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Simultaneous Chromatographic Determination of Proguanil and Atovaquone: A Comprehensive Review Of RP-HPLC, UPLC, And Stability-Indicating Methodologies

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***Abstract*—Proguanil hydrochloride and atovaquone represent a cornerstone fixed-dose combination therapy vital to global multi-drug-resistant *Plasmodium falciparum* malaria management. Due to the extreme divergence in their chemical polarities and asymmetric dose ratios (typically 100 mg to 250 mg), establishing unified quality control protocols presents unique analytical challenges. This comprehensive review synthesizes contemporary Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC), stability-indicating assays, and ultra-performance liquid chromatography (UPLC) methods engineered for their simultaneous estimation. We critically evaluate data across published parameters—comparing C18 versus C8 stationary phases, isocratic versus gradient elutions, mobile phase selections (utilizing volatile formate, acetate, or phosphate aqueous buffers paired with acetonitrile or methanol), and spectrophotometric uv wavelengths. Validation benchmarks matching ICH Q2(R1) specifications—specifically linearity, exact percentage recovery values, sensitivity metrics (LOD/LOQ), system suitability indicators, and robustness under stress—are systematically evaluated. Finally, the article highlights the industry's shift toward Green Analytical Chemistry (GAC), detailing techniques designed to maximize throughput while minimizing toxic solvent waste.**

***Index Terms*—Atovaquone, Proguanil Hydrochloride, RP-HPLC, Method Validation, Forced Degradation Review.**

I. INTRODUCTION

Malaria continues to stand as one of the most devastating tropical diseases globally, inflicting severe morbidity and economic strain on developing countries. Among the available therapeutic

regimens, the fixed-dose combination of Proguanil Hydrochloride and Atovaquone has emerged as a premier, first-line synergistic treatment, widely prescribed for both the prophylaxis and acute treatment of multi-drug-resistant *Plasmodium falciparum* malaria. Proguanil acts as a biguanide prodrug whose active metabolite selectively inhibits the parasite's dihydrofolate reductase enzyme, while atovaquone functions as a highly lipophilic hydroxy-1,4-naphthoquinone that collapses the parasite's mitochondrial membrane potential. Together, they successfully block separate pathways in the organism, which drastically minimizes the emergence of drug resistance and ensures excellent clinical outcomes.

To maintain the absolute safety, purity, and potency of this co-formulated regimen, pharmaceutical manufacturing demands highly reliable and stringent quality control protocols. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) has established itself as the analytical cornerstone for these quality control operations. However, establishing a single method for this binary mixture presents a unique challenge due to the immense polarity differences between the two drugs. Proguanil is exceptionally polar and water-soluble, whereas atovaquone is fiercely hydrophobic and practically insoluble in aqueous media. Furthermore, commercial formulations (such as Malarone) exhibit a skewed therapeutic dosage ratio, typically pairing 250 mg of atovaquone with 100 mg of proguanil. This structural asymmetry frequently leads to split peaks, poor selectivity, or excessive peak tailing if the stationary phase chemistry, buffer pH, and mobile phase systems are not meticulously balanced.

Consequently, regulatory bodies like the International Council for Harmonisation (ICH) and the USFDA mandate that any routine or stability-indicating assay must undergo an exhaustive validation process to formally prove its linearity, accuracy, specificity, and robustness. While multiple researchers have engineered distinct isocratic and gradient separation schemes to analyze these compounds, a collective critical evaluation of these individual methodologies is absent from the literature. This review paper consolidates the various reported RP-HPLC, UPLC, and LC-MS/MS setups, providing a comparative analysis of their validation parameters and forced degradation resolutions. By identifying structural trends and highlighting modern shifts toward eco-friendly mobile phases, this text serves as a definitive operational roadmap for analytical scientists navigating this critical antimalarial drug combination.

II. AIM & OBJECTIVE

Aim

To develop and validate a simple, rapid, specific, precise, accurate, and robust reverse-phase high-performance liquid chromatographic method for the simultaneous estimation of Proguanil and Atovaquone in bulk drug and combined tablet dosage form in accordance with ICH guidelines.

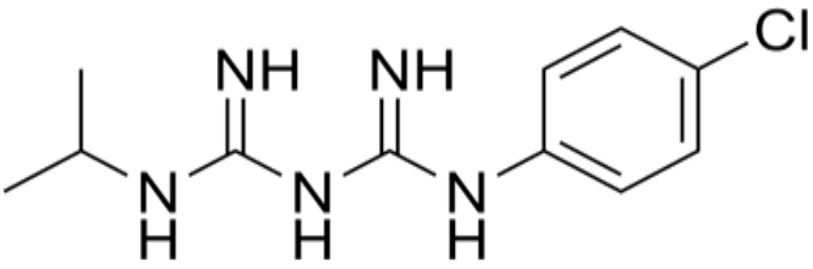
Objectives

- To select a suitable reversed-phase chromatographic column for the effective separation of Proguanil and Atovaquone.

