Research Article

Biomedical Chromatography

Received: 13 February 2008, Revised: 18 April 2008, Accepted: 22 April 2008 Published online 21 July 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/bmc.1083

Determination of glutathione disulfide levels in biological samples using thiol–disulfide exchanging agent, dithiothreitol

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ABSTRACT: A reverse-phase HPLC method incorporating dithiothreitol (DTT) reduction for quantitative determination of oxidized glutathione (GSSG) in biological samples is described here. This method is based on our previous enzymatic reduction technique that uses N-1-(pyrenyl) maleimide (NPM) as a derivatizing agent. In our earlier method, glutathione disulfide (GSSG) was measured by first reducing it to GSH with glutathione reductase (GR) in the presence of NADPH. However, this is a very costly and time-consuming technique. The method described here employs a common and inexpensive thiol–disulfide exchanging agent, DTT, for reduction of GSSG to GSH, followed by derivatization with NPM. The calibration curves are linear over a concentration range of 25–1250 nM ($r^2 > 0.995$). The coefficients of variations for intra-run precision and inter-run precision range from 0.49 to 5.10% with an accuracy range of 1.78–6.15%. The percentage of relative recovery ranges from 97.3 to 103.2%. This new method provides a simple, efficient, and cost-effective way of determining glutathione disulfide levels with a 2.5 nM limit of detection per 5 μL injection volume. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: GSH; GSSG; DTT; thiol–disulfide exchange; HPLC

Introduction

Glutathione (GSH), an important thiol tripeptide antioxidant (γ-glutamylcysteinylglycine) in cells, exists in two forms: reduced as GSH and oxidized as glutathione disulfide (GSSG) (Meister and Anderson, 1983). GSH is significantly favored over GSSG under healthy physiological conditions. However, pathological conditions causing oxidative stress have been found to result in a decreased GSH/GSSG ratio (Noctor *et al*., 2002). Therefore, evaluation of GSSG levels under stress-inducing conditions may provide valuable information regarding the oxidative stress a biological system is experiencing (Kleinman and Ritchie, 2000; Hernanz *et al*., 2000; Asensi *et al*., 1999).

Several spectrophotometric and HPLC methods have already been developed for determining GSSG and total GSH (GSH + GSSG) levels (Rahman *et al*., 2006; Tietz, 1969; Newton *et al*., 1981; Martin and White, 1991; Svardal *et al*., 1990; Martensson, 1987; Mopper, 1984). The current spectrophotometric determination of GSSG includes a glutathione reductase (GR) coupled method that uses 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in a recycling assay. In most HPLC methods, GSSG is measured by reducing it to GSH with the enzyme GR in the presence of NADPH. The difference between the total and initial GSH values is used to determine the GSSG concentration in the sample. Although GR makes the assay selective for glutathione, it is insufficiently sensitive in some cases. Moreover, exposure to light reduces DTNB and increases color intensity in spectrophotometric methods, which can lead to erroneous results (Walmsley *et al*., 1987).

Another approach involves a preliminary step in which the thiol group of GSH is blocked using 2-vinylpyridine (2-VP) (Griffith, 1980). The subsequent derivatization reaction occurs only with GSH formed during the *in vitro* GSSG reduction step. We previously reported a highly sensitive HPLC method which used 2-VP as a masking agent for GSH and *N*-1-(pyrenyl) maleimide (NPM) as a fluorescent derivatizing agent (Winters *et al*., 1995). We also demonstrated few distinct advantages of using NPM over other derivatizing agents. Although 2-VP does not inhibit GR and need not be removed from the sample, masking the GSH requires 40–60 min incubation at room temperature (Griffith, 1980). The toxic properties of 2-VP also make it difficult to work with. In addition, these methods are quite expensive, considering the cost of GR and NADPH. We have, therefore, focused our attention on the readily available and inexpensive reducing agent dithiothreitol for GSSG reduction. Dithiothreitol (Cleland's reagent, DTT) is an unusually strong reducing agent, owing to its high conformational propensity to form a six-membered ring with an internal disulfide bond (Griffith, 1980). The reduction of a typical disulfide bond proceeds by two sequential thiol–disulfide exchange reactions (Gilbert, 1990). The intermediate mixed-disulfide state is unstable (i.e., poorly populated) because the second thiol of DTT has a high propensity to close the ring, forming oxidized DTT and leaving behind a reduced disulfide bond (Cleland, 1964; Gilbert, 1995). Therefore, GSSG is reduced to 2 molecules of GSH in the presence of DTT by these two sequential thiol–disulfide exchange reactions.

