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DETERMINATION OF ACTIVE PHARMACEUTICAL INGREDIENTS IN A COMBINED INFUSION SOLUTION BY HPLC: METHOD DEVELOPMENT AND VALIDATION

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Annotation. Two HPLC methods for the determination of monoammonium glycyrrhizinate, glycine, and L-cysteine hydrochloride in the combined infusion solution «GEPA-SEEM» were developed and fully validated in accordance with ICH Q2(R1); the current approach was also considered in the context of ICH Q2(R2) and ICH Q14. Both methods demonstrated high precision (RSD ≤ 0.22%), accuracy (recovery 98.5–101.5%), and linearity ($R^2 > 0.999$). Based on the results, an in-house pharmacopoeial monograph was established for the product.

Keywords. HPLC; method validation; monoammonium glycyrrhizinate; infusion solution; hepatoprotective agent; ICH Q2(R2).

How to Cite

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Introduction. Ensuring the safety and clinical effectiveness of parenteral medicines depends fundamentally on rigorous quality control. Solutions administered by intravenous infusion are held to particularly demanding standards, being required to be sterile, apyrogenic, and precisely dosed with respect to their active pharmaceutical ingredients (APIs) [1, 2]. Consequently, the establishment of validated

analytical procedures and pharmacopoeial specifications is an essential step in bringing any new pharmaceutical product to registration.

The subject of this study, «GEPA-SEEM» (JV LLC «SAMARKAND-ENGLAND ECO-MEDICAL», Uzbekistan), is a newly developed combined infusion solution formulated with three active ingredients: monoammonium glycyrrhizinate (MAG, 0.8 mg/mL), glycine (8.0 mg/mL), and L-cysteine hydrochloride (0.6 mg/mL). MAG is known to produce a marked anti-inflammatory, antioxidant, and membrane-stabilizing effect on hepatocytes [3], while glycine, an amino acid engaged in hepatic detoxification pathways, contributes cytoprotective properties [4]. L-cysteine hydrochloride, for its part, is a sulfur-bearing amino acid acting as a glutathione precursor and thereby supplying intracellular antioxidant protection [5]. The combined presence of these three components defines the formulation of the product.

A review of the available literature revealed no validated quantitative HPLC procedure capable of determining all three APIs, either simultaneously or sequentially, within a combined infusion matrix. Accordingly, this work set out to develop two dedicated HPLC methods, to validate them in line with ICH Q2(R1) [6], taking into account the current principles of ICH Q2(R2) [7] and ICH Q14 [8], and to establish an in-house pharmacopoeial monograph for «GEPA-SEEM».

Research results

Test sample and composition

The «GEPA-SEEM» infusion solution manufactured by JV LLC «SAMARKAND-ENGLAND ECO-MEDICAL» (batch EX0110423, manufacture date 04/2023) served as the test article for this study. The quantitative composition of the product, expressed per 1 mL, is given in Table 1.

Table 1. Quantitative composition of the «GEPA-SEEM» infusion solution (per 1 mL).

Component	Amount	Role
Monoammonium glycyrrhizinate	0.80 mg	Active substance
Glycine	8.00 mg	Active substance
L-Cysteine hydrochloride	0.60 mg	Active substance
Sodium sulfite	0.80 mg	Excipient
Calcium disodium EDTA monohydrate	0.08 mg	Excipient
Sodium chloride	5.00 mg	Excipient
Water for injection	to 1 mL	Solvent

Chromatographic separation and specificity

The HPLC chromatograms obtained under Method 1 are shown in Figure 1. The MAG peak eluted at approximately 7.6 min, 18α-glycyrrhizinic acid at around 10.3 min (relative retention ~1.36), and Impurity-A at roughly 5.1 min (relative retention ~0.72). Blank and placebo chromatograms showed no interfering peaks at the MAG retention time, supporting the specificity of the method. 18α-glycyrrhizinic acid and Impurity-A were reported as additional chromatographic separation indicators; separate limits of detection or quantification for these impurities were not evaluated within this validation study.

