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Research Article

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Posted Date: March 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8964977/v1

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Additional Declarations: No competing interests reported.

HPTLC Screening of Specific Corticosteroid Adulterants: Prednisolone, Dexamethasone, Triamcinolone Acetonide, Betamethasone-17-Valerate, and Daucosterol in Herbal Medicines

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ABSTRACT

Adulteration of herbal medicines with corticosteroids remains a critical concern for product safety and quality control. Here we report a validated high-performance thin-layer chromatography (HPTLC) method for simultaneous detection of five steroid standards: daucosterol, prednisolone, betamethasone-17-valerate, dexamethasone, and triamcinolone acetonide. Twenty mobile phases were systematically evaluated, yielding five systems that provided reproducible resolution. Steroids were clearly separated and visualized under UV (254/366 nm) and visible detection with retention factors of 0.19–0.79. The method showed low limits of detection (333–603 ng/spot) and quantification (1009–1829 ng/spot), good linearity (0.2–1.0 mg/mL), and acceptable repeatability and intermediate precision. Densitometric evaluation supported both qualitative screening and quantitative determination. Applicability was confirmed in five spiked herbal medicine matrices, and slope-ratio analysis indicated limited matrix effects across the five mobile-phase systems. This protocol uses inexpensive solvents and readily available instrumentation, providing a robust and cost-effective tool for routine monitoring of steroid adulteration in herbal formulations.

Keywords: HPTLC (High-Performance Thin-Layer Chromatography); Corticosteroid adulteration; Herbal medicines; Method validation; Simultaneous detection

1. Introduction

Steroids have attracted increasing global attention due to their misuse as performance-enhancing drugs and their illicit distribution [1,2]. Beyond intentional misuse, synthetic steroids have been illegally incorporated into a broad range of products—including prescription medicines, dietary supplements, traditional medicines, and herbal remedies—to enhance perceived therapeutic efficacy [3,4]. Although steroids play an essential role in modern pharmacotherapy, their unauthorized presence in non-regulated products poses significant health risks.

The global consumption of herbal and traditional medicines continues to rise, driven by accessibility, affordability, cultural acceptance, and a growing preference for natural therapies. In response to market demand, some manufacturers adulterate herbal products with synthetic steroids despite regulatory prohibitions. Prolonged or unsupervised exposure to steroids can result in severe adverse effects, particularly immunosuppression, which increases susceptibility to fungal, bacterial, and viral infections, including oral candidiasis. Frequently reported adulterants include betamethasone dipropionate, betamethasone valerate, clobetasol propionate, dexamethasone, hydrocortisone acetate, mometasone furoate, prednisolone, and triamcinolone acetonide [5-13]. These concerns underscore the urgent need for robust and accessible analytical strategies to detect steroid adulteration and ensure the safety and integrity of herbal medicines.

Steroid hormones and synthetic corticosteroids are commonly analyzed using high-resolution techniques such as liquid chromatography–mass spectrometry and UV–VIS spectroscopy [14-17]. While these methods provide high sensitivity and selectivity, their routine implementation is often limited by

complex sample preparation, high operational costs, and extended analysis times. Consequently, there is growing interest in complementary approaches that enable rapid and cost-effective screening. Thin-layer chromatography (TLC), and particularly high-performance thin-layer chromatography (HPTLC), has emerged as a powerful alternative for steroid analysis, offering simplicity, parallel sample processing, and reliable analytical performance [18-25].

Here, we report the development of an HPTLC-based method for the chemical profiling of steroids, enabling the simultaneous identification and quantification of five representative standards: daucosterol, prednisolone, dexamethasone, triamcinolone acetonide, and betamethasone-17-valerate (**Fig. 1**). The method was optimized through systematic mobile-phase selection and validated using qualitative and quantitative performance criteria, including spike recovery experiments in herbal remedy matrices. Compared with LC–MS and UV-based techniques, the proposed approach offers a practical balance between analytical robustness, operational simplicity, and cost-effectiveness. As such, this method provides a valuable tool for routine quality control and pharmaceutical screening, particularly in settings with limited analytical infrastructure.

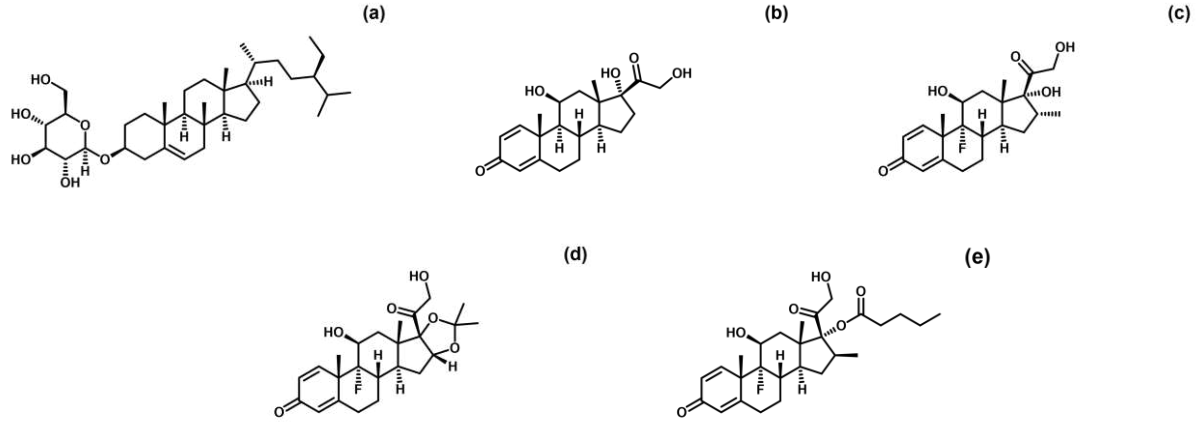


Fig. 1 Chemical structures of (a) daucosterol, (b) prednisolone, (c) dexamethasone, (d) triamcinolone acetonide and (e) betamethasone-17-valerate

2. Materials and Methods

2.1 Chemicals and reagents

All organic solvents used in this study, including acetonitrile (ACN, Scharlau), chloroform ($CHCl_3$, Scharlau), dichloromethane (DCM, Scharlau), isopropyl alcohol (IPA, Scharlau), *n*-hexane (Scharlau), methanol (MeOH, Scharlau), toluene (Scharlau), *tert*-butyl methyl ether (Scharlau), formic acid (98–100%, Scharlau), acetone (Aldrich), ethyl acetate (EtOAc, Aldrich), ethanol (EtOH, Aldrich), glacial acetic acid (CH_3COOH, Aldrich), sulfuric acid (H_2SO_4, Aldrich), *p*-anisaldehyde, and petroleum ether (Lab Scan Asia), were of analytical grade and used without further purification for the preparation of the mobile phase. The analytical standards for prednisolone (analytical standard, HPLC 99.0%), dexamethasone (analytical standard, HPLC 99.5%), triamcinolone acetonide (minimum 99%), betamethasone-17-valerate (analytical standard, HPLC 99.3%) and β-sitosterol-β-D-glucoside (daucosterol, analytical standard, HPLC ≥ 75.0%) were obtained from Aldrich.

