# Validated HPLC-UV Method for the Analysis of Glutathione and its Impurities

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## Background

Compounding pharmacies serve an important role for the specific needs of patients that cannot be met by FDA-approved drug products. However, patients have been harmed by inappropriately compounded drugs in the past. FDA works with the compounding community to continue to develop an appropriately balanced approach to regulation. As such, FDA is working to develop the 503A and 503B bulk drug substance lists for compounders to use.

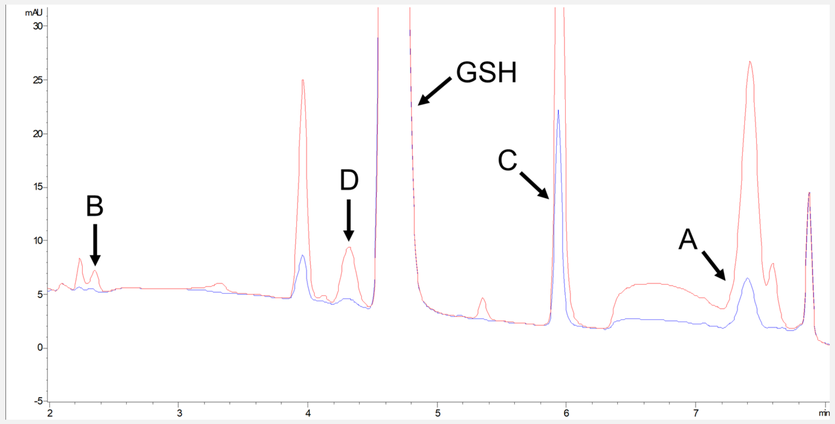
Glutathione (GSH) is a publicly nominated bulk drug substance to be evaluated for the 503B bulks list. GSH has become increasingly administered in compounding regimens. Unfortunately, several incidences of toxicity have occurred in recent years due to contaminated glutathione in compounded drugs. Current validated analytical methods to assess GSH substances and products are time and reagent-intensive and focus only on the detection of GSH and one impurity. We sought to develop an improved HPLC-UV method to assay and measure four related impurities in GSH bulk drug substances.

## Objectives

- To develop and validate a rapid, sensitive HPLC-UV method to detect and quantify glutathione and its four impurities A-D.
- To assay and measure impurities in commercial GSH bulk drug substances.

## Identification of Analytes

**Figure 1.** Analytes were identified in HPLC-UV spectra at 1x (blue) and 10x (red) test concentrations.




Following method development and optimization, single analyte and pooled analyte samples were prepared for GSH and impurities A-D at concentrations ranging from 2.5 µg/mL to 2.5 mg/mL for GSH and from 2.5 to 25 µg/mL for impurities. Appropriate test concentrations were determined to be 2.5 mg/mL for GSH and 2.5 µg/mL (1x) and are displayed in **Figure 1** along with 10x concentrations. Resolution factors ($R_f$, **Table 1**) and relative retention times (RRT, **Table 2**) were calculated from retention times (RT) for each analyte to account for potential chromatographic shifts.

**Table 1.** Resolution factors for each analyte in GSH reference standard samples.

| Analytes | $R_f$ |
|---|---|
| Impurity B to Impurity D | 20.1 |
| Impurity D to GSH RS | 3.5 |
| GSH RS to Impurity C | 15.3 |
| Impurity C to Impurity A | 15.3 |

**Table 2.** Relative retention times for each analyte in GSH reference standard samples.

| Analytes | RT | RRT |
|---|---|---|
| GSH | 4.7 | 1.0 |
| Impurity A | 7.4 | 1.6 |
| Impurity B | 2.3 | 0.5 |
| Impurity C | 5.9 | 1.3 |
| Impurity D | 4.3 | 0.9 |

## Method Validation

The method was validated successfully for precision, linearity, range, limit of quantitation, and accuracy for GSH (**Table 3**) and all four impurities (**Table 4**).