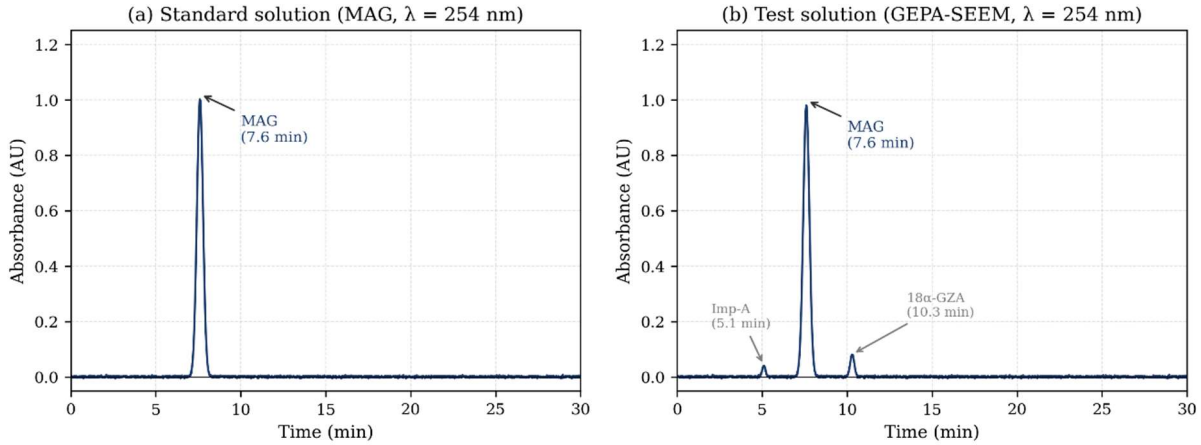


Fig. 1. Representative HPLC chromatograms by Method 1 (λ = 254 nm): (a) MAG standard solution; (b) «GEPA-SEEM» test solution. MAG — monoammonium glycyrrhizinate, 18α-GZA — 18α-glycyrrhizinic acid, Imp-A — Impurity-A.

Chromatograms recorded under Method 2 are presented in Figure 2. Glycine and L-Cys·HCl were separated to baseline (resolution factor greater than 3), with retention times of approximately 5.1 and 6.6 min, respectively. No interference from excipients was detected in the placebo chromatograms.

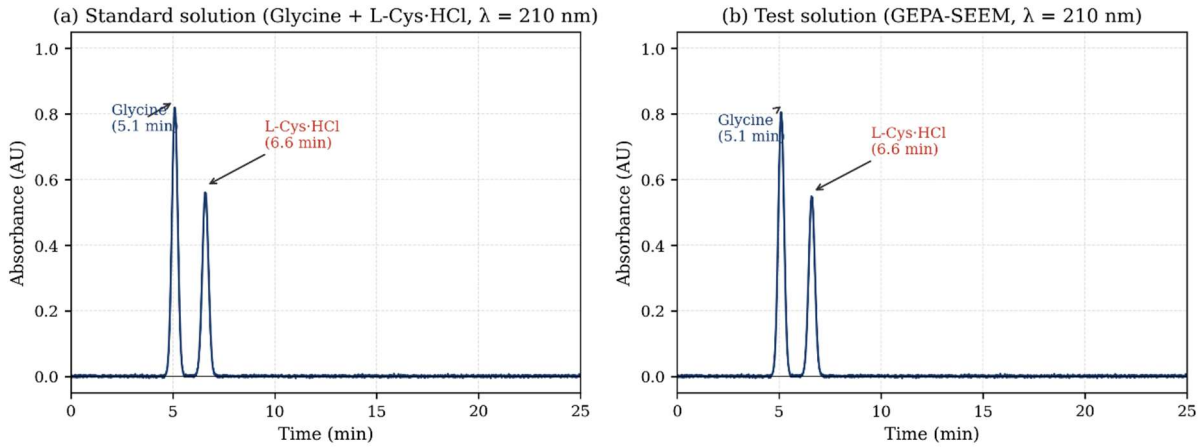


Fig. 2. Representative HPLC chromatograms by Method 2 (λ = 210 nm): (a) mixed standard solution (glycine + L-Cys·HCl); (b) «GEPA-SEEM» test solution.

System suitability and precision

Table 2 summarizes the system suitability parameters together with the precision data obtained for both methods.

Table 2. System suitability parameters and precision results.