In the present study, considering the cost, time, toxicity and large-scale screening of biological samples, we report a rapid, cost-effective and sensitive method to determine GSSG levels from total GSH using thiol–disulfide exchange reactions by DTT.

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Experimental

Reagents and Chemicals

Acetonitrile, acetic acid and *o*-phosphoric acid (all HPLC-grade) were purchased from Fisher Scientific (Houston, TX, USA). GSH, GSSG, DTT and NPM were purchased from Sigma (St Louis, MO, USA).

HPLC System. The HPLC system (Thermo Electron Corporation) consisted of a Finnigan™ SpectraSYSTEM SCM1000 vacuum membrane degasser, a Finnigan™ SpectraSYSTEM P2000 gradient pump, a Finnigan™ SpectraSYSTEM AS3000 autosampler and a Finnigan™ SpectraSYSTEM FL3000 fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 376 nm). The injection volume was 5 μL for all samples. The HPLC column was a Reliasil ODS-1 C_{18} column (5 μm packing material) with 250 × 4.6 mm i.d. (Column Engineering, Ontario, CA, USA). Two mobile phases were used in the gradient separation program: mobile phase A was composed of 0.05% acetic acid in 70:30 acetonitrile–HPLC-H_2O (v:v) and mobile phase B was composed of 0.4% *o*-phosphoric acid in 70:30 acetonitrile–HPLC-H_2O (v:v). The gradient program (Table 1) was used for optimal separation and quantification of GSH (or GSSG).

Preparation of Calibration Solutions. Calibration curves were constructed by plotting integrated peak areas vs GSH or GSSG concentrations (25, 125, 250, 500 and 1250 nM). In order to prepare indicated GSH standards, the required volume of GSH stock solution was combined with serine borate buffer (SBB) (100 mM Tris buffer containing 10 mM borate and 5 mM serine with 1 mM diethylenetriaminepentaacetic acid; pH = 8.3) to make a 250 μL solution. A 750 μL aliquot of 1 mM NPM (in acetonitrile) solution was added to this, and the solution incubated at room temperature for 5 min. A 10 μL aliquot of 2 M HCl was added to stop the reaction. The final pH of the solution should be less than 2.0, which is important for stabilizing the fluorescent derivatives. Derivatized samples were filtered through 0.45 μm nylon filters (Advantec MFS Inc., Dublin, CA, USA) and injected directly onto the HPLC system. For preparation of GSSG standards, 125 μL of 2 mM DTT, SBB and appropriate volume of GSSG stock solution were combined to make a 250 μL solution, which was then incubated for 30 min at room temperature. The sample tubes were sealed tightly to prevent any possible oxidation. At the end of 30 min, derivatization with NPM was performed as indicated above. The samples were filtered and injected onto the HPLC.

Table 1. Gradient program of mobile phases used in the analysis

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0.0	100		0.7
6.0	100		0.7
6.1		100	1.7
13		100	1.7
13.1	100		0.7
15	100		0.7

Mobile phase A: 0.05% acetic acid in 70:30 acetonitrile–HPLC-H_2O (v:v).
Mobile phase B: 0.4% *o*-phosphoric acid in 70:30 acetonitrile–HPLC-H_2O (v:v).

Sample Preparation and Derivatization. Tissue samples, ranging from 0.3 to 0.5 g, were minced and homogenized in 1 mL SBB (pH = 7.2). The buffer used contains L-serine and borate, which can inhibit γ-glutamyl transpeptidase (GGT), an enzyme that exists in some biological tissues such as liver and lung, and may hydrolyze GSH. SBB is therefore always used during homogenization of tissue samples to prevent loss of GSH. The homogenate was centrifuged for 5 min at 5000***g***. For GSH analysis, 20 μL of supernatant and 230 μL of SBB were added to 750 μL of 1 mM NPM solution and incubated at room temperature for 5 min. A 10 μL aliquot of 2 M HCl was used to stop the reaction. Derivatized tissue samples were filtered through 0.45 μm nylon filters and injected directly onto the HPLC system.

