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Article

Development and Validation of a Novel RP-HPLC Method for the Analysis of Reduced Glutathione

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The objective of this study was the development, optimization, and validation of a novel reverse-phase high-pressure liquid chromatography (RP-HPLC) method for the quantification of reduced glutathione in pharmaceutical formulations utilizing simple UV detection. The separation utilized a C18 column at room temperature and UV absorption was measured at 215 nm. The mobile phase was an isocratic flow of a 50/50 (v/v) mixture of water (pH 7.0) and acetonitrile flowing at 1.0 mL/min. Validation of the method assessed the methods ability in seven categories: linearity, range, limit of detection, limit of quantification, accuracy, precision, and selectivity. Analysis of the system suitability showed acceptable levels of suitability in all categories. Likewise, the method displayed an acceptable degree of linearity ($r^2 = 0.9994$) over a concentration range of 2.5–60 μg/mL. The detection limit and quantification limit were 0.6 and 1.8 μg/mL respectively. The percent recovery of the method was 98.80–100.79%. Following validation the method was employed in the determination of glutathione in pharmaceutical formulations in the form of a conjugate and a nanoparticle. The proposed method offers a simple, accurate, and inexpensive way to quantify reduced glutathione.

Introduction

Glutathione, a nonprotein tripeptide thiol, L-γ-glutamyl-L-γ-cysteinylglycine, is the most abundant thiol of low molecular weight (307.3 g/mol) found in animal cells (1). Glutathione is synthesized from glutamate, cysteine, and glycine in a two step processes that is catalyzed by two cytosolic enzymes; γ-glutamylcysteine synthetase and glutathione synthetase (2). First γ-glutamylcysteine synthetase catalyses the first, and rate limiting step; the reaction of L-glutamate and L-cysteine to form the intermediate L-γ-glutamyl-L-cystine (3). This intermediate is then combined with glycine by glutathione synthetase to produce glutathione (4). While hepatic cells produce the most glutathione, almost all cells in the human body produce glutathione to some extent (1). Glutathione serves multiple roles critical to sustaining the life of the cell including restoring molecules via hydrogen donation, maintaining thiols in various proteins in their reduced state, regulating critical homeostasis pathways, and functioning as an antioxidant (1, 5). The role that glutathione is best known for is protecting cells from oxidative stress. Because of this role, glutathione is believed to be linked to many diseases including influenza, HIV, AIDS, various types of cancer, cystic fibrosis, diabetes, and many others (1, 5–8). The molecular structure of the tripeptide glutathione is shown in Figure 1.

Many methods exist for the quantification of glutathione in biological matrices utilizing UV absorbance, florescence, spectrophotometry, electrochemical, and tandem mass spectroscopy methods for detection (9–15). Many of these methods utilize a separation via HPLC prior to detection. The most common methods for the quantification of glutathione in pharmaceutical formulations utilize UV absorbance, fluorescence detection, or spectrophotometric methods; however, these techniques require a derivatization prior to analysis (16–20). The reagents used for many of these derivatizations require extreme reaction conditions, and any excess reagent must be removed by an extraction in order to eliminate interference before beginning chromatography. The proposed method utilizes UV absorbance as the means of detection. UV absorbance detectors are relatively inexpensive and already widely employed in pharmaceutical laboratories. Moreover, the proposed method, unlike those published previously, does not require derivatization prior to analysis. The proposed method uses water as the aqueous component of the mobile phase, eliminating the need to prepare buffers. This is advantageous over many of the published methods which utilize phosphate salts in the mobile phase. Phosphate salts have been known to precipitate inside the column and HPLC system, potentially damaging the equipment (10). Because the proposed method utilizes the common UV detector, does not require any additional equipment such as a column over, uses a simple water: acetonitrile mobile phase, and allows for relatively short run times this method is able to economically analyze a large number of samples over a wide concentration range without sacrificing accuracy.

The objective of this research was to develop and validate a simple, cost effective, rapid, accurate, and widely applicable reverse phase high pressure (performance) liquid chromatography method for quantifying reduced glutathione in pharmaceutical formulations. After the chromatographic conditions were optimized the method was validated. Finally, the proposed method was used to assay the amount of glutathione present in two pharmaceutical formulations; a conjugate and a nanoparticle solution.