Parameter	Method 1 (MAG)	Method 2 (Gly/Cys)	Criterion
Symmetry factor (USP)	1.124	1.18 / 1.15	≤ 2.5
Theoretical plates (1st inj.)	6888	5430 / 4870	≥ 2000
RSD, 5 injections (%)	0.10	0.12 / 0.14	≤ 2.0
Intermediate precision RSD (%)	0.20	0.18 / 0.22	≤ 2.0

Both methods satisfied all the required system suitability criteria. RSD values remained at or below 0.22% for both intra-day and inter-session measurements, indicating excellent repeatability.

Linearity and range

Calibration curves were constructed across 50–150% of the nominal working concentration of each API: 0.004 mg/mL for MAG (0.002–0.006 mg/mL), 0.004 mg/mL for glycine (0.002–0.006 mg/mL), and 0.003 mg/mL for L-Cys·HCl (0.0015–0.0045 mg/mL). Relative to the nominal concentrations in the product, these working concentrations correspond to overall dilution factors of 200-fold for MAG and L-Cys·HCl and 2000-fold for glycine. Representative linearity plots for these ranges appear in Figure 3.

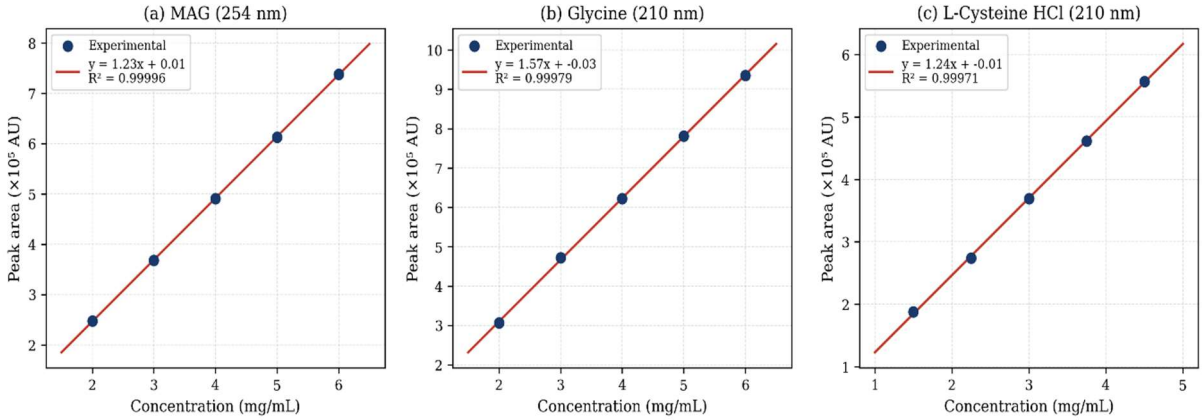


Fig. 3. Linearity plots for the three APIs: (a) MAG (Method 1, λ = 254 nm); (b) glycine (Method 2, λ = 210 nm); (c) L-cysteine hydrochloride (Method 2, λ = 210 nm). Each point represents the mean of triplicate injections; regression lines are shown.

Table 3. Linearity parameters for the three analytes.

Analyte	Range (mg/mL)	Slope	Intercept	R^2
MAG	0.002–0.006	1.23×10^8	2.1×10^3	> 0.999
Glycine	0.002–0.006	1.56×10^8	3.4×10^3	> 0.999
L-Cys·HCl	0.0015–0.0045	1.23×10^8	1.9×10^3	> 0.999

Precision and accuracy

Precision (RSD) and accuracy (percent recovery) data are shown in Figure 4. Across the three spiking levels tested (80%, 100%, and 120% of nominal concentration), recovery for each of the three APIs fell within 98.5–101.5%, satisfying the ICH Q2(R1) acceptance criteria, while RSD values stayed below 0.22% both within a day and between sessions.

