ELSEVIER

Journal of Chromatography B, 696 (1997) 175–182

JOURNAL OF CHROMATOGRAPHY B

# Liquid chromatographic assay of dityrosine in human cerebrospinal fluid[1]

Maged Abdelrahim[a], Elena Morris[a], Jeannean Carver[b], Stephen Facchina[c], Alison White[c], Ajay Verma[c,*]

[a]*Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, DC, USA*
[b]*Critical Care Division, Ohio State Medical Center, Columbus, OH, USA*
[c]*Department of Neurology, Uniformed Services University of the Health Sciences, National Naval Medical Center, 4301 Jones Bridge Road, Bethesda, MD 20814, USA*

Received 30 October 1996; revised 1 April 1997; accepted 14 April 1997

**Abstract**

A micro-scale method for separation and measurement of dityrosine in human cerebrospinal fluid (CSF) is described utilizing liquid–liquid extraction and ion-paired, reversed-phase high-performance liquid chromatography with fluorimetric detection. A mobile phase containing 1-heptanesulfonic acid linearly increased in methanol from 0 to 100% over 30 min allows the resolution of dityrosine from other fluorescent compounds with excitation at 285 nm and emission at 410 nm. As little as 0.15 ml CSF sample can be utilized with a detection limit of 60 pg dityrosine on the column. This method facilitates the use of CSF dityrosine as a measure of free radical mediated protein damage in the central nervous system. © 1997 Elsevier Science B.V.

*Keywords:* Dityrosine

## 1. Introduction

Oxygen free radicals are implicated in the pathogenesis of atherosclerosis, pulmonary toxicity, reperfusion injury, cataractogenesis, macular degeneration, sepsis, neoplastic transformation and neurodegenerative disease [1–4]. The central nervous system may be particularly sensitive to free radical mediated injury due to its high rate of oxygen utilization and relatively low amounts of antioxidants as compared with other organs. Free radical mediated nervous system injury has been implicated in the pathophysiology of stroke, Parkinsonism, Alzheimer's disease, Amytrophic Lateral Sclerosis, epilepsy and neuroinflammatory processes [4]. The growing importance of free radicals in the pathogenesis of human disease has led to an increased need for measurements of surrogate markers for free radicals. In spite of a broad array of existing analytical methods, no routine clinical diagnostic tests are currently available.

Free radicals are extremely reactive and short

*Corresponding author.

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

0378-4347/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
*PII* S0378-4347(97)00248-X

lived making direct measurement very difficult. Lipids, nucleic acids, carbohydrates and proteins can all be damaged by free radicals and the end products of free radical attack on these and other molecules can serve as surrogate markers for free radical injury. Several analytical techniques have been developed to measure these end products though only a few are clinically applicable. Typical clinical samples include urine, blood and CSF.

Lipid peroxidation is the most widely studied process but many of the assays employed are semiquantitative measures of damage to a broad class of molecules [5]. Studies of nucleic acid [6] and protein oxidation products [2] are attracting increasing attention. Oxidative damage to proteins results in the modification of several amino acids. One of these modifications involves the abstraction of the phenolic hydrogen atom from tyrosine residues. The resulting tyrosyl radical is relatively long-lived and can combine with another tyrosyl radical to generate a stable, covalent, carbon–carbon bond resulting in the formation of 1,3-dityrosine, a highly fluorescent molecule that is resistant to acid hydrolysis and protease activity (Fig. 1a) [7,8]. We have recently found dityrosine to occur in human CSF by measuring its fluorescence in unfractionated samples and have shown a dramatic increase in dityrosine levels after exposing CSF to a variety of free radical generating systems in vitro [9]. These observations suggest that CSF dityrosine measurements may serve as a clinical index of protein oxidation by free radicals in the central nervous system.