2.2 HPTLC Instrumentation

The steroid standard solutions were automatically applied onto HPTLC Silica Gel 60 F254 glass plates (10 x 10 cm, Merck, Germany) using a 100 µL Camag Linomat syringe and a TLC sampler (Camag Linomat 5, Switzerland), operated by WinCATS software (version 1.3.4, Camag). The chromatographic separation was achieved by linear ascending development in a 10 x 10 cm^2 twin trough glass chamber with a stainless-steel lid (Camag, Muttenz, Switzerland). Following the development, the HPTLC plate was derivatized using a solution of *p*-anisaldehyde-sulfuric acid reagent and subsequently heated on a TLC Plate Heater III (Camag, Muttenz, Switzerland). Visualization of the samples on the HPTLC plate was performed under UV irradiation at 254 nm and 366 nm, as well as under visible light, using a Camag Reprostar 3 densitometer equipped with a camera (Switzerland), operated by VideoStore software

(version 3.10.7, Synoptic Ltd., Camag). Densitometric scanning was conducted at 254 nm using a Camag TLC Scanner 3 (Switzerland), controlled by WinCATS software (version 1.4.3.6336, Camag).

2.3 Preparation of standard solutions and spiked sample

Stock solutions of the steroid standards (prednisolone, betamethasone-17-valerate, dexamethasone, and triamcinolone acetonide) were prepared by dissolving each compound in ethanol at a concentration of 5 mg/mL, whereas daucosterol was prepared at 2.5 mg/mL. All stock solutions were subsequently diluted to obtain working solutions of 1 mg/mL. Herbal medicine samples were obtained from the Manufacturing Unit of Herbal Medicines and Products, Faculty of Medicine Siriraj Hospital, which operates under Good Manufacturing Practice (GMP) standards certified by PIC/S guidelines. Five herbal preparations (Derris scandens (A), Thai green herbal (Keaw-hom, B), Prasachangdaeng (C), Sahas Thara (D), and Andrographis paniculata (E)) were spiked with steroid standards at final concentrations of 0.4 and 0.8 mg/mL. Each 100 mg portion of herbal material was extracted with 1 mL of ethanol, sonicated at 35 °C for 60 min, and centrifuged at 12,500 rpm for 30 min at 4 °C. The resulting supernatants were collected and stored at –80 °C until analysis

2.4 Application

The developed HPTLC method was applied to analyze a steroid standard solution at a concentration of 1 mg/mL, with a 3 µL aliquot (3000 ng/spot) applied at a flow rate of 80 nL/s. Sample application produced an 8 mm band. The origin of application was maintained at 10 mm from both the lower and lateral edges of the plate. For quantitative determination of the limit of detection (LOD), injection volumes of 3, 6, 10, and 12 µL were tested, corresponding to standard concentrations ranging from 0.01 to 1 mg/mL. Calibration curves for all five steroid standards were established over the range of 0.2–1.0 mg/mL (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). To ensure accuracy and reproducibility, the distance between adjacent application tracks was maintained at 11 mm.

2.5 Chromatography

Before chromatography was performed, the HPTLC plate was pre-washed three times with MeOH and then dried at room temperature for 10 minutes. The chamber was saturated with the desired mobile phase at room temperature for 30 minutes. The distance from the bottom edge of the plate to the developed method was set at 80 mm. Each mobile phase system was prepared with a total volume of 15 mL per run. The derivatizing reagent, *p*-anisaldehyde-sulfuric acid, was prepared by mixing *p*-anisaldehyde, glacial acetic acid, MeOH, and sulfuric acid in a volume ratio of 0.5: 10: 85: 5. The HPTLC plate was then soaked in this reagent and heated at 180°C for 30 minutes. After derivatization with the spraying reagent, the HPTLC plates were scanned, and the peak areas corresponding to the steroid spots were quantified.

2.6 Validation of method

The analytical method was validated in accordance with the International Council for Harmonisation (ICH) guidelines [26], with assessments performed for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. Linearity was established over the concentration range of 0.2–1.0 mg/mL for all five steroid standards. The LOD and LOQ were calculated from the standard deviation (SD) of the blank response and the slope of the calibration curve, using the equations LOD = 3.3SD/slope and LOQ = 10SD/slope. To evaluate precision and accuracy of the HPTLC method within the optimized mobile phase system, a spiking approach was employed. Five herbal medicines (Derris scandens (A), Thai green herbal (Keaw-hom, B), Prasachangdaeng (C), Sahas Thara (D), and Andrographis paniculata (E)) were spiked with the steroid standards at concentrations of 0.4 and 0.8 mg/mL.

Qualitative precision was assessed by repeatability (intra-day precision, determined from three consecutive trials within a single day) and intermediate precision (evaluated over three consecutive days).

Precision was quantified by relative standard deviation (%RSD) and changes in retention factor (ΔR_f). Acceptance criteria were defined as %RSD ≤ 2% and $\Delta R_f \leq 0.01$ for repeatability, and %RSD ≤ 15% and $\Delta R_f \leq 0.05$ for intermediate precision. For quantitative analysis, accuracy was determined as percent recovery (%recovery) from spiked samples, with acceptance criteria set at 90–107%, in accordance with AOAC guidelines [27]. Precision was reported as %RSD, with limits of ≤ 5.3% for repeatability and ≤ 8% for intermediate precision.

3. Results and Discussion

3.1 Optimization of chromate graphic conditions

Thirteen solvents were evaluated to design mixed mobile phases for HPTLC separation based on polarity index and miscibility. In total, 20 systematically varied mobile phase systems were investigated to achieve effective separation of five steroid standards—prednisolone, dexamethasone, triamcinolone acetonide, betamethasone-17-valerate, and daucosterol. Formic acid was incorporated at a fixed volume ratio of 0.5 in all systems, as it significantly enhanced steroid solubility and separation efficiency. The chromatographic performance of all 20 mobile phases, including polarity index, retention factors (R_f), run times, and solvent costs per experiment, is summarized in **Table 1**. Steroid standards were applied under optimized HPTLC conditions and visualized under UV irradiation at 254 nm. Prednisolone, dexamethasone, triamcinolone acetonide, and betamethasone-17-valerate were readily detected, whereas daucosterol was not UV-active at this wavelength. Consequently, p-anisaldehyde–sulfuric acid was employed as a derivatization reagent, followed by heating at 180 °C for 30 min. Distinct coloration was observed for each steroid—daucosterol (violet), prednisolone (dark green), dexamethasone (light brown), triamcinolone acetonide (yellow-green), and betamethasone-17-valerate (dark blue). After derivatization, all compounds appeared as dark spots under UV irradiation at 366 nm. Representative HPTLC images are

157 shown in **Fig. 2**, while complete chromatograms for all mobile phase systems are provided in **Figs. S1–**
158 **S20**.

159 Across all tested mobile phases, the five steroids exhibited a consistent elution order:
160 betamethasone-17-valerate < triamcinolone acetonide < dexamethasone < prednisolone < daucosterol.
161 Although R_f values and separation quality varied among systems **(Table 1**; **Figs. S1–S20**), all mobile
162 phases produced reproducible migration patterns, highlighting the strong influence of solvent composition
163 on chromatographic resolution. Mobile phase performance was further assessed using a simplified
164 desirability-based evaluation adapted from Derringer's approach. Selection criteria included (1)
165 differences in retention factors (ΔR_f), with larger values indicating superior separation; (2) R_f values
166 within the optimal range of 0.2–0.8; (3) separation time (20–35 min); and (4) solvent cost per experiment
167 (USD 0.19–0.54). Systems failing to meet these criteria were excluded. The overall desirability values (D)
168 for all systems are summarized in **Table S27**, with the top five candidates presented in **Table 2**.