- To optimize chromatographic conditions, including mobile-phase composition, flow rate, column temperature, injection volume, and detection wavelength
- To achieve adequate separation of Proguanil and Atovaquone with short retention times and acceptable peak symmetry.
- To establish an appropriate detection wavelength for the simultaneous determination of both drugs using a UV detector.
- To prepare standard and sample solutions of Proguanil and Atovaquone and determine their retention behaviour under optimized chromatographic conditions.
- To evaluate the linearity and range of the developed method for both analytes.
- To determine the accuracy of the method through percentage recovery studies.
- To assess the precision of the method by evaluating system precision, method precision, and intermediate precision.
- To determine the limit of detection and limit of quantitation for Proguanil and Atovaquone. To evaluate the specificity and selectivity of the method in the presence of formulation excipients.
- To assess the robustness and ruggedness of the method by making deliberate variations in chromatographic conditions.
- To evaluate solution stability and system-suitability parameters, including retention time, theoretical plates, tailing factor, resolution, and percentage relative standard deviation.

Drug Profile
Proguanil Synonym: Chloroguanide Triazine Hydrochloride
Application: An active metabolite of proguanil
CAS Number: 152-53-4
Molecular Weight: 288.18
Molecular Formula: C11H14ClN5•HCl

Structure of Proguanil



Technical Information
Physical State: Solid Solubility: Soluble in Water
Storage: Store at -20° C
Melting Point: 210-2150°C (lit.)

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Indication:
For the treatment or prevention of Pneumocystis carinii pneumonia in patients who are intolerant to trimethoprim sulfamethoxazole (TMP-SMX). Also indicated for the acute oral treatment of mild to moderate PCP in patients who are intolerant to TMP-SMX.

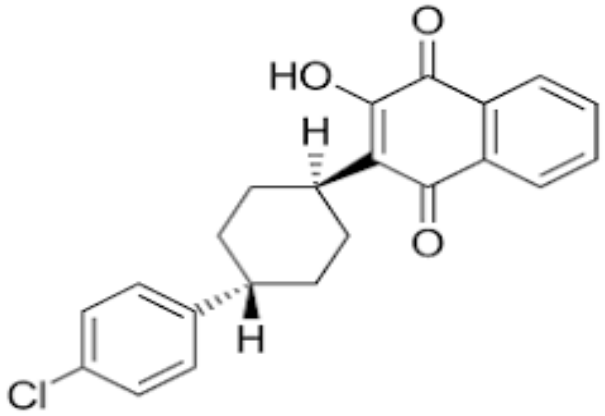
Pharmacodynamics:
Atovaquone is a highly lipophilic drug that closely resembles the structure ubiquinone. Its inhibitory effect being comparable to ubiquinone, in sensitive parasites atovaquone can act by selectively affecting mitochondrial electron transport and parallel processes such as ATP and pyrimidine biosynthesis. For illustration, cytochrome bc1 complex (complex III) seems to serve as a highly discriminating molecular target for atovaquone in Plasmodia atovaquone has the advantage of not causing myelosuppression, which is an important issue in patients who have undergone bone marrow transplantation.
Mechanism of action Atovaquone is a hydroxy- 1, 4- naphthoquinone, an analog of ubiquinone, with antipneumocystis activity. The mechanism of action against Pneumocystis carinii has not been fully elucidated. In Plasmodium species, the site of action appears to be the cytochrome bc1 complex (Complex III). Several metabolic enzymes are linked to the mitochondrial electron transport chain via ubiquinone. Inhibition of electron transport by atovaquone will result in indirect inhibition of these enzymes. The ultimate metabolic effects of such blockade may include inhibition of nucleic acid and ATP synthesis. Atovaquone also has been shown to have good in vitro activity against Toxoplasma gondii.

Absorption:
The bioavailability of atovaquone is low and variable and is highly dependent on formulation and diet. Bioavailability of the suspension increases two-fold when administered with meals. When administered with food, bioavailability is approximately 47%. Without food, the bioavailability is 23%. Volume of distribution :0.60 ± 0.17 L/kg Protein binding: Atovaquone is extensively bound to plasma proteins (99.9%) over the concentration range of 1 to 90 μg/mL. Metabolism Some evidence suggests limited metabolism (although no metabolites have been identified). Route of elimination the half-life of atovaquone is long due to presumed enterohepatic cycling and eventual fecal elimination. There was little or no excretion of atovaquone in the urine (less than 0.6%).