**Table 3.** Validation results for GSH. Acceptance criteria are shown in parentheses.

| GSH | |
|---|---|
| **Precision** | |
| **System (n=6) (RSD ≤ 2%)** | 0.1 |
| **Intra-Day (n=6) (RSD ≤ 2%)** | 1.1 |
| **Intermediate (n=12) (RSD ≤ 5%)** | 1.2 |
| **Linearity** | |
| **Range (mg/mL)** | 0.05 – 2.5 |
| **$R^2$ ($R^2$ ≥ 0.995)** | 0.997 |
| **LOQ (mg/mL)** | 0.04 |
| **Accuracy (Spike Recovery %)* - (98.0 – 102.0% & RSD ≤ 5%)** | |
| **80% Spike Level (n=3)** | 101.7 ± 0.7 (RSD = 0.2) |
| **100% Spike Level (n=3)** | 100.4 ± 1.0 (RSD = 1.0) |
| **120% Spike Level (n=3)** | 100.2 ± 0.3 (RSD = 0.3) |

**Table 4.** Validation results for GSH impurities A-D. Acceptance criteria are shown in parentheses.

| | Impurity A | Impurity B | Impurity C | Impurity D |
|---|---|---|---|---|
| **Precision** | | | | |
| **System (n=6) (RSD ≤ 5%)** | 3.3 | 0.0 | 4.3 | 2.5 |
| **Intra-Day (n=6) (RSD ≤ 10%)** | 3.8 | 3.3 | 8.4 | 2.7 |
| **Intermediate (n=12) (RSD ≤ 10%)** | 4.8 | 3.6 | 5.4 | 3.8 |
| **Linearity** | | | | |
| **Range (µg/mL)** | 1.25 – 3.75 | 1.25 – 3.75 | 1.25 – 3.75 | 1.25 – 3.75 |
| **$R^2$ ($R^2$ ≥ 0.98)** | 0.994 | 0.994 | 0.992 | 0.994 |
| **LOQ (µg/mL)** | 0.69 | 0.73 | 0.81 | 0.69 |
| **Accuracy (Spike Recovery %)* - (80.0-120.0% & RSD ≤ 10%)** | | | | |
| **80% Spike Level (n=3)** | 94.4 ± 5.1 (RSD = 5.4) | 118.5 ± 6.7 (RSD = 5.7) | 98.7 ± 3.4 (RSD = 3.4) | 101.9 ± 2.2 (RSD = 2.2) |
| **100% Spike Level (n=3)** | 89.8 ± 7.0 (RSD = 7.8) | 109.5 ± 5.8 (RSD = 5.3) | 97.6 ± 3.8 (RSD = 3.9) | 109.8 ± 0.0 (RSD = 0.0) |
| **120% Spike Level (n=3)** | 93.6 ± 5.6 (RSD = 5.9) | 85.9 ± 4.5 (RSD = 5.2) | 95.3 ± 3.5 (RSD = 3.7) | 92.8 ± 2.0 (RSD = 2.1) |

## Method Implementation for Bulk Substances

The method was implemented to test concentrations of GSH and impurities A-D in four commercially available GSH bulk substances labeled 1 through 4. $R_f$ (**Table 5**) and RRT values (**Table 6**) were comparable between the GSH reference standard samples and the bulk substance samples.

**Table 5.** Resolution factors for each analyte in GSH bulk substance samples.

| | GSH Bulk Substance Sample | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Analytes | $R_f$ | $R_f$ | $R_f$ | $R_f$ |
| Impurity B to Impurity D | 19.4 | 21.5 | 18.0 | 21.8 |
| Impurity D to GSH RS | 3.5 | 2.7 | 3.3 | 2.6 |
| GSH RS to Impurity C | 12.3 | 15.8 | 13.1 | 15.7 |
| Impurity C to Impurity A | 14.3 | 15.6 | 14.4 | 25.0 |

