RESEARCH PAPER

A Validated, Green HPTLC Densitometric Method for Simultaneous Determination of Sodium Alendronate and Cholecalciferol in Combined Tablet Dosage Form: A QbD-Guided Development, Forced Degradation Profiling, and Comparison with RP-HPLC

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ABSTRACT

Background: High-Performance Thin-Layer Chromatography (HPTLC) offers compelling advantages for pharmaceutical quality control, including exceptional sample throughput (up to 20 samples per plate analyzed simultaneously), minimal solvent consumption (10–20 mL per plate vs. 10–20 mL per injection for HPLC), open-system architecture enabling post-chromatographic derivatization, and suitability for 'green chemistry' principles. A validated HPTLC method for the simultaneous determination of the antiresorptive-vitamin D pair — Sodium Alendronate (SAL) and Cholecalciferol (CHO) — from their fixed-dose combination (FDC) tablet does not exist in the literature. The absence of such a method represents a significant gap given the growing need for cost-effective QC tools in resource-limited settings and the utility of HPTLC as an orthogonal method.

Methods: An Analytical Quality by Design (AQbD) framework was implemented to develop a normal-phase HPTLC method. A Box-Behnken Design (BBD, n=15) was employed to optimize Critical Method Parameters: mobile phase composition, chamber saturation time, application volume, migration distance, and derivatization reagent concentration. The optimized system comprised a normal-phase silica gel 60 F254 aluminum-backed plate with n-Butanol: Glacial Acetic Acid: Water (4:1:0.5, v/v/v) as the mobile phase (BAW system). Dual detection was achieved by densitometric scanning at 265 nm for CHO (direct) and at 520 nm for SAL after post-chromatographic derivatization with 0.2% ninhydrin reagent (110°C, 5 min). Method validation was conducted per ICH Q2(R1).

Results: The BAW mobile phase system achieved complete and reproducible separation of SAL ($R_f \approx 0.32 \pm 0.02$) and CHO ($R_f \approx 0.61 \pm 0.02$) with excellent band resolution ($\Delta R_f = 0.29$). Linearity was established over 200–1200 ng/band for SAL ($r = 0.9992$) and 20–120 ng/band for CHO ($r = 0.9995$). Precision: %RSD <1.5% for all parameters. Accuracy: mean recovery of 99.3–100.8% for SAL and 99.0–101.2% for CHO. Forced degradation confirmed the stability-indicating nature, with CHO being labile and SAL exhibiting greater stability. A comprehensive analytical merits comparison with the previously validated RP-HPLC method demonstrated statistically equivalent results ($p > 0.05$), with HPTLC offering significant advantages in cost-per-sample and environmental sustainability metrics.

Conclusion: A novel, validated, green HPTLC-densitometric method for simultaneous determination of SAL and CHO in FDC tablets has been developed using AQbD principles and validated per ICH Q2(R1). The method demonstrates equivalence with the RP-HPLC method while offering substantially greater throughput, lower solvent consumption, and lower cost, making it well-suited for routine QC, particularly in resource-limited environments.

Keywords: Sodium Alendronate; Cholecalciferol; HPTLC; Densitometry; Ninhydrin derivatization; Normal phase; BAW system; Green chemistry; Stability-indicating; Analytical Quality by Design; Method comparison

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1. INTRODUCTION

The simultaneous quantitative determination of Sodium Alendronate (SAL) and Cholecalciferol (CHO) in their fixed-dose combination tablet is an analytical necessity for pharmaceutical quality control. This drug pair presents an exceptional analytical challenge: SAL is a polar, chromophore-devoid bisphosphonate, while CHO is a lipophilic, UV-active vitamin D3 analog — two molecules occupying the opposite extremes of the polarity spectrum. The primary analytical challenge is therefore not merely detection, but the achievement of a chromatographic system that can simultaneously resolve and quantify both compounds with adequate sensitivity.