169 Based on these criteria, five mobile phase systems were selected for detailed analysis:
170 dichloromethane (DCM): isopropanol (IPA): acetonitrile (ACN): formic acid (7.5: 1.5: 0.5: 0.5); DCM:
171 petroleum ether: ethanol: formic acid (4.5: 3: 2: 0.5); chloroform ($CHCl_3$): IPA: petroleum ether: formic
172 acid (4: 3: 2.5: 0.5); $CHCl_3$: ethanol: n-hexane: formic acid (5: 2: 2.5: 0.5); and DCM: *tert*-butyl methyl
173 ether: methanol: formic acid (7.5: 1: 1: 0.5). Although these systems were not uniformly ranked highest
174 across all criteria, their selection was driven by superior separation efficiency, as reflected by large ΔR_f
175 values. Some mobile phases showed reduced performance at higher steroid concentrations and were
176 therefore excluded.

177 Among the optimized systems, DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5) provided the
178 most favorable overall separation of the five steroid standards. The enhanced performance is attributed to
179 the role of formic acid in improving steroid solubility and modulating interactions with the silica gel

stationary phase, leading to improved resolution. Well-defined and clearly separated bands were obtained across the tested concentration range (0.2–1.0 mg/mL), with consistent R_f values and no evidence of band overlap or concentration-dependent migration effects as shown in **Fig. 2**. The corresponding densitometric chromatograms showed a proportional increase in peak intensity with increasing concentration while maintaining stable peak positions and symmetrical peak shapes, confirming reliable chromatographic separation. This observation is consistent with previous reports demonstrating the effectiveness of formic acid as an additive for resolving structurally similar steroids in chromatographic systems.

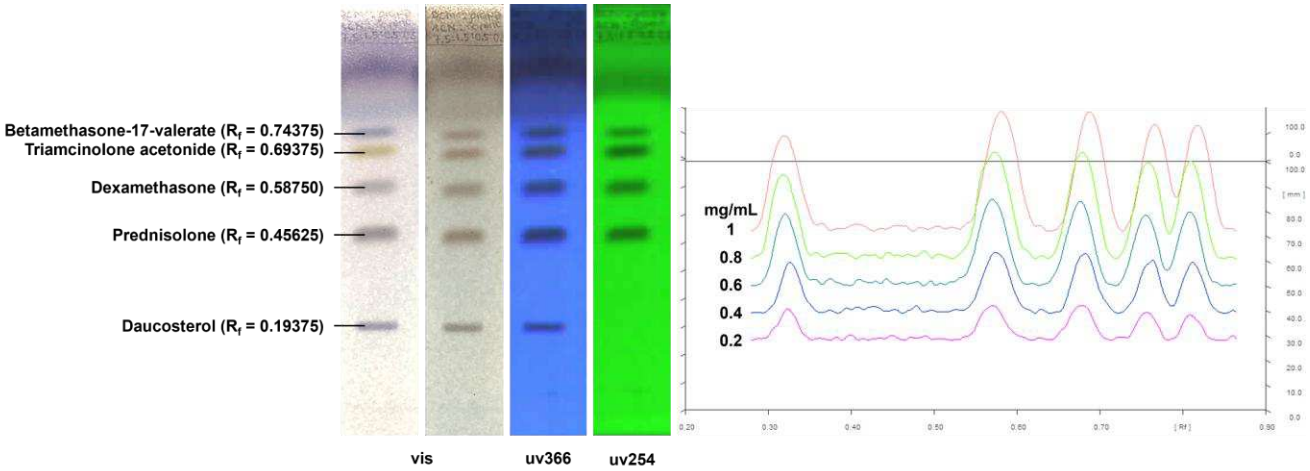


Fig. 2 Visualized HPTLC plate under UV irradiation at 254 and 366 nm and under visible light, together with the corresponding densitometric chromatograms, after development using the DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5) mobile phase system

3.2 Method validation

The HPTLC method was validated in accordance with International Council for Harmonisation (ICH) guidelines (ICH, 2023), evaluating linearity, limits of detection (LOD) and quantification (LOQ), precision, and accuracy. Validation was conducted for five steroid standards using five optimized mobile-phase systems, with densitometric detection.

3.2.1 Linearity

Linearity was assessed over the concentration range of 0.2–1.0 mg/mL (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) for all five steroid standards. Samples were applied at 10 µL per spot with an application rate of 80 nL s^{-1}, followed by densitometric scanning under optimized chromatographic conditions. All analytes exhibited linear responses between peak area and concentration across the selected mobile phases, with correlation coefficients meeting ICH acceptance criteria. Regression parameters and representative densitograms are summarized in **Table 3**, with full calibration data provided in **Tables S1–S5 and Figs. S21–S25**.

3.2.2 Limit of detection (LOD)

Visual detection limits were initially evaluated at concentrations of 0.01–0.90 mg/mL. At 0.01 and 0.03 mg/mL, steroid bands were not observable under UV (254 or 366 nm) or visible light. Increasing the application volume up to 12 µL did not enable detection at 0.01 mg/mL. Consequently, 0.03 mg/mL at 10 µL per spot (300 ng/spot) was established as the minimum visually detectable concentration for all steroids. Instrumental LODs were calculated using LOD = 3.3SD/slope, derived from the standard deviation of the blank response and the calibration slope. Across the five mobile phases, compound-specific LOD ranges were: Daucosterol: 0.0344–0.0402 mg/mL; Prednisolone: 0.0333–0.0392 mg/mL; Dexamethasone: 0.0339–0.0464 mg/mL; Triamcinolone acetonide: 0.0433–0.0564 mg/mL, and Betamethasone-17-valerate: 0.0411–0.0603 mg/mL. Overall, LODs ranged from 0.0333 to 0.0603 mg/mL, corresponding to 333–603 ng per spot, closely matching the visually established detection threshold.

3.2.3 Limit of quantification (LOQ)

Limits of quantification were calculated using LOQ = 10SD/slope under identical experimental conditions. The compound-specific LOQ ranges obtained across the five mobile phases were: Daucosterol: 0.1042–0.1220 mg/mL; Prednisolone: 0.1009–0.1187 mg/mL; Dexamethasone:

0.1026–0.1406 mg/mL; Triamcinolone acetonide: 0.1312–0.1709 mg/mL, and Betamethasone-17-valerate: 0.1247–0.1829 mg/mL. These values correspond to 1009–1829 ng per spot, confirming that the method supports reliable quantification of low-level steroids relevant to adulteration screening. Full numerical results are reported in **Table 3** and **Tables S1–S5**.

