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Optimization stacking by transient pseudo-isotachophoresis for capillary electrophoresis: example analysis of plasma glutathione

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Abstract

Low concentrations of both reduced form glutathione (GSH) and oxidized form glutathione (GSSG) in diabetic nephropathy (DN) patient's plasma were measured with transient pseudo-isotachophoresis. The plasma samples were deproteined with acetonitrile and centrifuged. The method was performed at constant voltage of 5 kV using a 300 m*M* borate buffer (pH 8.0), with a fused-silica capillary of 21 cm×75 μm. The sample length can reach 25% of the efficient length of the capillary, and the sensitivities of GSH and GSSG increased 15–20-fold. The method was also systematically optimized, and the results show that this type of stacking offers good repeatability for routine clinical assay of glutathione in DN plasmas.

Keywords: Transient pseudo-isotachophoresis; Stacking; Glutathione

1. Introduction

Glutathione (G), the major thiol in almost all cells and tissues, is found to be related to a variety of biological processes [1] and diseases, such as diabetes [2], and chronic renal failure [3], to name a few. The ratio of reduced form glutathione (GSH) to oxidized form glutathione (GSSG) is also a useful biological marker of cellular oxidative stress. Therefore, a powerful analytical method for measuring of both GSH and GSSG in biological fluids simultaneously would potentially benefit clinical diagnosis at the early stages of disease.

Unfortunately, measurement of G in plasma with capillary electrophoresis (CE) is not straightforward, since G lacks a strong chromophore and the concentration of glutathione in plasma is about μmol/l. To obtain lower detection limits, sensitivity flow cell [4], chemical derivatization [5–8] or electrochemical detection [9,10] was chosen. Stacking techniques, however, are also a useful and simpler way for improving the detection limits.

The acetonitrile–salts-stacking technique, which was introduced by Shihabi [11–15], has the advantage of stacking biosamples, which contained higher concentration of salts and proteins than the desired compounds and always deproteined with acetonitrile.

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The technique permits a larger injection volume (filling about 10–30% of the capillary volume to the detector) than in the classical capillary zone electrophoresis (CZE) mode (sample volume was within 1% of the capillary volume to the detector). The likely mechanism of the technique was proposed to be a transient pseudo-isotachophoresis (tp-ITP) procedure [13]. Shihabi had used this method to determine glutathione in dog myocardial tissue and red blood cells [12].

In our work, the tp-ITP method, coupled with CZE, was used to determination low concentration of G in diabetic nephropathy (DN) patient's plasma. The influences of sample matrix, buffer pH and concentration, applied voltage and capillary temperature on stacking and separation were systematically investigated. Under the optimum condition, about 25% of the efficient capillary length (inlet to the detector) was injected with sample, and the sensitivities of GSH and GSSG increased 15–20-fold.

2. Materials and methods

2.1. Chemicals

Glutathione (both reduced and oxidized forms) was purchased from Sigma (St. Louis, MO, USA). Borate, sodium chloride and acetonitrile (HPLC-grade agents) were purchased from Tianjin Chemical Reagent Company (Tianjin, China). All the solutions and samples were prepared in redistilled water daily and filtered through a 0.22-μm filter before use.

2.2. Instrumentation

All CE experiments were performed on a Beckman P/ACE MDQ system (Beckman, Fullerton, CA. USA) equipped with a UV–Vis detector, an autosampler and a temperature controller (15–60±0.1 °C). Instrument control and data analysis were carried out by Beckman P/ACE system software (Ver. 2.3) on a personal computer. For pH measurements, a pH meter (pHS-25, Weiye, Shanghai, China) calibrated with a precision of 0.01 pH unit was employed. Centrifugation was performed on a Biofuge status (Heraeus, Germany).

2.3. Preparation of plasma samples and standard samples

The plasma samples, obtained from the DN patients who were diagnosed by classical clinical method, were immediately stored at −20 °C. The plasma samples were deproteined with acetonitrile at the rate of 7:3 (v/v) (acetonitrile–plasma) and then centrifuged for 15 min at 10 000 rpm. The supernatants were injected directly into the CE system.

