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On-column preconcentration of glutathione and glutathione disulfide using pH-mediated base stacking for the analysis of microdialysis samples by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) has become a useful analytical tool for the analysis of microdialysis samples. However, CE with UV detection (CE-UV) does not provide detection limits sufficient to quantify glutathione (GSH) and glutathione disulfide (GSSG) in biological samples such as liver microdialysates, because of the small optical path length in the capillary. To overcome this limitation, an on-column preconcentration technique, pH-mediated base stacking, was used in this study to improve the sensitivity of CE-UV. This stacking technique allowed large volumes of high ionic strength sample injection without deterioration of the separation efficiency and resolution. A 26-fold increase in sensitivity was achieved for both GSH and GSSG using the pH-mediated base stacking, relative to normal injection without stacking. The limit of detection for GSH and GSSG was found to be 0.75 μM ($S/N = 6$) and 0.25 μM ($S/N = 6$), respectively. The developed method was used to analyze GSH and GSSG in liver microdialysates of anesthetized Sprague Dawley male rats. The basal concentrations of GSH and GSSG in the liver microdialysates of male rats were found to be 4.73 ± 2.08 μM ($n = 7$) and 5.52 ± 3.66 μM ($n = 7$), respectively.

Keywords: Capillary electrophoresis; Glutathione; Glutathione disulfide; pH-mediated stacking; On-column preconcentration; Microdialysis samples

1. Introduction

Glutathione is a thiol found intracellularly at high concentrations (1–10 mM) [1] and is also present in small amounts in the extracellular fluid. Glutathione exists as reduced glutathione (GSH) and in an oxidized form as glutathione disulfide (GSSG). GSH is a tripeptide of glycine, glutamate, and cysteine and GSSG is a dimer of GSH, where two GSH molecules are linked through a disulfide bond. A deficiency of glutathione is thought to be associated with a variety of diseases, such as cancer, neurodegenerative disorders, cystic fibrosis, lung diseases, HIV, and liver diseases [1,2]. One of the important functions of GSH in biological systems is antioxidant activity where GSH is oxidized to GSSG as it scavenges reactive oxygen species [2–4]. Therefore, the simultaneous determination of GSH and GSSG in biological fluids, such as microdialysates, is important since the ratio of GSH to GSSG concentration may be used as a biomarker of oxidative stress [2,5].

High performance liquid chromatography (HPLC) has been used widely for the analysis of thiols and disulfides in biological samples [6–10], but this separation technique requires a sample volume in the microliter range. Capillary electrophoresis (CE), however, uses only a few nanoliters of sample and is therefore a good choice to couple to microdialysis sampling which produces samples of only a few microliters total volume. A few papers have reported the analysis of biologically important thiols in microdialysis samples using CE with fluorescence and electrochemical (EC) detection [11,12].

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UV detection is widely used in CE since it is easy to operate and assemble to the CE system. However, it has poor concentration detection limits because of the small optical path length (25–75 μm) limited by the inner diameter of the capillary and small sample injection volumes (∼nL). To improve the detection limits of UV detection, the analytes can be chemically derivatized by UV labeling agents with strong UV absorption [13]. EC detection offers lower detection limits, but the integration of an EC detection cell with an electrophoretic separation system can be problematic because the separation current has to be separated effectively from the detection circuit [14,15]. Laser-induced fluorescence (LIF) detection offers excellent detection limits, but requires chemical derivatization with fluorescent labels when the analytes do not have native fluorescence.

A simple way to improve the sensitivity of CE with UV detection (CE-UV) without using any chemical derivatization is to use an on-column preconcentration technique so that a larger sample volume may be injected onto the capillary without losing separation efficiency and resolution. The commonly used on-column preconcentration techniques [16–19] are field-amplified stacking [20,21], large-volume sample stacking [22,23], pH-mediated stacking [24–27], transient isotachophoresis (t-ITP) [28–30], transient pseudo-isotachophoresis (tp-ITP) [31,32], and dynamic pH junction [33,34].

pH-mediated stacking has proven to be a simple and useful on-column preconcentration technique for the analysis of high ionic strength samples, such as microdialysates [24–27]. Using this technique, a large volume of high ionic strength sample can be injected directly into the CE capillary without prior dilution or any sample pretreatment. Acid stacking is used for cationic analytes and base stacking for anionic analytes. Both stacking techniques have been used successfully to increase the sensitivity of CE-UV for a variety of analytes in high ionic strength matrices [24–27]. A 66-fold enhancement in sensitivity has been reported using the pH-mediated base stacking method for the analysis of anions, such as *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and syringic acid [25].