3.2.4 Precision (repeatability and intermediate precision)

Precision was evaluated using five herbal matrices spiked with steroid standards at 0.4 and 0.8 mg/mL. Repeatability (intra-day precision) was assessed from three replicate analyses within one day, while intermediate precision was determined over three consecutive days. For qualitative assessment, retention factor (R_f) precision was calculated from nine measurements (triplicate analyses over three days). Intra-day %RSD values ranged from 0.58 to 1.90, satisfying the acceptance criterion of ≤2%, although ΔR_f values (0.0125–0.0250) slightly exceeded conventional thresholds. Inter-day precision yielded %RSD values of 0.95–3.36 and ΔR_f values of 0.0187–0.0438, all within accepted limits (%RSD ≤15%, $\Delta R_f \leq 0.05$), demonstrating stable chromatographic performance (**Tables S11–S15**). Quantitative precision showed intra-day %RSD values between 1.17 and 4.71, with most results complying with AOAC guidelines (≤5.3%). Inter-day %RSD values ranged from 1.65 to 3.84, fully meeting the AOAC criterion of ≤8% (**Table 4** and **Tables S16–S20**). Although intra-day ΔR_f values slightly exceeded the stringent internal criterion (≤0.01), the observed variability is consistent with the inherent characteristics of HPTLC and remains acceptable for qualitative evaluation, as supported by low %RSD values, consistent band positions, and reproducible densitometric responses.

3.2.5 Accuracy

Accuracy was assessed by percent recovery (%recovery) using spiked herbal samples at 0.4 and 0.8 mg/mL. Mean intra-day recoveries ranged from 92.38 ± 1.34% to 103.66 ± 1.85%, while inter-

day recoveries ranged from 93.00 ± 1.72% to 103.41 ± 2.25%, all within the AOAC acceptance range of 90–107%.

Partial co-migration between spiked steroids and endogenous herbal constituents was observed in some matrices, attributed to the use of *p*-anisaldehyde–sulfuric acid as the derivatization reagent. This reagent reacts with multiple compound classes, including sugars, phenols, and terpenes, which may generate overlapping signals after heating (**Figs. S26–S30**; **Tables S6–S10**). Matrix effects were further evaluated using matrix-matched calibration curves, with slope ratios close to unity indicating minimal matrix influence (**Table 5** and **Tables S21–S25**).

Although LC–MS remains the benchmark for molecular selectivity and sub-ng sensitivity, the HPTLC strategy developed here addresses a complementary analytical need by providing a rapid, cost-effective, and high-throughput screening platform for routine surveillance of corticosteroid adulteration in herbal medicines. Consistent performance across five mobile-phase systems yielded densitometric LODs of 0.0333–0.0603 mg/mL and LOQs of 0.1009–0.1829 mg/mL (333–603 and 1009–1829 ng per 10 μL spot), together with acceptable precision and accuracy in chemically complex herbal matrices. Crucially, the method enables the concurrent quantification of daucosterol and four synthetic corticosteroids within a single analytical workflow—a capability not previously reported—thereby expanding the practical scope of steroid analysis for pharmaceutical quality control and regulatory monitoring.

4. Conclusion

This study establishes a validated HPTLC platform for the simultaneous screening of daucosterol, prednisolone, dexamethasone, triamcinolone acetonide, and betamethasone-17-valerate. Systematic optimization of the mobile phase yielded high-fidelity separation of these structurally diverse steroids

within complex herbal matrices, surpassing traditional thin-layer protocols in both reproducibility and speed. While visual detection provides a cost-effective frontline defense for regulatory oversight, the method's adaptability to densitometry offers a scalable pathway for high-precision quantification. Ultimately, this work delivers a practical, high-throughput solution for combating pharmaceutical adulteration in global supply chains, offering a standardized framework for public health protection and on-site regulatory screening.

Supporting Information

The retention factor (R_f) and visualized of five steroid standards under uv254, 366 nm and visible light under 20 mobile phase system. The calibration curve, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ) of five steroid standard with 0.2-1.0 mg/mL. The observation, repeatability and intermediate precision of spiked steroid standard in five herbal medicine sample under 5 selected mobile phase system, the slope ratio and the solvent cost. (PDF)

Acknowledgments

The herbal medicine samples in this publication were supported by Center of Applied Thai Traditional Medicine and the Department of pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

Author contributions

Chomtisa Borsap: investigation, methodology, validation, visualization, writing–original draft, and writing–review & editing; Dollaporn Mamaethong: writing–review & editing; Suveerawan Limsuvan:

writing–review & editing; Pravit Akarasereenont: conceptualization, data curation, project administration, supervision, and writing–review & editing.

Data availability

All recent data are available from the corresponding author upon reasonable request.

Declaration

Conflicts of Interest

The authors declare no conflicts of interest.

Ethical approval

This article does not contain any studies with human or animal subjects.

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Table 1 Results of retention factor (Rf) for Daucosterol, prednisolone, dexamethasone, triamcinolone acetonide and betamethasone-17-valerate by the developed HPTLC method.

No.	Mobile phase	Polarity index (PI)	Retention factor (R_f)					Run time (min)	Solvent cost/time (USD)
			Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone -17-valerate		
1	DCM: Acetone: *n*-Hexane: Formic acid (4.5: 3: 2: 0.5)	3.2250	0.18750	0.36875	0.44375	0.51875	0.57500	20	0.2514
2	DCM: IPA: ACN: Formic acid (7.5: 1.5: 0.5: 0.5)	3.5000	0.19375	0.45625	0.58750	0.69375	0.74375	20	0.2930
3	EtOAc: DCM: *n*-Hexane: Formic acid (5: 4: 0.5: 0.5)	3.7400	0.13750	0.31250	0.43750	0.48750	0.54375	20	0.3297
4	DCM: EtOAc: Acetone: Formic acid (4: 4: 1.5: 0.5)	3.5900	0.23750	0.47500	0.56250	0.60000	0.64375	20	0.3075
5	Toluene: IPA: *n*-Hexane: Formic acid (5: 3: 1.5: 0.5)	2.6700	0.38750	0.50000	0.55625	0.60000	0.64375	23	0.1903
6	DCM: Petroleum ether: EtOH: Formic acid (4.5: 3: 2: 0.5)	2.7650	0.46250	0.57500	0.66250	0.73750	0.79375	25	0.2790
7	Toluene: EtOAc: Formic acid (5: 4.5: 0.5)	3.4800	0.12500	0.28750	0.37500	0.41250	0.48125	20	0.2715
8	Toluene: IPA: MeOH: Formic acid (7: 2: 0.5: 0.5)	3.0150	0.40625	0.48750	0.52500	0.56875	0.61250	22	0.1893
9	EtOAc: $CHCl_3$: Petroleum ether: Formic acid (5.5: 3: 1: 0.5)	3.9600	0.15000	0.31250	0.41250	0.46250	0.52500	23	0.3003
10	$CHCl_3$: IPA: Petroleum ether: Formic acid (4: 3: 2.5: 0.5)	3.1350	0.43125	0.58125	0.66250	0.75000	0.80625	35	0.2098
11	$CHCl_3$: Acetone: Petroleum ether: Formic acid (4.5: 3: 2: 0.5)	3.6950	0.27500	0.43750	0.48750	0.55625	0.60625	23	0.2148
12	$CHCl_3$: Acetone: MeOH: Formic acid (6: 3: 0.5: 0.5)	4.5450	0.34375	0.55000	0.59375	0.65000	0.68750	23	0.2122
13	Toluene: Acetone: Petroleum ether: Formic acid (4.75: 2.75: 2: 0.5)	2.8625	0.31250	0.37500	0.41250	0.45625	0.49375	20	0.1971
14	$CHCl_3$: EtOH: *n*-Hexane: Formic acid (5: 2: 2.5: 0.5)	3.3900	0.42500	0.51875	0.57500	0.66250	0.71875	25	0.2409
15	$CHCl_3$: EtOAc: ACN: Formic acid (6: 2: 1.5: 0.5)	4.5000	0.16875	0.33750	0.40000	0.47500	0.51875	23	0.2634
16	DCM: *tert*-butyl methyl ether: MeOH: Formic acid (7.5: 1: 1: 0.5)	3.3850	0.28750	0.47500	0.54375	0.63125	0.67500	20	0.3422
17	Toluene: *tert*-butyl methyl ether: ACN: Formic acid (5: 2.5: 2: 0.5)	3.2850	0.21875	0.40000	0.45625	0.51250	0.56875	20	0.3393
18	EtOAc: $CHCl_3$: *tert*-butyl methyl ether: Formic acid (4: 3: 2.5: 0.5)	3.9150	0.15625	0.33750	0.45000	0.48750	0.55625	25	0.4059
19	*tert*-butyl methyl ether: *n*-Hexane: Acetone: Formic acid (6.5: 2: 1: 0.5)	2.4350	0.18125	0.37500	0.46250	0.51250	0.60625	25	0.5397
20	DCM: Toluene: MeOH: Formic acid (7: 2: 0.5: 0.5)	3.2050	0.23125	0.29375	0.33750	0.41250	0.43750	20	0.2896