Standard stock solutions of GSH (3.6 m*M*) and GSSG (1.9 m*M*) were prepared quantitatively with water and stored at 4 °C until use. The working standard solutions were used after the dilution of stock solutions, and finally contained 20 m*M* pH 8.0 borate buffer, 45 m*M* sodium chloride and 70% (v/v) acetonitrile (or as specified).

2.4. Electrophoresis procedure

All the separations were performed on a fused-silica capillary of 31 cm (effective length 21 cm)×75 μm I.D. (Yongnian Photoconductive Fibre Factory, Hebei, China), using a 300 m*M* borate buffer (pH 8.0, the counter ion was sodium ion) as the stacking and separation buffer, at 20 °C with a constant voltage of +5 kV. The samples were injected in pressure mode at inlet (0.5 p.s.i. for 30 s or as specified) (1 p.s.i.=6894.76 Pa). The detection wavelength was set to 200 nm. Before each run, the capillary was rinsed with borate buffer at 20 p.s.i. for 1 min to equilibrate the capillary inner wall.

3. Results and discussion

As a new useful method, tp-ITP has the advantage of permitting the injection of large volume of biosamples, which would contain high concentration of salt. However, several parameters affected the real stacking result. In this section, the stacking and separation conditions are systematically optimized, and the mechanism of the procedure also discussed.

3.1. Effects of sample matrix in tp-ITP

The conditions of the sample matrix (such as concentration of NaCl and ACN) were important for

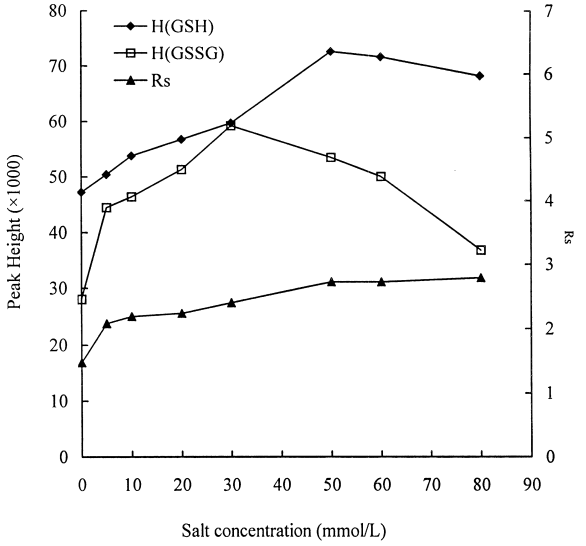

Fig. 1. Influence of salt concentration in samples on peak height and R_s. The samples were injected in the pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH 8.0), voltage: +5 kV, temperature: 25 °C, detected at 200 nm.

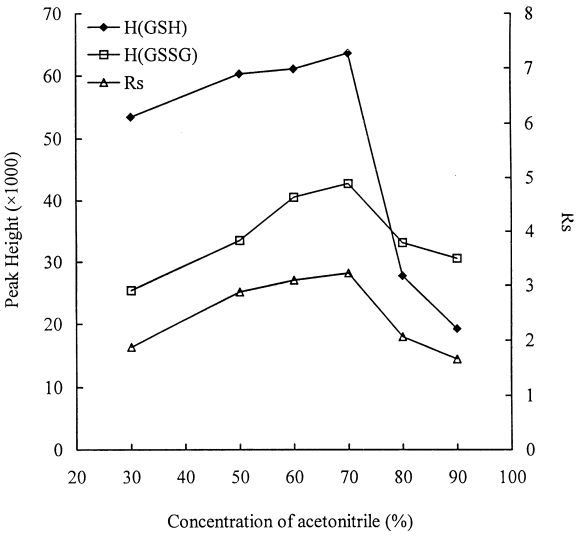

Fig. 2. Effect of acetonitrile concentration in samples on peak heights and R_s. The samples were injected in the pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH 8.0), voltage: +5 kV, temperature: 25 °C, detected at 200 nm.

