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Original research paper

Application of green analytical principles in the HPLC analysis of prednisolone derivatives: Method optimization and validation for nasal powder formulations

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ABSTRACT

Glucocorticoids are a group of drugs increasingly used in modern medical practice due to their pronounced anti-inflammatory and immunosuppressive properties. In this study, prednisolone disodium phosphate and prednisolone acetate were analysed, with the aim of developing and validating an HPLC method in accordance with ICH Q2(R2) guidelines and quantifying their content in the active pharmaceutical ingredient powder and a model in-house sample. By applying the HPLC-DAD method with gradient elution, effective separation of the analytes was achieved. The method met all validation parameter requirements. The obtained results showed that the content of both analytes in the tested samples (bulk API powders and in-house prepared model formulation) was within the prescribed limits according to current pharmacopoeial standards. The proposed HPLC-DAD method was assessed for its applicability and environmental profile utilizing a range of green and blue metric tools. This comprehensive evaluation confirms that the method adheres to green analytical principles, making it suitable for sustainable pharmaceutical analysis.

Keywords: prednisolone disodium phosphate, prednisolone acetate, HPLC-DAD, validation, green analytical chemistry

INTRODUCTION

Prednisolone is structurally defined as 11β,17α,11-trihydroxypregna-1,4-diene-3,20-dione. It is a steroid compound featuring a hydroxyl group at the C11 position (Fig. 1a) (1). From a pharmacokinetic standpoint, prednisolone is a corticosteroid with a short plasma half-life ranging from approximately 2 to 4 hours, as reported by Bergmann *et al*. (2). It exhibits significant plasma protein binding; 70–90 % of the circulating drug is bound primarily to albumin and transcortin. In addition, prednisolone is a substrate for P-glycoprotein transporters and is extensively metabolized by the cytochrome P450

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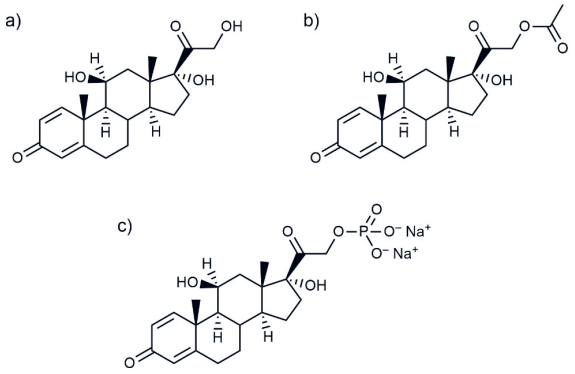

Fig. 1. Chemical structures of: a) prednisolone; b) prednisolone acetate; c) prednisolone disodium phosphate.

enzyme CYP3A4 (3). For therapeutic application, it is administered in esterified forms – prednisolone acetate and prednisolone disodium phosphate – which undergo enzymatic ester hydrolysis to release the pharmacologically active compound, prednisolone. Renal excretion constitutes the primary route of elimination, predominantly in the form of sulfate and glucuronide conjugates.

Prednisolone acetate is an ester derivative of prednisolone and it functions as its prodrug. Its chemical structure is depicted in Fig. 1b. Structurally, it differs from prednisolone by the substitution of the hydroxyl group at the C21 position with an acetate group, resulting in a compound with increased lipophilicity, which facilitates the epithelial permeation (4). As previously mentioned, enzymatic ester hydrolysis is required to convert this prodrug into its active form.

Prednisolone disodium phosphate (Fig. 1c) is also a prodrug of prednisolone. The hydroxyl group at the C21 position is replaced with a phosphate moiety, while the rest of the molecule remains unchanged. The presence of the phosphate group significantly enhances aqueous solubility, rendering it up to 30 times more soluble in water than prednisolone itself. Owing to this high aqueous solubility, it is particularly suitable for formulating the solutions.