When GSSG or total GSH (tGSH) was assayed, 20 μL of supernatant and 105 μL of SBB (pH = 8.3) were added to 125 μL of DTT (2 mM) and incubated at room temperature for 30 min. The mixture was then derivatized as per the aforementioned method. The GSSG area was calculated from the tGSH and GSH areas.

Comparison with Enzymatic Reduction Method

GSSG levels were determined in the liver, lung and brain samples obtained from Sprague–Dawley rats using our enzymatic reduction method as well as DTT method. Briefly, our enzymatic reduction method was carried out as summarized below. A 40 μL aliquot of straight tissue homogenate and 44 μL of HPLC water were incubated with 16 μL of 2-vinyl pyridine (6.25% 2-VP in 95% ethanol) for 1 h at room temperature in order to block all free thiol groups. After 1 h, 95 μL of a 2 mg/mL solution of NADPH and 5 μL of 2 units/mL glutathione reductase solution were mixed with the original solution. A 100 μL aliquot of this solution was then quickly removed and mixed with 150 μL of HPLC grade water and 750 μL of NPM (1 mM in acetonitrile). After a 5 min incubation period, the reaction was stopped by addition of 10 μL of 2 M HCl. The samples were filtered through a 0.45 μm filter and injected onto the HPLC system (Winters *et al*., 1995).

Determination of Protein

Protein levels of the tissue samples were measured by the Bradford method (Bradford, 1976). Concentrated Coomassie blue (Bio-Rad, Hercules, CA, USA) was diluted 1:5 (v/v) with distilled water, and then 2.5 mL of this diluted dye were added to 50 μL of diluted tissue supernatant. The mixture was incubated at room temperature for 5 min and the absorbance measurement was taken at 595 nm using a UV–vis spectrophotometer. Bovine serum albumin (BSA) was used as protein standard.

Accuracy, Precision and Recovery

Accuracy was determined by analyzing three replicates of tissue samples containing 125, 250, 500 and 1250 nM of GSSG. The mean values for the three replicates at each concentration in each matrix were calculated and the relative deviation of the mean from the true value served as the measure of accuracy.

Intra-run precision was determined by analyzing three replicate GSSG-spiked tissue samples at concentration levels ranging from 125 to 1250 nM in one analytical run, and comparing the GSSG

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concentration calculated from the peak areas of the three replicates in each matrix. [The average calibration curves used in the calculations were, for GSSG, $y = 46.877x + 317.27$, $r^2 = 0.998$.] Inter-run precision was obtained by derivatizing three replicate GSSG-spiked tissue samples at concentration levels ranging from 25 to 1250 nM in three different analytical runs and comparing the GSSG concentration calculated from the peak areas of the three replicates in each matrix. The coefficients of variation were calculated in each matrix and served as the measure of precision.

Relative recovery was determined by spiking the liver, lung, and brain samples with 125, 250, 500 and 1250 nM of GSSG in three replicates. Recoveries were calculated by comparing the analytical results for those three spiked samples with the un-spiked pure standards at the abovementioned four concentrations that represent 100% recovery.

Sensitivity

The limit of detection (LOD) was determined by analyzing three replicates of blank samples without the interference of noise (signal-to-noise, S/N = 3:1). The limit of quantitation (LOQ) was the concentration of GSSG when its peak area was 10 times that of the peak area of the blank (S/N = 10).

Results

Calibration Curve

Calibration curves were plotted using integrated peak areas vs standard GSH and GSSG concentrations (Fig. 1). Linearity in each curve was achieved over a concentration range of 25–1250 nM ($r^2 > 0.995$). The GSSG area was nearly twice the GSH area of the corresponding concentration.

Figure 2(a) and (b) represent chromatograms of DTT alone and 500 nM of GSSG, respectively. As is shown, the GSSG peak is well separated from the DTT peak. This GSSG peak actually belongs to the GSH formed after the reduction of GSSG. These peaks were used for the calibration curve.