Dityrosine has been measured in isolated protein hydrolysates and, more recently, in human serum using DEAE cellulose chromatography, high performance liquid chromatograpy and gas chromatograpy–mass spectrometry [8,10–13]. The methods for dityrosine identification described to date involve lengthy purification from protein hydrolysates and precolumn derivatization. This hampers rapid analyses of multiple samples. In this paper, we describe a simple and accurate method for the rapid determination of dityrosine levels in multiple human CSF samples based on liquid–liquid extraction, reversed-phase chromatography and fluorescence detection. This method does not rely on pre-purification or pre-derivatization and displays high sensitivity. We have optimized this method to handle small clinical samples and to distinguish dityrosine from other fluorescent CSF molecules.

## 2. Experimental

### 2.1. Reagents

The methanol used was Burdick and Jackson brand purchased from Baxter (Chicago, IL, USA). The water used was HPLC grade obtained from a Millipore (Bedford, MA, USA) water system. The paired ion chromatography (PIC-7, 1-heptanesulfonic acid) reagent was purchased from Waters (Milford, MA, USA). Chloroform was purchased from Fisher Scientific (Pittsburg, PA, USA). Trifluoroacetic acid (TFA) and all other reagents were obtained from Sigma (St. Louis, MO, USA).

### 2.2. Fluorescence measurements

Dityrosine displays a strong fluorescence emmision maximum at 410 nm. At pH approximately 10, this peak has a fluorescence excitation maximum between 320 and 325 nm, while at pH approximately 2, the excitation maximum is between 285 and 290 nm. This pH dependent excitation maximum shift and emission maximum are unique characteristics of dityrosine [7]. Fluorescence measurements during dityrosine synthesis were made using a Perkin–Elmer LS 50-B Fluorimeter and those during HPLC analysis were made using a Waters Model 470 in-line fluorimeter.

### 2.3. Synthesis of dityrosine standard

Authentic dityrosine was synthesized and purified essentially as previously described [7] with minor modifications. Briefly, 250 ml of a 20 m*M* tyrosine solution in 0.2 *M* ammonium hydroxide (pH 12.0) was oxidized with the addition of 2 ml of 3% $H_2O_2$ and 4 mg of horseradish peroxidase (type VI, Sigma). The reaction was allowed to proceed for 12 h at 37°C and then stopped with the addition of 5 m*M* sodium metabisulfite. The reaction mix was then lyophilized to complete dryness. The residue was then dissolved in acidified methanol (methanol–HCl, 95:5, v/v) and