Both GSH and GSSG are anions at physiological pH, hence, base stacking was used in this study. In base stacking, the EOF is reversed using a cationic surfactant in the BGE and the separation is performed with reverse polarity in order to obtain electromigration of anions and EOF in the same direction. The background electrolyte (BGE) consists of salt of a weak base (e.g. NH_4^+). A large volume sample injection is followed by the injection of a strong base. This results in the titration of BGE cations (e.g. ammonium ions in NH_4^+/NH_3 buffer) by hydroxyl ions and creates a low conductivity sample zone where anions move faster and stack at the interface of the sample and highly conductive background electrolyte [25,27].

This paper describes the optimization of a CE-UV method with pH-mediated base stacking for the simultaneous analysis of GSH and GSSG in high ionic strength sample matrix and the application of the developed method to quantify GSH and GSSG in the liver microdialysates of anesthetized male Sprague Dawley rats.

2. Materials and methods

2.1. Materials

Reduced glutathione (∼98%), glutathione disulfide (∼98%), and tetradecytrimethylammonium bromide (99%) were purchased from Sigma-Aldrich Company (St. Louis, MO). All other compounds were reagent grade or better. Distilled-deionized-water (Water Pro Ps, Labconco, Kansas City, MO) was used in the preparation of all solutions. The anesthetics (Isoflurene, Xylazine, and Ketamine) used for the animal studies were supplied by the Animal Care Unit at the University of Kansas.

2.2. Sample preparation

The CE background electrolyte was ammonium buffer and consisted of 100 mM ammonium chloride with 0.5 mM tetradecyltrimethylammonium bromide (TTAB) adjusted to pH 8.4 with 0.1 M sodium hydroxide solution. The Ringer's solution was composed of 155 mM NaCl, 5.5 mM KCl, and 2.3 mM $CaCl_2$ at pH 7.4. Both GSH (10 mM) and GSSG (5 mM) stock solutions were prepared in BGE. GSH stock solution was prepared daily and GSSG stock solution was prepared weekly and stored in the refrigerator. Standard solutions of GSH and GSSG were prepared daily from their stock solutions by multiple dilutions with Ringer's solution. Buffer and Ringer's solutions were bubbled with Argon gas for 20 min to remove dissolved oxygen. All solutions were filtered through a 0.22 μm pore size syringe filter (Millipore Millex™ GP, Fisher Scientific) prior to use. The microdialysis samples were analyzed without any pretreatment.

2.3. CE-UV apparatus

A lab-built CE system with a SpectraPhysics UV1000 UV detector (Thermoseparation, San Jose, CA) was used for this study. Polyimide coated fused silica capillary (Polymicro Technologies, Phoenix, AZ) with 50 μm i.d. and 360 μm o.d. was cut to a total length of 60 cm long with a 45 cm effective length. The analysis was performed in reversed EOF mode using a cationic surfactant, TTAB, in the background electrolyte. Both GSH and GSSG were detected on-column by UV absorbance at a wavelength of 214 nm. A voltage of −10 kV was applied across the capillary using a high voltage power supply unit (CZE1000R, Spellman High Voltage Electronics, Hauppauge, NY, USA) to drive the electrophoresis. All sample injections into the capillary were made electrokinetically at −10 kV, and analyses

were performed at ambient temperature. Data acquisition was through an PCI-MIO-16XE-50 A/D computer card and programming was performed in-house using LabView software (National Instruments, Austin, TX).

2.4. Microdialysis sampling

A CMA/100 microinjection pump purchased from CMA/Microdialysis AB, (Stockholm, Sweden) was used to deliver Ringer's solution at a flow rate of 1 μL/min flow rate through the microdialysis probe. Linear probes of 10 mm active length were made from polyimide tubing with 127 μm o.d. and 100 μm i.d. (MicroLumen Inc., Tampa, FL) and Spectra/Por® in vivo microdialysis hollow fibers of regenerated cellulose (Spectrum, Rancho Dominguez, CA) with a 216 μm o.d., a 200 μm i.d., and a molecular weight cut-off of 18,000 Daltons. Probe calibration was performed by no net flux (NNF) experiment in vivo [35].