Approved medicinal products containing prednisolone or its ester derivatives are available in various pharmaceutical forms, depending on the therapeutic indication and route of administration. They are mostly formulated as ophthalmic suspensions, solutions and ointments, but are also available as topical skin solutions, as well as oral and rectal solutions and tablets. Prednisolone acetate, due to its lipophilic properties, is predominantly used in suspension formulations intended for the treatment of inflammatory ocular conditions. In contrast, prednisolone disodium phosphate, being hydrophilic and highly water-soluble, is employed in solution-based formulations designed for rapid and effective absorption, including pediatric preparations. Advanced nasal delivery of corticosteroids represents a growing area of research (5–7). Prednisolone acetate and prednisolone disodium phosphate can both be formulated in nasal dry powders, thereby circumventing issues related to the physical and chemical instabilities commonly associated with aqueous formulations (6, 8).

According to the available literature, the analytical methods used in the analysis of prednisolone and its derivatives are commonly classified into two main categories: spec-

trometric and chromatographic techniques. Among the spectroscopic methods, ultraviolet-visible (UV-Vis) spectroscopy (9) and Fourier transform infrared (FTIR) spectroscopy are the most frequently employed. FTIR spectroscopy is primarily used for the identification of functional groups and the confirmation of chemical structure and purity, including that of prednisolone acetate and prednisolone disodium phosphate (10). In contrast, UV-Vis spectrophotometry, as described in the European Pharmacopoeia (11), is used for determining the content of active pharmaceutical ingredients (APIs). It is a simple, rapid, and cost-effective method, suitable for routine analysis and testing of raw materials, particularly when dealing with simple matrices (9). However, its major limitation lies in limited selectivity, especially in the presence of multiple excipients or in complex formulations.

In the analysis of prednisolone and its prodrugs, including prednisolone acetate and prednisolone disodium phosphate, chromatographic methods play a dominant role due to their high selectivity and sensitivity. The most widely used technique is high-performance liquid chromatography (HPLC), particularly in the reversed-phase, as recommended by the European Pharmacopoeia (11). This method allows for the simultaneous determination of multiple components with high accuracy. Additionally, other chromatographic techniques have been reported, including normal-phase HPLC (12), thin-layer chromatography (TLC) (13), and advanced hyphenated methods such as LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) (14) and GC-MS (gas chromatography-mass spectrometry) (15). These techniques are particularly valuable in bioanalytical and pharmacokinetic studies, where high sensitivity and selectivity are essential.

The aim of this research was to widen the scope of the HPLC-DAD method applicability by modifying a simple and reliable method for simultaneous determination of prednisolone acetate and prednisolone disodium phosphate. The modified method allows for a novel application in efficient analysis of these two corticosteroids in in-house prepared formulations intended for intranasal drug delivery. The method was designed to be both sustainable and economically viable, supporting the high-throughput analysis required for further product development.

EXPERIMENTAL

Standards and solvents

Prednisolone disodium phosphate certified reference material, pharmaceutical secondary standard (PHR2816), and prednisolone acetate certified reference material, pharmaceutical secondary standard (PHR1630) were purchased from Sigma-Aldrich (USA).

Acetonitrile, ethanol and methanol (HPLC grade, HiPerSolv CHROMANORM) were obtained from VWR Chemicals (Belgium), while tetrahydrofuran (HPLC grade) was purchased from Roth (Germany). Ultrapure water with a resistivity of 18.2 MΩ cm (25 °C) was obtained using a Millipore purification system (USA).

Samples

Bulk API powder, prednisolone acetate and prednisolone disodium phosphate (Biosynth, Slovakia) were used. Prednisolone disodium phosphate loaded hyaluronate/mannitol microparticles were tailored for an in-house prepared dry powder model sample for nasal administration.

Preparation of standard solutions

Separate standard stock solutions of prednisolone disodium phosphate (mass concentration 500 μg mL^{-1}, molar concentration 1.032 mmol L^{-1}) and prednisolone acetate (mass concentration 500 μg mL^{-1}, molar concentration 1.242 mmol L^{-1}) were prepared by dissolving the standards in 70 % ethanol using distinctive Class A volumetric flask. Appropriate aliquots of the two individual stock solutions were combined and subsequently diluted with the same solvent to the calibration mark to obtain the mixed standard solution. Working solutions were prepared by appropriate dilutions of the standard mixture, covering two distinct concentration ranges for each analyte: low (1.2–6.4 μg mL^{-1}) and high (5–100 μg mL^{-1}). All solutions were stored at 4 °C, protected from light until use.