DTT Reduction of GSSG in Biological Samples

In order to investigate if our DTT reduction method worked for biological samples, liver samples were used. A known quantity of liver sample was first homogenized in SBB. An identical volume of homogenate was put into two separate test tubes. One was directly derivatized with NPM and the resulting chromatogram is shown in Fig. 3(a). The second aliquot was treated with DTT before derivatization with NPM. The chromatogram shown in Fig. 3(b) was obtained from this sample. The height of the GSH peak significantly increased in Fig. 3(b) due to the reduction of GSSG by DTT.

Comparison with Enzymatic Reduction Method

Table 2 compares values of GSSG in the liver, lung and brain samples estimated using our enzymatic reduction method and DTT method. The values obtained using DTT method are comparable to those obtained by our enzymatic reduction method. In both liver and brain samples, there is less than 10% difference in the GSSG levels measured by DTT reduction method

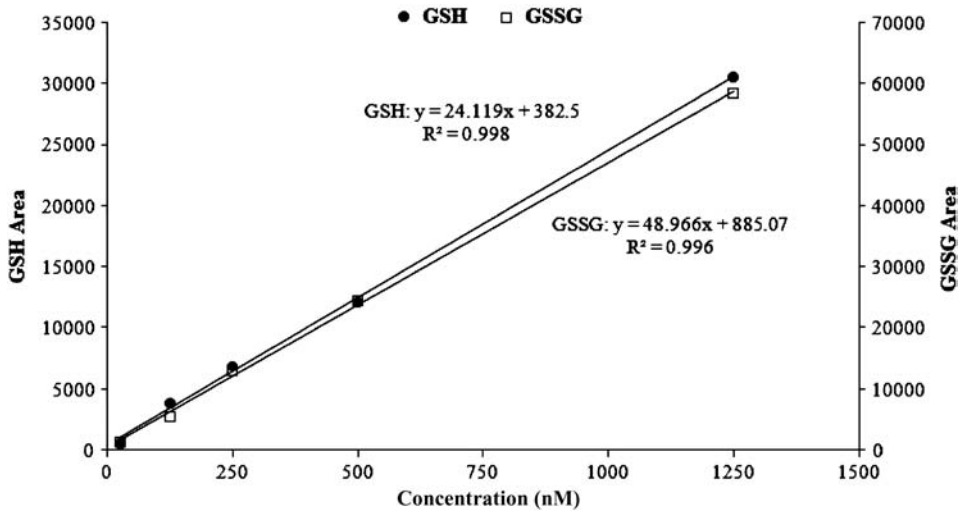

Figure 1. Overlay of standard curves of GSH and GSSG. The concentrations range of GSH and GSSG used were identical. The area under the peak of a particular concentration of GSSG is twice the area under the peak of the same GSH concentration.

Table 2. Comparison of GSSG level in liver, lung and brain samples obtained from Sprague–Dawley rats, using enzymatic reduction method and DTT method

Tissue ($n = 3$)	GSH (nmol/mg protein)	Enzymatic reduction method GSSG (nmol/mg protein)	DTT method GSSG (nmol/mg protein)
Liver	48.19 ± 2.97	3.09 ± 0.13	2.44 ± 0.29
Lung	36.16 ± 2.51	6.96 ± 0.22	6.12 ± 0.43
Brain	18.30 ± 0.76	0.32 ± 0.04	0.39 ± 0.06

