Evaluation of BEH C_{18}, BEH HILIC, and HSS T3 (C_{18}) Column Chemistries for the UPLC–MS–MS Analysis of Glutathione, Glutathione Disulfide, and Ophthalmic Acid in Mouse Liver and Human Plasma

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Abstract

Glutathione (GSH), glutathione disulfide (GSSG), and ophthalmic acid (OA) are important biological oxidative stress biomarkers to be monitored in pathological and toxicological studies. With the advent of liquid chromatography tandem mass spectrometry (LC–MS–MS) technology, sensitive and selective analysis of these biomarkers in biological samples is now being performed routinely. Due to the hydrophilic and polar natures of GSH and its endogenous derivatives, achieving good retention, resolution, and peak shape is often a chromatographic challenge. In this study, three ultra-performance (UP) LC column chemistries (namely, BEH C_{18}, BEH HILIC, and HSS T3 [C_{18}]) are evaluated for the UPLC–MS–MS analysis of GSH, GSSG, and OA extracted from mouse liver and human plasma samples. The chromatographic parameters evaluated are retentivity, tailing factor, MS sensitivity, and resolution of the three analytes. Based on the optimized method for each column chemistry, our results indicate that the HSS T3 (C_{18}) column chemistry affords the best retention and separation of these analytes when operated under the ultra high-pressure chromatographic conditions.

Introduction

Reduced glutathione (GSH, Figure 1A) and its oxidized form, glutathione disulfide (GSSG, Figure 1B) are often used as oxidative stress biomarkers in several human diseases such as cancer, diabetes mellitus, and HIV (1–2). In most of the diseased states, it was often reported that the level of GSH declined while that of GSSG increased. This resulted in a reduced GSH/GSSG ratio, which in turn is an important index of oxidative stress. Hence, many research and clinical groups measure GSH and/or GSSG in their respective oxidative stress-related studies. Recently, Soga et al. (3) conducted a metabolomics study on acetaminophen-

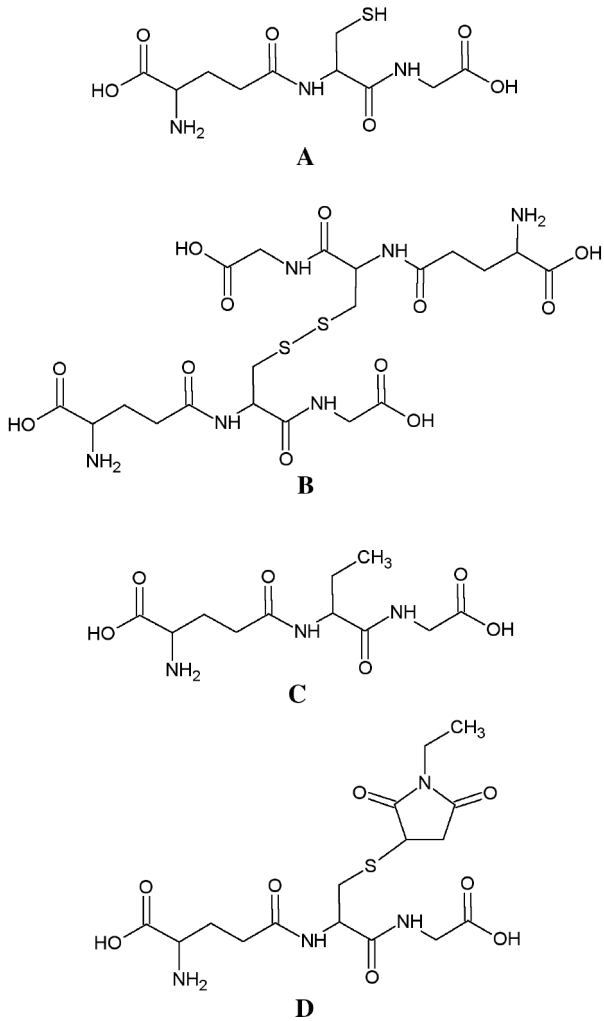

Figure 1. Chemical structures of GSH (A), GSSG (B), OA (C), and GSH-NEM derivative (D).