Preparation of prednisolone derivative-loaded microparticles as the dry powder model sample

Prednisolone derivative-loaded microparticles were prepared by spray-drying the aqueous solution containing prednisolone disodium phosphate, sodium hyaluronate and mannitol. A concentrated aqueous solution of sodium hyaluronate (0.5 %, *m*/*m*) was prepared by dissolving sodium hyaluronate in purified water with continuous stirring on a magnetic stirrer. Mannitol and prednisolone disodium phosphate were weighed separately and dissolved in purified water, after which the required amount of the sodium hyaluronate solution was added. The mixture was then homogenized using a magnetic stirrer until a uniform dispersion was obtained. The concentration of prednisolone disodium phosphate, sodium hyaluronate and mannitol in the final solution were 0.1, 0.05 and 6.0 % (*m*/*m*), resp.

Prednisolone disodium phosphate-loaded microparticles were prepared by spray-drying using Büchi Mini Spray Dryer B-290 (Büchi, Switzerland) equipped with an ultrasonic nozzle (Büchi). The process parameters were set as follows: aspirator rate at 100 %, compressed airflow at 500 kPa, power of the ultrasonic nozzle at 60 %, inlet air temperature at 120 °C and feed pump setting at 5 %. Outlet temperature ranged between 67 and 70 °C.

HPLC-DAD

The analysis of prednisolone acetate and prednisolone disodium phosphate was performed using an Agilent 1260 liquid chromatograph equipped with a diode array detector (DAD), following a modified method based on report of Finšgar *et al.* (16). Chromatographic separation was carried out on a Kinetex C18 column with a particle size of 5 μm and dimensions of 150 × 4.6 mm (Phenomenex, USA), using gradient elution with varying mobile phase composition. Mobile phase A consisted of acetonitrile/ultra-pure water/tetrahydrofuran (15:75:10, *V*/*V*/*V*), while mobile phase B consisted of acetonitrile/ultra-pure water (80:20, *V*/*V*). The chromatographic separation was conducted using the following gradient program: initial conditions corresponded to 100 % mobile phase A, from 0 to 9 min, the proportion of phase B was linearly increased to 10 %, between 9 and 18 min, the phase B content was further increased to 60 %, at 20 min, the system returned to the initial conditions (0 % mobile phase B), allowing for full column re-equilibration before the next injection. The flow rate was set at 0.8 mL min^{-1}, and the column was thermostated at 50.0 °C. The injection volume was 20 μL. Absorbance was measured at 254 nm, with the spectral range set from 190 to 590 nm and a scan step of 2 nm. The total run time was 20 min.

Validation of the chromatographic method

Described HPLC method was validated in accordance with the current ICH Q2(R2) guidelines (17). The evaluated validation parameters included selectivity, linearity, working range, limit of detection (*LOD*), limit of quantification (*LOQ*), accuracy, precision and robustness.

Each of these parameters was calculated individually for each analyte, thereby ensuring the method's reliability in the quantification of prednisolone derivative components within complex matrices. An in-house prepared model formulation intended for nasal administration was used as the test sample, alongside a blank formulation that did not contain prednisolone derivatives.

Preparation of prednisolone derivative sample from active pharmaceutical ingredient powder

A precisely weighed portion of the API bulk powder was quantitatively transferred into a volumetric flask and dissolved in ethanol/ultra-pure water (70:30, *V*/*V*). The dispersion was thoroughly mixed using a vortex mixer (ZX3, Velp Scientifica, Italy) and subsequently brought to volume with the same solvent. To ensure complete dissolution of the analyte, the volumetric flask was placed in an ultrasonic bath (Elmasonic XtraTT, Elma Schmidbauer, Germany). The resulting solution was filtered through a membrane filter with a pore size of 0.45 μm (Chromafil Xtra 0.45 μm, 25 mm, Macherey-Nagel, Germany). The filtrate obtained in this manner was used directly as the analytical test solution.

