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DEVELOPMENT AND VALIDATION OF AN ANALYTICAL RP-HPLC METHOD FOR THE DETERMINATION OF RELATED IMPURITIES IN MESALAMINE

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Related Impurities, Method
Validation, Quantification and Purity
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ABSTRACT: Mesalamine is a key therapeutic agent used in the treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Ensuring its purity and controlling related impurities is critical for drug safety and efficacy. This study focuses on the development and validation of a robust reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous detection and quantification of three structurally related impurities Impurity G, Impurity K, and Impurity L in mesalamine pharmaceutical formulations. The chromatographic separation was achieved using an Agilent C18 column with an isocratic mobile phase composed of methanol and 0.1% acetic acid (52:48, v/v), at a flow rate of 0.85 mL/min, with detection at 229 nm. The method was validated in accordance with ICH Q2(R2) guidelines, covering critical parameters such as specificity, linearity ($R^2 \geq 0.999$), precision (%RSD ≤ 0.65), accuracy (% recovery 94.8%–96%), robustness, limit of detection (0.20 $\mu\text{g/mL}$), and limit of quantification (0.62 $\mu\text{g/mL}$). The assay demonstrated high reliability for quality control applications and routine analysis of mesalamine products. Additionally, the method showed effective impurity resolution without the need for stress-degradation studies, making it stability-neutral. Overall, this validated RP-HPLC method serves as a regulatory-compliant and practical analytical tool for the pharmaceutical industry, ensuring the consistent quality and safety of mesalamine-based therapies.

INTRODUCTION: Mesalamine (5-aminosalicylic acid, 5-ASA) is a widely used anti-inflammatory agent in the treatment and management of inflammatory bowel disease (IBD), especially ulcerative colitis and Crohn's disease ¹⁻³. Its therapeutic effect is exerted locally in the colonic mucosa, where it inhibits the cyclooxygenase and

lipoxygenase pathways, suppresses prostaglandin and leukotriene synthesis, and neutralizes free radicals ^{3, 4}. Beyond anti-inflammatory action, mesalamine also modulates nuclear factor- κB (NF- κB) signaling and reduces interleukin expression, contributing to mucosal healing ^{5, 6}.

Clinical and pharmacological reviews confirm its efficacy and favourable safety profile in both induction and maintenance phases of IBD therapy ^{1, 2, 3, 7}. Furthermore, recent molecular studies have identified mesalamine's potential anticancer properties, particularly in colorectal cancer prevention, through regulation of cell proliferation and apoptotic pathways ⁶.

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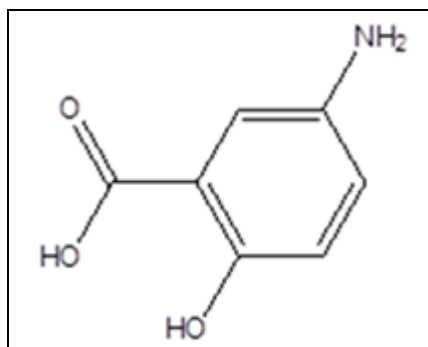