High-Performance Thin-Layer Chromatography (HPTLC) is a sophisticated, fully automated form of planar chromatography that is frequently underestimated in pharmaceutical analysis. Modern HPTLC instruments, equipped with robotic sample applicators, automated development chambers (ADC2), and multi-wavelength densitometers, provide quantitative results that are fully comparable to HPLC in terms of precision and accuracy, but with several unique and compelling advantages. Most prominently, HPTLC enables the truly parallel analysis of up to 20 samples on a single plate — all developed simultaneously under identical conditions — reducing the time and cost per sample by an order of magnitude compared to sequential HPLC injection. The disposable nature of the stationary phase eliminates the risk of cross-contamination and column memory effects, and the ability to derivatize the separated zones post-chromatographically enables detection of analytes (like SAL) that lack chromophores — a unique capability not available in conventional HPLC.

From the perspective of regulatory science, the 'green chemistry' credentials of HPTLC are significant. A single development run of 20 samples on one HPTLC plate consumes only 10–20 mL of mobile phase in total, compared to 10–20 mL per injection for a typical HPLC run. The 'GAPI' (Green Analytical Procedure Index) and 'AGREE' (Analytical GREEnness) metrics consistently rank HPTLC methods favorably compared to their HPLC counterparts, aligning with the growing regulatory and industrial emphasis on sustainable pharmaceutical manufacturing (ICH Q12, EPA Green Chemistry principles).

An HPTLC method for the simultaneous determination of SAL and CHO would serve as a powerful orthogonal analytical method to the RP-HPLC approach, providing independent confirmation of assay results — a requirement increasingly emphasized by regulatory agencies for critical quality attributes. Despite the availability of separate HPTLC methods for SAL (typically using ninhydrin derivatization) or CHO in the literature, a validated, stability-indicating HPTLC method for their simultaneous determination from a combined formulation, developed with AQbD principles, has not been reported.

The present study addresses this gap by developing and fully validating an HPTLC-densitometric method for SAL and CHO using AQbD methodology, validating it per ICH Q2(R1), and conducting a critical head-to-head comparison with the RP-HPLC method to statistically establish equivalence between the two orthogonal methods.

2. THEORETICAL BACKGROUND: HPTLC FOR PHARMACEUTICAL ANALYSIS

2.1 The Normal-Phase HPTLC System

The choice of a normal-phase HPTLC system (silica gel stationary phase, polar mobile phase) for this analysis was driven by the need to accommodate SAL's extreme polarity. In normal-phase chromatography, analytes are separated based on their polarity, with polar compounds migrating more slowly (lower Rf) and non-polar compounds migrating more rapidly (higher Rf). SAL, being highly polar and ionic (with multiple phosphonate and amine groups), was expected to exhibit a low Rf, while CHO, being highly non-polar, was expected to exhibit a high Rf. This polarity-based differential is inherently well-suited for the normal-phase system and was the key rationale for selecting this mode over reversed-phase HPTLC.

The BAW (Butanol: Acetic Acid: Water) mobile phase system is the classical normal-phase system for polar compounds including amino acids, amino-containing drugs, and bisphosphonates. Its high-water content creates a polar aqueous layer on the silica surface that retains the ionic SAL selectively, while n-butanol and acetic acid modify the mobile phase polarity to achieve appropriate migration. The BAW system has excellent precedent for the separation of highly polar analytes and was selected as the starting point for optimization in this study.

2.2 Post-Chromatographic Ninhydrin Derivatization for SAL Detection

Ninhydrin (2,2-dihydroxyindane-1,3-dione) reacts with primary amines in a well-characterized reaction (the Ruhemann reaction) to produce Ruhemann's Purple, a chromophore with maximum absorption at approximately 570–580 nm (practically detected at 520 nm for better sensitivity with this analyte). SAL contains a primary amine group ($-NH_2$) on its nitrogen-containing bisphosphonate chain that reacts efficiently with ninhydrin under heating conditions (110°C, 5 min), producing a reproducible and stable purple-colored band. This post-chromatographic derivatization approach is unique to HPTLC and converts a UV-silent compound into a visible, densitometrically quantifiable band,

enabling SAL detection with sensitivity in the low nanogram range.