Extraction of prednisolone derivative from model sample

An accurately weighed portion of the finely milled and homogenized, in-house prepared model sample, formulated for nasal administration, was quantitatively transferred into a volumetric flask. Subsequently, 8 mL of ethanol/ultra-pure water (70:30, *V*/*V*) was added as the extraction solvent. The mixture was vigorously mixed for 5 min using a vortex mixer (ZX3, Velp Scientifica). To ensure efficient extraction of the analyte, the volumetric flask was placed in an ultrasonic bath for 15 min. Following sonication, the mixture was centrifuged using a mini G centrifuge (Ika, Germany) for 5 min to separate particulate matter. The resulting supernatant was filtered through a 0.45-μm pore-size membrane. The filtrate was transferred to the volumetric flask, and extraction solvent was added to dilute the solution to the mark. The filtrate obtained using this procedure was used directly as the analytical test solution for further analysis.

To assess the selectivity of the method, a blank sample solution was prepared by the same procedure.

Assessment of sustainability of the proposed method

The sustainability of the proposed method is evaluated based on two fundamental parameters: environmental friendliness and practicability. For this purpose, four distinct open-source and web applications were employed: the Analytical GREEnness Metric Approach and Software (AGREE) (18), the Analytical GREEnness Metric for Sample Preparation (AGREEprep) (19), the Complementary Modified Green Analytical Procedure Index (ComplexMoGAPI) (20), and the Blue Applicability Grade Index (BAGI) (21).

Statistical analysis

All collected data were analyzed and statistically evaluated using Microsoft Office Excel 2019. A significance level of 5 % (α = 0.05) was applied throughout all statistical analyses.

RESULTS AND DISCUSSION

The developed HPLC method was specifically optimized for the simultaneous separation and quantification of prednisolone acetate and prednisolone disodium phosphate in real samples, namely, the formulation and bulk samples.

According to the monographs of the European Pharmacopoeia (Ph. Eur.) (11) and the British Pharmacopoeia (BP) (22), the content of prednisolone acetate and prednisolone disodium phosphate is commonly determined using UV-Vis spectrophotometry, whereas the United States Pharmacopeia (USP) (23) recommends an HPLC-based approach. For chromatographic separation, a C18 reversed-phase column was selected due to its broad applicability, retention capacity for moderately polar corticosteroids, and overall robustness.

Furthermore, to achieve improved baseline resolution of prednisolone derivatives, the mobile phase composition was modified from the pharmacopoeial method and employed as proposed by Finšgar *et al*. (16). Different columns, including Zorbax C18 and C8, were initially evaluated; however, the Kinetex C18 column demonstrated the best long-term durability and was consequently chosen for further investigation. Final method optimization was carried out on a Kinetex C18 column (5 μm, 150 × 4.6 mm), using a mobile phase consisting of acetonitrile/tetrahydrofuran/water (15:10:75, *V*/*V*/*V*) as mobile phase A, and acetonitrile/water (80:20, *V*/*V*) as phase B.

Based on the UV-Vis spectra of both prednisolone derivatives with maximum absorption (λ_{max}) at 254 nm (Fig. S1), this wavelength was selected for detection, considering the presence of conjugated double bond systems typical of steroidal structures.

Following the optimization of chromatographic conditions, the proposed HPLC method was validated in accordance with the relevant regulatory guidelines (17).

Validation of the analytical method

Selectivity. – Method selectivity was evaluated to confirm that the analytes could be accurately identified and quantified without interference from each other, excipients, matrix constituents, or other structurally related substances. Standard working solutions for low and high concentration range contained equal concentrations of both analytes (prednisolone disodium phosphate and prednisolone acetate). HPLC-DAD analysis was performed on a working solution containing 6.4 μg mL^{-1} each of prednisolone disodium phosphate and prednisolone acetate. The resulting chromatogram showed distinct peaks at retention times of 8.04 min for disodium phosphate and 11.46 min for the acetate (Fig. 2).