FIG. 1: MESALAMINE

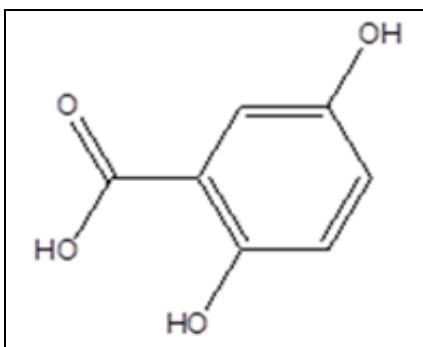


FIG. 2: MESALAMINE IMPURITY G

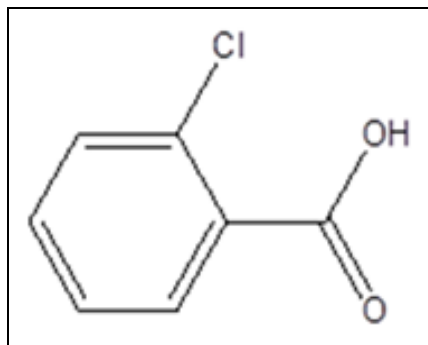


FIG. 3: MESALAMINE IMPURITY L

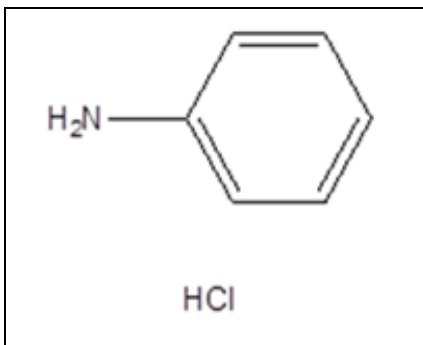


FIG. 4: MESALAMINE IMPURITY K

Pharmaceutical formulations of mesalamine, especially delayed-release tablets, must be rigorously monitored for the presence of related impurities. During synthesis, processing, or storage, mesalamine is prone to forming structurally related substances. Of particular importance are Impurity-G, Impurity-K, and Impurity-L, which may originate from synthetic intermediates or byproduct reactions^{8, 9}. Their accumulation, even at low concentrations, can affect product stability and patient safety. Regulatory frameworks such as ICH Q3A(R2) and Q3B(R2) require that such impurities be identified, quantified, and controlled using validated analytical procedures¹⁰.

For impurity profiling, reversed-phase high-performance liquid chromatography (RP-HPLC) is the most commonly used technique due to its selectivity, accuracy, and compatibility with regulatory expectations^{11, 12}. Numerous studies have investigated HPLC-based methods for mesalamine quantification. Alhadad and Al-Salman developed an RP-HPLC spectrophotometric method for mesalamine analysis in bulk and formulations¹³. Trivedi and Patel presented a UPLC method to detect genotoxic/aniline-related impurities with enhanced sensitivity⁸, while a separate study by the same authors evaluated

mesalamine impurity profiling using a validated reversed-phase approach⁹. Piponski *et al.* focused on impurities A and C using a fast HPLC method tailored to both raw and finished products¹⁴. Gatkai *et al.* proposed a stress-based stability indicating assay for mesalamine under various degradation conditions¹⁵, further supported by a related study validating assay performance in both bulk and tablet forms¹⁶. Similarly, Sahoo *et al.*¹⁷ and Moharana *et al.*¹⁸ reported RP-HPLC methods for estimating mesalamine, focusing on content uniformity and impurity detection. However, many existing methods either lack coverage of Impurities G, K, and L or were primarily designed for degradation studies.

More recent approaches such as those by Tsamis *et al.* integrated HPLC-DAD and LC-MS for mesalamine and related actives in rectal and oral products¹⁹. Additional dual-analyte RP-HPLC methods, such as those involving mesalamine with prednisolone²⁰ or curcumin²¹, offer insights into separation challenges in multicomponent formulations. At the same time, studies investigating mesalamine's dissolution discrepancies²², bioanalytical detection in plasma²³, and food effect pharmacokinetics²⁵ underline the complexity of its behaviour in both analytical and physiological environments.

Despite these developments, gaps remain in the availability of a routine, validated, impurity-focused RP-HPLC method specifically designed for the quantification of mesalamine alongside Impurities G, K, and L in finished pharmaceutical products. Most literature methods either target general degradation pathways^{15, 16} plasma-level detection²³, or method combinations with other actives^{20, 21}.

The ICH Q2(R2) guideline outlines essential criteria for method validation, including specificity, linearity, precision, accuracy, robustness, detection limits, and system suitability^{10, 11}. Recent reviews highlight that while HPLC remains a gold-standard analytical tool, there is increasing emphasis on the application of Design of Experiments (DoE) for method optimization and regulatory readiness²⁴. Furthermore, evolving perspectives on HPLC theory and practice continue to highlight improvements in resolution, speed, and system adaptability²⁴⁻²⁶.

