Validation of a Stability-Indicating Method for Methylseleno-L-Cysteine (L-SeMC)

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Abstract

Methylseleno-L-cysteine (L-SeMC) is a naturally occurring amino acid analogue used as a general dietary supplement and is being explored as a chemopreventive agent. As a known dietary supplement, L-SeMC is not regulated as a pharmaceutical and there is a paucity of analytical methods available. To address the lack of methodology, a stability-indicating method was developed and validated to evaluate L-SeMC as both the bulk drug and formulated drug product (400 µg Se/capsule). The analytical approach presented is a simple, nonderivatization method that utilizes HPLC with ultraviolet detection at 220 nm. A C18 column with a volatile ion-pair agent and methanol mobile phase was used for the separation. The method accuracy was 99–100% from 0.05 to 0.15 mg/mL L-SeMC for the bulk drug, and 98–99% from 0.075 to 0.15 mg/mL L-SeMC for the drug product. Method precision was <1% for the bulk drug and was 3% for the drug product. The LOQ was 0.1 µg/mL L-SeMC or 0.002 µg L-SeMC on column.

Introduction

Selenium is known to be both essential and toxic to humans, depending on its species and quantity (1–3). Thus, quantifying selenium in medications, treatments and dietary supplements is critical, as below a threshold it may prove to have beneficial effects, but above which it can be deleterious. Methylseleno-L-cysteine (L-SeMC) is a naturally occurring amino acid analogue believed to act as an antioxidant, as well as having chemopreventive actions towards reduction and incidence of various cancers (2). As a result, L-SeMC has been studied as a potential agent in cancer chemoprevention and as a general dietary supplement.

Despite the advancing clinical study knowledge of L-SeMC, used alone or in combination with other agents, further work remains in the field of analytical support (4–8). As a known dietary supplement, L-SeMC is not regulated as a pharmaceutical and thus there are concerns about the quality, stability, storage conditions and selenium content in selenium supplements (3).

For analytical support of Phase I clinical studies, validation of stability-indicating methods for both the bulk drug and formulated drug product (400 µg Se/capsule) was performed. The chromatographic validation consisted of system suitability, specificity, filter compatibility, solution stability, method precision, accuracy, linearity, method robustness, intermediate precision and limits of quantitation and detection (LOQ) and (LOD). The method validation plan and results are presented herein.

Experimental

Instrumentation and materials

HPLC was performed using both a Shimadzu LC-2010CHT Liquid Chromatograph equipped with an internal ultraviolet (UV) detector, while intermediate precision included use of a Waters 2695 Separations Module equipped with an external Waters 2487 Dual λ Absorbance detector. Detection was at 220 nm. Results were acquired and processed with the PerkinElmer TotalChrom™ chromatography data system. For the specificity analysis, a Shimadzu LC-2010CHT Liquid Chromatograph equipped with an SPD-M10Avp Diode Array Detector (190–400 nm) was used, with processing by Shimadzu Class-VP™. HPLC separation was conducted using a Phenomenex® Gemini C18, 150 × 4.6 mm, 3.0 μm column.
Se-methylseleno-L-cysteine (i.e., L-SeMC), 98%, Lot No. A0294726, supplied by Acros Organics, was used as the reference standard/bulk drug for the study. The solutions were prepared by weighing 5.0 (±0.5) mg of L-SeMC into a 50-mL volumetric flask, adding mobile phase to ~30% of the flask volume and mixing the solution with a vortexer for ~30 s. The contents of the flask were then diluted to volume with mobile phase and mixed well.

L-SeMC capsules (400 µg selenium), Lot 56789-155, supplied by Pharm Ops, Inc., were also used for this validation study. Each L-SeMC capsule contains ~400 µg of selenium in the form of L-SeMC (i.e., ~0.9 mg of L-SeMC, with a ratio of 78.96/182.08 for Se/L-SeMC) and 215 mg of excipients (including microcrystalline cellulose, crospovidone and magnesium stearate).

The placebo material used in this study was prepared according to the formulation record for the drug product. The placebo mixture was prepared in a screw-cap scintillation vial by weighing 14.1 (±0.1) g of microcrystalline cellulose, 750.0 (±50.0) mg of crospovidone and 75.0 (±7.0) mg of magnesium stearate, which is essentially the content of 70 capsules. The scale-up by 70 was performed to allow accurate weighing of all the excipients on the analytical balance, and to provide enough material to use throughout the method validation study. The contents of the vial were mixed thoroughly by shaking.