The elution order of the two analytes reflects their relative polarity. As already mentioned, prednisolone disodium phosphate is a more polar molecule than prednisolone acetate which enhances its solubility in mobile phases with higher aqueous content.

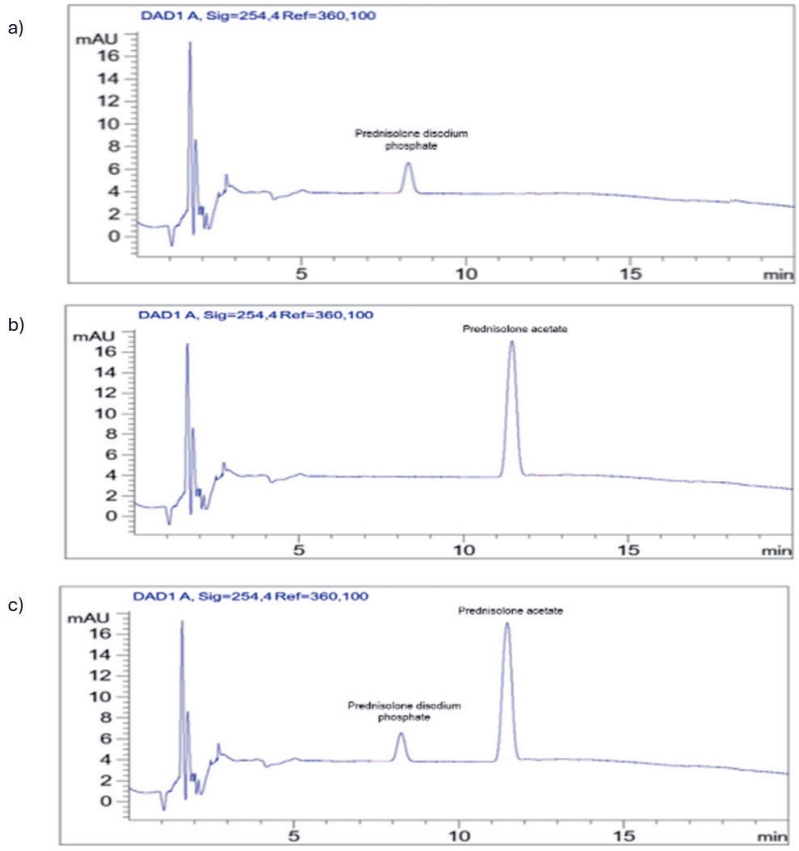

Fig. 2. Representative chromatograms: a) prednisolone disodium phosphate; b) prednisolone acetate; c) standard mixture containing both prednisolone derivatives, at a concentration of 6.4 μg mL^{-1} under optimized conditions.

The column's ability to resolve the analytes under defined chromatographic conditions was subsequently evaluated to ensure clear peak separation and to rule out any potential overlap. The peak widths at half height were measured to be 0.27 min for prednisolone disodium phosphate and 0.32 min for prednisolone acetate (concentration level 2.4 μg mL^{-1}), resp., and the calculated resolution (R_s) was 6.46. Given that a resolution value of 1.5 indicates baseline separation, with peak overlap below 0.3 %, it is evident that the peaks were well-resolved. Additionally, the resolution between the prednisolone derivatives and their nearest neighboring peaks exceeded 1.5, further confirming the satisfactory selectivity of the method under the optimized chromatographic conditions.

Peak purity values were 999.9 for prednisolone acetate and 999.8 for prednisolone disodium phosphate (Table I), indicating high spectral homogeneity. These results confirm that the column resolution is satisfactory, thereby supporting the method's selectivity and its suitability for quantitative analysis.