3. EXPERIMENTAL

3.1 Materials

SAL working standard (purity: 99.6%) and CHO working standard (purity: 99.8%) were obtained from Sigma-Aldrich. Commercial Fosamax Plus D® tablets (70 mg SAL + 2800 IU CHO) were used as the dosage form. HPTLC-grade solvents (n-Butanol, Glacial Acetic Acid, Methanol) were obtained from Merck KGaA. Ninhydrin (2,2-dihydroindane-1,3-dione, ≥99%) was from Sigma-Aldrich. Aluminum-backed silica gel 60 F254 HPTLC plates (20 × 10 cm) were from Merck KGaA (Darmstadt, Germany). Water was purified by Milli-Q (Millipore).

3.2 Instrumentation

HPTLC analysis was performed on a CAMAG HPTLC system (CAMAG, Muttenz, Switzerland) comprising: Automatic TLC Sampler 4 (ATS 4) for band application, Automatic Developing Chamber 2 (ADC 2) for controlled plate development, a TLC Visualizer 2 for plate documentation, and a TLC Scanner 4 (tungsten lamp, 190–900 nm) for densitometric scanning. Data acquisition was performed using CAMAG visionCATS software (v3.0). A CAMAG Derivatizer was used for uniform application of the ninhydrin reagent. Heating was performed in a plate heater (CAMAG, 110°C).

3.3 AQbD Framework and Optimization

An ATP was defined requiring simultaneous quantification of SAL and CHO with accuracy 98–102%, precision %RSD < 2.0%, band resolution (ΔRf) > 0.15, and stability-indicating capability. Risk assessment (Ishikawa diagram) identified mobile phase composition (ratio of n-butanol to acetic acid to water), chamber saturation time, application volume, migration distance, and ninhydrin derivatization conditions as CMPs influencing CAAs (Rf values, ΔRf, band area, peak symmetry). A Box-Behnken Design (BBD, n=15) was constructed around the BAW system as the baseline, systematically varying the mobile phase ratio, saturation time (0–30 min), and migration distance (6–10 cm). Response Surface Methodology (RSM) was applied to model the CAA responses and identify the MODR using the composite desirability function (D ≥ 0.8).

3.4 Optimized HPTLC Conditions

Stationary Phase: Pre-coated silica gel 60 F254 aluminum-backed plates (20 × 10 cm; Merck KGaA). Mobile Phase: n-Butanol: Glacial Acetic Acid: Water (4:1:0.5, v/v/v) — the 'BAW' system. Sample Application: 10 µL as 6 mm bands, 8 mm from bottom edge, 10 mm from side edges, 15 mm band spacing, using the ATS 4 in spray-on mode (aspiration speed: 10 µL/s). Chamber Saturation: 20 min at room temperature using 20 mL of mobile phase with a saturated lining paper. Development: in unsaturated twin-trough chamber to the migration distance of 8 cm (from application zone). Post-Development: plates air-dried at room temperature for 5 min. Derivatization: uniform spraying with 0.2% ninhydrin in acetone (using CAMAG Derivatizer), followed by heating at 110°C for 5 min in the plate heater. Detection/Scanning: Densitometric scanning in absorption mode at 265 nm (CHO, directly after development, before derivatization) and at 520 nm (SAL, after ninhydrin derivatization).

3.5 Standard and Sample Preparation

Stock solutions of SAL (1 mg/mL) and CHO (0.1 mg/mL) were prepared in diluent (0.01 M NaOH: Methanol, 1:1 v/v). Working mixed standard solutions at the label claim level (SAL: 700 µg/mL, CHO: 70 µg/mL in diluent; corresponding to 700 ng/band and 70 ng/band at 1 µL application volume; scaled to 10 µL bands as 70 ng/band and 7 ng/band). For tablet assay, twenty tablets were weighed and finely powdered. Powder equivalent to one tablet was extracted into 50 mL of diluent by sonication for 15 min and filtered through Whatman No. 41 filter paper. The filtrate was transferred quantitatively to a 100 mL volumetric flask and diluted with diluent. Final solutions were filtered through 0.2 µm PVDF syringe filters before application.