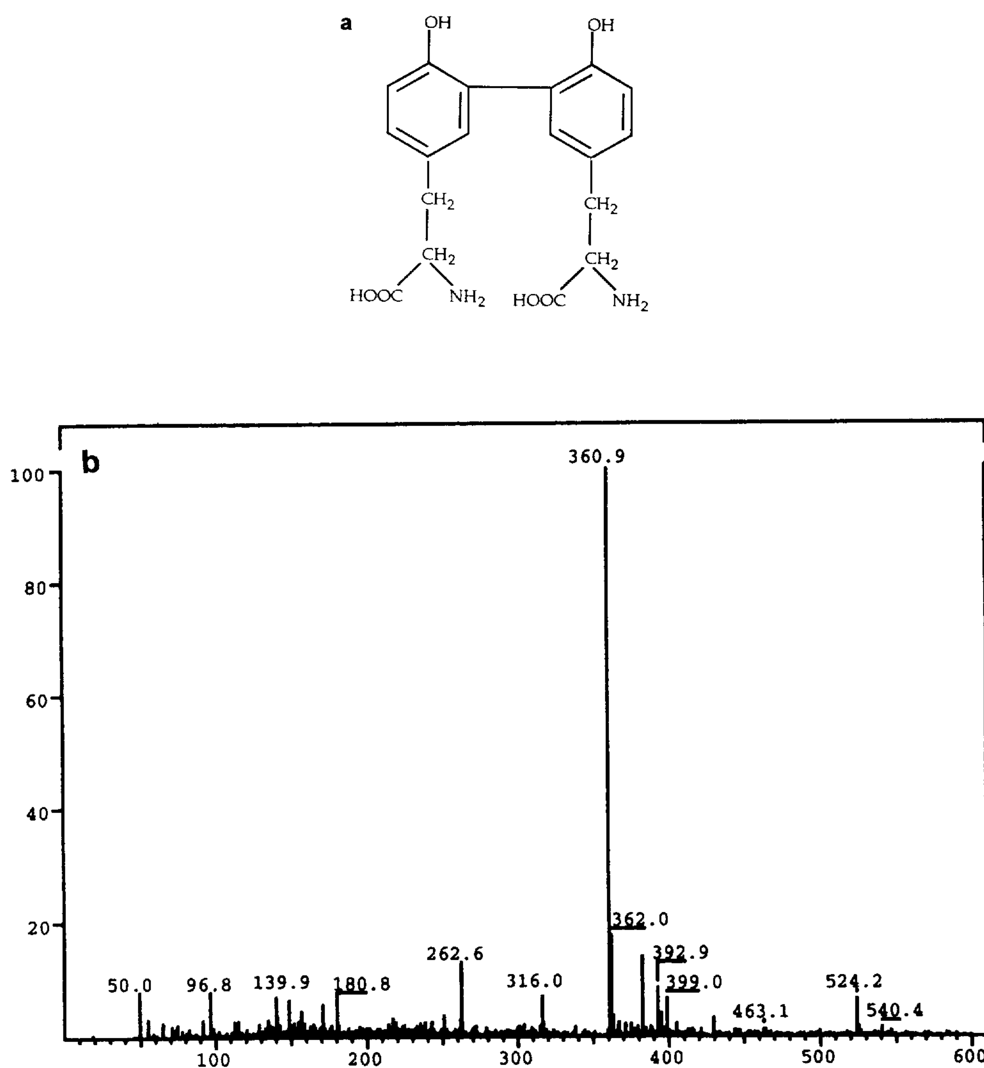


Fig. 1. (a) Chemical structure of dityrosine. (b) Mass spectrometry analysis of dityrosine standard. Analysis was performed as described in Section 2. The standard had a molecular mass of 361 and was >98% pure.

filtered over a 0.22 μm filter to remove most of the unreacted tyrosine precipitate. The filtered solution was then reduced in volume to 20 ml using a rotory evaporator under vacuum and applied to a 2.5×70 cm CP-11 cellulose phosphate (Whatman Scientific, Maidstone, UK) column pre-equilibrated with 0.2 *M* acetic acid. The column was washed with 2 l of 0.2 *M* acetic acid and eluted with 0.2 *M* acetic acid containing 0.5 *M* NaCl. Fractions with flourescence characteristics similar to dityrosine in acidic conditions (excitation maximum 285 nm, emission maximum 410 nm) were collected, dried down in a rotory evaporator under vacuum and redissolved in distilled, deionized water. The insoluble material was discarded and the soluble material was again concentrated using a rotory evaporator under vacuum and loaded onto a Dowex 50-X8 cation exchange column preequilibrated in 0.2 *M* $NH_4OH$. The column was washed with 500 ml of distilled deionized water and eluted with 300 ml of 2 *M* $NH_4OH$. Fractions of 3 ml were collected and monitored for fluorescence characteristic of dityrosine in basic conditions (exci-

tation maximum 325 nm, emission maximum 410 nm). Fluorescent fractions were monitored for purity by HPLC (see below) and those with a single peak were collected and pooled. The solvent was removed by several evaporations in a speed vac. Final purity was assessed by mass spectrometry.

### 2.4. Mass spectrometry

The spectrum of dityrosine was obtained on triple quadrupole mass spectrometer Model TSQ-700 from Finnigan-Mat (San Jose, CA, USA). The source used with this instrument was an electrospray interface manufactured by Finnigan-Mat. The electron multiplier was set at 1200 V. Sample preparation for analysis by mass spectrometer: 1 μl of dityrosine (1.354 mg/ml) from the preparation described before was added to 1 ml of methanol and water (1:1) in 0.1% acetic acid. The sample was infused into the mass spectrometer at a flow-rate of 3 μl/min using a Harvard pump (Southnatik, MA, USA).