Therefore, the aim of this study is to develop and validate a novel RP-HPLC method for the quantification of mesalamine and its related impurities Impurity-G, Impurity-K, and Impurity-L in pharmaceutical dosage forms. The method is designed to be stability-neutral, does not rely on forced degradation, and is validated in full compliance with ICH Q2(R2). This work provides a robust and regulatory-compliant tool for routine impurity profiling and quality control of mesalamine-based drug products.

Experimental:

Chemical and Reagents: Mesalamine drug substance, working reference standards of IMP-G, IMP-K, and IMP-L were acquired from B.L Chemicals, Mumbai. Potassium Di Hydrogen Ortho Phosphate (HPLC grade, Merck, Mumbai), Ortho Phosphoric Acid (AR grade, Merck, Mumbai), Acetonitrile (HPLC grade, Merck, Mumbai) and Milli Q water.

Instrumentation: The Agilent Tech. Gradient System HPLC (Model No: 1100), equipped with an online degasser, column thermostat, automatic sampler, and UV-DAD detector (G13148, S.NO. DE71365875). Data integration and analysis were performed using Agilent ChemStation software.

This setup enabled efficient separation and reliable detection under controlled chromatographic conditions.

Chromatographic Conditions: The Agilent Tech. Gradient System HPLC), with a quaternary gradient pump auto-injector, and UV-DAD detector, was used for the analysis of Mesalamine and its related impurities (G, K, L). Data integration was performed using Agilent ChemStation software. Chromatographic separation was achieved on a reverse-phase Agilent C18 column (4.6 × 250 mm, 5 µm particle size) using an isocratic mobile phase of Methanol and 0.1% Acetic Acid (52:48% v/v) at a flow rate of 0.85 mL/min. The column was maintained at 25°C, with detection at 229 nm.

Preparation of Stock Standard Solutions:

Preparation of Mesalamine:

1. Std Mesalamine 10 mg in 10 ml Methanol = 1000 µg/ml Mesalamine - Stock -I
2. Take 0.1 from stock I. and make vol. with Diluent 10 ML = 10 µg/ml Mesalamine
3. Take 0.2 from stock I. and make vol. with mobile phase 10 ML = 20 µg/ml Mesalamine
4. Take 0.3 from stock I. and make vol. with mobile phase 10 ML = 30 µg/ml Mesalamine
5. Take 0.4 from stock I. and make vol. with mobile phase 10 ML = 40 µg/ml Mesalamine
6. Take 0.5 from stock I. and make vol. with mobile phase 10 ML = 50 µg/ml Mesalamine

Preparation of Impurities:

Stock Solution I: (1000 µg/mL for Impurities G, K, L):

Weigh 10 mg of each impurity (G, K, L) and dissolve in 10 mL Methanol to obtain 1000 µg/mL stock solutions.

Preparation of Working Solutions:

- ❖ 10 µg/mL Impurity (G, K, L): Take 0.1 mL from Stock I and dilute to 10 mL with diluent.

- ❖ 20 µg/mL Impurity (G, K, L): Take 0.2 mL from Stock I and dilute to 10 mL with mobile phase.
- ❖ 30 µg/mL Impurity (G, K, L): Take 0.3 mL from Stock I and dilute to 10 mL with mobile phase.
- ❖ 40 µg/mL Impurity (G, K, L): Take 0.4 mL from Stock I and dilute to 10 mL with mobile phase.
- ❖ 50 µg/mL Impurity (G, K, L): Take 0.5 mL from Stock I and dilute to 10 mL with mobile phase.

Preparation of Mesalamine and Impurities (Combined):

Stock-I: Weigh 10 mg of Mesalamine, Impurity G, Impurity K, and Impurity L separately and dissolve in 10 mL Methanol to obtain 1000 µg/mL stock solutions for each.

Preparation of Combined Working Solution (20 µg/mL each):

1. Take 0.2 mL from Mesalamine Stock-I (1000 µg/mL).
2. Take 0.2 mL from Impurity G Stock-I (1000 µg/mL).
3. Take 0.2 mL from Impurity K Stock-I (1000 µg/mL).
4. Take 0.2 mL from Impurity L Stock-I (1000 µg/mL).
5. Make up the volume to 10 mL with mobile phase.