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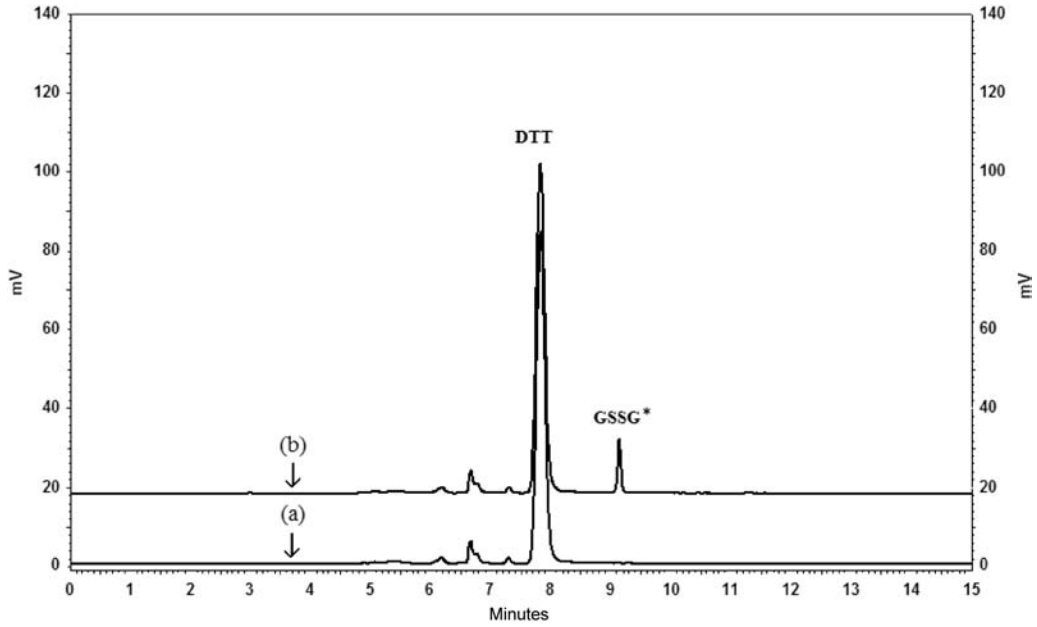

Figure 2. Chromatogram showing GSSG standard treated with DTT and then derivatized with NPM. (a) Chromatogram of DTT only. (b) Chromatogram of 500 nM GSSG treated with DTT. * This peak belongs to newly produced GSH after the reduction of GSSG by DTT.