gaining the tp-ITP phenomena. Absence of either NaCl or ACN would destroy the stacking. Fig. 1 shows the influence of salt concentration in sample on the peak height (H) and resolution (R_s) between GSH and GSSG. A salt concentration range of 30–50 m*M* was considered to be favorable for stacking both GSH and GSSG (the H and R_s in this range differed little from each other). It was reported that the total salt concentration of plasma was about 150 m*M* [15]. When the DN plasma samples were treated as described in Section 2.3, the salt concentration in the treated plasma was about 45 m*M*, which was just in the favorite range of the optimized salt concentration. This permitted the advantage of directly injecting the treated plasma without the need to add additional salts to the treated plasma sample or dilution. The concentration of ACN in the sample was adjusted from 30 to 90% (v/v), and the influences of ACN concentration in sample on the H and R_s are shown in Fig. 2. An acetonitrile concentration of 70% was selected as the best concentration for the highest H and R_s values. The likely mechanism was: the Cl^- of NaCl in the sample, which was in high concentration and had a high mobility, moved quickly ahead of the analytes as a wide band and acted as leading ions [13]; ACN, provided a high field strength for its low conductance [13], may play the role as a traditional terminating ion, or the borate ion, which has the lowest mobility may also act as terminating ion; GSH and GSSG migrated behind the leading ion (Cl^-) as sharp bands being pushed by the high field strength of the terminating ions.

Since the injection times (volumes) directly influenced the sensitivity, a larger-volume injection would be appreciated for assay of lower concentration samples. However, in the CZE mode, separation was destroyed as the injection volume increased. When it came to the tp-ITP mode, even when 60% of the capillary was filled with the sample, the separation result was really sound. Fig. 3 shows the effects of the injection time on migration times, R_s and theoretical plate number (N). When the sample zone became longer, the conductance of the whole fluid in the capillary became lower, which decreased the electric field intensity of both the sample zone and the background electrolyte (BGE), as a result, the velocity of the electroosmotic flow (EOF) decreased and the migration times of GSH and GSSG were delayed. The decreasing N and R_s

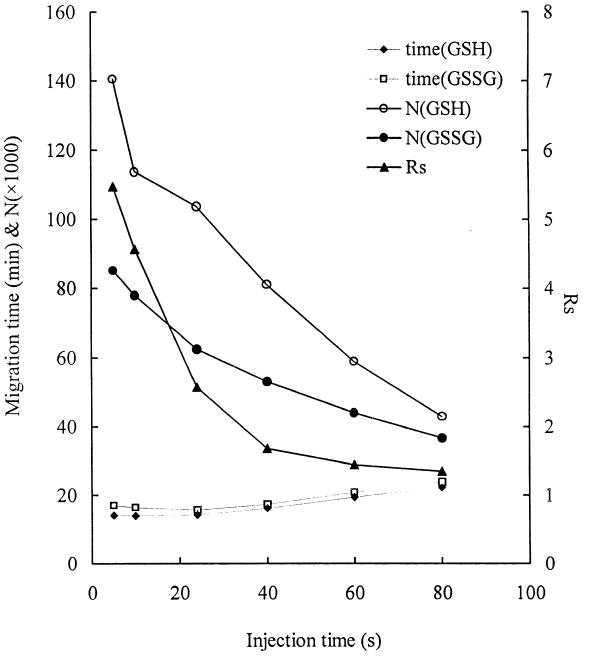

Fig. 3. Effect of injection time on stacking. The samples were injected in pressure mode (from 0.3 p.s.i. for 3 s to 0.5 p.s.i. for 80 s) to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH 8.0), voltage: +5 kV, temperature: 25 °C, detected at 200 nm.