Table 2 The top five overall D values of 20 mobile phase screening from Derringer's desirability.

Ranking	Mobile phase system	Separation (maximum, weight = 1)			0.2-0.8 (maximum, weight = 1)			Price (USD) (minimum, weight = 0.5)			Time (min) (minimum, weight = 0.5)			Overall D
		Data	d_i	D	Data	d_i	D	Data	d_i	D	Data	d_i	D	
1	DCM: EtOAc: Acetone: formic acid (4: 4: 1.5: 0.5)	7	0.75	0.75	in	1	1	0.31	0.6	0.77	20	1	1	0.8344
1	DCM: *tert*-butyl methyl ether: MeOH: Formic acid (7.5: 1: 1: 0.5)	7	0.75	0.75	in	1	1	0.34	0.6	0.77	20	1	1	0.8344
1	Toluene: *tert*-butyl methyl ether: ACN: Formic acid (5: 2.5: 2: 0.5)	7	0.75	0.75	in	1	1	0.34	0.6	0.77	20	1	1	0.8344
2	DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5)	10	1	1	out	0.5	0.5	0.29	0.8	0.89	20	1	1	0.7647
3	$CHCl_3$: Acetone: MeOH: Formic acid (6: 3: 0.5: 0.5)	6	0.625	0.625	in	1	1	0.21	0.8	0.89	23	0.6	0.77	0.7565
4	DCM: EtOH: Petroleum ether: formic acid (4.5: 2: 3: 0.5)	7	0.75	0.75	in	1	1	0.28	0.8	0.89	25	0.4	0.63	0.7514
5	$CHCl_3$: EtOH: *n*-Hexane: Formic acid (5: 2: 2.5: 0.5)	6	0.625	0.625	in	1	1	0.24	0.8	0.89	25	0.4	0.63	0.7071

Table 3 The correlation coefficient, regression equation, limit of detection (LOD) and limit of quantification (LOQ).

a) DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5)

	Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone-17-valerate
Correlation coefficient	0.9906	0.9920	0.9930	0.9918	0.9954
Regression equation	y = 16389x + 2172.6	y = 15524x + 732.85	y = 15075x + 1424.1	y = 10781x + 161.16	y = 13370x - 1166.4
LOD	0.0371	0.0392	0.0403	0.0564	0.0455
LOQ	0.1124	0.1187	0.1222	0.1709	0.1378

b) DCM: petroleum ether: EtOH: formic acid (4.5: 3: 2: 0.5)

	Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone-17-valerate
Correlation coefficient	0.9921	0.9918	0.9922	0.9905	0.9942
Regression equation	y = 14216x + 884.36	y = 14684x +125.95	y = 13513x - 360.69	y = 9209.4x - 112.81	y = 8097.9x + 289.05
LOD	0.0344	0.0333	0.0372	0.0531	0.0603
LOQ	0.1042	0.1009	0.1126	0.1608	0.1829

c) $CHCl_3$: IPA: Petroleum ether: Formic acid (4: 3: 2.5: 0.5)

	Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone-17-valerate
Correlation coefficient	0.9904	0.9913	0.9913	0.9912	0.9921
Regression equation	y = 12290x + 662.81	y = 14021x - 86.37	y = 11113x + 1150.1	y = 8888.6x + 636.47	y = 10004x + 138.49
LOD	0.0402	0.0353	0.0445	0.0556	0.0494
LOQ	0.1220	0.1069	0.1349	0.1686	0.1498

d) $CHCl_3$: EtOH: *n*-hexane: formic acid (5: 2: 2.5: 0.5)

	Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone-17-valerate
Correlation coefficient	0.9920	0.9906	0.9906	0.9912	0.9935
Regression equation	y = 13745x + 628.6	y = 15012x + 377.1	y = 15401x - 149.3	y = 12047x + 53.04	y = 12676x + 40.05
LOD	0.0379	0.0347	0.0339	0.0433	0.0411
LOQ	0.1150	0.1053	0.1026	0.1312	0.1247

e) DCM: *tert*-butyl methyl ether: MeOH: formic acid (7.5: 1: 1: 0.5)

	Daucosterol	Prednisolone	Dexamethasone	Triamcinolone acetonide	Betamethasone-17-valerate
Correlation coefficient	0.9910	0.9920	0.9964	0.9911	0.9967
Regression equation	y = 16518x + 14.08	y = 15149x + 1124	y = 12281x - 136.72	y = 11206x - 480.47	y = 13176x - 126.22
LOD	0.0345	0.0376	0.0464	0.0509	0.0433
LOQ	0.1045	0.1140	0.1406	0.1541	0.1311

Table 4 The %RSD (precision) and %recovery (accuracy) of five steroid standards in five spiked herbal medicines under different five mobile phase system.

a) DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5)