2.5. Surgical procedure

Prior to the surgery, male Sprague-Dawley rats (350–400 g weight) were pre-anesthetized using Isoflurene and then fully anesthetized by intra-muscular injection of ketamine (100 mg/kg dose) and xylazine (10 mg/kg dose) mixture. Additional doses of ketamine (1/4 of original dose) were injected as needed to keep the rats anesthetized throughout the entire experiment. The anesthetized rats were placed on top of a heated pad to maintain body temperature during the surgery and sampling. The liver was exposed after the abdominal incision. The linear probe was implanted in the liver and perfused with Ringer's solution at a flow rate of 1 μL/min in all experiments. The implanted probe was flushed with Ringer's solution for 30 min before collecting microdialysate at 10 min intervals. The concentrations of GSH and GSSG in collected microdialysates were monitored for 60–120 min to achieve steady basal levels.

3. Results and discussion

3.1. Optimization of sample and base injection length

One way to improve the sensitivity of UV detection is to inject a large volume sample. However, this leads to peak broadening or destacking unless employing an on-column preconcentration technique. Fig. 1 shows that both sensitivity and resolution are very low for GSH and GSSG with 3 s and 30 s sample injections for samples in Ringer's solution. Normal injection without stacking does not provide sufficient detection limits for the analysis of GSH and GSSG in biological samples even when injecting large volume samples (e.g. 30 s sample injection). The poor sensitivity and separation efficiency are the results of the small optical path length (50 μm) in the capillary and the destacking phenomena of high ionic strength samples. Sample destacking occurs when the sample zone has a higher conductivity than that of the BGE. The high ionic strength samples have high conductivity and charged analytes migrate slowly in the sample zone. During the CE separation, the analytes migrate faster when they enter the BGE zone from the sample zone, thus resulting in sample destacking or band broadening. The extent of destacking is higher with the longer sample injection times and higher ionic strength samples [24,25,27].

Sample injection followed by 0.1 M NaOH injection (pH-mediated base stacking) increased the sensitivity and efficiency of both GSH and GSSG (Fig. 2 and Table 1). The amount of base to be injected into the capillary is dependent on the amount of sample injected. Hence, sensitivity increases as a function of base injection time until the base amount reaches an optimum. The electrokinetic injection of OH^- results in the titration of NH_4^+ to NH_3, creating a low conductivity sample zone through which the analyte anions migrate faster and stack at the interface of the titrated sample and BGE zones. Peak tailing is the result

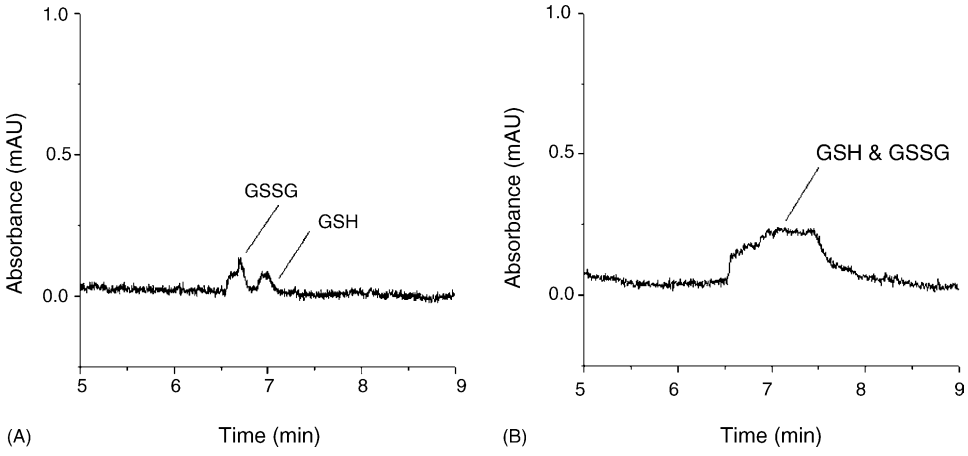

Fig. 1. GSH and GSSG detection without pH-mediated stacking: (A) 3 s electrokinetic (EK) injection of sample (mixture of 10 μM GSH and 10 μM GSSG in Ringer's solution), (B) 30 s EK injection of sample. Conditions: 50 μm i.d. capillary ×60 (45) cm, 100 mM NH_4C1 BGE with 0.5 mM TTAB at pH 8.4, EK injection at −10 kV, separation at −10 kV, and detection at 214 nm.