**Table 6.** Relative retention times for each analyte in GSH bulk substance samples.

| | GSH Bulk Substance Sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | |
| Analytes | RT | RRT | RT | RRT | RT | RRT | RT | RRT |
| GSH | 4.6 | 1.0 | 4.8 | 1.0 | 4.6 | 1.0 | 4.8 | 1.0 |
| Impurity A | 7.1 | 1.5 | 7.8 | 1.6 | 7.1 | 1.5 | 7.8 | 1.6 |
| Impurity B | 2.2 | 0.5 | 2.2 | 0.5 | 2.2 | 0.5 | 2.2 | 0.5 |
| Impurity C | 5.8 | 1.2 | 6.2 | 1.3 | 5.8 | 1.2 | 6.2 | 1.3 |
| Impurity D | 4.2 | 0.9 | 4.5 | 0.9 | 4.2 | 0.9 | 4.5 | 0.9 |

The four GSH bulk substances were then tested for GSH (**Table 7**) and GSH impurity A-D (**Table 8**) concentrations. Total impurity concentrations are displayed in **Table 9**. All four drug substances passed assessment for assay and impurity given previously defined USP criteria which specify that GSH measured concentrations must range from 98.0 to 102.0% of the prepared concentration, impurity C must comprise no more than 1.5% of the total measured GSH concentration, and total impurities must comprise no more than 2.0% of the total measured GSH concentration.

**Table 7.** Assay results for each GSH bulk substance sample.

| GSH Bulk Substance Sample | Assay ± STD (%) (RSD%) |
|---|---|
| 1 | 100.2 ± 1.3 (RSD = 1.3) |
| 2 | 99.9 ± 3.8 (RSD = 3.8) |
| 3 | 100.1 ± 1.3 (RSD = 1.3) |
| 4 | 98.7 ± 0.5 (RSD = 0.5) |

**Table 8.** Individual impurity results for each GSH bulk substance sample.

| GSH Bulk Substance Sample | Impurity | Impurity Test Result ± STD (%) |
|---|---|---|
| 1 | A | 0.06 ± 0.00 (RSD = 2.4) |
| | B | 0.08 ± 0.01 (RSD = 8.7) |
| | C | 0.25 ± 0.00 (RSD = 0.5) |
| | D | 0.23 ± 0.00 (RSD = 1.0) |
| 2 | A | 0.09 ± 0.01 (RSD = 6.2) |
| | B | < 0.008 (below LOD) |
| | C | 0.55 ± 0.00 (RSD = 0.7) |
| | D | < 0.008 (below LOD) |
| 3 | A | 0.02 ± 0.00 (RSD = 10.0) |
| | B | 0.02 ± 0.00 (RSD = 10.4) |
| | C | 0.37 ± 0.00 (RSD = 0.0) |
| | D | 0.48 ± 0.02 (RSD = 3.4) |
| 4 | A | 0.52 ± 0.01 (RSD = 2.8) |
| | B | < 0.008 (below LOD) |
| | C | 0.22 ± 0.00 (RSD = 1.9) |
| | D | 0.29 ± 0.00 (RSD = 1.0) |

**Table 9.** Total impurity amounts for each GSH bulk substance.

| GSH Bulk Substance sample | Total Impurities (%) |
|---|---|
| 1 | 0.7 |
| 2 | 0.1 |
| 3 | 1.4 |
| 4 | 1.0 |

## Conclusions

- A sensitive HPLC-UV method was developed and validated for identification, precision, linearity, range, limits of detection and quantitation, and accuracy to assess GSH and its four major impurities.
- GSH was quantifiable at concentrations as low as 0.04 mg/mL, while the impurities were quantifiable at concentrations ranging from 0.69 to 0.81 µg/mL (0.3% w/w of GSH).
- Four GSH bulk substances were assayed and tested for impurities. The products tested within the acceptance criteria for both GSH and impurities. Total impurity amounts ranged from 0.1 to 1.4% of the measured GSH concentration.

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## Disclaimer

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