Table I. Chromatographic system suitability data[a]

	Prednisolone acetate		Prednisolone disodium phosphate		Reference values (17, 23)
	Value	RSD (%)[b]	Value	RSD (%)[b]	
Retention time, t_R (min)	11.46	1.8	8.04	1.9	RSD < 2.0 %
Peak area (mAU s)	268.56	0.5	47.42	1.3	RSD < 2.0 %
Symmetry, A_s	1.18	1.8	1.11	1.9	0.8–1.2
Peak purity, P_p	999.8	0.01	999.9	0.01	> 999.0
Peak capacity, P_c	62.31	1.3	73.74	0.03	N/A

N/A – not applicable, RSD – relative standard deviation; [a] System suitability data was performed on the prepared working solution, which contained each prednisolone disodium phosphate and prednisolone acetate at a concentration of 6.4 μg mL^{-1}; [b] n = 6.

Linearity. – In this study, linearity was confirmed across two working ranges: a low concentration range (1.2–6.4 μg mL^{-1}) and a high concentration range (5–100 μg mL^{-1}), each tested at five different concentration levels. All measurements were performed in triplicate and in parallel for both analytes, thereby ensuring the reliability and precision of the results. The obtained data are presented in Table II.

Table II. Linearity and sensitivity data

	Prednisolone acetate		Prednisolone disodium phosphate	
	Low concentration range	High concentration range	Low concentration range	High concentration range
Regression analysis data[a]				
Linearity range (μg mL^{-1})	1.2–6.4	5–100	1.2–6.4	5–100
Slope	42.238	41.404	7.517	17.969
Intercept	–0.975	24.484	–0.079	18.399
SE of the slope	0.190	0.275	0.032	0.124
SE of the intercept	0.767	15.281	0.130	6.906
Correlation coefficient (R)	0.9997	0.9999	0.9999	0.9999
Regression SS	23,853.24	13,178,514.97	762.629	2,482,118.53
Residual SS	0.968	2325.65	0.028	474.941
Total SS	23,854.20	13,180,840.61	762.657	2,482,593.47
Sensitivity				
LOD (μg mL^{-1})[b]	0.06	1.22	0.06	1.27
LOQ (μg mL^{-1})[b]	0.18	3.69	0.17	3.84

SE – standard error, SS – sum of squares; [a] n = 5; [b] Limit of detection (*LOD*) and limit of quantitation (*LOQ*) were calculated from calibration curve using the standard deviation of the response and the slope of the curve.

Linearity is expressed through the correlation coefficient (R) of the regression line. The correlation coefficient for both prednisolone derivatives was 0.9999 across both concentration ranges, confirming a strong linear relationship between the peak area and the analyte concentration, being compliant with ICH Q2(R2) guidelines criterion of $R > 0.999$ (17).

Moreover, regression analysis showed that the standard errors of the slope and intercept were low in all cases, confirming the high precision of the regression models. Additionally, the regression sum of squares (SSreg) accounted for more than 99.98 % of the total variance, while the residual sum of squares (RSS) was negligible, further confirming the validity and robustness of the models.

To further support the linearity assessment, a residual plot was constructed (Fig. 3). This plot illustrates the differences between experimentally observed and predicted values as a function of concentration. The residuals were randomly distributed around the zero line and were of similar magnitude, thus confirming that the relationship between concentration and response is linear.

Accuracy. – The accuracy of the method was determined using two complementary methods. The first method involved the assessment of accuracy through the application of the regression equation. Three concentration levels of the analyte – low, medium, and high, for both working ranges – were analyzed, with each level measured in triplicate. Analytical recovery was evaluated, and the results for prednisolone derivatives are presented in Table III. In all cases, the analytical recovery exceeded 96.6 %, with the highest value reaching 103.7 %. In accordance with the ICH Q2(R2) guidelines (17), the acceptable range for analytical recovery was obtained.

