitating solution, vortexed, and frozen on dry ice. When all of the samples had been collected, they were thawed and centrifuged at 13 000*g* for 15 min. The supernatants from the above samples were removed from the pellets. The pellets were washed three times with 200 μl of the acetate buffer, and resuspended in 190 μl of acetate buffer with sonication. A 400 μl aliquot of reducing reagent was added followed by 10 μl of 5.0 μM bis-(pantoyl-β-aminoethyl) disulfide. The samples were incubated at 37°C for 60 min and vortexed every 10 min. A 400 μl aliquot of precipitating solution was added, and the samples were then vortexed. (If required, lens homogenate samples were diluted to reduce the actual pantetheine homogenate concentration to within the range of the standard curve.) The samples were then processed as described in Section 2.5.3.

2.5.5. Total supernatant pantetheine assay (Fig. 3B)

This assay was identical to that outlined in Section 2.5.4. except that following centrifugation a 20 μl aliquot of each supernatant was added to 180 μl of the acetate buffer (20 mM, pH 4.8). The samples were then processed as described, starting with the reduction.

2.5.6. Free supernatant pantetheine assay (Fig. 3C)

Lens homogenate samples containing 100 μM of pantetheine were prepared as described in Section 2.5.4. A 20 μl aliquot of each homogenate was removed after 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105 and 120 min and added to 180 μl of precipitating solution. Following centrifugation at 13 000*g* for 15 min, 20 μl of each supernatant was added to 10 μl of 5.0 μM pantoylcysteamine, 580 μl of acetate buffer (20 mM, pH 4.8) and 400 μl of precipitating solution in a 1.5 ml polypropylene microcentrifuge tube. Samples were then processed as described in Section 2.5.3.

2.6. Supernatant pantethine assay (Fig. 3D)

2.6.1. Chromatographic conditions

The mobile phase consisted of a phosphate buffer (50 mM, pH 2.8 with 0.2 mM EDTA) and acetonitrile (86%:14%, v/v). The flow was set to 0.7 ml min^{-1}. A dual Hg/Au working electrode (Bioanalytical Systems) was prepared as described in Section 2.3. The LC44-C detector was set to −1.200 V (upstream electrode), +0.150 V (downstream electrode), 50 nA and 0.10 Hz.

2.6.2. Standards and quality control

Two stock solutions of pantethine in methanol were independently prepared and stored at 4°C. The standards and quality control sample were prepared before each use by diluting the stock solutions. A 40 μl aliquot of each standard or quality control was added to 1.5 ml polypropylene microcentrifuge tubes followed by a 160 μl aliquot of precipitating reagent and 20 μl of 200 μM bis-(pantoyl-β-aminoethyl) disulfide. Samples were centrifuged at 13 000*g* for 15 min. The supernatant was filtered using a 0.2 μm, 1.5 ml microcentrifuge filter for 5 min at 3000*g*.

2.6.3. Supernatant pantethine assay (Fig. 3D)

A 10 μl aliquot of a 100 μM solution of pantethine in water was added to 90 μl of lens homogenate in duplicate. After 1 min, the samples were processed as described for the pantethine standards and quality control. This process was repeated for the following time points: 2, 4, 6, 8, 10, 15, 20, 25, and 30 min.

2.7. Calculations and data analysis

A standard curve was prepared by plotting the peak height ratio vs. concentration. The slope (m), standard error of the slope (se_m), intercept (b), standard error of the intercept (se_i), and correlation coefficient (r) of the standard curve were determined using linear regression and analysis of variance. If the origin was not statistically different from zero, a linear regression through the origin was used. S-Plus was used for nonlinear-least-squares analysis of the data (StatSci, Seattle, WA).