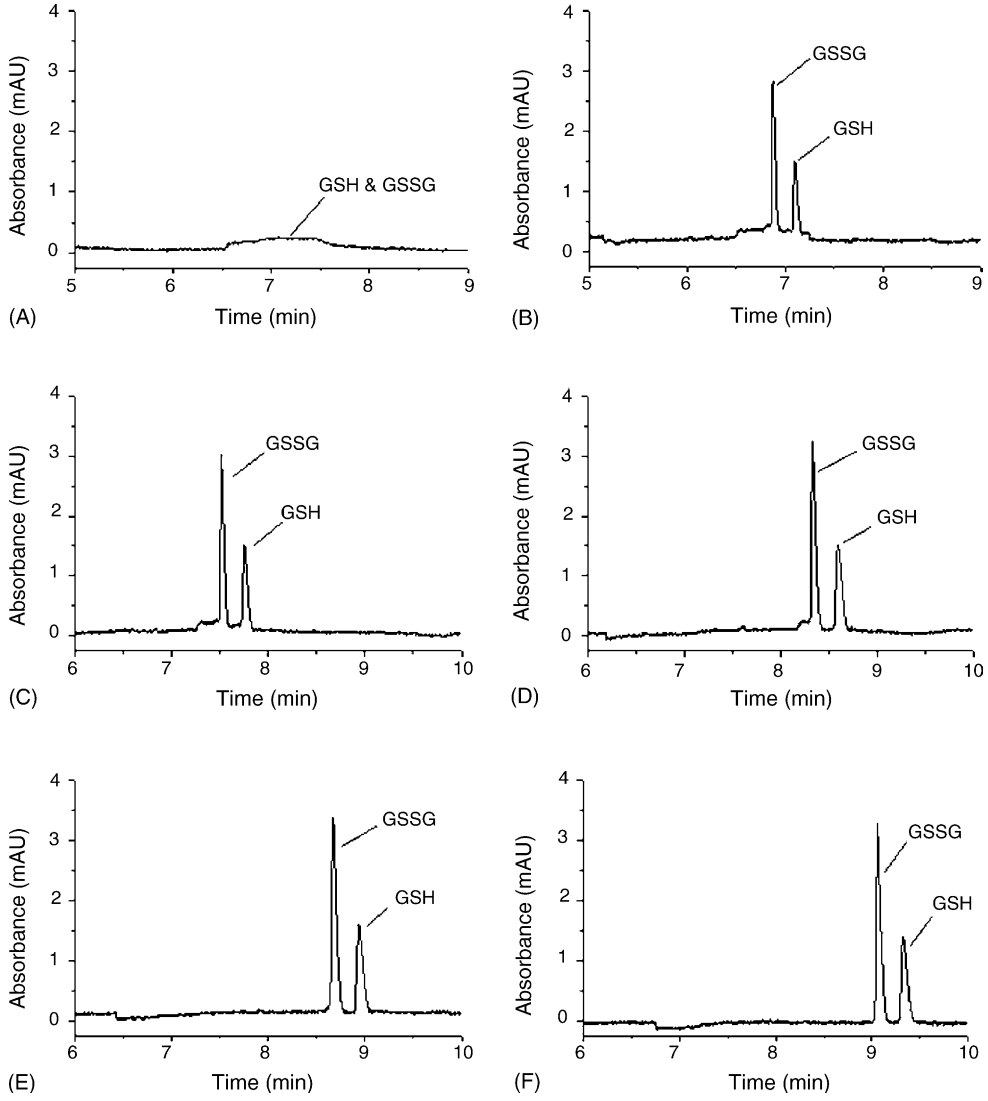

Fig. 2. Optimization of injection ratio with ammonium buffer system: (A) 30 s EK injection of sample (mixture of 10 μM GSH and 10 μM GSSG in Ringer's solution)/0 s EK injection of NaOH, (B) 30 s EK sample /30 s EK NaOH, (C) 30 s EK sample/40 s EK NaOH, (D) 30 s EK sample /50 s EK NaOH, (E) 30 s EK sample/60 s EK NaOH, (F) 30 s EK sample 70 s EK NaOH. Conditions: same as in Fig. 1.