Steroid standard	Conc. (µg/mL)	%RSD				%Recovery			
		Intra-day (mean±SD), n=3			Inter-day (mean±SD), n=9	Intra-day (mean±SD), n			Inter-day (mean±SD), n
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
Betamethasone-17-valerate	0.4	2.6428 ± 1.4048	4.3202 ± 1.5754	3.0015 ± 0.9100	3.3215 ± 1.3806	94.4870 ± 2.9538, 15	95.6632 ± 4.0165, 15	95.1099 ± 2.7678, 15	95.0867 ± 3.2531, 45
	0.8	2.9934 ± 1.8743	3.5809 ± 0.5780	2.7117 ± 1.1396	3.0953 ± 1.1975	96.8074 ± 3.1887, 15	98.6525 ± 3.9552, 15	96.9480 ± 4.1110, 15	97.4693 ± 3.7828, 45
Triamcinolone acetonide	0.4	2.9046 ± 1.5809	3.3258 ± 0.9059	3.8454 ± 1.4781	3.3586 ± 1.2421	97.3528 ± 4.6421, 12	97.4382 ± 4.3908, 12	97.6026 ± 3.6563, 12	97.4645 ± 4.1285, 36
	0.8	3.7092 ± 0.5774	2.1286 ± 0.4315	3.6840 ± 1.7141	3.1739 ± 1.2162	97.0126 ± 3.4373, 12	97.5813 ± 3.2556, 12	98.8840 ± 3.5288, 12	97.8260 ± 3.4043, 36
Dexamethasone	0.4	3.8134 ± 1.7065	4.2880 ± 0.3413	3.4093 ± 1.0521	3.8369 ± 1.0858	98.6854 ± 3.9572, 15	100.5470 ± 4.3229, 15	96.4800 ± 3.3037, 15	98.5708 ± 4.1506, 45
	0.8	1.8932 ± 1.2414	2.8039 ± 1.0639	2.0948 ± 0.3109	2.2639 ± 0.9295	99.6652 ± 2.2714, 15	99.7208 ± 2.8341, 15	101.7856 ± 2.8351, 15	100.3906 ± 2.7840, 45
Prednisolone	0.4	2.8308 ± 1.2180	2.9945 ± 1.0431	2.6992 ± 0.3466	2.8415 ± 0.8303	101.9066 ± 2.8411, 15	101.3344 ± 3.2940, 15	101.7425 ± 2.7777, 15	101.6612 ± 2.9214, 45
	0.8	2.1952 ± 0.5520	3.8176 ± 0.6780	3.3016 ± 0.3159	3.1048 ± 0.8552	97.8969 ± 2.3664, 15	103.3515 ± 3.8312, 15	100.1974 ± 3.2638, 15	100.4819 ± 3.8671, 45
Daucosterol	0.4	2.7975 ± 0.4083	1.9155 ± 0.2692	2.2489 ± 0.7120	2.3206 ± 0.5790	102.0986 ± 2.9249, 15	103.4712 ± 1.8681, 15	101.8959 ± 2.3622, 15	102.4886 ± 2.4716, 45
	0.8	2.3290 ± 0.3540	2.1877 ± 0.9030	2.5374 ± 0.4360	2.3514 ± 0.5531	99.5749 ± 2.3499, 15	97.0356 ± 2.2485, 15	100.4608 ± 2.8501, 15	99.0238 ± 2.8470, 45

b) DCM: petroleum ether: EtOH: formic acid (4.5: 3: 2: 0.5)

Steroid standard	Conc. (µg/mL)	%RSD				%Recovery			
		Intra-day (mean±SD), n=3			Inter-day (mean±SD), n=9	Intra-day (mean±SD), n			Inter-day (mean±SD), n
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
Betamethasone-17-valerate	0.4	3.5454 ± 1.2321	1.9685 ± 0.7579	2.0859 ± 0.6858	2.5333 ± 1.1043	100.5710 ± 4.0795, 15	101.2360 ± 2.1505, 15	101.5686 ± 2.7836, 15	101.1252 ± 3.0673, 45
	0.8	1.8214 ± 0.2956	2.6440 ± 0.4786	3.3571 ± 1.0470	2.6075 ± 0.8922	100.7022 ± 2.8230, 15	100.7056 ± 2.7104, 15	99.7592 ± 3.2273, 15	100.3890 ± 2.8965, 45
Triamcinolone acetonide	0.4	2.6105 ± 0.9148	2.1233 ± 0.8671	1.7898 ± 0.6422	2.1746 ± 0.7925	96.1946 ± 2.7028, 12	93.7313 ± 2.5439, 12	92.9850 ± 1.6077, 12	94.3036 ± 2.6602, 36
	0.8	3.9220 ± 1.3526	3.5020 ± 0.8689	2.4130 ± 0.5178	3.2790 ± 1.0808	97.1767 ± 3.8190, 12	95.3571 ± 3.1625, 12	94.2851 ± 2.1075, 12	95.6063 ± 3.2540, 36
Dexamethasone	0.4	2.9948 ± 0.8543	2.1778 ± 0.5777	1.9195 ± 0.6031	2.3640 ± 0.7702	102.8494 ± 3.1291, 12	102.0941 ± 2.6512, 12	102.4224 ± 2.2448, 12	102.4553 ± 2.6398, 36
	0.8	3.3477 ± 0.8734	2.8720 ± 1.3470	2.5695 ± 0.9146	2.9297 ± 0.9843	100.2275 ± 4.1619, 12	100.8793 ± 2.8397, 12	102.4313 ± 2.7040, 12	101.1794 ± 3.3399, 36
Prednisolone	0.4	2.2423 ± 1.0055	1.6847 ± 0.4994	2.1306 ± 0.8937	2.0192 ± 0.7616	102.9419 ± 2.5942,12	103.6618 ± 1.8487, 12	103.6308 ± 2.3526, 12	103.4115 ± 2.2455, 36
	0.8	3.0140 ± 0.2938	2.8680 ± 1.3937	2.8906 ± 1.1784	2.9242 ± 0.9268	97.3603 ± 2.7361, 12	95.6916 ± 2.7621, 12	97.6942 ± 3.8529, 12	96.9154 ± 3.1946, 36
Daucosterol	0.4	2.0593 ± 0.7149	1.6175 ± 0.8331	2.1077 ± 0.7216	1.9282 ± 0.6972	101.5110 ± 2.0212, 15	102.1412 ± 2.3753, 15	103.1595 ± 2.0967, 15	102.2706 ± 2.2284, 45
	0.8	3.4131 ± 1.5879	2.7148 ± 0.5982	2.6250 ± 0.5558	2.9176 ± 0.9678	98.9893 ± 4.1477, 15	97.2684 ± 3.6035, 15	99.3514 ± 2.5598, 15	98.5364 ± 3,5404, 45

c) $CHCl_3$: IPA: Petroleum ether: Formic acid (4: 3: 2.5: 0.5)