Method Validation: The method validation was performed as per ICH guidelines, covering accuracy, precision, linearity, LOD, LOQ, robustness, and system suitability to ensure the reliability of the analytical procedure.

Linearity: Linearity is the ability of an analytical method to produce results that are directly proportional to the concentration of the analyte within a specified range. It is an essential parameter that ensures accurate quantification of the analyte. A calibration curve is typically constructed by plotting the response against the concentration, and

the linear regression equation is used to determine the correlation coefficient (R^2). A high R^2 value confirms the method's linearity over the specified range. Linearity is crucial for pharmaceutical analysis, ensuring precise dosing and reliable quantification. Generally, at least five concentration levels are used to establish linearity, and the correlation coefficient should ideally be ≥ 0.99 .

Precision: Precision refers to the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of the same homogeneous sample. It is expressed as the relative standard deviation (%RSD) and is assessed at different concentration levels. Precision is categorized into repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability evaluates consistency under the same conditions, while intermediate precision assesses reproducibility under different conditions, such as different days, analysts, or instruments. Precision is crucial for ensuring reliable and consistent results, and the %RSD should typically be within acceptable limits ($\leq 2\%$) for pharmaceutical methods.

Accuracy: Accuracy is the closeness of the measured value to the true or accepted reference value and is typically expressed as % Recovery. It is determined through recovery studies by spiking known amounts of the analyte at different levels, usually 80%, 100%, and 120% of the target concentration. The percentage recovery should be within an acceptable range (typically 98–102%) to confirm that the method accurately quantifies the analyte. Accuracy is essential in pharmaceutical analysis to ensure that the method provides correct results, preventing potential dosing errors.

Repeatability: Repeatability is a subset of precision that measures the consistency of results under identical operating conditions over a short period. It is evaluated by analysing the same sample multiple times and calculating the %RSD. Repeatability ensures that the method produces stable and reliable results when performed under identical conditions. Typically, the %RSD should be $\leq 2\%$ in pharmaceutical methods to confirm the method's reliability.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Limit of Detection (LOD) and Limit of Quantification (LOQ) define the sensitivity of the method. LOD is the lowest concentration of the analyte that can be detected but not necessarily quantified, whereas LOQ is the lowest concentration that can be quantified with acceptable precision and accuracy. These values are calculated using standard formulas involving the standard deviation and slope of the calibration curve. LOD and LOQ are critical for determining the method's ability to detect and quantify trace amounts of the analyte, which is particularly important in impurity detection and low-concentration analysis.

Robustness: Robustness refers to the ability of an analytical method to remain unaffected by small, deliberate variations in method parameters. It is assessed by introducing minor changes to critical factors such as flow rate, mobile phase composition, and detection wavelength. A robust method ensures consistent performance despite minor variations, making it more practical for use across different laboratories. Robustness testing typically involves changing one parameter at a time while keeping others constant to evaluate the method's stability.

Assay: Assay is the determination of the percentage of the active ingredient (analyte) in a sample compared to the label claim. It is performed at a specific concentration to verify the accuracy and precision of the method. The assay ensures that the method can reliably determine the correct

amount of active pharmaceutical ingredient (API) in formulations, which is essential for quality control and regulatory compliance. The %RSD should be within the acceptable range (typically $\leq 2\%$) to confirm the accuracy and precision of the assay

RESULTS AND DISCUSSION:

Method Development: The RP-HPLC analysis was conducted using an Agilent Technologies Gradient System (Model No. 1100) with an auto-injector, ensuring precise sample introduction. A UV-DAD detector (G13148, S.No. DE71365875) was utilized for detection, while a quaternary gradient pump (G130A, S. No. DE9180834) facilitated efficient mobile phase delivery. The system was operated using Agilent ChemStation software for data acquisition and processing. The chromatographic separation was performed on an Agilent C18 column (4.6×250 mm, $5 \mu\text{m}$ particle size packing), providing high-resolution analysis. The mobile phase consisted of methanol (MeOH) and 0.1% acetic acid in a 52:48 (% v/v) ratio, ensuring optimal analyte separation. The detection wavelength was set at 229 nm, and the flow rate was maintained at 0.85 mL/min, with a column temperature of 25°C to ensure reproducibility. The sample injection volume was 20 μL , and the diluent used was a mixture of 60% buffer (0.1% OPA in water) and 40% MeOH, ensuring proper solubility and stability of the analyte. This method was optimized to achieve precise, accurate, and reproducible results, making it suitable for pharmaceutical analysis.