Experimental

Materials and methods

Acetonitrile was obtained from V.W.R. International (Batavia, IL). Reduced glutathione (GSH) (> 98%) was purchased from M.P. BioMedicals (Solon, OH). The HPLC system was purchased from

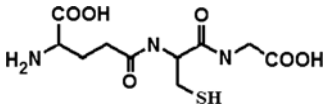

Figure 1. The molecular structure of the tripeptide glutathione; N-(N-L-γ-glutamyl-L-cysteinyl) glycine.

Perkin Elmer (Waltham, MA). The column and guard column were from Agilent Technologies (Santa Clara, CA). Methanol and 1,4-butanediol diglycidyl were obtained from Fisher Scientific (Pittsburg, PA). Autosampler vials (2 mL) were obtained from Perkin Elmer (Waltham, MA). Polyethylene glycol (MW 4,000) was purchased from Hampton Research (Aliso Viejo, CA). Chromatographic-grade water was produced by running water collected from a NANOpure Water System™ (Dubuque, IA) through Whatman Filter Paper (Fisher Scientific, Pittsburg, PA). Mobile phase solvents were degassed with a vacuum pump from Transcat (Rochester, NY). All other solvents were purchased as HPLC grade, and used without further purification.

GSH standard solutions

Standard solutions of GSH were prepared by solubilizing 7–10 mg of GSH in the appropriate amount of mobile phase to make a stock solution containing 1.0 mg/mL GSH. After vortexing for thirty seconds, aliquots of the stock solution (5.0, 10, 20, 60, and 120 μL) were then diluted with the appropriate amount of mobile phase to create working solutions with volumes of 2 mL (2.5, 5, 10, 30, and 60 μg/mL). These working solutions were then vortexed for thirty seconds, and 1 mL was transferred into a 2 mL vial, which was placed in the autosampler for testing. Promega GSH-Glo Glutathione Assay™ (Promega Corporation, Madision, WI) was used for detection of specifically GSH (reduced glutathione) in the sample and this assay does not detect oxidized glutathione (21, 22). This GSH assay was used to confirm that the glutathione in the standard solutions was in its reduced state and did not oxidize. The working solutions were stored at 4°C and protected from light for the duration of the day. Fresh stock and working solutions were then prepared on each subsequent day of testing.

Analytical procedure

The HPLC system was made up of a Perkin Elmer HPLC, Flexar binary pump, Flexar Autosampler and UV/visible detector. The separations was performed on a C18 (100 x 4.6 mm id, 3.6 μm) Zorbax column and a Zorbax (5 μm) 4.6 x 12.5 mm guard column, both kept at room temperature (24°C). Two pre-injection flush cycles were run prior to each injection, followed by one post-injection flush cycle. The speed of each flush cycle was set to "medium." Each sample was tested by injecting 20 μL into the column. Samples were kept at 22°C while inside the Autosampler. The mobile phase consisted of a mixture of water (pH 7.0) and acetonitrile. The ratio of the mobile phase was 50/50 (v/v), and the flow rate was set to 1.0 mL/min. The peaks were determined by UV detection, which was set at a wavelength of 215 nm. Various chromatographic conditions were systematically tested including; mobile phase composition (solvent ratio as well as solvent composition) flow rate, detection wavelength, injection volume, and autosampler temperature, in order to optimize the procedure before validation.

Calibration curves

Calibration curves were constructed by plotting the area of the peak against the known concentration. The known concentrations used to make the external calibration curves were 2.5, 5, 10, 30, and 60 μg/mL. Linear regression tests were performed using Microsoft Excel 2007 via the least squares method. The equation for the respective calibration curve was extrapolated to allow for determination of all subsequent calculations.

Validation of the proposed method

After the chromatographic conditions were optimized, the method was then validated. For validation the following parameters were evaluated: range, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and specificity.

Linearity and range

Linearity refers to the ability of the assay to give the analyst data that is directly proportional to the amount of analyte that the sample contains (23). Similarly, the range refers to the highest and lowest amount of the analyte that the method can detect with an appropriate amount of accuracy, precision, and linearity (23). The range and linearity were evaluated by the construction of calibration curves using standard solutions with concentrations ranging from 2.5 to 60 μg/mL. This range of concentrations was selected based on the target concentration of the method, which was established as 10 μg/mL. Each injection was done in six replicates.