In practice, the drug product sample is prepared by pooling the contents of 10 capsules in a mortar, homogenizing the content with a pestle, and then weighing an amount equivalent to 2.5 times the average capsule fill weight into a 25-mL volumetric flask; for the method validation, mock drug product samples were prepared by combining accurately weighed portions of reference standard and placebo mixture in a volumetric flask. Weighed 540.0 (±50.0) mg of placebo mixture and 2.5 (±0.2) mg of L-SeMC into a 25-mL volumetric flask, which is essentially the content of 2.5 capsules. The contents of the flask were diluted with mobile phase to ~50% of the volume of the flask, mixed by vortexer for ~30 s, then diluted to the full volume of the flask and mixed well.

Methanol (HPLC grade, Burdick and Jackson), purified water (18.2 mΩcm, Labconco), phosphoric acid (HPLC grade, Fluka) and sodium 1-octanesulfonate monohydrate (ion-pairing grade, ≥99.0%, Sigma) were used to prepare the mobile phase and diluent. Sodium hydroxide (NaOH, 0.1 N solution, Fluka), hydrochloric acid (HCl, 0.1 N solution, Alfa Aesar) and hydrogen peroxide (H2O2, 30% solution, Sigma) were used to prepare the mobile phase and diluent. The UV spectrum of L-SeMC, showing the optimum wavelength of detection at 220 nm, is presented in Supplementary Material, Figure S1.

The total run time was 35 min, with L-SeMC eluting at ~16 min.

### Method validation plan

The following characteristics were evaluated for the method validation: chromatographic system suitability, specificity, filter compatibility, solution stability, method precision, accuracy, linearity, method robustness, intermediate precision and LOQ and LOD.

**System suitability.** The chromatographic system suitability evaluations included determination of standard accuracy and system precision (% RSD), including overall system precision. The retention time average of L-SeMC was also noted. These parameters were assessed from replicate injections of standard solutions containing 0.1 mg/mL L-SeMC (i.e. 0.04 mg/mL Se).

**Specificity.** The specificity of the method was evaluated by comparing the chromatography of a placebo solution, a mobile phase blank, a bulk drug solution, a 0.1% bulk drug solution and a mock drug product solution, and by evaluating the L-SeMC peak for homogeneity by photodiode array detection from 190 to 400 nm. Degraded solutions were also analyzed.

**Forced degradation** was performed via decomposition studies of a mock drug product sample solution, evaluated under basic, acidic and oxidative conditions for ~24 h. A mock drug product sample solution was prepared by adding 1080.00 (±100) mg of placebo and 5.0 (±0.5) mg of L-SeMC to a 25-mL volumetric flask. The contents of the flask were diluted with mobile phase to ~50% of the volume of the flask, mixed by vortexer for ~30 s, then diluted to the full volume of the flask and mixed well. Using a mechanical pipette, 5.0 mL portions of this solution were transferred to four individual glass scintillation vials. For the control sample, 5.0 mL of mobile phase was added to the vial. For the degradation samples, 5.0-mL of the specified solution (i.e. 0.1 N NaOH, 0.1 N HCl, or 0.003% H2O2) was added to the vial. The contents of the vials were mixed well and stored at ambient conditions with uncontrolled lighting for ~24 h. When removed for analysis, each solution was filtered through a 0.45-µm PTFE filter into an autosampler vial.

**Filter compatibility.** Filter compatibility was assessed by examining three types of filters on a mock drug product sample solution. Pall 0.45 µm PVDF, Pall 0.45 µm Nylon and Pall 0.45 µm PTFE filters were evaluated. A mock drug product sample solution was prepared and a portion of this solution was vialed for analysis after centrifugation.

**Solution stability.** The solution stability of a bulk drug and mock drug product sample solution was assessed periodically up to 1 week after initial preparation. Portions of the solutions were stored at ambient conditions for 24 h and the solutions were analyzed using the described method and the same conditions as used for the stability study.
laboratory temperature in the presence and absence of ambient laboratory lighting (i.e. uncontrolled lighting conditions) and at refrigerated temperature (i.e. 2–8°C) in the absence of light. Two reference standard solutions were prepared at each assessment time for comparison of the L-SeMC response factors.

**Method precision.** The precision of six bulk drug and drug product sample solutions was evaluated.

For the bulk drug, six independent sample solutions were prepared as stated in Instrumentation and materials.