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induced liver injury and suggested ophthalmic acid (OA, Figure 1C), a GSH endogenous derivative, as a novel oxidative stress biomarker. Hence, the development of an analytical method for the measurement of GSH, GSSG, and OA is paramount. The conventional methods for quantitating these analytes and/or their derivatives include spectrophotometric, fluorometric, bioluminescence, and high-performance liquid chromatography (HPLC) coupled to UV, flurometric, or electrochemical detectors (2). In recent years, these analyses have also been extended to mass spectrometry (MS) coupled with gas chromatography or liquid chromatography (2,4–6). Liquid chromatography–tandem mass spectrometry (LC–MS–MS), in particular, is becoming the method of choice as it averages the sensitivity of tandem MS and the selectivity of chromatography, which improves the accuracy of quantitating low concentrations of GSH and GSSG in blood and tissue samples (7–10). Due to the hydrophilic and polar natures of these analytes, various types of LC column chemistries, including hydrophilic interaction chromatography (HILIC) (5), mixed mode anion exchange (6), ion exchange (7), amide (8), C_{18} (9–10), and amino (11–13) have been explored for the quantitation of GSH and GSSG. The total analysis time of most studies is long (ranging from 10–35 min), except for Steghens et al. (6), who reported a fast 6 min mixed mode chromatography single ion monitoring LC–MS assay for the measurement of GSH and GSSG in whole blood after the derivatization of GSH with N-ethylmaleimide (NEM).

In the present work, three ultra-performance (UP) LC column chemistries; namely, bridged ethylsiloxane/silica hybrid (BEH) C_{18}, BEH hydrophilic interaction chromatography (HILIC), and high strength silica (HSS) T3 (C_{18}) were evaluated and compared for the fast, selective, and simultaneous analysis of GSH, GSSG, and OA. A rapid and sensitive UPLC–MS–MS method was optimized for each column for the determination of GSH, GSSG, and OA in mouse liver and human plasma. To prevent the auto-oxidation of GSH to GSSG during the sample work-up, GSH in mouse liver and human plasma was rapidly derivatized with NEM to form the stable derivative, GSH-NEM (Figure 1D). The NEM derivatization step is important to circumvent the overestimation of GSSG and the underestimation of GSH (6,11–13). The evaluation of the three column chemistries is further discussed in the paper.

Experimental

Chemicals

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Leicestershire, UK) and Tedia Company, Inc. (Fairfield, OH), respectively. Dichloromethane (DCM), AR grade, was purchased from Merck (Merck Pte. Ltd., Singapore). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). GSH, GSSG, NEM, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO). OA was purchased from Bachem (Bubendorf, Switzerland). Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$) with > 99.8% purity was obtained from Duchefa Biochemie B.V. (Haarlem, The Netherlands). All other chemicals and reagents used for the experiments were of analytical grade.

UPLC–quadrupole linear ion trap MS conditions

A Waters ACQUITY UPLC system (Waters, Milford, MA) was interfaced with a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAPMS) equipped with TurboIonSpray ESI source (2000 QTRAP, Applied Biosystems, Foster City, CA). Details of the intermodule communication are described in our previous study (14). In this paper, the influences of ACQUITY UPLC BEH C_{18}, BEH HILIC, and HSS T3 (C_{18}) columns (Waters) on the LC–MS–MS analysis of GSH, GSSG, and OA were investigated. The columns used in the study were all 100 × 2.1 mm i.d., 1.7 µm particle size, with the exception of the HSS T3 (C_{18}) column, which has 1.8 µm particle size. The column heater and sample manager were kept at 60ºC and 4ºC, respectively. As comparisons were performed on three different column chemistries, the LC conditions were optimized differently to achieve the best chromatographic parameters for the analytes. For BEH C_{18} and HSS T3 columns, the flow rate was 0.45 mL/min and the mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The optimized elution conditions are described in Table I. Each wash cycle consisted of 1000 µL of weak wash solvent (100% water) and 1000 µL of strong wash solvent (100% acetonitrile). For the BEH HILIC column, the mobile phases consisted of acetonitrile (solvent A) and 10mM ammonium formate in water, pH 3 (solvent B). The optimized elution conditions are described in Table II. Each wash cycle consisted of 1000 µL of weak wash solvent (100% acetonitrile) and 1000 µL of strong wash solvent (100% water). The injection mode for all analyses was partial loop with needle overfill and the injection volume was 3 µL. All the MS experiments were performed using electrospray positive ionization mode (ESI+). Multiple reaction monitoring (MRM) experiments, using *m/z* transitions of 433.0 to 304.0, 613.0 to 355.0, and 290.1 to 161.1 were performed to profile GSH-NEM, GSSG, and OA in the mouse liver and human plasma samples. The optimized MS conditions for the MRM experiments are summarized in Table III. All data were acquired at unit resolution, and data processing was performed using Analyst 1.4.1 software (Applied Biosystems).