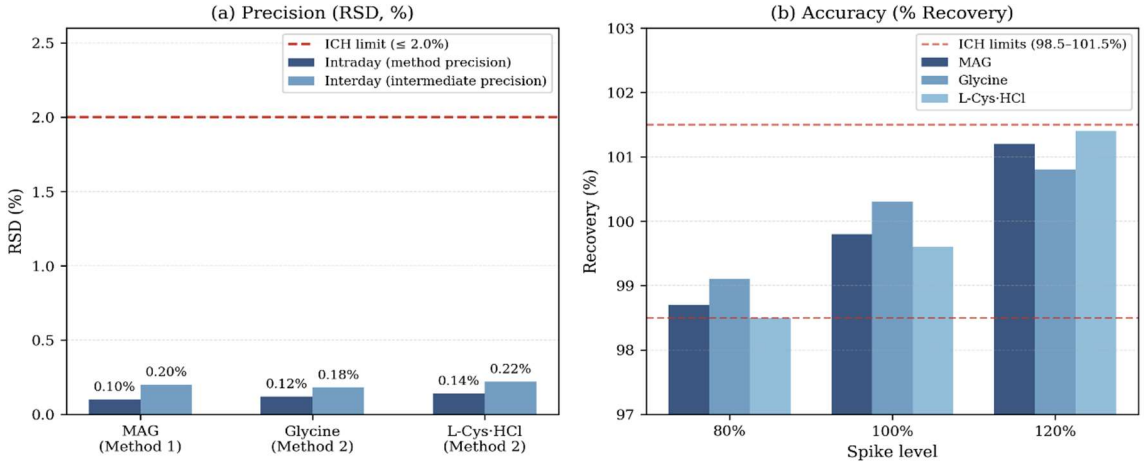


Fig. 4. (a) Precision expressed as RSD (%) for intra-day and inter-session measurements; the red dashed line indicates the ICH limit (2.0%). (b) Accuracy expressed as percent recovery at three spiking levels (80%, 100%, 120%); red dashed lines indicate the ICH acceptance range (98.5–101.5%).

Table 4. Accuracy results (percent recovery, n = 3 per level).

Analyte	Spike 80% (%)	Spike 100% (%)	Spike 120% (%)
MAG	98.7 ± 0.4	99.8 ± 0.3	101.2 ± 0.5
Glycine	99.1 ± 0.5	100.3 ± 0.4	100.8 ± 0.4
L-Cys·HCl	98.5 ± 0.6	99.6 ± 0.5	101.4 ± 0.6

Robustness

Robustness of Method 1 was assessed by deliberately altering the flow rate (1.1 and 1.3 mL/min), the column temperature (25 and 35 °C), and the proportion of organic modifier (±10%); the corresponding results appear in Figure 5 and Table 5.

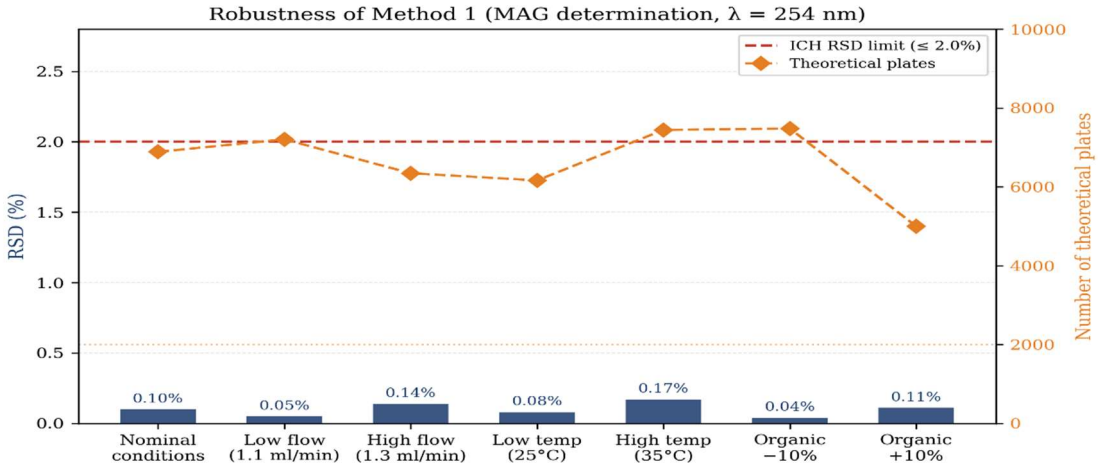


Fig. 5. Robustness of Method 1 (MAG, λ = 254 nm). Bars show RSD (%) under the various conditions (left axis); diamonds indicate the number of theoretical plates (right axis). The red dashed line marks the ICH RSD limit (2.0%); the orange dotted line marks the minimum theoretical-plate criterion (2000).