### 2.5. Source of human cerebrospinal fluid

The research was approved by the WRAMC Clinical Investigations Committee. The CSF used in our study was obtained from the Walter Reed Army Medical Center, Department of Neurology under Department of Clinical Investigations protocol WU 7160. The CSF sample used for dityrosine recovery studies was obtained from a patient with a surgical shunt of the lateral cerebral ventricle. The shunt allowed access to freshly produced ventricular CSF which reduced the contribution of endogenous dityrosine in the recovery studies. Adult CSF samples used to explore endogenous dityrosine levels were lumbar puncture specimens left over from clinical laboratory measurements. All samples were stored frozen at −20°C in 1–5 ml aliquots and were used only following patient discharge.

### 2.6. HPLC analysis

The high-performance liquid chromatography system used was equipped with an autosampler Model 715, $C_{18}$ Nova pack column (60 Å, 4 μm, 3.9×150 mm), and fluorescence detector Model 470 set at excitation 285 nm and emission 410 nm, all from Waters. The mobile phase consisted of 100% water with PIC-7 reagent as part A and 100% methanol with PIC-7 reagent as part B of the gradient. The mobile phase was degassed continuously with helium during the experiment. The mobile phase was decreased from 100% of part A at the initial condition to 0% at the final condition with the increase of part B from 0% at the initial condition to 100% at the final condition. The duration of this gradient was 30 min using a linear curve (preprogrammed curve 6). The flow-rate of the mobile phase was maintained at 1.0 ml/min, and the column temperature was kept at 25°C. Equilibration time at the end of the gradient was 10 min.

### 2.7. Preparation of standard curves and CSF samples

Since we found dityrosine to occur naturally in human CSF, it was necessary to use water instead of a CSF matrix to establish the standard curves. This eliminated any contribution of the naturally occurring dityrosine in CSF to the values used to construct the standard curves. Known aqueous dityrosine standards were extracted for HPLC analysis as follows. A 150-μl volume of dityrosine solutions prepared in water at different concentrations (33.80, 16.90, 8.45, 4.22, 1.69, 0.845 and 0.422 ng/ml) was placed into 12×75 mm glass tubes followed by addition of 0.1% trifluoroacetic acid (TFA) to make the final volume equivalent to 1 ml (pH 2.30). A 1-ml volume of chloroform was added and the mixture was vortex-mixed for 20 min followed by centrifugation at 3000 *g* for 20 min at 10°C. A 700-μl aliquot of the aqueous phase was then transferred to 1.5-ml eppendorf tubes, and evaporated with a speed vac. The residue was dissolved in 220 μl of 0.1% TFA, vortex-mixed for 10 min, centrifuged for 5 min at 450 *g*, and 200 μl injected onto the HPLC column.

For preparation of clinical cerebrospinal fluid samples, 150 μl of CSF was placed into glass tubes, adjusted to 1 ml with 0.1% TFA and processed exactly as described above for the dityrosine standards.

### 2.8. Precision studies

Within-run precision was assessed by six replicate determination of three different control dityrosine

solutions (12.675, 2.112 and 0.845 ng/ml) prepared in water. The values of these controls were calculated from a standard curve prepared as described above on the same day as the controls. The values of these controls chosen represented the extremes of the usual standard curve to cover the high, middle and lower ranges.

Between-run precision was determined on six different days, by the construction of complete standard curves and by the analysis each day of three controls representing high (12.675 ng/ml), medium (2.112 ng/ml) and low (0..845 ng/ml) concentrations of dityrosine.

### 2.9. Recovery studies

The recovery of dityrosine from water and CSF was determined by comparing the peak area of known concentration of pure dityrosine standard with those of water and CSF spiked with three different amounts (50.79, 8.46 and 3.39 ng) of dityrosine. Three replicates of each dityrosine concentration were assayed and the water and CSF samples were treated with chloroform as described above.

### 2.10. Statistical methods

Standard methods were used to calculate the mean, standard deviation, standard error of the mean, coefficient of variation and linear regression parameters. One-way analysis of variance was used to test for differences among the recovery rates of the three different dityrosine concentrations.