Mouse liver and human plasma sample processing

Whole liver of one male C57BL/6 mouse was harvested, snap-frozen in liquid nitrogen immediately, and kept frozen at –80°C until sample preparation. One hundred twenty milligrams of the liver were accurately weighed out and 600 µL of the derivatizing solution (solution A) was added immediately to the weighed liver in a 2-mL Eppendorf tube and homogenized for 1–2 min (Heidolph Instruments, Schwabach, Germany). Solution A was prepared freshly by mixing NEM and $Na_2EDTA.2H_2O$ in water–methanol (3/2, v/v) and adjusting the pH of the solution to 7.4 with solid $NaHCO_3$. The final concentrations of NEM and $Na_2EDTA.2H_2O$ in solution A were 250mM and 1.5 mg/mL, respectively.

Under these conditions, the auto-oxidation of GSH was prevented during tissue homogenization by the formation of a stable GSH derivative, GSH-NEM, while GSSG and OA remained unchanged. 240 µL of 600 g/L TCA was added to precipitate the protein and the mixture was vortexed at high speed for 20 s. The mixture was then centrifuged at 13,000 rpm for 5 min at 4°C. 750 µL of the supernatant was collected into a clean tube and

subjected to liquid–liquid extraction (LLE) using 2 mL of DCM. The mixture was vortexed at high speed for 1 min and centrifuged subsequently at 13,200 rpm for 8 min at 4°C. 500 µL of the supernatant was collected into a clean Eppendorf tube and evaporated to dryness for 2 h at 35°C under a gentle flow of nitrogen gas (TurboVap LV, Caliper Life Science, Hopkinton, MA). For the purpose of this study, a human plasma sample obtained from the National University Hospital in Singapore was spiked at a final concentration of 5µM each of GSH, GSSG, and OA. 25 µL of solution A were added to 100 µL of the spiked plasma and vortexed gently for 20 s. 25 µL of 600 g/L TCA were added to precipitate the protein, vortex-mixed at high speed for 20 s, and 350 µL of cold water were subsequently added. The mixture was then centrifuged at 13,000 rpm for 5 min at 4°C. 450 µL of the supernatant were collected into a clean tube and subjected to LLE using 1500 mL of DCM. The mixture was vortexed at high speed for 1 min and centrifuged subsequently at 13,000 rpm for 8 min at 4°C. 300 µL of the supernatant were collected into a clean Eppendorf tube and evaporated to dryness for 1.5 h at 35°C under a gentle flow of nitrogen gas using TurboVap LV. For analyses on the BEH C_{18} and HSS T3 (C_{18}) columns, the residue obtained from the liver and plasma samples was reconstituted with 100 µL of water, vortex-mixed, and centrifuged at 13,000 rpm for 5 min. For analyses on the BEH HILIC column, each residue was reconstituted with 100 µL of methanol–acetonitrile (1:3, v/v), vortex-mixed, and centrifuged at 13,000 rpm for 5 min. For all samples, 80 µL of the supernatant were transferred into the HPLC vial, and 3 µL were injected for LC–MS–MS analysis.

Data analysis

For each analysis, the retention time (RT), tailing factor (TF), MS sensitivity expressed as signal-to-noise ratio (S/N), and resolution (R_S) were calculated and compared. The TF was calculated using the following equation (USP method):

$$TF = (a + b) / 2a \qquad \text{Eq. 1}$$

where a is the distance from the leading edge of the peak to the midpoint, and b is the distance from the point at peak midpoint to the trailing edge, both measured at 5% of the peak height. S/N ratios (S/N × 3 standard deviation), which indicate the MS sensitivity of the method, were calculated using Analyst 1.4.1 software. R_S, which defines the amount of separation between two adjacent peaks, was calculated using the following equation:

$$R_S = (t_{R2} - t_{R1}) / (1/2)\ (W_1 + W_2) \qquad \text{Eq. 2}$$

where t_{R1} and t_{R2} are the elution times of the first and second peak, respectively; while W_1 and W_2 are the widths of the first and second peaks at the base line, respectively.