Steroid standard	Conc. (µg/mL)	%RSD				%Recovery			
		Intra-day (mean±SD), n=3			Inter-day (mean±SD), n=9	Intra-day (mean±SD), n			Inter-day (mean±SD), n
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
Betamethasone-17-valerate	0.4	2.7708 ± 1.6834	2.8544 ± 0.8594	2.2198 ± 1.2925	2.6150 ± 1.1832	103.5883 ± 3.4635, 15	102.9871 ± 2.8093, 15	102.4286 ± 2.3480, 15	103.0013 ± 2.8830, 45
	0.8	2.2140 ± 0.6177	3.2665 ± 1.2256	2.4221 ± 1.3043	2.6342 ± 1.0626	102.6097 ± 2.2895, 15	101.2941 ± 3.4603, 15	103.1764 ± 3.1461, 15	102.3601 ± 3.0435, 45
Triamcinolone acetonide	0.4	2.2141 ± 0.8311	2.2708 ± 0.1205	1.5593 ± 0.4256	2.0147 ± 0.5821	94.9504 ± 2.5843, 12	93.5760 ± 2.0033, 12	93.1014 ± 1.4017, 12	93.8759 ± 2.1471, 36
	0.8	2.2500 ± 0.6654	2.5263 ± 0.9981	2.6545 ± 0.4423	2.4770 ± 0.6638	92.8459 ± 2.0847, 12	92.8220 ± 2.2890, 12	93.5316 ± 2.3078, 12	93.0665 ± 2.1904, 36
Dexamethasone	0.4	3.2747 ± 1.2941	2.3764 ± 1.1349	2.4432 ± 0.3627	2.6981 ± 0.9805	100.1407 ± 3.3313, 15	98.7851 ± 2.5900, 15	99.0976 ± 2.5282, 15	99.3411 ± 2.8360, 45
	0.8	2.9447 ± 0.4606	2.8024 ± 0.8828	2.3325 ± 0.7960	2.6932 ± 0.6952	100.4477 ± 3.4883, 15	100.6879 ± 2.7702, 15	101.0285 ± 3.5570, 15	100.7214 ± 3.2245, 45
Prednisolone	0.4	2.7851 ± 0.4177	4.1106 ± 0.7904	3.4854 ± 1.5130	3.4604 ± 1.0497	101.6958 ± 2.8528, 15	100.4935 ± 4.0259, 15	99.1720 ± 3.7065, 15	100.4538 ± 3.6338, 45
	0.8	2.7319 ± 0.5598	3.2309 ± 0.9630	2.7963 ± 0.4414	2.9197 ± 0.6435	98.3705 ± 2.6263, 15	98.3275 ± 3.1960, 15	96.3792 ± 2.5744, 15	97.6924 ± 2.9044, 45
Daucosterol	0.4	2.6953 ± 1.0736	2.7475 ± 1.4411	2.4464 ± 0.5690	2.6297 ± 0.9527	99.7805 ± 2.6853, 15	100.6990 ± 2.7864, 15	99.5882 ± 2.3210, 15	100.0226 ± 2.5921, 45
	0.8	2.3354 ± 0.9121	4.1408 ± 0.8584	1.9797 ± 1.3294	2.8186 ± 1.3569	101.1636 ± 2.5620, 15	99.6771 ± 4.2591, 15	100.3316 ± 3.6309, 15	100.3908 ± 3.5261, 45

d) $CHCl_3$: EtOH: *n*-hexane: formic acid (5: 2: 2.5: 0.5)

Steroid standard	Conc. (µg/mL)	%RSD				%Recovery			
		Intra-day (mean±SD), n=3			Inter-day (mean±SD), n=9	Intra-day (mean±SD), n			Inter-day (mean±SD), n
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
Betamethasone-17-valerate	0.4	1.7890 ± 0.1486	3.2135 ± 1.2256	1.8502 ± 0.7825	2.2842 ± 1.0102	102.7695 ± 2.9330, 15	102.0482 ±3.3513, 15	102.9074 ± 1.9080, 15	102.5750 ± 2.7594, 45
	0.8	3.3140 ± 2.1819	2.1588 ± 0.7686	3.2922 ± 0.6190	2.9217 ±1.3271	96.5484 ± 3.3912, 15	95.8088 ± 3.3774, 15	99.8858 ± 3.6873, 15	97.4143 ± 3.8512, 45
Triamcinolone acetonide	0.4	1.6554 ± 0.3352	1.4296 ± 0.1897	1.8774 ± 0.1925	1.6542 ± 0.2897	93.0509 ± 1.9194, 12	92.3809 ± 1.3400, 12	93.5757 ± 1.7657, 12	93.0025 ± 1.7170, 36
	0.8	3.4516 ± 1.9055	2.1412 ± 0.7888	1.9958 ± 0.8812	2.5295 ± 1.3189	94.7900 ± 3.3831, 12	94.3014 ± 2.0925, 12	93.4094 ± 2.1594, 12	94.1669 ± 2.6028, 36
Dexamethasone	0.4	3.6630 ± 0.4461	2.5187 ± 1.0284	2.4376 ± 0.6799	2.8731 ± 0.8843	100.9254 ± 3.4774, 12	100.5983 ± 2.7959, 12	102.1604 ± 2.8946, 12	101.2280 ± 3.0588, 36
	0.8	2.5057 ± 1.4057	2.8017 ± 0.5170	2.6604 ± 0.1307	2.6559 ± 0.7626	97.4304 ± 3.0471, 12	97.7671 ± 2.8587, 12	99.6204 ± 2.6295, 12	98.2726 ± 2.9348, 36
Prednisolone	0.4	2.5528 ± 1.5099	2.5447 ± 0.2295	3.2596 ± 0.5051	2.7857 ± 0.8793	102.1709 ± 3.5063, 12	99.5752 ± 3.1962, 12	101.5962 ± 3.0562, 12	101.1141 ± 3.3593, 36
	0.8	3.8456 ± 0.7461	2.9970 ± 0.8211	2.1568 ± 0.6465	2.9998 ± 0.9731	98.5206 ± 3.4783, 12	100.1946 ± 2.8189, 12	100.8125 ± 2.3151, 12	99.8425 ± 2.9914, 36
Daucosterol	0.4	2.5680 ± 0.9168	2.2311 ± 1.3558	3.2692 ± 0.2824	2.6894 ± 0.9487	103.0691 ± 2.5995, 15	102.8573 ± 2.6216, 15	100.6304 ± 3.1382, 15	102.1856 ± 2.9521, 45
	0.8	2.0601 ± 0.5207	2.3594 ± 0.9851	1.5033 ± 0.1764	1.9742 ± 0.6780	101.7322 ± 2.1891, 15	103.0937 ± 2.4024, 15	102.6884 ± 1.7655, 15	102.5047 ± 2.1648, 45

e) DCM: *tert*-butyl methyl ether: MeOH: formic acid (7.5: 1: 1: 0.5)