## 3. Results

### 3.1. Dityrosine synthesis

Dityrosine preparations were 98% pure with a molecular mass of 361 by mass spectrometry (Fig. 1b).

### 3.2. HPLC analysis

Mean coefficient of determination ($R^2$) for seven separate dityrosine standard curves in water was 0.999412 with a mean equation of $y=1.994523x$. Fig. 2a shows a typical chromatogram obtained from a 33.86 ng/ml standard sample of dityrosine in water. At a flow-rate of 1.0 ml/min, retention time for dityrosine was about 15.5 min. Fig. 2b shows another chromatogram with a sample of 169 pg/ml. Since only 200 μl is applied to the column, this measurement represents 33.8 pg of dityrosine applied to the column.

Analytical recovery of dityrosine extracted from water ranged from 85.35 to 87.45% (S.E.M. 0.51), and was independent of concentration over the range from 32.32 to 2.15 ng on column. The recovery of dityrosine from CSF ranged from 93.23 to 98.88% (S.E.M. 0.89) over the same concentration range (Table 1). Human CSF samples demonstrated several endogenous fluorescent species with the 285/410 nm excitation/emmision wavelength pair used in our acidic chromatography system. Use of ion-pairing helped to resolve the dityrosine peak from these other peaks (Fig. 3) while employing other HPLC protocols for identifying dityrosine [13,15] with CSF samples failed to resolve dityrosine from several other peaks (data not shown).

Within-run coefficient of variability (C.V.) values ranged from 3.25 to 5.65% and were independent of the amount of dityrosine analyzed (Table 2). Between-run C.V. values ranged from 3.06 to 5.64%, and were also unrelated to the amount of dityrosine analyzed (Table 3). Day-to-day variation in standard curve parameters were minimal and not statistically significant. Mean coefficient of determination ($R^2$) for seven separate dityrosine standard curves in water was 0.999412 with a mean equation of $y=1.994523x$. The limit of quantitation was defined as the lowest concentration (0.169 ng/ml) used on the standard curves. Statistics derived from the signal generated by this concentration of dityrosine using the area under the curve from 8 different standard curves had a coefficient of variability of less than 10%. No changes in dityrosine concentrations were observed in individual samples stored at −20°C for up to one year.

## 4. Discussion

Dityrosine groups are known to form on proteins as a result of oxidation of tyrosine residues. Oxidized proteins are also known to be rapidly hydrolyzed by endogenous proteases with the release of constituent