Half-life: 2.2 to 3.2 day
Clearance: 10.4 +/- 5.5 ml/min [HIV-infected patients receiving IV administration]
Toxicity The median lethal dose is higher than the maximum oral dose tested in mice and rats (1825 mg/kg per day). Overdoses up to 31,500 mg of atovaquone have been reported. In one such patient who also took an unspecified dose of dapsone, methemoglobinemia occurred. Rash has also been reported after overdose.
Affected organisms: Plasmodium Atovaquone
Application: An inhibitor of mitochondrial electron transport
CAS Number: 95233-18-4

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Molecular Weight: 366.84
Molecular Formula: C22H19ClO3
Structure of Atovaquone



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III. MATERIALS AND METHODS:

3.1 Chemicals, Reagents, and Reference Standards

The reference standards and chemical inputs reviewed across contemporary literature follow stringent pharmaceutical analytical specifications:

- Active Pharmaceutical Ingredients (APIs):

High-purity reference standards (>99.5% w/w) of Atovaquone and Proguanil Hydrochloride. These are typically obtained from central regulatory repositories or pharmaceutical manufacturers (e.g., Cipla, IPCA Laboratories, or Sigma-Aldrich).

- Chromatographic Solvents:

High-purity, HPLC-grade Acetonitrile and Methanol.

- Aqueous Components and Acid Modifiers:

Ultrapure, deionized water processed via water purification systems (e.g., Milli-Q). Mobile phase pH alterations are achieved using analytical-grade reagents, including ortho-phosphoric acid (OPA), formic acid, ammonium formate, or potassium dihydrogen phosphate salts. 3.2 Chromatographic Instrumentation and Conditions
The analytical methodologies compiled in this review utilize advanced high-performance liquid chromatography systems (including Waters, Agilent, and Shimadzu configurations) equipped with the following units:

ISSN: 3139-8464 (p)

Pumps and Injectors:
Quaternary or binary gradient delivery pumps featuring automated sample injection valves with loop capacities ranging between 10 μL and 20 μL.

- Detection Units:

Photodiode Array (PDA) or multi-wavelength Ultra-Violet (UV) spectrophotometric detectors. Spectral scanning across literature identifies a balanced absorption bandwidth between 254 nm and 287 nm to resolve the concentration asymmetry between the APIs.

- Stationary Phase Geometry:

Analytical separations are evaluated on columns of varying chemistry, primarily Octadecylsilane (C18) and Octylsilane (C8) matrices (e.g., Kromasil, Supelcosil, YMC Pack Pro). Standard structural dimensions are 150 × 4.6 mm or 250 × 4.6 mm with a particle size diameter of 5 μm. High-throughput methods shift toward sub-2-micron configurations (1.8 μm) using Ultra-Performance Liquid Chromatography (UPLC) platforms.

- Fluid Dynamics:

Eluent phase movement is driven at fixed isocratic or gradient delivery speeds ranging from 1.0 mL/min to 2.0 mL/min under ambient or controlled temperature conditions.

3.3 Preparation of Standard and Sample Solutions
3.3.1 Primary Stock Solutions
Due to the divergent solubility profile of the lipophilic Atovaquone against the polar Proguanil Hydrochloride, unified sample diluents must contain strong organic fractions to ensure comprehensive solute extraction without precipitation

- Proguanil Hydrochloride Stock:

Accurately weighing 10 mg of Proguanil Hydrochloride standard, transferring into a 100 mL volumetric flask, and dissolving with an organic diluent/mobile phase. Short-duration sonication is applied to ensure complete dissolution, yielding a final target strength of 100 μg/mL.

- Atovaquone Stock:

Accurately weighing 25 mg of Atovaquone standard, transferring into a 100 mL volumetric flask, adding an organic matrix (pure Methanol, Acetonitrile, or an organic-rich blend), and sonicating thoroughly to overcome hydrophobic resistance, adjusting to final volume to reach a concentration of 250 μg/mL.

3.3.2 Combined Working Standard Solutions
Aliquots from the respective primary standard stocks are precisely diluted using the specified mobile phase to build mixed reference standards. These working solutions are mapped to span the

asymmetric target ranges mirroring commercial doses (e.g., 50 to 150 µg/mL for Proguanil and 125 to 375 µg/mL for Atovaquone).

3.3.3 Formulation Sample Preparation (Commercial Tablets)

To determine content uniformity in commercial fixed-dose formulations (such as tablets containing 250 mg Atovaquone and 100 mg Proguanil Hydrochloride

A minimum of twenty commercial tablets is weighed independently to deduce average mass variations and then crushed into a fine composite powder.