Table I. Optimized Elution Conditions for BEH C18 and HSS T3 (C18) Columns: Curves 3, 6, and 9 Represent Convex, Linear, and Concave Gradients, Respectively

Time (min)	%B	Curve
0.00	0.1	–
2.00	2.0	9
3.50	30.0	3
3.51	95.0	6
4.79	95.0	6
4.80	0.1	6
6.00	0.1	6

Table II. Optimized Elution Conditions for BEH HILIC Column; Curve 6 Represents a Linear Gradient

Time (min)	%B	Curve
0.00	5	–
3.50	50	6
3.51	95	6
5.49	95	6
5.50	5	6
12.00	5	6

Results and Discussion

The UPLC BEH C18 column (Figure 2A) incorporates trifunctional ligand bonding chemistries on the 1.7 µm BEH particles that produces stability over a wide pH range. The BEH chemistry utilizes new endcapping processes that ensure good peak shape for basic analytes. On the other hand, the UPLC BEH HILIC column contains the 1.7 µm unbonded BEH particles as shown in Figure 2B. As unbonded silica stationary phases often used in HILIC are prone to dissolution at neutral pH, the BEH HILIC overcomes this problem by affording greater chemical stability. The BEH HILIC column may be advantageous in retaining and separating very polar basic compounds, alternating selectivity as compared to reversed-phase chromatography, increasing ESI–MS sensitivity, and facilitating sample preparation. The new UPLC HSS T3 column incorporates 100% silica particles (Figure 2C) and was designed for use in applications up to 15000 psi. The HSS T3 (C18) 1.8 µm bonded phase was fabricated to retain and separate small,

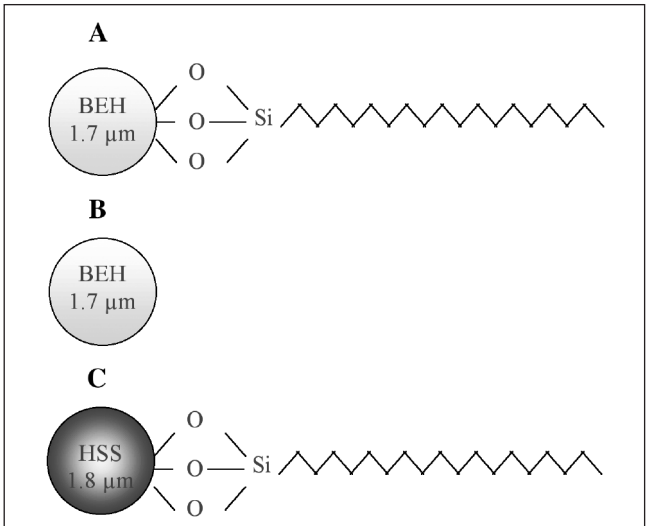

Figure 2. Illustrations of BEH C18 (A), BEH HILIC (B), and HSS T3 (C_{18}) (C) column chemistries.

water-soluble polar organic compounds. GSH, GSSG, and OA are highly polar organic compounds containing basic moieties, and we predicted that the three UPLC column chemistries may be applicable in their own ways to retain and separate them. It is intriguing from an application perspective to explore and understand the influence of the three column chemistries on the chromatographic behaviors of these polar analytes.

Retentivity

As shown in Figures 3 and 4, GSH-NEM derivative, GSSG, and OA were generally well-retained on all three columns for the mouse liver samples (RT > 1 min). For human plasma samples, similar observations were made on the retentivity of these compounds (Figure 5). The RTs typically ranged from 1.3 to 3.0 min. While the retentivities of these compounds were not significantly different among the three columns, it was apparent that BEH C_{18} and HSS T3 (C_{18}) column chemistries afforded a higher level of retention for GSH-NEM derivative, while BEH HILIC rendered better retention for both GSSG and OA. Between HSS T3 (C_{18}) and BEH C_{18} column chemistries, the former provided a more superior retention of GSSG and OA. One notable difference in the retention of these compounds lay in the elution sequence of these analytes on the BEH HILIC column as compared to the BEH C_{18} and HSS T3 (C_{18}) columns. In the BEH HILIC column, GSH-NEM was eluted first, followed by OA and GSSG (Figure 4). Over a decade ago, Alpert (15) first described HILIC in which a hydrophilic stationary phase is eluted with a more hydrophobic mobile so that RTs increase with the hydrophilicity of the solutes. HILIC is a variant of normal-phase chromatography, in which the presence of water in the mobile phase is crucial for the establishment of a stagnant enriched layer on the surface of the stationary phase, into which the analytes may selectively partition. Retention involves a mixed mode mechanism involving both partitioning and cation-exchange mechanisms. Our observations made on the UPLC BEH HILIC column were not unexpected, as the polar hydrophilic GSSG and OA eluted after the less polar GSH-NEM derivative. On the other hand, as expected, OA was eluted first, followed by GSSG and GSH-NEM on both the BEH C_{18} and HSS T3 (C_{18}) columns. This clearly demonstrated the difference in the selectivities of HILIC and C_{18} column chemistries in the analysis of GSH-NEM, GSSG, and OA. This difference in chromatographic selectivity is important for the UPLC analysis of polar compounds to meet different application needs. However, for the BEH HILIC column, it was also observed during method development that while the gradient time required for separation of the analysis was short (3.5 min), the equilibration time had to be sufficiently long (more than 6 min for 100 × 2.1 mm i.d. column) to achieve good RT reproducibility and peak shape of the analytes. This explains the common use of isocratic separations for the HILIC columns and the relatively longer analysis time.