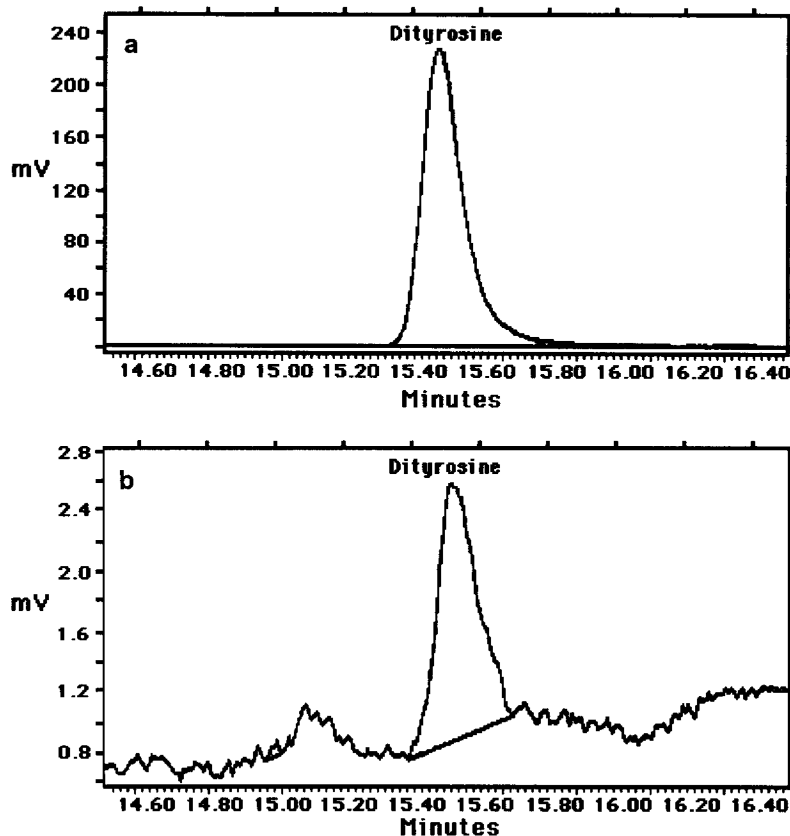


Fig. 2. HPLC analysis of dityrosine standard. HPLC analysis was performed as described in Section 2 and results from 2 seperate runs which are shown using 200 μl of 33.86 ng/ml dityrosine (a) and 169 pg/ml dityrosine (b).

amino acids and dityrosine [8]. Dityrosine is a very stable molecule with strong fluorescence properties and thus may potentially serve as a clinically useful marker to monitor oxidative damage to proteins [11,12]. While dityrosine measurements have long been used to monitor protein oxidation in vitro [7,8], only recently have these measurements been applied to human physiological fluids [11,12,14]. Detection methods used to measure dityrosine vary from measurements of overall fluorescence in unfractionated samples to extensive purification of dityrosine from unknown samples prior to measurement. Fluorescence measurements in unfractionated samples may be confounded by the presence of other molecules with similar fluorescent properties such as tryptophan, lipid peroxidation products, lipofucin, carotenes and salicylates, or by the presence of molecules absorbing near 400 nm such as heme. Extensive purification procedures, on the other hand, impede rapid analysis of small endogenous quantities of dityrosine found in limited clinical samples such

Table 1
Dityrosine % recovery from water vs. CSF

| Matrix | Water | CSF |
|---|---|---|
| 50.79 ng spike ($n$=3) | 87.45 | 98.88 |
| 8.46 ng spike ($n$=3) | 86.59 | 93.65 |
| 3.39 ng spike ($n$=3) | 85.35 | 93.23 |
| Average | 86 | 95 |
| S.D. | 1.056 | 3.148 |
| C.V. | 1.221 | 3.305 |
| S.E.M. | 0.51 | 0.89 |

Water or CSF were spiked with the indicated amounts of dityrosine standard and extracted as described in the Section 2. Mean percent recoveries and statistics for each dityrosine spike ($n$=3) is shown for the two different matrices.

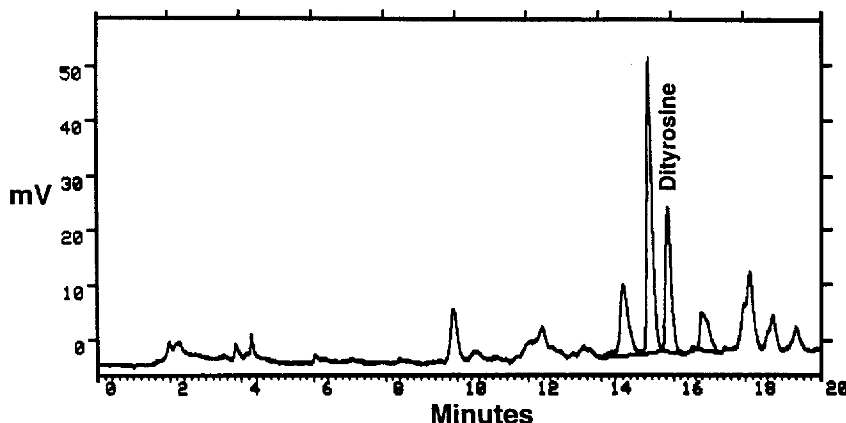


Fig. 3. Detection of endogenous dityrosine in human CSF. Human CSF samples were processed and analyzed by HPLC as described in Section 2. A typical CSF sample analysis is shown.