Steroid standard	Conc. (µg/mL)	%RSD				%Recovery			
		Intra-day (mean±SD), n=3			Inter-day (mean±SD), n=9	Intra-day (mean±SD), n			Inter-day (mean±SD), n
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
Betamethasone-17-valerate	0.4	2.7022 ± 2.2449	2.3984 ± 0.6046	2.4036 ± 1.0624	2.5014 ± 1.2869	102.5283 ± 3.2756, 12	101.5240 ± 2.4208, 12	100.4270 ± 2.3817, 12	101.4931 ± 2.7846, 36
	0.8	2.4650 ± 0.2361	1.1683 ± 0.7274	2.9056 ± 0.4260	2.1797 ± 0.8963	102.1140 ± 2.2992, 12	103.3004 ± 2.0502, 12	101.7880 ± 2.7101, 12	102.4008 ± 2.3927, 36
Triamcinolone acetonide	0.4	3.4834 ± 0.1821	2.3897 ± 1.4853	2.5782 ± 1.4251	2.8171 ± 1.1506	95.6795 ± 3.1153, 15	94.7463 ± 3.2689, 15	93.8675 ± 2.6787, 15	94.7644 ± 3.0547, 45
	0.8	2.1850 ± 0.6562	3.0009 ± 0.0801	2.3920 ± 0.4973	2.5260 ± 0.5532	93.6041 ± 2.0850, 15	94.6933 ± 2.6795, 15	92.8814 ± 2.2684, 15	93.7262 ± 2.4233, 45
Dexamethasone	0.4	4.7059 ± 0.9241	3.0813 ± 0.5561	3.7346 ± 1.3899	3.8406 ± 1.1291	99.1532 ± 4.3049, 12	100.9882 ± 2.9767, 12	100.1068 ± 3.6436, 12	100.0827 ± 3.6550, 36
	0.8	2.9272 ± 1.1674	2.3094 ± 1.7739	2.8602 ± 1.2121	2.6989 ± 1.2573	101.4773 ± 3.0382, 12	102.7433 ± 3.4274, 12	102.7273 ± 3.1433, 12	102.3160 ± 3.1717, 36
Prednisolone	0.4	3.1152 ± 0.4986	1.9472 ±1.0895	2.3885 ± 0.8813	2.4836 ± 0.9022	99.8738 ± 2.9298, 15	98.1237 ± 2.6178, 15	98.2994 ± 2.4123, 15	98.7657 ± 2.7196, 45
	0.8	1.4631 ± 0.5789	2.5958 ± 0.5350	2.2139 ± 1.1622	2.0909 ± 0.8614	98.8340 ± 2.6771, 15	96.5972 ± 2.3943, 15	95.7802 ± 2.1367, 15	97.0705 ± 2.6947, 45
Daucosterol	0.4	2.9407 ± 2.9387	2.7374 ± 1.1052	2.2125 ± 0.3501	2.6302 ± 1.6127	101.2554 ± 3.8696, 15	102.4983 ± 2.9106, 15	101.8918 ± 2.2296, 15	101.8818 ± 3.0504, 45
	0.8	2.0732 ± 0.2482	2.4047 ± 0.3117	1.5313 ± 0.2325	2.0031 ± 0.4461	98.7056 ± 3.2768, 15	95.9457 ± 2.6752, 15	96.7115 ± 1.9006, 15	97.1209 ± 2.8683, 45

Table 5 The slope ratio (mean±SD) of five steroid standards in five spiked herbal medicines (derris scandens (A), Thai green herbal (Keaw-hom, B), Prasachangdaeng (C), Sahas Thara (D) and Andrographis paniculata (E)) under different five mobile phase system.

a) DCM: IPA: ACN: formic acid (7.5: 1.5: 0.5: 0.5)

Steroid standard	A	B	C	D	E
Betamethasone-17-valerate	1.0159 ± 0.0778	1.0266 ± 0.0903	0.9974 ± 0.0527	0.9996 ± 0.0648	0.9532 ± 0.0765
Triamcinolone acetonide	1.0308 ± 0.0490	0.9761 ± 0.0603	0.9318 ± 0.0943	0.9888 ± 0.0878	N/A
Dexamethasone	1.0195 ± 0.1072	1.0254 ± 0.0723	1.0255 ± 0.0428	1.0208 ± 0.0754	1.0222 ± 0.0635
Prednisolone	1.0200 ± 0.0841	1.0184 ± 0.0874	0.9750 ± 0.0773	0.9596 ± 0.0699	0.9922 ± 0.0887
Daucosterol	0.9376 ± 0.0365	0.9438 ± 0.0715	0.9590 ± 0.0586	0.9684 ± 0.0824	0.9691 ± 0.0566

b) DCM: petroleum ether: EtOH: formic acid (4.5: 3: 2: 0.5)

Steroid standard	A	B	C	D	E
Betamethasone-17-valerate	0.9891 ± 0.0765	0.9676 ± 0.0603	1.0161 ± 0.0750	1.0376 ± 0.0521	0.9722 ± 0.1002
Triamcinolone acetonide	0.9347 ± 0.0611	0.9762 ± 0.0782	0.9812 ± 0.0519	0.9842 ± 0.0667	N/A
Dexamethasone	0.9806 ± 0.0722	1.0106 ± 0.0701	1.0044 ± 0.0924	1.0005 ± 0.0813	N/A
Prednisolone	0.9016 ± 0.0728	0.8917 ± 0.0546	0.9164 ± 0.0891	N/A	0.9071 ± 0.0674
Daucosterol	0.9364 ± 0.0739	0.9524 ± 0.0668	0.9418 ± 0.0781	0.9411 ± 0.0950	0.9684 ± 0.0778

c) $CHCl_3$: IPA: Petroleum ether: Formic acid (4: 3: 2.5: 0.5)

Steroid standard	A	B	C	D	E
Betamethasone-17-valerate	1.0213 ± 0.0638	1.0096 ± 0.0617	1.0039 ± 0.0483	1.0297 ± 0.0504	1.0124 ± 0.0929
Triamcinolone acetonide	0.9329 ± 0.0492	0.9162 ± 0.0442	0.9132 ± 0.0442	0.9280 ± 0.0567	N/A
Dexamethasone	1.0139 ± 0.0666	0.9856 ± 0.0823	1.0301 ± 0.0907	1.0305 ± 0.0664	1.0449 ± 0.0867
Prednisolone	0.9176 ± 0.0514	0.9943 ± 0.0601	0.9347 ± 0.0743	0.9546 ± 0.0857	0.9453 ± 0.0399
Daucosterol	0.9857 ± 0.0923	0.9762 ± 0.0661	0.9989 ± 0.0660	1.0467 ± 0.0482	1.0304 ± 0.0852

d) $CHCl_3$: EtOH: *n*-hexane: formic acid (5: 2: 2.5: 0.5)

Steroid standard	A	B	C	D	E
Betamethasone-17-valerate	0.9270 ± 0.0527	0.9273 ± 0.0929	0.9471 ± 0.0959	0.9285 ± 0.0809	0.8828 ± 0.0884
Triamcinolone acetonide	0.9444 ± 0.0520	0.9368 ± 0.0543	0.9758 ± 0.0447	0.9563 ± 0.0786	N/A
Dexamethasone	0.9512 ± 0.0560	0.9634 ± 0.0968	0.9318 ± 0.0602	0.9663 ± 0.0664	N/A
Prednisolone	0.9898 ± 0.0619	0.9807 ± 0.0546	1.0082 ± 0.0654	N/A	0.9637 ± 0.0772
Daucosterol	1.0255 ± 0.0573	1.0243 ± 0.0424	1.0249 ± 0.0762	1.0378 ± 0.0567	1.0288 ± 0.0409

e) DCM: *tert*-butyl methyl ether: MeOH: formic acid (7.5: 1: 1: 0.5)

Steroid standard	A	B	C	D	E
Betamethasone-17-valerate	1.0239 ± 0.0507	1.0376 ± 0.0484	1.0301 ± 0.0560	1.0407 ± 0.0686	N/A
Triamcinolone acetonide	0.9231 ± 0.0487	0.9338 ± 0.0476	0.9290 ± 0.0573	0.9012 ± 0.0607	0.9472 ± 0.0528
Dexamethasone	1.0594 ± 0.0651	1.0589 ± 0.0670	1.0234 ± 0.0753	1.0403 ± 0.0740	N/A
Prednisolone	0.9516 ± 0.0548	0.9587 ± 0.0455	0.9621 ± 0.0700	0.9379 ± 0.0549	0.9583 ± 0.0450
Daucosterol	0.9472 ± 0.0741	0.9325 ± 0.0905	0.9290 ± 0.0667	0.8933 ± 0.0558	0.9160 ± 0.0518

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