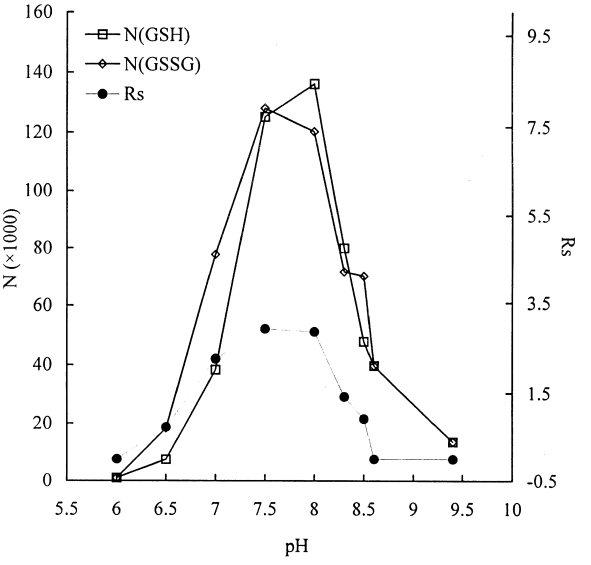

Fig. 4. Effect of pH on *N* and R_s. The samples (contain 30 m*M* sodium chloride, 70% acetonitrile and 20 m*M* borate buffer of a corresponding pH to the BGE) were injected in the pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate, voltage: +5 kV, temperature: 25 °C, detected at 200 nm.

was mainly caused by the decreasing efficient capillary length (the results of increasing sample length). Finally, an injection time of 30 s (at 0.5 p.s.i.) was decided for plasma sample analysis, as the interference of the unknown compounds in plasma sample was limited and a better sensitivity achieved.

3.2. Influence of buffer pH and concentration on stacking

Fig. 4 shows the influence of pH on *N* and R_s. It was found that when the pH of BGE was lower (higher) than 6.5 (8.5), stacking would not appear properly, and that, in the pH range of 7.0–8.0, GSH and GSSG could be well stacked and separated (N=140 000 and R_s=3.0). The pH 8.0 buffer was chosen for its capacity for larger injection volume.

BGE concentration varied from 40 to 350 m*M* (Fig. 5). The results indicated that stacking would not take place properly at low BGE concentration (<100 m*M*). When the BGE concentration became higher (from 100 to 300 m*M*), stacking phenomenon appeared. However, when the buffer concentration was higher than 300 m*M*, the electric current (the capillary was filled with BGE) did not increase linearly and the Joule heat broadened the peak and reduced the *N* and R_s (Fig. 5). Finally, a concentration of 300 m*M* BGE, which had high R_s and *N*, was selected for further studies.

3.3. Effect of voltage and capillary temperature in tp-ITP

A voltage of 5 kV seemed to be best for stacking (Fig. 6). When the voltage is low (1–3 kV), the velocities of the analytes in BGE and EOF were too small, the migration times were delayed (above 100 min at 1 kV), and the diffusion ruined the separation. As the voltage became higher (10–15 kV), the EOF became larger and the migration time decreased quickly (2.5 min at 15 kV). And the times needed for

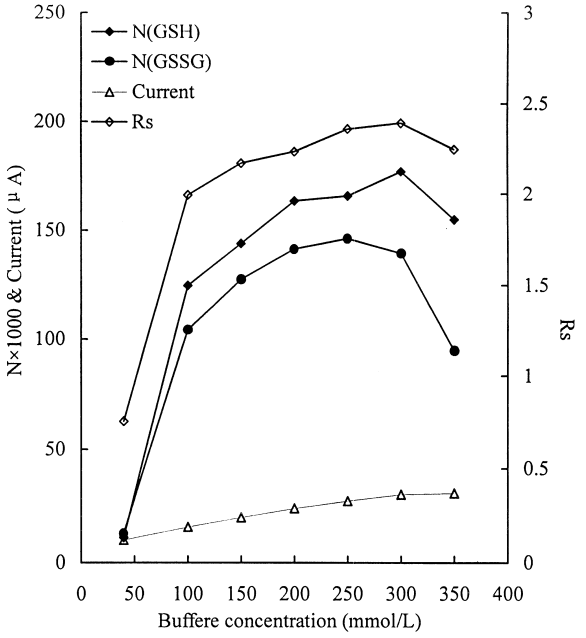

Fig. 5. Effect of buffer concentration on stacking. The samples were injected in pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: borate (pH 8.0), voltage: +5 kV, temperature: 25 °C, detected at 200 nm.