of incomplete titration of the sample zone as an insufficient amount of base relative to sample is injected. It has been shown that too long of a base injection results in the deterioration of the separation resolution because most of the capillary length is used for stacking and only a small portion of the capillary is left for the separation [25].

Table 1
Separation efficiency as a function of injection ratio

Injection ratio (sample/NaOH)	*S/N*		Efficiency (×1000)	
	GSH	GSSG	GSH	GSSG
30/0 s	A broad unresolved peak (Fig. 2A)			
30/30 s	28	56	130	189
30/40 s	30	62	107	236
30/50 s	30	66	90	208
30/60 s	31	69	97	224
30/70 s	30	70	103	201

It has been shown that the optimum base injection time is a function of capillary length, the ionic strengths of sample and BGE, and the analyte electrophoretic mobility [25,27]. Using the ammonium buffer system (100 mM NH_4Cl with 0.5 mM TTAB, pH 8.4) and a 30 s sample injection, sensitivity and efficiency increased with an increase in base injection time up to 60 s. Base injections longer than 60 s did not improve the separation. Analysis of 10 μM GSH and 10 μM GSSG standard in Ringer's solution using the optimized injection protocol of a 30 s sample injection followed by a 60 s injection of 0.1 M NaOH (30/60 s) showed a 26-fold increase in sensitivity for both GSH (Fig. 3A and B) and GSSG (Fig. 3C and D) in comparison to normal electrokinetic injection (3 s injection without stacking).