Limit of detection and quantification

The lowest amount of the analyte that the proposed method is able to detect, but is not able to quantify with acceptable levels of accuracy or precision is known as the limit of detection (LOD) (23). The limit of detection was calculated for the proposed method using the formula $LOD = 3.3\delta/S$ where δ is the standard deviation and S is the slope of the calibration curve. This was then confirmed by visual observation of the chromatograms generated from replicate injections of this concentration as well as concentrations slightly below it. Similar to the limit of detection, the limit of quantification (LOQ) is the smallest amount of the substance that is being measured that the proposed procedure is able to detect with acceptable degrees of accuracy and precision (23). The limit of quantification was likewise calculated using the formula $LOQ = 10\delta/S$ as well as observation of the chromatograms generated by replicate injections of solutions with known amounts of GSH at and slightly below the calculated LOQ concentration.

Accuracy and precision

Accuracy, also called trueness, is the degree of difference that the data points exhibit when compared to the actual value

(23). Precision is the degree of difference among multiple data points taken from the same homogeneous sample under the same conditions (23). There are three levels of precision: repeatability (also known as intra-assay precision), intermediate precision, and reproducibility. Repeatability is an expression of the precision of an assay when run using the same conditions and equipment over a short period of time (23). Intermediate precision is the degree of difference between measurements when the analytical procedure is performed by different analysts, using different equipment, on different days (23). Intermediate precision shows the variations of the assay when preformed within the same laboratory, while reproducibility expresses the variation that exists between multiple laboratories (23). The accuracy of the proposed method was determined by the percent recovery study, and the precision was evaluated based on the inter-day and intra-day variations. Intra-day variation was evaluated by running six replicate injections of the five standard concentrations on day one. Then six replicate injections of the five standard solutions were run on different days to obtain the inter-day variation. The relative standard deviation (RSD) was calculated for both the intra-day and inter-day injections.

The percent recovery study was conducted by the spiking method. Briefly, an external calibration curve was constructed using the proposed method. Once the equation of the line for the calibration curve was established, six injections of each of the three spiked solutions were injected consecutively. The amount that the solution was spiked with was equivalent to 70%, 100%, and 130% of the concentration of the original sample (10 μg/mL) as prescribed by Murali *et al.* (24). The amount of sample that was recovered was calculated using the external calibration curve established that day.

Specificity

Specificity is defined as the ability of the proposed method to accurately assess the amount of analyte in a sample when other components, such as products formed by the degradation of the sample, are present and may cause interference (23). Visual evaluation of the GSH peak showed that the peak was well shaped and free of interfering peaks.

System suitability

The chromatograms that the proposed method generated were evaluated and the tailing factor (T_f) and asymmetry factor (A_s) were calculated along with the theoretical plate number (N) and the height equivalent to a theoretical plate (HETP). The equations used were: $T_f = (a + b)/2a$ and $A_s = b/a$. Where A is the width of the front half of the peak and B is the width of the back half of the peak. The width of the peak was determined at 5% of the peak height for the tailing factor, and at 10% of the peak height for the asymmetry factor. The theoretical plate number was calculated using the van Deemter equation: $N = [8*\ln(2) * (T_R{}^2)] / (W_{0.5}{}^2)$ where T_R is the retention time and $W_{0.5}$ is the width of the peak at half the peak height. HEPT was then calculated using HEPT = L / N with L being the length of the column in centimeters.

Figure 2. The molecular structure of the pharmaceutical PEG conjugate synthesized using the epoxy-oxirane activation method. The epoxy-oxirane employed was 1,4-butanediol diglycidyl ether.

Table I
Regression Analysis Data for the Proposed Method*

Parameter	Results
Linearity Range	2.5–60 μg/mL
Regression Equation	Y = 6756.3x − 9841.44
Slope*	6756.3
Intercept*	−9841.44
Coefficient of Determination (r^2)	0.9994
Limit of Detection	0.6 μg/mL
Limit of Quantification	1.8 μg/mL

*Regression equation y = ax + b, a: slope, b: intercept.

Pharmaceutical samples

The GSH was conjugated to polyethylene glycol (PEG) after activation of the PEG using the epoxy-oxirane activation method described elsewhere (25). Briefly, solubilized PEG (MW 4,000) was activated using the epoxy-oxirane 1,4-butanediol diglycidyl ether. At alkaline pH the hydroxyl group of PEG reacts with the epoxy-oxirane. The reactive oxirane, now joined to PEG, was then reacted with the amine group of GSH to form the conjugate as seen in Figure 2. A 1.1 mg sample of the lyophilized conjugate was solubilized in acetonitrile. The sample was then used for replicate analysis.