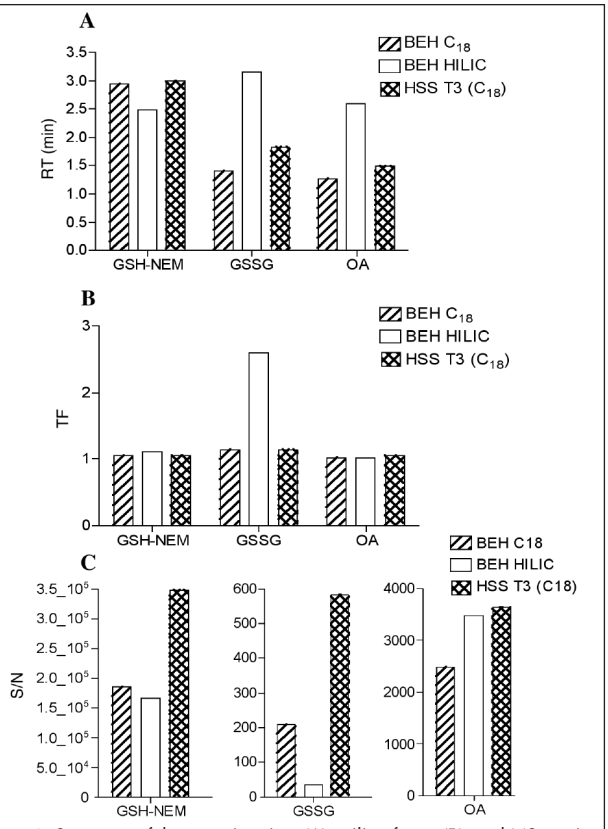

Figure 3. Summary of the retention time (A), tailing factor (B), and MS sensitivity (C) for the BEH C_{18}, BEH HILIC, and HSS T3 (C_{18}) columns. All calculations were made based on data obtained from the mouse liver samples.