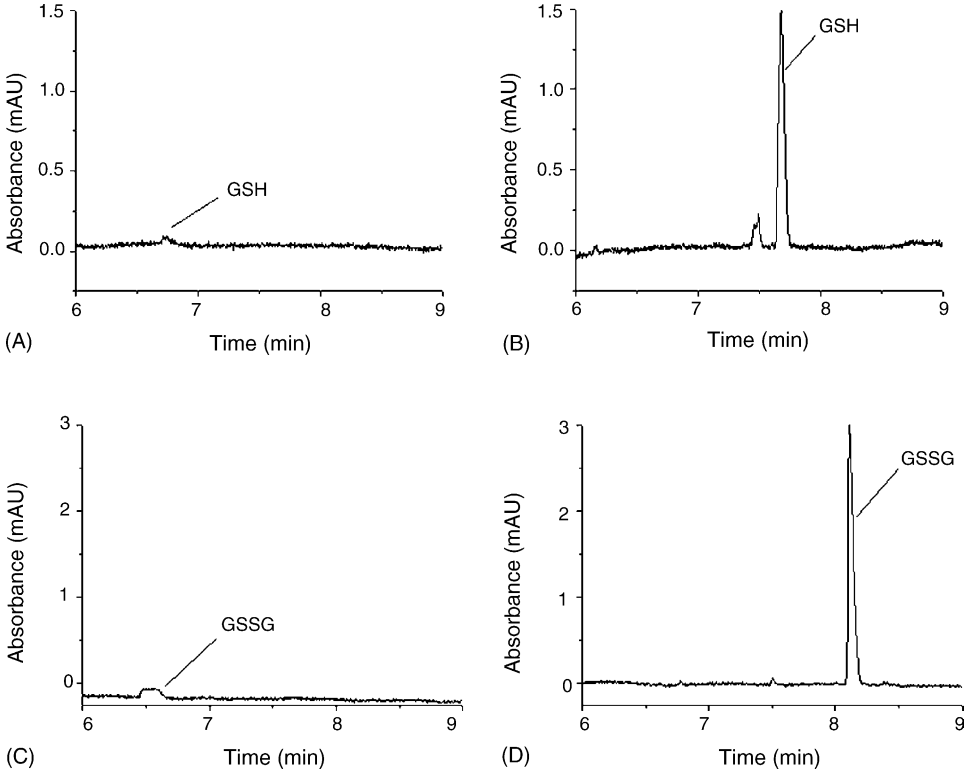

Fig. 3. Effect of pH-mediated base stacking on sensitivity. (A) GSH without stacking; 3 s EK injection of 1 μM GSH in Ringer's solution/0 s EK injection of NaOH, (B) GSH with stacking, 30 s EK 10 μM GSH in Ringer's solution/60 s EK NaOH, (C) GSSG without stacking; 3 s EK 10 μM GSSG in Ringer's solution/0 s EK NaOH, (D) GSSG with stacking; 30 s EK 10 μM GSSG in Ringer's solution/60 s EK NaOH. Conditions: same as in Fig. 1.

3.2. Method validation

The performance of the method in terms of reproducibility of migration time and sensitivity was evaluated by comparing migration time and sensitivity between analyses of 10 μM GSSG standard solutions in the same capillary. The reproducibility of migration time (7.17 ± 0.082 min) and sensitivity (0.257 ± 0.008 mAU/μM) was acceptable, with R.S.D.s below 5% (1.14 and 3.08%, respectively and $n = 12$) for the analysis within the same day. The resolution of GSH and GSSG was found to be 2.53 ± 0.171 ($n = 12$).

The relationship between peak height (mAU) and concentration (μM) for standards prepared in Ringer's solution was evaluated by linear regression analysis. A series of standard solutions of both GSH and GSSG ranging from 0.25 to 80 μM were tested. The limits of detection for the analysis of GSH and GSSG were found to be 0.75 μM ($S/N = 6$) and 0.25 μM ($S/N = 6$), respectively. Electropherograms near the limits of detection of GSH and GSSG are shown in Fig. 4. The response for GSH was linear over the range of 0.75–40 μM ($n = 6$). The response for GSSG was linear over the range of 0.25–50 μM ($n = 7$). The equations for the

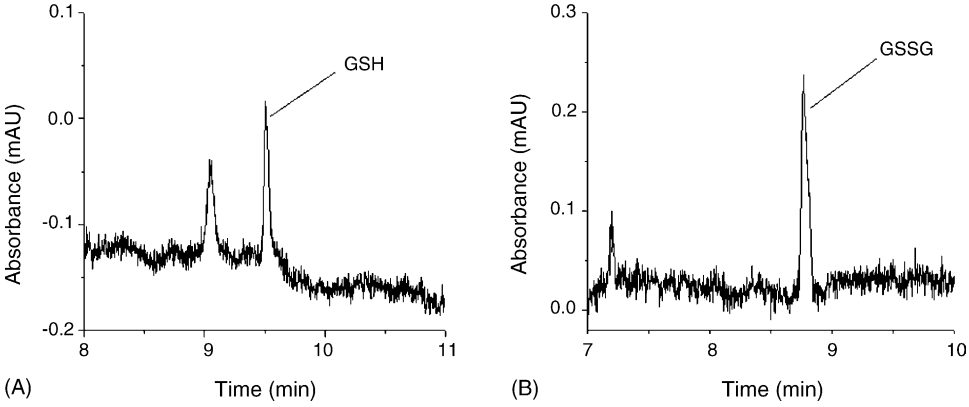

Fig. 4. Electropherograms of GSH and GSSG near the detection limit: (A) 0.75 μM GSH in Ringer's solution, (B) 0.25 GSSG in Ringer's solution. Conditions: same as in Fig. 1.