3. Results

A chromatographic method was developed for pantetheine to measure total pellet pantetheine, total supernatant pantetheine and free supernatant pantetheine (Figs. 4A–4C). The reduction of pantethine and bis-(pantoyl-β-aminoethyl) disulfide using TCEP in acetate buffer resulted in 100% conversion to pantetheine and pantoyl cysteamine. The standard curve for pantetheine was linear from 0.07–4.0 μM and for a typical standard curve $r = 0.999$, $m = 1.99$, $se_m = 0.02$, and $b \cong 0$. The expected concentrations for each of the three quality control samples were 0.10, 0.40 and 1.6 μM. The interassay average concentrations and standard deviations for three quality control samples were 0.12 μM $\pm$ 0.01 μM ($n = 5$), 0.41 μM $\pm$ 0.01 μM ($n = 7$), and 1.58 μM $\pm$ 0.04 μM ($n = 7$).

Another chromatographic method was developed for pantethine (Fig. 4D) to measure free supernatant pantethine. The standard curve was linear from 0.5–125 μM ($r = 0.999$, $m = 0.0401$, $se_m = 0.0004$ and $b \cong 0$). The interassay average concentration for the 125 μM quality control sample was 128 μM $\pm$ 5 μM (standard deviation, $n = 4$).

The majority of the added pantethine (98.5%) is found in the supernatant and the supernatant total pantetheine concentration is not a function of time under the conditions reported here (Fig. 5). The supernatant total pantetheine concentration (Fig. 3B) is the sum of the free pantetheine concentration plus pantetheine resulting from the reduction of pantethine and mixed disulfides of pantetheine. The average supernatant total pantetheine concentration is 191 μM $\pm$ 4 μM (standard deviation, $n = 12$) for all the samples (six time points in duplicate).

The concentration of supernatant pantethine decreases exponentially with time (Eq. (1)). The rate constant is 0.25 min^{-1} with a standard error of 0.01 min^{-1}. The calculated initial value was 103 μM with a standard error of 3 μM.

$$C_{\text{pantethine}} = 103e^{-0.25t} \quad (1)$$

The concentration of supernatant pantetheine (Fig. 5) increases in a pseudo first order manner (Eq. (2)). Supernatant-free pantetheine concentration (Fig. 3C) was determined without reduction and so pantoylcysteamine (Fig. 1), a thiol, was used as the internal standard. The rate constant is 0.16 min^{-1} with a standard error of 0.02 min^{-1}. The concentration of pantetheine plateaus at 208 μM with a standard error of 8 μM. The free supernatant pantetheine concentration in lens homogenate does not change in the time period from 30–120 min.

$$C_{\text{pantetheine}} = 208(1 - e^{-0.16t}) \quad (2)$$

Total pellet pantetheine concentration is basically 1.5% of the total pantethine concentration added to the lens homogenate and constant as a function of time (Fig. 5). Reduction of the resuspended pellet yielded the thiols of the internal standard and protein mixed disulfides of pantetheine (Fig. 3A). The average pellet total pantetheine concentration is 2.9 μM $\pm$ 0.4 μM (standard deviation, $n = 12$) for all the samples (six time points in duplicate).