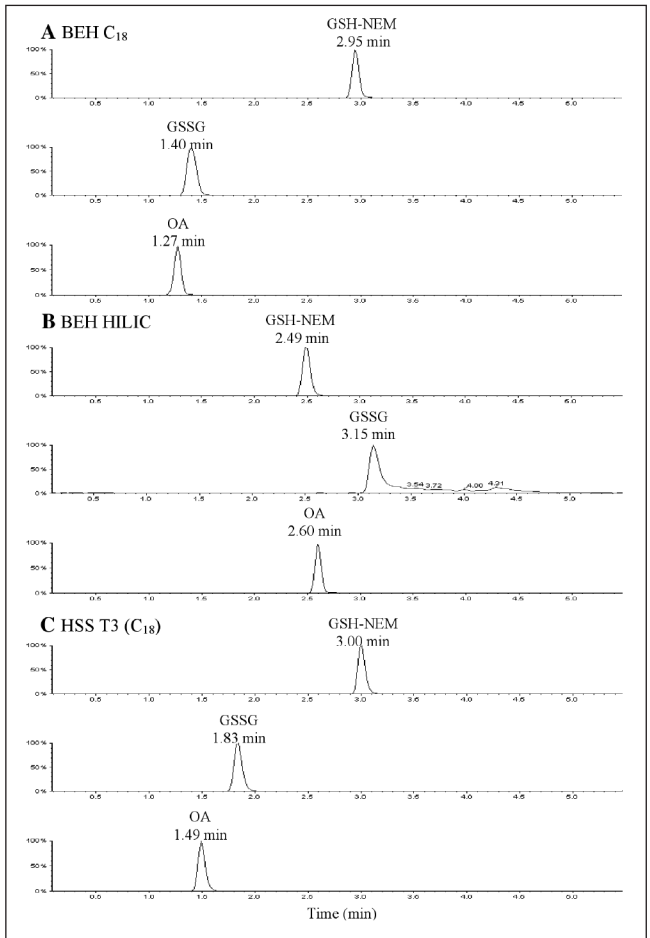

Figure 4. Extracted chromatograms of GSH-NEM, GSSG, and OA in the mouse liver sample as analyzed on the BEH C_{18} (A), BEH HILIC (B), and HSS T3 (C_{18}) (C) columns.

Tailing factor

The tailing factor (TF) for GSH-NEM and OA on all three columns was approximately 1, demonstrating that there was hardly any peak tailing for these two analytes. As compared to other HPLC studies (5,9–10), all UPLC columns in general afforded superior peak resolution (average peak width of approximately 0.2 min) and shape with minimal peak tailing. This is the advantage of using sub-2 µm particles for LC separation, as the peak resolution and shape were enhanced significantly. However, for GSSG, the tailing was prominent (Figures 3 and 4) on the BEH HILIC column (TF = 2.60), while no significant tailing was observed on both the BEH C_{18} and HSS T3 (C_{18}) columns. As peak tailing compromises the accurate measurement of an analyte and negatively affects the sensitivity of the assay, BEH HILIC chemistry is not suitable for analyzing this series of polar compounds, particularly GSSG.

Sensitivity

Among the three columns, HSS T3 (C_{18}) afforded the best MS sensitivity for GSH-NEM, GSSG, and OA with the highest S/N ratios (Figure 3). As compared to the BEH C_{18} column, the higher sensitivity obtained on the HSS T3 (C_{18}) column may be due to the greater retention of the analytes, leading to higher MS ionization efficiency with increasing organic content of the mobile phase. One will expect the sensitivity of BEH HILIC to be the best because the analysis comprised of a highly organic mobile phase, which was expected to enhance the ESI ionization efficiency of the analytes. However, to obtain good peak shape and avoid non-retention on the HILIC column, the sample matrix needs to have a high organic content. Hence, in this study, the dried residues of the mouse liver and human plasma were reconstituted in a highly organic solvent mixture consisting of methanol–acetonitrile (1/3 v/v). However, GSH-NEM, GSSG, and OA are polar hydrophilic compounds and were found not to be fully solubilized when reconstituted with the highly organic solvent mixture. As such, the MS sensitivity of the BEH HILIC assay was greatly compromised. This was prominent for GSSG in the human plasma sample (Figure 5), where the peak was below the limits of detection on the column. Chromatographers should be mindful of this possible limitation of the BEH HILIC column chemistry in the analysis of GSH and its derivatives in biological samples.