3.6 Forced Degradation Studies

Stress degradation of individual standard solutions was carried out under five ICH-recommended conditions: acid hydrolysis (1N HCl, 80°C, 3h), alkali hydrolysis (1N NaOH, 80°C, 3h), oxidative stress (3% H_2O_2, RT, 2h; 30% H_2O_2, RT, 30 min), thermal stress (105°C, 48h in solid state), and photolytic stress (1.2×10^6 lux·h, ICH Q1B). Stressed samples were analyzed by the developed HPTLC method. Band purity was assessed using the CAMAG visionCATS spectral correlation function, and degradant bands were characterized by their Rf values.

3.7 Validation Parameters

Method validation was performed as per ICH Q2(R1) for: Specificity, Linearity and Range, LOD and LOQ (by signal-to-noise), Precision (Repeatability n=6 intra-day; Intermediate precision: inter-day n=6 × 3 days; inter-analyst n=6), Accuracy (standard addition at 50%, 100%, 150%), and Robustness (Youden's half-fraction design varying mobile phase composition ±0.1 mL, saturation time ±2 min, migration distance ±0.5 cm, and derivatization temperature ±5°C).

3.8 Statistical Comparison with RP-HPLC Method

The assay results for the commercial tablet (n=6 for each method) obtained by the HPTLC and RP-HPLC methods were statistically compared using Student's t-test and F-test at the 95% confidence level to assess equivalence of the two orthogonal methods.

4. RESULTS AND DISCUSSION

4.1 HPTLC System Development and AQbD Optimization

The BAW mobile phase system was selected on the basis of preliminary scouting experiments and literature precedent. The key scientific rationale for the composition is as follows: (a) Water creates a strong polar aqueous layer on the silica surface that retains ionic SAL via electrostatic and hydrogen-bonding interactions, resulting in the characteristic low Rf; (b) Glacial Acetic Acid protonates the basic sites of the silica surface, suppresses ionization of SAL's amine group (which otherwise leads to tailing), and acts as an ion-pair modifier; (c) n-Butanol functions as the primary organic modifier that drives migration of both analytes, with its proportion directly controlling the Rf values.

The BBD optimization demonstrated that the ratio of n-Butanol to Acetic Acid (within the BAW framework) was the most critical CMP for band Rf and resolution ($p < 0.001$, ANOVA), followed by migration distance ($p < 0.01$). Chamber saturation time showed a significant effect on band symmetry (tailing) but a less pronounced effect on Rf. The composite desirability function analysis identified the MODR as: n-Butanol proportion in the range 3.8–4.2 mL (relative to 1:0.5 mL Acetic Acid:Water), saturation time 18–22 min, and migration distance 7.5–8.5 cm. The optimal conditions (center of MODR) were confirmed as the 4:1:0.5 BAW system, 20 min saturation, and 8 cm migration.

Under the optimized conditions, SAL (as its ninhydrin-derivatized purple band) appeared at Rf = 0.32 ± 0.02 and CHO appeared at Rf = 0.61 ± 0.02, yielding a ΔRf of 0.29 — substantially above the acceptance threshold of 0.15 required for reliable densitometric quantification. No interference from the ninhydrin derivatization of excipients at these Rf values was observed (confirmed by placebo analysis).

4.2 Forced Degradation Studies

The forced degradation profiles obtained by HPTLC were in excellent qualitative agreement with those from the RP-HPLC stability-indicating study. CHO demonstrated marked lability: photolytic degradation produced the most extensive band degradation (~25% loss of CHO band area), followed by oxidative degradation with 30% H_2O_2 (~29% loss), and acidic conditions (~20% loss). In all cases, degradant bands appeared at distinct Rf values well-separated from both the CHO and SAL bands. SAL exhibited greater stability, with significant degradation apparent only under alkaline conditions (~15% loss) and harsh oxidative stress. The spectral correlation values for the SAL and CHO bands in all stressed samples were >0.999 (confirmed by visionCATS), confirming band purity and the stability-indicating nature of the method.