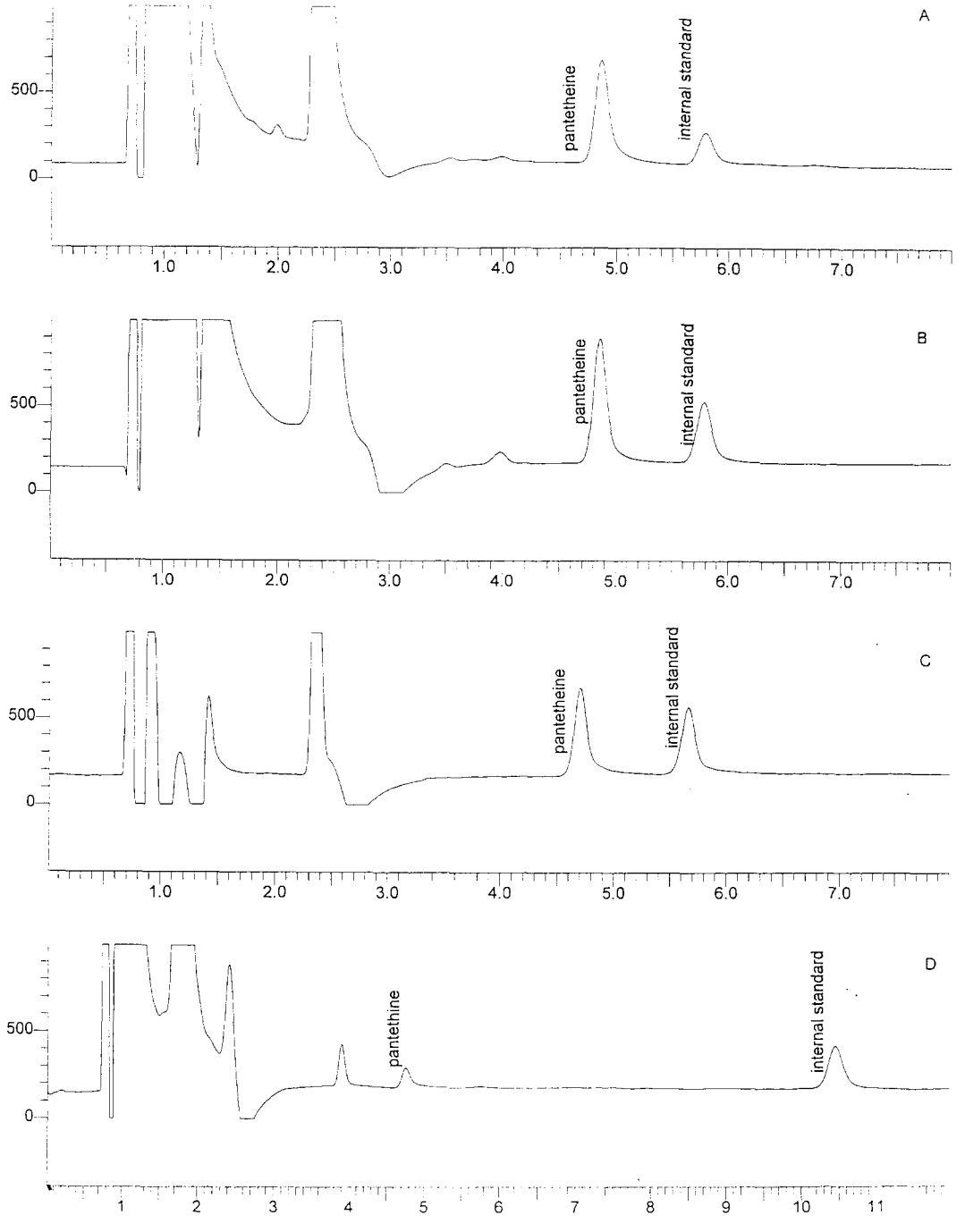


Fig. 4. Representative HPLC/EC chromatograms: (A) total pantetheine in the pellet; (B) total supernatant pantethine; (C) free supernatant pantetheine; (D) supernatant pantethine.