A nanoparticle sample was prepared from poly(lactic-co-glycolic acid) (PLGA) using the nanoprecipitation method described elsewhere (26). Briefly, PLGA (120 mg) was dissolved in 3 mL acetone before being added drop-wise into 6mL of water. This solution was stirred for 2 hours at 40°C before adding 20 mg of the lyophilized PEG-GSH conjugate. After the PEG-GSH was allowed to stir in the NP solution for 45 minutes at room temperature the solution was centrifuged (4,500 rpms for 40 min) and the supernatant was separated from the NP. The free GSH present in the supernatant was measured by the developed HPLC method. The nanoparticles were then allowed to dry overnight. A sample of the dried nanoparticles was dissolved in acetonitrile and the GSH present on the surface of the nanoparticle was measured by the HPLC method.

Results

The calibration curves constructed for GSH show that the method was linear from 2.5 to 60 μg/mL, corresponding to 25% and 600% of the target concentration (Table I), and the correlation coefficient value was close to 1 (Table II). A regression line was fit using the equation y = ax + b at the concentrations of 2.5, 5, 10, 30, and 60 μg/mL (Figure 3). The limits of detection and quantification for the method were determined using the aforementioned equations, and then these values were experimentally tested. The LOD was determined to be 0.6 μg/mL, and the LOQ was found to be 1.8 μg/mL, and this was supported by the experimental chromatograms. The

Table II
Coefficients of Determination for Six Linear Curves Fitted to the Data Using the Least Squares Method

Run Number	Coefficient of Determination (r^2)	Run Number	Coefficient of Determination (r^2)
1	0.9994	**4**	0.9993
2	0.9994	**5**	0.9993
3	0.9993	**6**	0.9993
	Mean	0.99932	

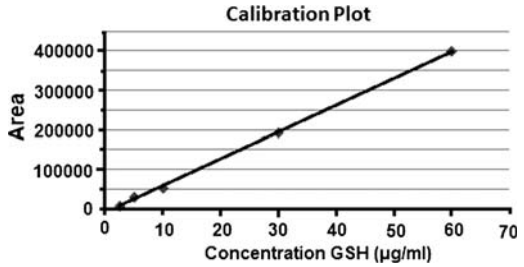

Figure 3. A plot of concentration (μg/mL) vs. peak area of GSH constructed with a linear trend line fitted using the least squares method.

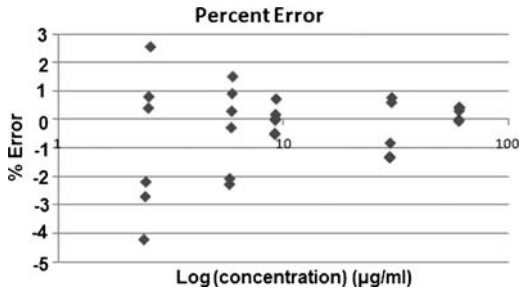

Figure 4. A graphical depiction of the percent error from six replicate injections. The log of the concentration (μg/mL) of GSH is plotted against the percent error for each injection.

graphical depiction of the percent error from six replicate injections is shown in Figure 4.

The precision of the method was evaluated by analysis of the relative standard deviation (RSD) (Table III). The average intra-day RSD for all five concentrations was 1.09% and the average inter-day RSD was 1.54% (n = 6). Evaluation of the full intermediate precision is beyond the scope of this study. The accuracy of the method was determined by the spiking method and corresponding percent recovery (Table IV). Three spiked samples were prepared by spiking the 10 μg/mL sample with 70%, 100%, and 130% of the original concentration (7, 10, and 13 μg/mL respectively). The average percent recovery for the 70%, 100%, and 130% spiked samples were found to be 100.79%, 99.64%, and 98.80% respectively (n = 6).