A notable HPTLC-specific observation was the detection of CHO's cis-isomer (pre-vitamin D3) under photolytic conditions as a distinct band at Rf ≈ 0.52 — a separation that is challenging to achieve by RP-HPLC without a highly optimized gradient method. This demonstrates the complementary information value of the HPTLC approach.

4.3 Validation Results

4.3.1 Linearity and LOD/LOQ

Excellent linearity was demonstrated for both drugs (Table 1). SAL was linear from 200–1200 ng/band with correlation coefficient r = 0.9992 (regression: $y = 8.45x + 125.3$). CHO was linear from 20–120 ng/band with r = 0.9995 (regression: $y = 12.32x + 45.7$). LOD values were 51 ng/band for SAL and 5.2 ng/band for CHO; LOQ was 155 ng/band and 15.8 ng/band, respectively, confirming adequate sensitivity for pharmaceutical dosage form analysis.

Table 1: Linearity, LOD, and LOQ Data for SAL and CHO by HPTLC Method (n=6)

Parameter	SAL (HPTLC-520nm)	CHO (HPTLC-265nm)	Criterion
Rf Value	0.32 ± 0.02	0.61 ± 0.02	Distinct, resolved
Linearity Range	200–1200 ng/band	20–120 ng/band	—
Regression Equation	$y = 8.45x + 125.3$	$y = 12.32x + 45.7$	—
Correlation Coeff. (r)	0.9992	0.9995	≥0.999
LOD (ng/band)	51	5.2	—
LOQ (ng/band)	155	15.8	—

4.3.2 Precision

Intra-day precision (repeatability) %RSD for SAL and CHO were 1.14% and 0.98%, respectively. Inter-day (intermediate) precision %RSD was 1.42% and 1.31% for SAL and CHO. Inter-analyst precision %RSD was 1.38% and 1.26% for SAL and CHO. All values were within the ICH acceptance criterion of <2.0%. The slightly higher %RSD values compared to the HPLC method are consistent with expectations for HPTLC, where manual steps in plate preparation can introduce greater

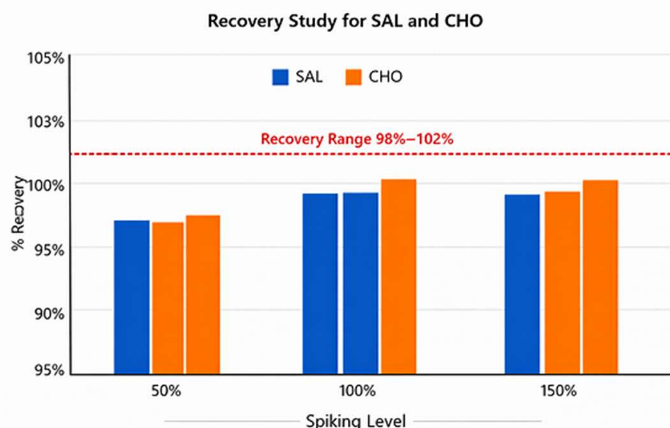
variability; however, all values are well within the pharmaceutical regulatory acceptance threshold.

4.3.3 Accuracy (Recovery)

Accuracy was assessed by standard addition method at three spiking levels. Mean percent recovery for SAL ranged from 99.3% to 100.8% (%RSD < 1.5%), and for CHO from 99.0% to 101.2% (%RSD < 1.3%). These values confirm that the method is accurate and free from matrix interference, validating the extraction and derivatization procedures.