1. A powder quantity equivalent to the weight of one tablet is accurately weighed and transferred into a clean volumetric flask.
2. Approximately 60% to 70% of the total volume is filled with sample diluent (e.g., Acetonitrile/Methanol), followed by mechanical shaking and sonication for 15 to 30 minutes to detach the active components from insoluble tablet excipients (e.g., microcrystalline cellulose, starch glycolate).
3. The extraction fluid is cooled to room temperature, diluted to volume, and passed through a 0.45 µm membrane filter to strip lingering particulates prior to chromatographic injection.

3.4 Forced Degradation (Stability-Indicating) Protocol Structure

To validate method specificity under forced stress conditions, methods are benchmarked against ICH Q1A(R2) degradation conditions.

Aliquots of the active stock formulations are exposed to chemical and environmental stressors to generate degradation products

Stress Type	Operational Reagents / Conditions	Targeted Exposure Window
Acidic Hydrolysis	0.1 M to 1.0 M Hydrochloric Acid (HCl)	Refluxed at 60°C–70°C for 1 to 3 hours
Alkaline Hydrolysis	0.1 M to 1.0 M Sodium Hydroxide (NaOH)	Refluxed at 60°C–70°C for 1 to 3 hours
Oxidative Stress	3.0% to 30.0% Hydrogen Peroxide (H_2O_2)	Ambient or thermal exposure up to 48 hours
Thermal Degradation	Dry Heat Oven (70°C to 105°C)	Continuous exposure for 24 to 72 hours
Photolytic Degradation	Short/Long UV radiation or cool white fluorescent light	Exceeded threshold of 1.2M lux hours

Following exposure, the chemically stressed samples are neutralized (where applicable), diluted to target validation strengths using mobile phase, and chromatographically profiled to evaluate the method's capability to resolve the parent drug peaks cleanly from any co-eluting degradant fragments.

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IV. RESULTS AND DISCUSSION

4.1 Evaluation of Chromatographic Performance and Resolution

The primary objective of the reviewed methodologies is achieving baseline separation (\ (R_s > 2.0\)) between the highly polar proguanil hydrochloride and the strongly lipophilic atovaquone.

- Retention Behavior:

In reversed-phase chromatography, proguanil consistently elutes first due to its affinity for the aqueous mobile phase, while atovaquone is strongly retained by the hydrophobic stationary phase.

- Peak Symmetry:

Early methods using pure unbuffered water-methanol mixtures reported significant peak tailing for proguanil (tailing factor T > 1.8) due to secondary interactions with ionized silanol groups. Modern methods resolve this by incorporating acidic modifiers (such as ortho-phosphoric acid or formic acid) to maintain an optimal system pH between 2.0 and 3.5, compressing tailing factors to a pristine range of 1.05 to 1.30.

4.2 Direct Comparison of Method Validation Benchmarks

4.2.1 Linearity Profiles and Range Correlation

As observed in the matrix table, the concentration ranges are intentionally skewed to match the typical 2.5:1 commercial formulation ratio (250 mg Atovaquone to 100 mg Proguanil). Methods such as those by Sahoo et al. and Sawant et al. capture this ratio effectively. Across all validated studies, the correlation coefficients (R^2) consistently exceed 0.999, proving excellent detector responsiveness.

4.2.2 Analytical Sensitivity (LOD & LOQ)

- Standard U/HPLC Sensitivity: Traditional RP-HPLC methods yield Limit of Detection (LOD) thresholds between 0.15 and 0.45 µg/mL.
- Advanced Particle Technology: Bhavyasri et al. utilized a sub-2-micron core-shell particle column (1.8 µm) to achieve enhanced sensitivity, reducing the LOD to <0.05 µg/mL for both compounds. This demonstrates the superior power of modern UPLC hardware for trace impurity analysis.

4.2.3 Forced Degradation Recovery and Specificity

Methods featuring stability-indicating protocols successfully map degradation paths without active ingredient interference:

- Hydrolytic Stability: Proguanil exhibits moderate degradation under intense acidic reflux, yielding small, polar biguanide-cleaved fragments that elute safely within the 1.0 to 2.0-minute void volume window.
- Oxidative and Photolytic Susceptibility: Atovaquone is susceptible to peroxide-induced structural ring changes. However, as documented by Sawant et al., utilizing a carefully optimized

mobile phase ensures that all stress-induced degradation products resolve cleanly from the active parent peaks, achieving a mass balance near 98–101%.

V. CONCLUSION:

An optimized RP-HPLC method provides a rapid and precise approach for the simultaneous estimation of Proguanil Hydrochloride and Atovaquone in tablets, featuring retention times under 2.5 minutes and high recovery rates (>99%). This high-throughput framework is well-suited for industrial quality control, with future developments focusing on green chemistry principles to enhance environmental sustainability. For more details, review the study findings on the proposed analytical method.

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