For the drug product, sample solutions were prepared from two capsule groups. As in practice, three sample solution preparations were made from each group. The groups were each prepared by weighing 10 capsules, then the capsule shells were separated and the contents were pooled into a mortar. Each pool was ground into a fine powder using a pestle. The capsule shells were cleared of any remaining fill material using house air, then reweighed to calculate the average fill weight of each group. The average fill weight was multiplied by 2.5 to obtain the amount of pooled material needed for each analytical sample preparation. This amount (i.e. ~540 mg) was then weighed into 25-mL volumetric flasks, with each capsule group producing three samples. Mobile phase was added to ~50% of the volume of the flasks, then the content of the flasks were mixed well by a vortexer and inversion for ~2 min. The flasks were then placed in an ultrasonic bath for 5 min. After sonication, the content of the flasks were mixed by a vortexer for 1 min, diluted to the full volume of the flasks with mobile phase, then mixed well for ~30 s. Finally, a portion of each solution was filtered through a 0.45-µm PTFE syringe filter, discarding the first few milliliters of filtrate, prior to analysis.

**Accuracy and linearity.** Accuracy and linearity were assessed by analyzing both the bulk drug and mock drug product sample solutions at various concentrations. Triplicate preparations at 50, 100 and 150% of the analytical concentration (i.e. 0.1 mg/mL L-SeMC or 0.04 mg/mL Se) were evaluated. The mock drug product was also evaluated at 70% of the analytical concentration after failing to meet the accuracy criteria at the 50% level.

**Robustness.** The robustness of the method was assessed by examining the effects of altered flow rate (±0.1 mL/min) and mobile phase composition (±2% methanol) on the theoretical plates and retention time of L-SeMC. A 0.1 mg/mL L-SeMC bulk drug solution was used for the determination.

**Intermediate precision.** Intermediate precision was performed by a second analyst preparing the system suitability standards, mobile phase, capsule pool and drug product sample solutions, and utilizing a separate HPLC system, column and detector. Three drug product sample solutions were prepared from a 10-capsule pool.

**Limits of quantitation and detection.** Serial dilutions of a bulk drug and mock drug product sample solution were made to determine the LOQ and LOD. Six injections of each solution were made to assess the precision of the response and the signal-to-noise value.

**Results**

**Method development**

Representative chromatograms of a mobile phase blank and L-SeMC reference standard solution are presented in Figure 1.

**Method validation results**

**System suitability**

The system suitability results met the specifications of system precision ≤2.0% RSD and standard accuracy 98–102%. The average retention time ranged from 14.7 to 18.0 min over the nearly 6-week study. Results are presented in Table I for each day of the analysis.

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![Figure 1. Chromatograms of L-SeMC reference standard (0.1 mg/mL) and diluent.](https://academic.oup.com/chromsci/article-abstract/54/1/22/2754734/122754734)
Specificity
Duplicate injections of each solution were analyzed and the results met the specification of no placebo or diluent blank peak eluting within the peak width of L-SeMC (resolution $\geq 1.5$), or the interference was $<0.1\%$ of the L-SeMC peak. The L-SeMC peak was homogeneous as determined by the PDA spectra taken at the up-slope, apex and down-slope of the peak. The percent degradation of L-SeMC was calculated from the difference between the average peak areas in the $T_0$ control sample vs. the degradation sample. Essentially, no degradation was observed for the acidic and basic conditions; however, nearly 50% degradation was achieved for the oxidative condition; this was expected due to the antioxidant properties of the molecule. Chromatograms of the $T_0$ control, 0.1 N HCl, 0.1 N NaOH and 0.003% H$_2$O$_2$ conditions from forced degradation are shown in Figure 2. The degradation by peroxide was performed on a later date than the acid and base degradation, which resulted in less retention of L-SeMC on the column; thus, the chromatogram ensuing from the peroxide degradation is offset by 3 min in the overlay to align the L-SeMC peaks.

Filter compatibility
Duplicate injections of the unfiltered and filtered solutions were made, with averaging of the L-SeMC peak areas obtained from the duplicate injections. The results for the nylon, PVDF and PTFE filters met the specification of $\leq 2.0\%$ difference compared with the unfiltered solution, with the chromatographic profiles appearing the same. The PTFE syringe filter was chosen for use in subsequent analyses since it provided the lowest percent difference in response (i.e. 0.2% difference).

Solution stability
A single injection of the bulk drug and filtered mock drug product sample solutions was obtained following initial preparation ($T_0$) and the determined concentration of selenium calculated. Two fresh system suitability standards were prepared for each subsequent time point. These standards, shown to meet the system suitability requirements, were used to quantitate the percent variance of the concentration of the stored solutions at the different time points.