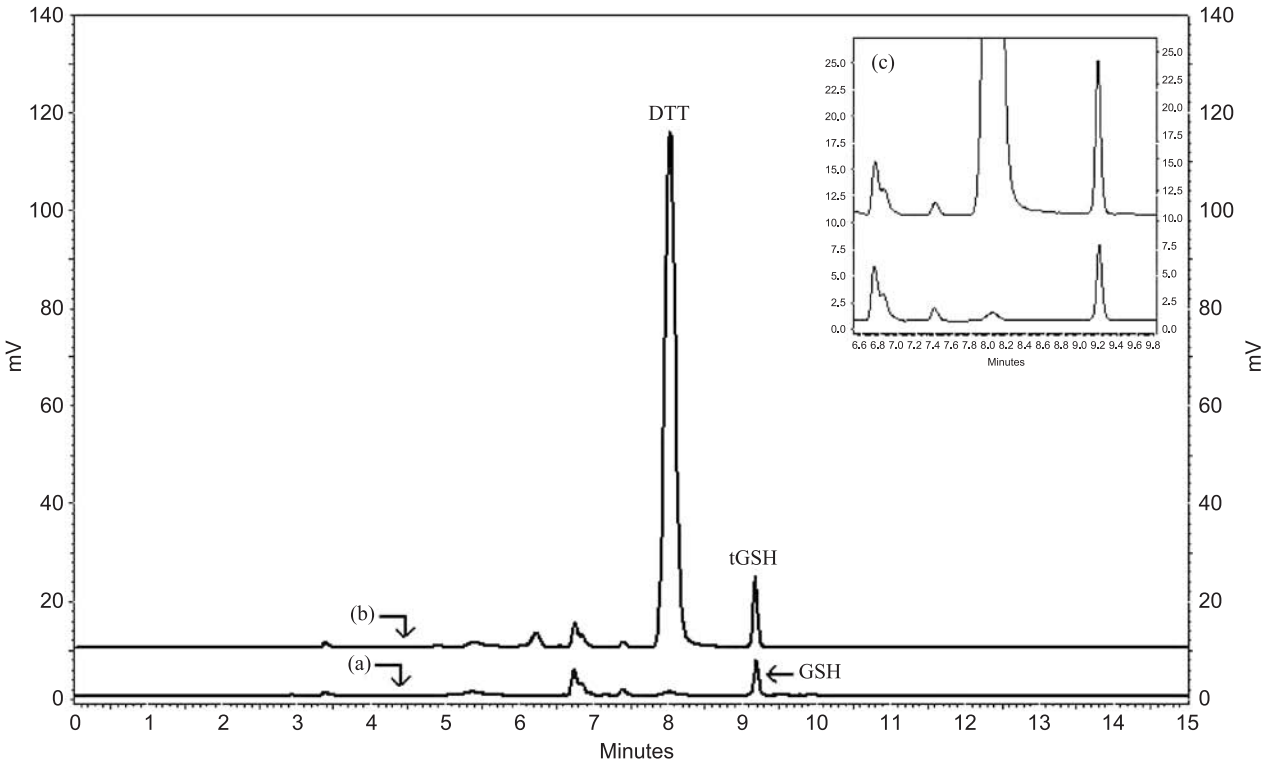

Figure 3. Chromatograms of liver samples obtained from Sprague–Dawley rats. (a) Liver sample derivatized with NPM (GSH assay). (b) Liver sample treated with DTT and then derivatized with NPM (GSSG assay). (c) GSH peaks magnified to highlight change in peak area.

and enzymatic reduction method. This difference is even smaller in lung samples (less than 5%). The slight differences in the values obtained may stem from (1) oxidation of GSH during sample preparation and (2) inadequate blocking of –SH groups by 2-VP.

Accuracy, Precision and Recovery

The coefficients of variation (CV) for inter-run and intra-run precision, the relative deviation for accuracy and relative recovery of the samples spiked with GSSG (125, 250, 500 and 1250 nM) in the

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Table 3. Inter- and intra-run precision, accuracy, and relative recovery of three samples spiked with GSSG (125, 250, 500 and 1250 nM) in sample matrices and standards

Sample matrix ($n = 3$)	Liver	Lung	Brain	Standard (%)
Inter-run precision (%)	2.70–5.36	1.44–4.18	1.88–5.10	0.84–2.92
Intra-run precision (%)	2.60–4.98	1.02–3.26	1.32–3.25	0.49–2.71
Accuracy (%)	2.69–5.43	1.78–5.03	2.47–6.15	2.38–3.35
Percentage relative recovery	97.3 ± 5.6	100.8 ± 3.7	103.2 ± 4.8	N/A

sample matrix (liver, lung, and brain) and standards are shown in Table 3. The CVs for intra-run precision and inter-run precision ranged from 0.49 to 5.10% and the relative deviations for accuracy ranged from 1.78 to 6.15%; the percentage of relative recovery ranged from 97.3 to 103.2%.

Sensitivity

The LOD and LOQ were determined by analyzing three replicates of blank samples without the interference of noise (S/N = 3:1 and S/N = 10), respectively. The LOQ of GSSG by this method was found to be 10 nM. The detection limit determined by this technique was found to be 2.5 nM (signal-to-noise = 3) with 5 μL injection volume.

Conclusion

Numerous GSSG detection methods have been reported in scientific literature, many of which involve the enzymatic reduction method, in which GSSG is reduced by GR in the presence of NADPH. Several of these methods have certain disadvantages such as use of toxic chemicals like 2-VP, requirement for expensive chemicals like NADPH and lengthy experimental protocol. Considering the need for large-scale screening studies involving biological samples, we have modified and developed a rapid, sensitive and reproducible HPLC method with fluorescence detection, using the common and inexpensive thiol-exchanging agent, DTT. With our new method, we have cut the cost of sample preparation from 39 cents per sample to only 0.13 cents, in addition to having decreased sample preparation time by 30 min. Further, it has been reported previously that several steps involved in sample preparation using the GSH method, such as acidification and deproteinization using chemicals like trichloro acetic acid (TCA), may contribute to oxidation of GSH and overestimation of disulfides (Rossi *et al*., 2002). Our method minimizes sample preparation steps and, therefore, better reflects the true values of GSH and GSSG. With these advantages, we believe this method can be extensively used in future studies.

Acknowledgements

The authors would like to thank Barbara Harris for carefully editing this manuscript. B. Ates was supported by the Turkish Scientific Technical Research Council. This work was also supported by NIH (R15DA023409-01A2).

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