Table 5. Robustness data for Method 1 (MAG determination).

Condition	Symmetry factor	Theoretical plates	RSD (%)
Nominal conditions	1.124	6888	0.10
Low flow (1.1 mL/min)	1.126	7205	0.05
High flow (1.3 mL/min)	1.118	6346	0.14
Low temperature (25 °C)	1.099	6164	0.08
High temperature (35 °C)	1.145	7444	0.17
Organic solvent −10%	1.357	7480	0.04
Organic solvent +10%	1.375	4999	0.11

Across every condition tested for Method 1, RSD stayed at or below 0.20%, symmetry factors remained within the acceptable range, and the theoretical plate count never fell below 4999 — well above

the minimum requirement of 2000. These findings support the robustness of Method 1 to minor variations in analytical conditions.

Solution and mobile-phase stability

Both standard and test solutions proved stable at room temperature over a 24-hour period, with peak area changes staying under 0.5% relative to the initial values. Likewise, the acetic-acid-based mobile phase used in Method 1 retained its chromatographic performance throughout the same 24-hour interval.

In-house pharmacopoeial monograph (specification)

Drawing on the validation outcomes and the requirements set out in the State Pharmacopoeia of the Republic of Uzbekistan, a proposed in-house pharmacopoeial specification was formulated for the 100 mL «GEPA-SEEM» infusion solution (Table 6). pH, osmolality, bacterial endotoxins, sterility, and shelf life were not directly evaluated within the HPLC validation study and are therefore presented as proposed quality specifications for the in-house monograph.

Table 6. Specification of the «GEPA-SEEM» infusion solution (in-house monograph).

Test	Requirement	Control method
Description	Clear, colorless or slightly yellowish solution	Visual
Identification (MAG, Gly, L-Cys·HCl)	HPLC retention time matches the standard	HPLC, SPh RUz 2.2.29
Clarity	Clear (compared with water for injection)	SPh RUz 2.2.1
Color	Colorless or not darker than reference solution Y3	SPh RUz 2.2.2
pH	5.0–7.0	Potentiometric, SPh RUz 2.2.3
Visible particles	None permitted	Visual, SPh RUz 2.9.20
Sub-visible particles (100 mL): ≥10 µm; ≥25 µm	≤ 6000 particles/vial; ≤ 600 particles/vial	SPh RUz 2.9.19
Related substances: 18α-GZA; Imp-A; Any other; Total	≤10.0%; ≤5.0%; ≤2.0%; ≤7.0%	HPLC, SPh RUz 2.2.29
Density	1.000–1.020 g/cm³	SPh RUz 2.2.5
Osmolality	260–320 mOsmol/kg	Cryoscopic, SPh RUz 2.2.35
Bacterial endotoxins	≤ 0.3 EU/mL	SPh RUz 2.6.14
Sterility	Must be sterile	Membrane filtration, SPh RUz 2.6.1
Assay (per 1 mL): MAG; Glycine; L-Cys·HCl	0.00072–0.00088 g; 0.0072–0.0088 g; 0.00054–0.00066 g	HPLC, SPh RUz 2.2.29
Shelf life	2 years	—

Discussion

The pair of HPLC methods developed here addresses the analytical challenge created by the compositional heterogeneity of «GEPA-SEEM»: its three APIs — a triterpenoid glycoside, an aliphatic amino acid, and a sulfur-containing amino acid — differ substantially in chemical character, so that adequate separation and sensitive detection could only be achieved by applying distinct chromatographic conditions to each.

Method 1 relies on a reversed-phase C18 column combined with an aqueous–organic–acidic mobile phase, a configuration that yields excellent retention and peak symmetry for MAG while also permitting

detection of its principal impurity, 18α-glycyrrhizinic acid (relative retention 1.36). Detection was carried out at 254 nm, providing selective determination of MAG under the selected chromatographic conditions.

Method 2, by contrast, relies on an ion-pairing chromatographic strategy in which tetrabutylammonium hydroxide serves as the ion-pairing reagent, a step that proves essential for retaining the polar amino acids on the C18 stationary phase. Detection at 210 nm, using the short-wavelength UV absorption of free amino acids, enables glycine and L-Cys·HCl to be quantified together in a single analytical run. The two amino acid peaks are resolved to baseline (resolution factor greater than 3), which allows accurate peak integration free of mutual interference.