The percent variance was calculated by the absolute difference between the concentration of the initial preparation ($T_0$) and the

<table>
<thead>
<tr>
<th>Table I. Interday System Suitability Results for L-SeMC</th>
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<tr>
<td><strong>Criteria</strong></td>
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<tr>
<td>Accuracy (%), 98–102</td>
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<tr>
<td>Initial system precision (% RSD, $\leq 2.0$)</td>
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<tr>
<td>Overall system precision (% RSD, $\leq 3.0$)</td>
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<tr>
<td>Average L-SeMC retention time (min) FYI only</td>
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<tr>
<td>Day 7  143  143  143  143  143  143  143  143  143  143  143  143  143  143  143</td>
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*Intermediate precision.

Figure 2. Forced degradation chromatograms.
concentration of the stored solution. The results met the criteria of (i) not $>3.0\%$ variance, (ii) no new impurity peaks $\geq 0.2\%$ of the main peak and (iii) any impurity peaks $\geq 0.2\%$ observed in the $T_0$ sample were still present in the stored solution. The bulk drug and filtered mock drug product sample solutions were shown to be stable for up to 7 days at ambient conditions in the presence of light.

Method precision

Single injections of the six bulk drug and drug product sample solutions were evaluated. The selenium content was assessed in comparison with the system suitability standards. The bulk drug assay was expressed as percentage of selenium determined compared with the theoretical amount weighed and the drug product assay was expressed as percentage of selenium determined per capsule compared with the product label claim (i.e. 400 $\mu$g of selenium per capsule). The results met the criteria of $\leq 3.0\%$ RSD for the bulk drug assay and $\leq 5.0\%$ RSD for the drug product assay, with 0.5 and 3.0\% RSD, respectively.

Accuracy and linearity

A single injection of each solution was analyzed. The accuracy ($\%$) of the solutions was evaluated by comparing the determined concentration of selenium, determined from bracketing reference standards, to the gravimetric concentration of selenium. The correlation coefficient ($r$) was determined by least squares linear regression analysis of the L-SeMC peak area responses ($y$) and the gravimetric concentrations ($x$).

Each individual preparation met the criteria of $97–103\%$ accuracy, with the bulk drug preparations ranging from 99.2 to 100.1\% and the mock drug product preparations ranging from 97.5 to 98.5\%.

The correlation coefficient ($r$) was 0.99995 for the bulk drug and 0.99658 for the mock drug product solutions. The slope, intercept and plot of residuals are presented in Supplementary Material, Figures S3 and S4.

Robustness

Duplicate injections of a bulk drug solution were made at each of the altered conditions. There was no criteria established; the data were collected for information only. The theoretical plate counts and retention times of L-SeMC are summarized in Table II.

Intermediate precision

The system suitability parameters were met, and the $\%$ RSD of the assay values was determined. The assay results ($n = 3$) met the criteria of $\leq 5.0\%$ RSD, with 3.7\%, and agreed within 1.6\% of the method precision results.

Limits of quantitation and detection

The results met the LOQ criteria of $\geq 10$ signal-to-noise ratio and $\%$ RSD $\leq 15$ for the response, with an LOQ of $0.1\%$, or 0.1 $\mu$g/mL L-SeMC (0.002 $\mu$g on-column) for both the bulk drug and drug product. The LOD criteria of $\geq 3$ signal-to-noise ratio was met, with an LOD of 0.01\% for the bulk drug and 0.02\% for the drug product.

Discussion

Validation assessment included chromatographic system suitability, specificity, filter compatibility, solution stability, method precision, accuracy, linearity, robustness, intermediate precision and LOQ and LOD. All validation parameters met the criteria, showing this method to be appropriate for its intended use as a stability-indicating method for analytical support of Phase I clinical studies.

The bulk drug regression data in Supplementary Material, Figure S2 were evaluated for lack of homoscedasticity since the residuals plot shows increasing variation with increasing concentration. While the data appear heteroscedastic, the scale of the residuals plot is insignificant, with a spread of $\pm 20,000$, which amounts to just 0.5\% of the maximum $y$ value; furthermore, the accuracy across the range of the bulk drug preparations was 99–100\% of the predicted value and the correlation coefficient was 0.99995. It was determined that the linear relationship is strong enough for the method’s intended purpose; if the method is to support Phase II clinical studies or beyond, then additional validation data may be needed to support the conclusion.

Conclusion

A reversed-phase ion-pairing HPLC method utilizing UV detection was developed and validated for the identification, assay and impurity profile analyses of L-SeMC bulk drug and 400 $\mu$g selenium capsules. The ability to utilize a non-derivatization method for the analysis of the amino acid L-SeMC proves to be a reliable and convenient alternative to existing techniques. The mobile phase is compatible with mass spectrometry, making the method adaptable for the analytical support of late phase clinical studies when the identification of impurities and degradants is essential.

Supplementary material


Funding

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