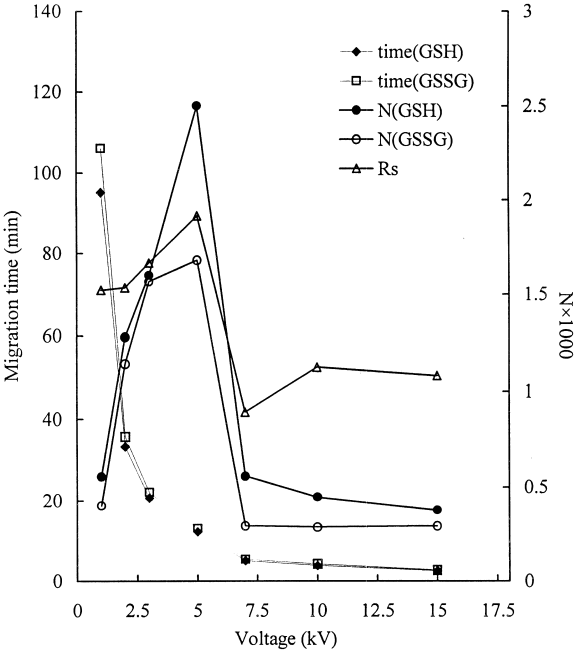

Fig. 6. Effect of running voltage on stacking. The samples were injected in the pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH 8.0), temperature: 25 °C, detected at 200 nm;.

separation step were limited, as a result, the separation was limited. The capillary temperature range of 15.5 to 20 °C was found to have the highest *N* and R_s values (Fig. 7). However, the temperature of 15.5 °C is close to the limit of the instrument supply (15 °C), which causes method unstablity (RSDs of the migration time of either GSH or GSSG were above 2.0%). Finally, the capillary temperature of 20 °C was selected for its better ruggedness (see Table 1).

3.4. Analysis of standard sample

A standard mixture of GSH (180 μmol/l) and GSSG (95 μmol/l) was analyzed under the optimum conditions (Fig. 8a). It indicated that, even when 25% of the efficient capillary length was filled with the mixture, GSH and GSSG were well stacked and separated within 15 min with a higher *N* (240 000) and R_s (3.4).

3.5. Reproducibility, calibration and limit of detection

Based on the good separation results, six replicates of the standard samples were used to test the repeatability of the method. Linearity ranges, using area as the function of the concentration, were obtained with analyzing the diluted stock standards (the injection time was set to 30 s at 0.5 p.s.i.). Limits of detection (LODs) of GSH and GSSG were tested at $S/N=5$. The assay results are shown in Table 1.

3.6. Assay of the DN plasmas

Fig. 8 shows the electropherograms of standard and DN plasma sample under the optimum conditions. It was found that the migration times of GSH and GSSG in plasma sample (Fig. 8b) are similar to that of the standard (Fig. 8a). The plasma sample

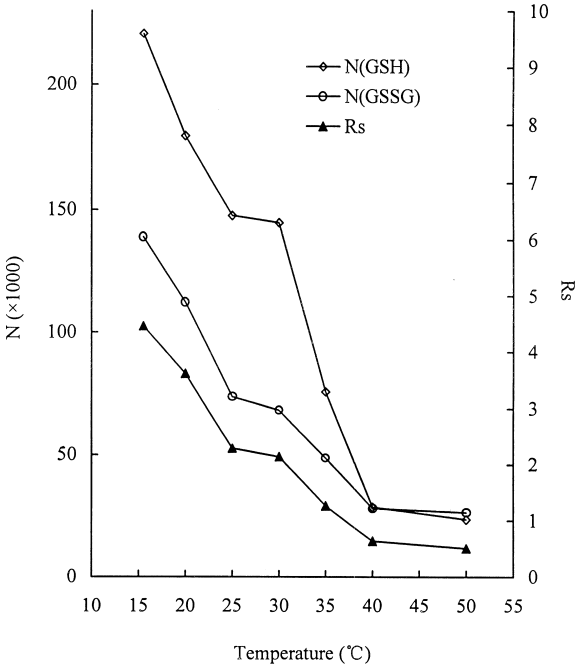

Fig. 7. Effect of capillary temperature on stacking. The samples were injected in the pressure mode at 0.5 p.s.i. for 24 s to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH 8.0), voltage: +5 kV, detected at 200 nm.