The proposed reverse-phase HPLC method was intended to be an accurate and economical way to quantify GSH in concentrations ranging from 2.5 to 60 μg/mL. Figure 5 illustrates an overlapping chromatogram of the five standard solutions (2.5, 5, 10, 30, and 60 μg/mL). The tailing factor and asymmetry factor of the 10 μg/mL peaks were 1.21 and 1.18, respectively. The theoretical plate number was determined to be greater than 2000, and the HEPT was 0.0046 cm. All of these values are

Table III
Precision Data: Relative Standard Deviation (RSD).*

Concentration (μg/mL)	Intra-Day* (μg/mL)	RSD (%)	Inter-Day* (μg/mL)	RSD (%)
2.5	2.49	2.69	2.38	3.98
5.0	5.86	1.17	5.87	1.8
10.0	9.20	0.48	9.22	0.84
30.0	29.76	0.91	29.93	0.91
60.0	60.18	0.20	60.09	0.20
	Mean	1.09	**Mean**	1.54

* mean of six values (n = 6).

Table IV
Accuracy Data: Percent Recovery of GSH Conducted by Spiking the 10 μg/mL Sample.

Sample Amount (μg/mL)	Amount Added* (μg/mL)	Amount Recovered† (μg/mL)	% Recovery (w/w)
10	7.0	7.06	100.79%
10	10	9.96	99.64%
10	13	12.84	98.80%
		Mean	99.74%

* 70%, 100%, and 130% of 5.0 μg/mL.
† mean of six values (n = 6).

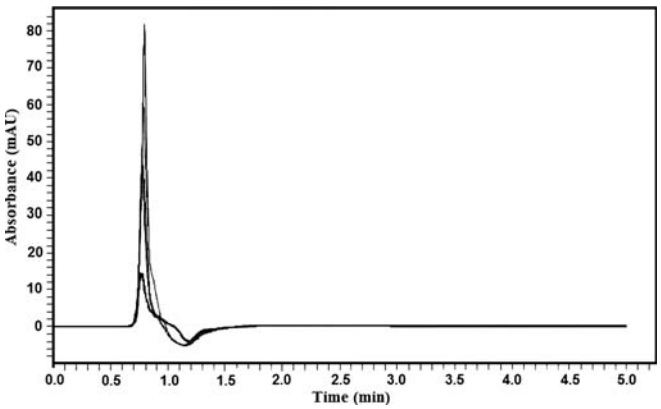

Figure 5. The chromatograms of the five standard solutions (2.5, 5, 10, 30, 60 μg/mL) of GSH overlaid on top of each other.

within the acceptable limits, and demonstrate the system suitability for the proposed analytical test (Table V). Resolution was not addressed because the chromatograms only displayed one peak as seen in Figures 5. The well shaped peaks in the chromatograms verify that the proposed method demonstrates satisfactory specificity.

Analysis of pharmaceutical formulations

The developed HPLC method was successfully applied for analysis of GSH in the PEG-GSH conjugate, nanoparticles, and nanoparticle supernatant. The PEG-GSH conjugate was determined to contain 4.21% (w/w) GSH (Table VI). The nanoparticles formulation contained 0.078 ± 0.017% (w/w) GSH (i.e. conjugated GSH) while the nanoparticles supernatant contained 0.376 ± 0.028% (w/v) GSH (i.e. free GSH) (Table VII). Using this data a nanoparticle was prepared containing the anti-cancer agent Paclitaxel and a 2% (w/w) coating of PEG-GSH conjugate which was subsequently employed in other ongoing studies (data not shown).

Discussion

Prior to validation the method was optimized, testing a variety of different parameters. The wavelength of 215 nm was chosen based on the amount of absorbance that a solution of GSH in acetonitrile displayed during a scan of wavelengths from 190 to 400nm (Figure 6). Likewise, different mobile phase compositions were analyzed prior to validation. These included varying the ratio of aqueous and organic solvents (75:25, 65:35, 50:50, and 35:65), as well as the use of methanol in place of acetonitrile. Solvent gradients were not used because the sample matrix was simple. When methanol was used in place of acetonitrile a drifting baseline signal resulted, which led to problems with integration. For this reason acetonitrile was selected as the organic solvent.

A common problem in RP-HPLC analysis is peak tailing. In many cases tailing is caused by the retention between the acidic silanol in the stationary phase's support structure and a positively charged analyte (27). This type of tailing can be seen when the analyte contains a protonated amine group. The amine group of GSH was protonated in the experimental conditions (pH 7), and was therefore expected to be the cause of some of the tailing seen. This hypothesis was supported by the decrease in tailing observed when samples of the PEG-GSH conjugate, in which glutathione's amine group was bonded to the epoxy-oxirane and unable to be protonated, were analyzed. The acid dissociation constant (pKa) for the amine group in GSH is 9.12, and therefore would require the use of a strongly basic mobile phase to remain deprotonated and neutrally charged (18). Since the majority of RP-HPLC columns are unable to withstand such alkaline pH no mobile phase compositions with pH values higher than 7 were tested.