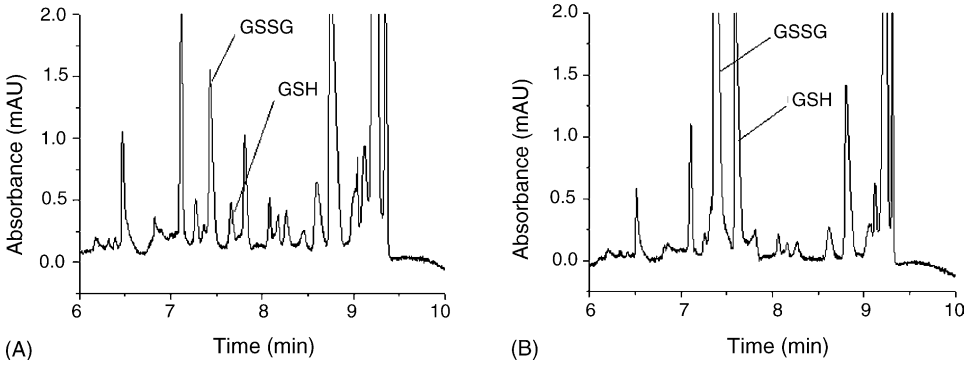

Fig. 5. Electropherograms of unspiked and spiked liver microdialysates. (A) liver microdialysate, (B) spiked liver microdialysate (5 μL microdialysate spiked with 1 μL of 100 μM GSH and 1 μL of 100 μM GSSG standard solutions). Conditions: same as in Fig. 1.

regression lines were $y = 0.130x + 0.012$ ($R^2 = 0.995$) for GSH and $y = 0.252x + 0.127$ ($R^2 = 0.997$) for GSSG.

4. Determination of GSH and GSSG in microdialysis samples

The optimized CE-UV method with pH-mediated base stacking was used to determine the basal concentrations of GSH and GSSG in liver microdialysates of anesthetized Sprague-Dawley male rats. A typical electropherogram of basal rat liver microdialysate is shown in Fig. 5A. The identity of the GSH and GSSG peaks in the electropherogram were confirmed by spiking with standards (Fig. 5). To perform spiking experiments, 5 μL of microdialysate was spiked with 1 μL of 100 μM GSH and 1 μL of 100 μM GSSG standard solutions. The overall peak heights of spiked microdialysate (Fig. 5B) were lowered in comparison to the unspiked microdialysate (Fig. 5A) due to the dilution effect in the spiked sample. The concentrations of GSH and GSSG were determined from GSH and GSSG calibration curves. The recoveries of GSH and GSSG determined by *in vivo* no net flux experiments were 49.5 ± 13.4 % ($n = 7$) and 47.0 ± 2.83% ($n = 7$), respectively. The concentrations of GSH and GSSG in the extracellular space of the liver varied from rat to rat (Table 2). Based on probe calibration results, the average basal concentrations of GSH and GSSG in the liver microdialysates of male rats were found to be 4.73 ± 2.08 μM ($n = 7$) and 5.52 ± 3.66 μM ($n = 7$), respectively. This variation may be due to the variation in the stress levels of different rats. In a previous study using CE with electrochemical detection, the basal concentration of GSH in liver microdialysates of anesthetized male Sprague-Dawley rats was found to be 4.7 ± 1.6 μM [12]. In another study using HPLC with fluorescence detection, the concentration of GSH in rat liver microdialysates was found to be in the range of 4.16–76.5 μM [36].

Table 2
Concentration of GSH and GSSH in rat liver microdialysate

Rat	GSH (μM)	GSSG (μM)
1	7.37 ± 2.04	9.06 ± 0.24
2	2.62 ± 1.37	9.78 ± 1.65
3	5.14 ± 0.24	8.54 ± 3.35
4	3.59 ± 0.25	1.86 ± 0.06
5	3.93 ± 0.10	1.53 ± 0.13
6	7.66 ± 2.21	5.70 ± 1.95
7	2.79 ± 0.11	2.14 ± 0.05

5. Conclusion

A CE-UV method with pH-mediated base stacking has been developed to analyze glutathione and glutathione disulfide simultaneously in high ionic strength sample matrices. This method provides a simple and effective way for the on-column preconcentration and detection of analytes in a single run, and sensitivity to GSH and GSSG detection was increased by 26-fold relative to normal sample injection without stacking. The limits of detection for GSH and GSSG in high ionic strength sample matrices were 0.75 μM ($S/N = 6$) and 0.25 μM ($S/N = 6$), respectively. The method was successfully used to determine GSH and GSSG in liver microdialysates of Sprague-Dawley male rats, and could be employed in the future to monitor the GSH and GSSG concentration change during oxidative stress (e.g. ischemia and reperfusion) for the better understanding of antioxidant activity of GSH.

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