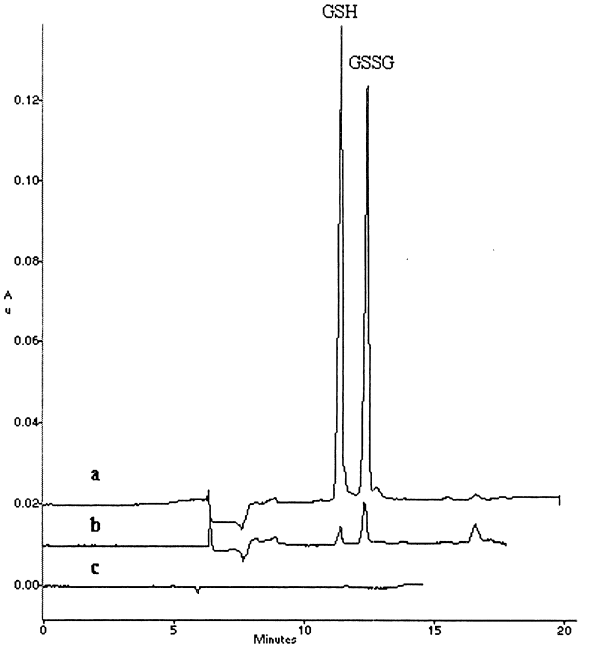

Fig. 8. Electropherograms of standard (a) and DN plasma sample. The sample was treated as described in Section 2.3 and were injected in the pressure mode at 0.5 p.s.i. for 30s (25%) (b) or 0.3 p.s.i. for 3 s (1%) (c) to a fused capillary (21 cm×75 μm I.D.), buffer: 300 m*M* borate (pH8.0), voltage: +5 kV, temperature: 20 °C, detected at 200 nm.

was also injected in the CZE mode (0.3 p.s.i., 3 s, about 1% of the efficient capillary length, Fig. 8c), as expected, both GSH and GSSG could not be found. Obviously, the detection limit was greatly proved using the tp-ITP method. The analysis results of the plasma were 6.3±0.8 and 8.8±0.5 μmol/l (n=3) for GSH and GSSG, respectively. The analysis results of plasma indicated that, the ratio of GSH to GSSG was 0.72, which was much lower than the normal value (higher than 54). This result proved that the abnormal level of oxidative stress, which may be caused by the high concentration of glucose, would harm the renal system of the DN patient.

Table 1
Characteristics of the method

	GSH	GSSG
Time (RSD, %) (n=6)	0.66	0.26
Height (RSD, %) (n=6)	1.6	2.2
Area (RSD, %) (n=6)	1.3	1.8
Linearity range (μmol/l)	1–90	1–60
r^2	0.9985	0.9993
LOD (μmol/l)	0.5	0.3

The samples (contain GSH: 180 μmol/l, GSSG: 95 μmol/l, 30 m*M* sodium chloride, 70% acetonitrile and 20 m*M* borate buffer of a corresponding pH to the BGE) were injected in the pressure mode at 0.5 p.s.i. for 30 s to a fused capillary (21 cm×75 μm I.D.), BGE buffer: 300 m*M* borate (pH 8.0), voltage: +5 kV, temperature: 20 °C, detected at 200 nm.

4. Conclusion

The tp-ITP method was applied as a new way to determine lower concentrations of GSH and GSSG in DN plasma. The parameters that influenced the stacking and separation were systematically investigated and optimized. Good repeatability and linearity were also obtained. It is hoped that the method presented here will be suitable for routine plasma assays in clinical study and helpful for therapy of DN.

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