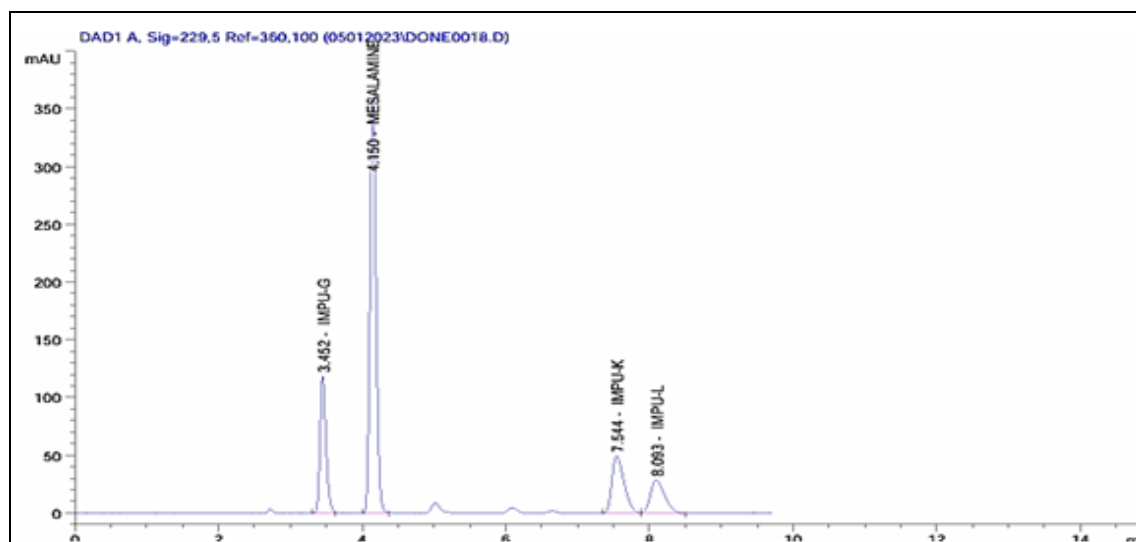


FIG. 5: CHROMATOGRAM SHOWING THE SEPARATION OF MESALAMINE

Linearity and Range:

Linearity: The method was evaluated using five different concentrations: 10, 20, 30, 40, and 50 µg/mL. Each concentration was injected into the HPLC system, and the corresponding peak areas were recorded.

The calibration curve plotted peak area against concentration, resulting in the linear regression equation: $Y = 44.66x - 34.57$. The slope of the equation, 44.66, represents the sensitivity of the method, while the y-intercept, -34.57, reflects the theoretical response at zero concentration. The correlation coefficient ($R^2 = 0.999$) confirms an

excellent linear relationship, indicating that the method is highly linear over the specified range.

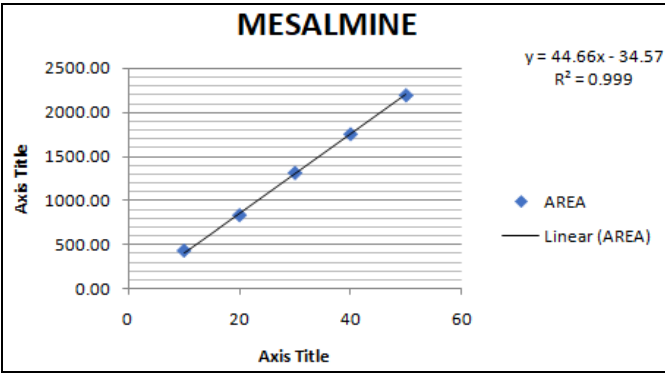


FIG. 6: CHROMATOGRAM FOR LINEARITY

TABLE 1: LINEARITY DATA FOR MESALAMINE AND ITS IMPURITIES

Concentration (µg/mL)	Area (Impurity G)	Area (Impurity L)	Area (Impurity K)
10	427.0869	427.7375	427.0869
20	830.6176	833.03	830.6176
30	1308.2819	1319.1252	1308.2819
40	1756.4877	1754.3034	1756.4877
50	2199.7202	2198.3667	2199.7202