Table V
System Suitability Parameters for the Proposed Method

Parameter	Results	Acceptable Limits
Asymmetry Factor	1.18	< 1.5
Tailing Factor	1.21	< 2.0
Plate Number	> 2000	> 2000
HEPT	0.0046cm	

Table VI
Application of the Proposed HPLC Method to Pharmaceutical Samples of the GSH Containing PEG-GSH Conjugate

Sample	Mean Concentration of GSH in the PEG-GSH conjugate (μg/mL)	%GSH in the PEG-GSH conjugate (%w/w)
1	45.92	4.59
2	39.63	3.96
3	40.87	4.08
	Mean	4.21
	Standard Deviation	0.33

Table VII
Application of the Proposed HPLC Method in Analysis of GSH in the Pharmaceutical Samples of PLGA Nanoparticles Containing GSH.

Sample	Mean Concentration GSH (μg/mL)	Conjugated GSH on the surface of nanoparticles (%w/w)	Free GSH in the nanoparticles supernatant (%w/v)
1	5.67	0.082	0.366
2	1.04	0.092	0.354
3	3.90	0.059	0.408

Conclusion

The proposed method was first developed and optimized; systematically testing different parameters. Each parameter was evaluated, and once all parameters had been tested the method was finalized and subjected to repeat testing in

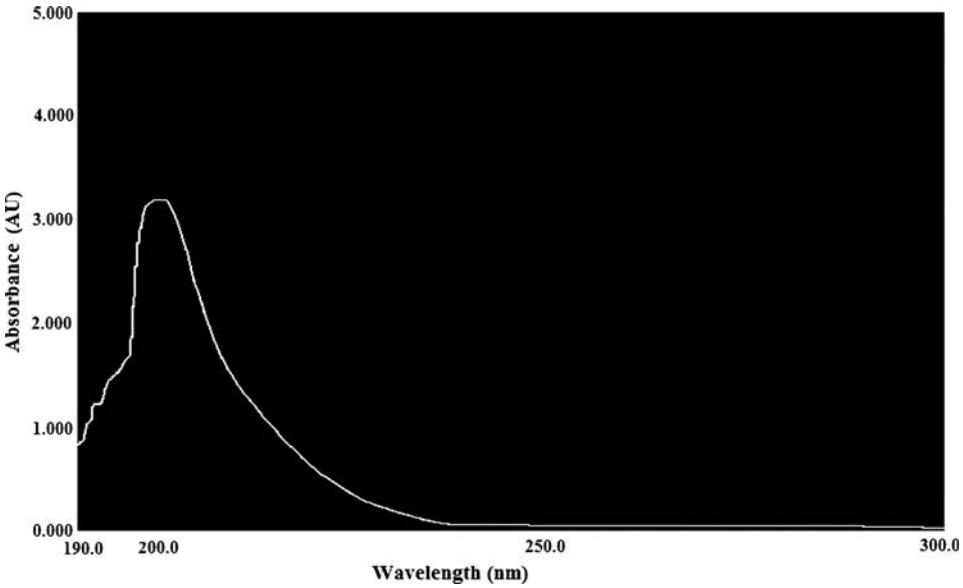

Figure 6. A scan of the amount of UV absorbance at varying wavelengths of GSH solubilized in acetonitrile. A solution of pure acetonitrile was used as the reference. Although this depiction only shows the absorbance values obtained between 190 and 300nm, the compound was scanned from 190–400 nm with no noteworthy absorbance at any wavelengths not shown here.

order to validate the procedure. Validation assessed the linearity, range, limit of detection, limit of quantification, accuracy, precision, and specificity of the proposed analytical method. After validation the method was applied for the analysis of two pharmaceutical preparations. Both preparations were analyzed without incident. The proposed validated RP-HPLC method for the analysis of GSH achieved acceptable levels of simplicity, cost, precision, linearity, sensitivity, reproducibility, selectivity, and accuracy. Likewise, all of the system suitability parameters are within the acceptable range, making this an acceptable method for quantifying GSH by RP-HPLC.

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