The proposed specification limits for API content are set at ±10% of nominal content. The pH range of 5.0–7.0 and osmolality range of 260–320 mOsmol/kg are presented as quality specifications; their physiological compatibility and conformity with an isotonic limit were not independently evaluated in the present study.

The methods described are ready for direct implementation in pharmaceutical quality-control laboratories and may further serve as a template for developing comparable analytical procedures for other multi-component hepatoprotective infusion products.

Experimental section

Reagents and reference standards

Reference standards: monoammonium glycyrrhizinate (batch AmGly04-03/23-02, purity 96.76%); glycine (batch Gly03-03/23-02, purity 99.90%); L-cysteine hydrochloride (batch Cys.Hyd02-03/23-02, purity 99.60%). Reagents: acetonitrile (HPLC grade, ≥99.9%, VWR Chemicals); tetrabutylammonium hydroxide solution 54–56% (Sigma-Aldrich); orthophosphoric acid (≥99.0%, Sigma-Aldrich); glacial acetic acid (Supelco); water for injection (conductivity ≤1.2 μS·cm^{-1}, produced by SEEM). All chemicals were used as received.

Preparation of standard and test solutions. The nominal working concentrations for the linearity range were calculated from the nominal product content: 0.004 mg/mL for MAG, 0.004 mg/mL for glycine, and 0.003 mg/mL for L-Cys·HCl. These values correspond to overall dilution factors of 200-fold, 2000-fold, and 200-fold, respectively. Accuracy testing at 80%, 100%, and 120% levels was performed with n = 3 at each level.

Equipment

Two Shimadzu HPLC systems were used: (1) Shimadzu LC-40 Nexera Lite (serial number L22155902420AE) and (2) Shimadzu LC-20 Prominence. Chromatographic separation was performed on a Shim-pack GIST C18 column (250 × 4.6 mm, 5 μm; serial number 21C22070). Additional equipment: AUY 120 D analytical balance (Shimadzu); KQ-100 DE ultrasonic bath; Sartorius Stedim filter device; FiveEasy F20 pH meter (Mettler Toledo). All equipment was calibrated within the established schedule.

Chromatographic methods

Method 1 (MAG, λ = 254 nm): mobile phase water–acetonitrile–glacial acetic acid (61:38:1, v/v/v), flow rate 1.2 mL/min, Shim-pack GIST C18 column (250 × 4.6 mm, 5 μm). Method 2 (glycine and L-Cys·HCl, λ = 210 nm): mobile phase tetrabutylammonium hydroxide buffer (pH 7.0)–acetonitrile (95:5, v/v), flow rate 0.5 mL/min.

Validation methodology

Both methods were validated in accordance with ICH Q2(R1) [6], taking into account the principles of ICH Q2(R2) [7] and ICH Q14 [8], for the following parameters: specificity (by comparing blank and placebo solutions with the standard); precision (intra-day and inter-session repeatability based on 5 injections); accuracy (spike-recovery method at 80%, 100%, and 120% of nominal concentration, n = 3 per level); linearity (calibration at no fewer than five points over 50–150% of nominal concentration); robustness for Method 1 (by deliberately varying flow rate, column temperature, and organic modifier content); and solution and mobile-phase stability (at room temperature over 24 hours).

Conclusion. In summary, two HPLC methods were developed and fully validated in accordance with ICH Q2(R1) for the quantitative determination of MAG (Method 1, λ = 254 nm) and of glycine together with L-cysteine hydrochloride (Method 2, λ = 210 nm) in the combined infusion solution «GEPA-SEEM». Both methods proved specific, precise (RSD ≤ 0.22%), accurate (recovery 98.5–101.5%), and linear across the working concentration range ($R^2 > 0.999$); the robustness experiment reported here was performed for Method 1 and gave satisfactory results. An in-house pharmacopoeial monograph specifying the quality requirements for «GEPA-SEEM» was compiled and may provide a regulatory foundation for registering the product in the Republic of Uzbekistan and for extending its use to other markets.

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