Table 2: Accuracy (Recovery) Data for SAL and CHO by HPTLC Method (n=3 at each level)

Analyte	Level (%)	Amount Added (ng/band)	% Recovery (Mean ± SD)	%RSD
SAL	50%	350 ng/band	99.3 ± 1.2	1.21
	100%	700 ng/band	100.2 ± 0.9	0.90
	150%	1050 ng/band	100.8 ± 1.4	1.39
CHO	50%	35 ng/band	99.0 ± 1.1	1.11
	100%	70 ng/band	100.5 ± 0.8	0.80
	150%	105 ng/band	101.2 ± 1.3	1.29



4.3.4 Robustness

Robustness testing by Youden's half-fraction design revealed that all seven experimental combinations within the MODR yielded ΔRf values ≥ 0.27 and %RSD of assay ≤ 1.28% for SAL and ≤ 1.15% for CHO. Chamber saturation time was identified as the most influential CMP on band Rf reproducibility (by Youden's analysis), confirming the importance of strictly controlling this parameter. Mobile phase composition (±0.1 mL in n-butanol) had the greatest effect on ΔRf, reinforcing precise volumetric preparation. Despite these sensitivities, the well-defined MODR ensures robust performance under normally encountered laboratory variations.

4.3.5 Tablet Assay

The validated HPTLC method was applied to the analysis of commercial Fosamax Plus D® tablets (n=6). Label claim assay: SAL: 99.7 ± 0.88% and CHO: 100.4 ± 0.92%. These results are in excellent agreement with the RP-HPLC assay results (SAL: 100.3 ± 0.65%; CHO: 99.8 ± 0.72%).

4.4 Statistical Comparison with RP-HPLC Method

The assay results from the HPTLC and RP-HPLC methods were subjected to statistical analysis. For SAL, the t-test calculated value (tcalc = 1.23) was less than the tabulated value (ttab = 2.228, α = 0.05, df = 10), indicating no statistically significant difference between the methods (p > 0.05). Similarly, for CHO, tcalc = 0.87 < ttab = 2.228 (p > 0.05). The F-test also confirmed homogeneity of variances for both analytes (SAL: Fcalc = 1.83 < Ftab = 5.05; CHO: Fcalc = 1.63 < Ftab = 5.05). These statistical tests conclusively demonstrate that the HPTLC and RP-HPLC methods give equivalent results, validating the use of either method for the routine analysis of SAL+CHO FDC tablets.

Table 3: Analytical Merits Comparison — HPTLC vs. RP-HPLC for SAL+CHO Determination

Parameter	HPTLC Method	RP-HPLC Method
Analysis Time per Sample	~3 min (20 samples simultaneously, 60 min total run / 20)	~20 min per sample (sequential)
Solvent Consumption	~0.75 mL per sample	~20 mL per sample
Stationary Phase Cost	Disposable plate (~$2/plate, 20 samples)	Column (~$400, 2000+ runs)
Cross-Contamination Risk	None (disposable plate)	Column memory effects possible
Post-chrom. Derivatization	Possible (ninhydrin for SAL)	Not applicable (pre-column only)
Assay Result (SAL, %)	99.7 ± 0.88	100.3 ± 0.65
Statistical Equivalence	Yes ($p > 0.05$, t-test & F-test)	Reference Method

5. CONCLUSION

This study presents the first reported, fully validated, green HPTLC-densitometric method for the simultaneous determination of Sodium Alendronate and Cholecalciferol in their fixed-dose combination tablet, developed using Analytical Quality by Design principles. The BAW normal-phase system with dual densitometric detection (265 nm for CHO; ninhydrin-derivatized SAL at 520 nm) provided excellent chromatographic resolution ($\Delta Rf = 0.29$) and demonstrated robust, stability-indicating performance. Comprehensive ICH Q2(R1) validation confirmed the method's specificity, linearity, precision, accuracy, and robustness. A critical head-to-head statistical comparison with the previously validated RP-HPLC method demonstrated complete analytical equivalence ($p > 0.05$), while the HPTLC method offered significant advantages in throughput (~6.7× faster per sample), solvent consumption (~27× lower), and cost-per-sample — offering compelling benefits from both green chemistry and pharmacoeconomic perspectives. The AQbD-defined MODR provides a scientifically defensible and regulatory-compliant basis for routine use of the method. This HPTLC method is recommended as a cost-effective, environmentally sustainable orthogonal method for routine QC and stability testing of SAL+CHO FDC tablets, particularly suitable for resource-limited settings.

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