Table 2
Within-run precision

| Standard (ng/ml) | 12.675 | 2.112 | 0.84 |
|---|---|---|---|
| Average measurement ($n$=6) | 12.31 | 2.20 | 0.86 |
| S.D. | 0.400 | 0.123 | 0.049 |
| C.V. | 3.25 | 5.57 | 5.65 |
| S.E.M. | 0.126 | 0.70 | 0.033 |

as CSF. We have developed a rapid, reliable, simple and sensitive assay for the determination of dityrosine in human CSF using a simple aqueous extraction step and paired-ion HPLC with fluorometric determination. Our assay allows the processing of large numbers of samples in a short amount of time with clinical sample volumes as little as 150 μl.

Dityrosine formation is known to result from free radical mediated attack on amino acids and proteins. Overall dityrosine fluorescence of unfractionated human CSF samples has been shown to increase following treatment of samples with free radical generating systems [9]. The use of our ion-pairing HPLC system reveals that there are several fluorescent peaks in human CSF with fluorescence characteristics resembling that of dityrosine under acidic conditions (Fig. 3). Thus, our HPLC system allows selective quantitation of CSF fluorescence attributable to dityrosine. The stability of dityrosine and our assay's ability to quantitate endogenous levels in human CSF (Fig. 3) should allow dityrosine to serve as a new clinical index for oxidative injury to proteins and amino acids in the nervous system. Such injury can result from CNS inflammation, ischemia/reperfusion, or toxic insults. Oxidative injury catalyzed by free radicals is also thought to represent a major pathophysiological mechanism in several neurodegenerative diseases [4]. The ability to monitor a stable, sensitive endogenous indicator of free radical mediated protein injury in small amounts of CSF should facilitate the exploration of this hypothesis and should also allow the clinical evaluation of antioxidant therapeutic approaches. Our assay should also be applicable to other body fluids.

Table 3
Between-run precision

| Standard (ng/ml) | 12.675 | 2.112 | 0.845 |
|---|---|---|---|
| Average measurement ($n$=6) | 12.721 | 2.154 | 0.891 |
| S.D. | 0.466 | 0.122 | 0.027 |
| C.V. | 3.666 | 5.643 | 3.066 |
| S.E.M. | 0.137 | 0.70 | 0.033 |

## Acknowledgments

This study was supported by Department of Clinical Investigation WU No. 7160, and by The

Defense and Veteran's Head Injury Program, Walter Reed Army Medical Center, Washington, DC, USA.

## References

[1] B. Halliwell, J.M.C. Gutteridge, Methods Enzymol. 186 (1990) 81.
[2] E.R. Stadtman, Science 257 (1992) 1220.
[3] J.A. Knight, Ann. Clin. Lab. Sci. 25 (1995) 111.
[4] C.W. Olanow, Trends Neurosci. 16 (1993) 439.
[5] A.E. Holley, K.H. Cheeseman, Br. Med. Bull. 49 (1993) 494.
[6] M.G. Simic, Cancer Res. 54 (1994) 1918s.
[7] R. Amado, R. Aeschbach, H. Neukom, Methods Enzymol. 107 (1984) 377.
[8] C. Giulivi, J.A. Davies, Methods Enzymol. 233 (1994) 363.
[9] A. Verma, M. Erickson, J. Carver, G. Carlson, Soc. Neurosci. Abstr. 20 (1994) 641.
[10] K. Zaitsu, S. Eto, Y. Ohkura, J. Chromatogr. 206 (1981) 621.
[11] J.W. Heinecke, W. Li, H.L. Daenke III, J.A. Goldstein, J. Biol. Chem. 268 (1993) 4069.
[12] J.W. Heinecke, W. Li, G.A. Francis, J.A. Goldstein, J. Clin. Invest. 91 (1993) 2866.
[13] E.H. Smail, P. Briza, A. Panagos, L. Berenfeld, Infect. Immun. 63 (1995) 4078.
[14] H.B. Nielsen, B. Hanel, S. Loft, H.E. Poulson, B.K. Pederson, M. Diamant, K. Vistisen, N.H. Sechler, J. Sports Sci. 13 (1995) 109.
[15] C. Giulivi, J.A. Davies, J. Biol. Chem. 268 (1993) 8752.