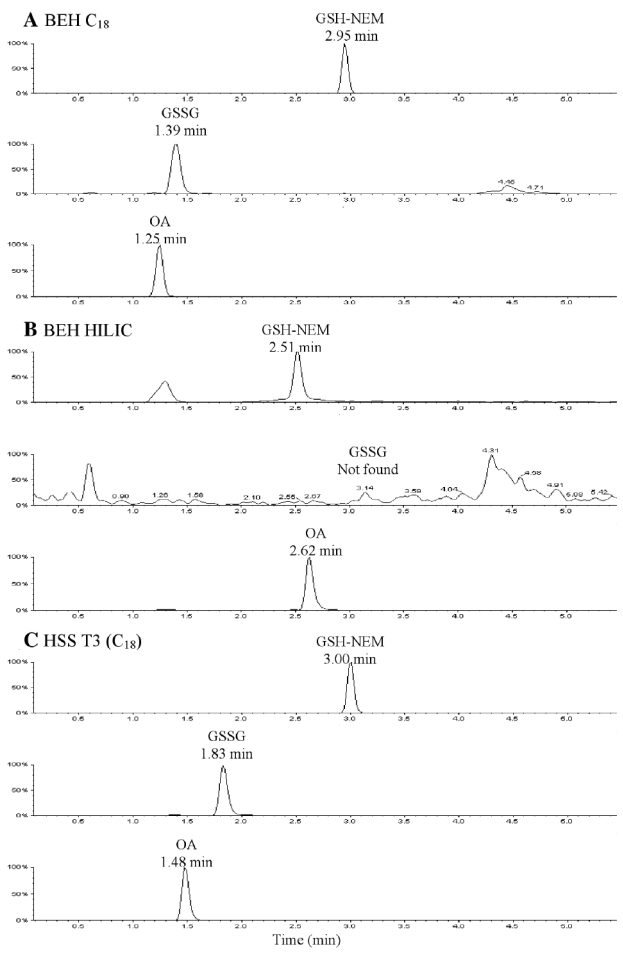

Figure 5. Extracted chromatograms of GSH-NEM, GSSG, and OA in the human plasma sample as analyzed on the BEH C_{18} (A), BEH HILIC (B), and HSS T3 (C_{18}) (C) columns.

Resolution

For a measurable separation to occur and to allow good quantitation, the minimum R_S value should be 1.5, while values of 1.7 or higher are generally desirable for robust methods. On the BEH C_{18} column, the R_S between the GSSG/GSH-NEM pair was more than 1.7, indicating good resolution. On the other hand, poor resolution was observed for GSSG and OA (R_S = 0.88). Hence, we confirmed that while the BEH C_{18} column was able to retain the three analytes, it failed to resolve the polar hydrophilic GSSG and OA. On the contrary, the R_S values among the pairs of GSH-NEM/GSSG and GSSG/OA were excellent (R_S > 1.7) for the HSS T3 (C_{18}) column. This was probably due to the accentuated retention of the polar analytes on the HSS T3 (C_{18}) column, leading to an improvement in the chromatographic resolution. For the BEH HILIC column, the R_S were good ($R_S \geq 1.5$), except the for the

Table III. Optimized MS Parameters for the Detection of GSH-NEM, GSSG, and OA

	Value		
Parameter	**GSH-NEM**	**GSSG**	**Ophthalmic acid**
Curtain gas (psi)	15	15	15
IonSpray voltage (V)	5000	5000	5000
Temperature (°C)	500	500	500
Gas 1 (psi)	60	60	60
Gas 2 (psi)	65	65	65
Interface heater	ON	ON	ON
CAD gas	Medium	Medium	Medium
Declustering potential (V)	60	60	45
Entrance potential (V)	8.50	8.50	5
Collision energy (V)	20	20	15
Collision cell exit potential (V)	3.50	3.50	3.00
Collision cell entrance potential (V)	35.05	29.11	16.00
Dwell time (ms)	250	350	350

GSH-NEM/OA pair (R_S = 0.8). As shown in Table II, a linear gradient profile was adopted for the separation of the analytes on the BEH HILIC column. During the method development, various gradient profiles have been investigated on this column, and unlike BEH C_{18} and HSS T3 (C_{18}) columns, it was found that this parameter had no significant impact on the separation of the analytes, especially GSH-NEM and OA. A similar phenomenon was also observed by Iwasaki et al. (5), where GSH-NEM was poorly resolved from the internal standard, γ-glutamyl glutamic acid, and GSSG. While some scientists may argue that poor resolution is not an issue in selective LC–MS–MS quantitative experiments, one cannot rule out the possibility of ion suppression due to co-elution of analytes. Ion suppression may in turn lead to poor accuracy and reproducibility of the assay. Hence, it is still paramount to achieve good chromatographic resolution whenever possible.

Conclusion

While all three UPLC columns chemistries were found to be suitable for the analysis of GSH-NEM, GSSG, and OA, the state-of-the-art HSS T3 (C_{18}) column proved to be most suitable for their analysis in terms of chromatographic retentivity, peak shape, MS sensitivity, and resolution. The optimized UPLC–MS–MS method is fast, selective, sensitive, and able to analyze GSH-NEM, GSSG, and OA simultaneously. As demonstrated by the analysis of these compounds in the mouse liver and human plasma samples, the novel HSS T3 (C_{18}) UPLC–MS–MS assay is potentially amenable for the quantitative bioanalysis of GSH-NEM, GSSG, and OA in an in vivo study. In the immediate future, the assay may be extended to measure other polar endogenous metabolites and is potentially applicable in oxidative stress-related metabolomics studies.

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