Precision: Precision was evaluated at three concentration levels 10, 30, and 50 µg/mL. Multiple injections were performed for each concentration, and the peak areas were recorded. The %RSD was calculated to assess variability. For repeatability (intra-day precision), the same concentration (30 µg/mL) was analysed multiple times under the same conditions, yielding a low

%RSD, indicating consistent results. For intermediate precision (inter-day precision), the same concentration was analysed on different days and under varied conditions. The %RSD values remained within the acceptable limit, demonstrating that the method provides reproducible results even under different experimental conditions.

TABLE 2: PRECISION RESULTS

Concentration (µg/mL)	Intra-day Mean Area	Intra-day %RSD	Inter-day Mean Area	Inter-day %RSD
10	425.79	0.65	425.82	0.01
30	1311.35	0.04	1312.73	0.16
50	2183.01	0.38	2183.86	0.04

Accuracy: Accuracy was determined through recovery studies at 80%, 100%, and 120% of the target concentration. At the 100% level, the measured recovery was close to 100%, confirming

that the method accurately quantifies the analyte. The recovery values across all levels remained within the acceptable range (98–102%), ensuring the correctness of the method.

TABLE 3: ACCURACY RESULTS

Concentration (µg/mL)	Amount Added	Amount Found	% Recovery
10	8	7.68	96.0
10	10	9.58	95.8
10	12	11.37	94.8

Repeatability: The repeatability of the method was assessed by analyzing the same concentration (30 µg/mL) six times. The %RSD value was found to be 0.14%, indicating that the method is highly repeatable.

TABLE 4: REPEATABILITY DATA FOR MESALAMINE AND ITS IMPURITIES

Concentration (µg/mL)	Mean Area	SD	%RSD
30	1313.08	1.86	0.14

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curve. The LOD and LOQ values were found to be 0.20 µg/mL and 0.62 µg/mL, respectively, indicating that the method is sensitive and capable of detecting and quantifying low concentrations of Mesalamine and its impurities.

Robustness: The robustness of the method was tested by making small changes to critical

parameters: flow rate (0.75 mL/min to 0.95 mL/min), mobile phase composition (methanol percentage changed from 50.9% to 52.9%), and wavelength (227 nm to 229 nm).

The impact of these variations on the peak area and %RSD was evaluated. The results showed minimal deviation, confirming that the method remains reliable under slight changes in analytical conditions.

TABLE 5: ROBUSTNESS DATA FOR MESALAMINE AND ITS IMPURITIES

Parameter	Variation	Mean Area	SD	%RSD
Flow Rate (mL/min)	0.75	1056.62	0.09	0.01
	0.95	820.31	0.66	0.08
Wavelength (nm)	227	1001.86	2.62	0.26
	229	932.80	2.02	0.22

Assay: The assay was performed at 50 µg/mL, and the percentage label claim was determined. The mean percentage label claim was found to be within the acceptable range, confirming that the method accurately quantifies the active ingredient in the pharmaceutical formulation. The %RSD was also within limits ($\leq 2\%$), ensuring precision and reliability in quantification.

CONCLUSION: The Present study successfully developed and validated a precise, accurate, and robust RP-HPLC method for the identification and quantification of mesalamine-related impurities, specifically impurities G, L, and K. The method exhibited excellent specificity, linearity, precision, accuracy, and robustness, meeting the stringent requirements of ICH guidelines. Optimized chromatographic conditions ensured effective separation of impurities, making the method highly suitable for routine quality control and regulatory compliance. The validated method provides a reliable analytical tool for ensuring the safety, efficacy, and purity of mesalamine formulations. Future studies may focus on enhancing sensitivity, expanding applicability to different formulations, and conducting long-term stability assessments to further support pharmaceutical development and regulatory submissions.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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