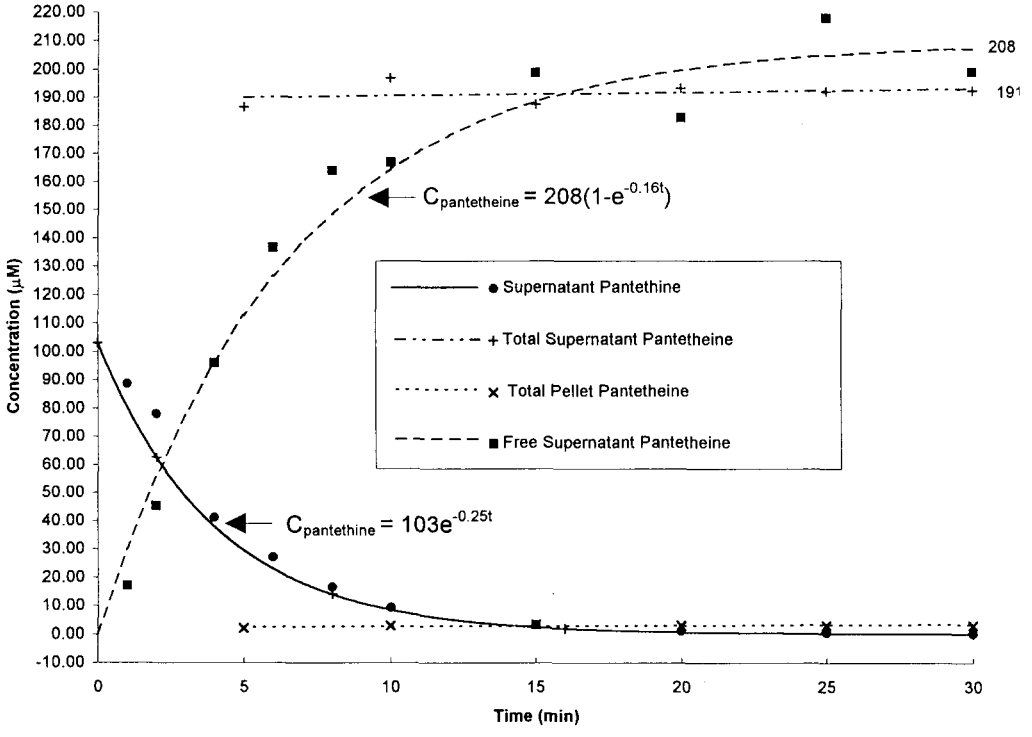


Fig. 5. Total pellet pantetheine, total supernatant pantetheine, free supernatant pantetheine and supernatant pantethine concentrations in lens homogenates plotted as a function of time.

4. Discussion

For this study, HPLC/EC provided a suitable method for studying the stability of pantethine in rabbit lens homogenates. Although the initial goal was to develop one isocratic method for pantetheine and pantethine, two isocratic chromatographic methods were developed because the capacity factor for pantethine is very large under mobile phase conditions that permit adequate resolution of pantetheine and its internal standard. Each method is accurate, precise and linear over two orders of magnitude.

The experimental design was such that pantethine was used as the standard for all three pantetheine assays. The primary reasons for this choice were that very pure preparations of pantethine are commercially available while pantetheine must be synthesized and the product is not as pure. Secondly, pantetheine is more unstable than its corresponding disulfide, pantethine. Thirdly, a measure of reduced pantethine was required for all three assays. However, using pantethine as the standard for free supernatant pantetheine required that the standards be treated in a manner slightly different from the samples. The pantethine in the standards was completely reduced by TCEP, while the pantethine added to the samples was reduced to varying degrees in the lens homogenate to pantetheine. (The lens homogenate sample were not reduced with TCEP.) For both standards and samples, pantoylcysteamine was used as the internal standard. The net result was that thiols were still being measured.

The decrease in pantethine concentration in lens homogenates is not due to pantethinase activity. Rabbit plasma pantethinase activity is known

to be very high, yielding a rapid terminal half-life for pantethine in plasma. However, pantethinase cleaves one equivalent of pantethine to yield two equivalents of pantothenic acid and one equivalent of cystamine (Fig. 2) but not two equivalents of pantetheine. In fact, pantetheine is also a substrate for pantethinase (Fig. 2). Free pantetheine is stable in lens homogenate for at least 2 h. Therefore, degradation due to pantetheinase is not significant.

The most plausible hypothesis is that pantethine is being rapidly and completely reduced to pantetheine under the conditions reported, as demonstrated by the essentially complete conversion of pantethine to pantetheine. The exact mechanism of this reduction remains to be elucidated. This means that about 87.5% (≈three half-lives) of the pantethine present in the lens is converted to pantetheine by the time the lens is thawed, homogenized and refrozen.

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