The second method for accuracy determination utilized the standard addition method. In HPLC analysis, this procedure is used when there is a risk that the sample

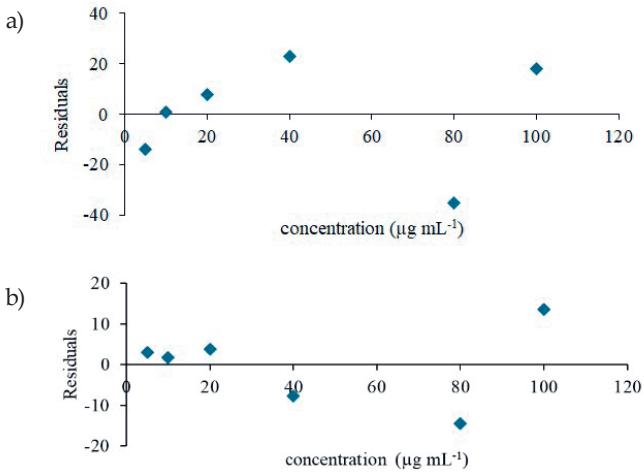

Fig. 3. Residual plots illustrating the regression model fit for: a) prednisolone acetate; b) prednisolone disodium phosphate. Panels show residuals for the high concentration range of these prednisolone derivatives.

Table III. Accuracy assessment of the method using the regression equation and the standard addition method

	Prednisolone acetate			Prednisolone disodium phosphate			Reference value
	Recovery (%)[a]	RSD (%)[a]	Accuracy error (%)[a]	Recovery (%)[a]	RSD (%)[a]	Accuracy error (%)[a]	Recovery (%)
Low concentration range							
Low (1.2 μg mL^{-1})	97.06	0.15	2.94	103.67	0.53	3.67	95–105
Medium (2.4 μg mL^{-1})	100.31	0.60	0.31	98.47	2.05	1.53	95–105
High (6.4 μg mL^{-1})	99.34	0.28	0.66	97.50	0.55	2.50	95–105
High concentration range							
Low (10 μg mL^{-1})	97.32	1.09	2.68	97.34	1.34	2.66	95–105
Medium (40 μg mL^{-1})	99.57	0.80	0.43	96.62	2.45	3.38	95–105
High (100 μg mL^{-1})	96.65	2.73	3.35	100.59	0.39	0.59	95–105
Standard addition method							
Low (1.2 μg mL^{-1})	97.69	0.15	2.37	98.41	0.53	1.59	95–105
Medium (2.4 μg mL^{-1})	101.70	0.60	1.70	96.92	2.05	3.08	95–105
High (6.4 μg mL^{-1})	99.77	0.28	0.23	97.44	0.55	2.56	95–105

RSD – relative standard deviation; [a] $n = 3$.

matrix may influence analyte quantification (*i.e.*, the matrix effect), potentially biasing results if the conventional external standard method is applied. As shown in Table III, the results for both analytes fell within the above-mentioned reference range (recoveries for prednisolone disodium phosphate were in the range from 96.9 to 98.4 % and for prednisolone acetate in the range from 97.7 to 101.7 %), thereby confirming the accuracy of the method through both evaluation approaches.

Precision. – The precision of the analytical method was evaluated by determining its repeatability and intermediate precision. Within a single day, the same samples were analyzed six times at different time intervals, with peak areas compared to assess intra-day variability. Repeatability in the low concentration range (1.2–6.4 μg mL^{-1}) was assessed by analyzing a standard solution (2.4 μg mL^{-1}) in six replicates on the same day. Intermediate precision was evaluated by repeating the same procedure over three consecutive days, each time using six replicates (Table IV). A comparable procedure was applied for the high concentration range (5–100 μg mL^{-1}), utilizing a standard solution of 40 μg mL^{-1} (Table IV).

Table IV. Precision evaluation: repeatability data

	Prednisolone acetate	Prednisolone disodium phosphate	Reference value (%) (17, 23)
Low concentration range[a]			
Repeatability (RSD, %)[b]	0.15	0.73	RSD < 2.0
Intermediate precision (RSD, %)[c]	1.24	1.93	RSD < 2.0
High concentration range[a]			
Repeatability (RSD, %)[b]	1.07	1.58	RSD < 2.0
Intermediate precision (RSD, %)[c]	1.32	1.89	RSD < 2.0

RSD – relative standard deviation; [a] Repeatability was assessed by analyzing a standard solution (2.4 μg mL^{-1}) for the low concentration range (1.2–6.4 μg mL^{-1}) and 40 μg mL^{-1} for the high concentration range (5–100 μg mL^{-1}) in six replicates within a single day. Intermediate precision was evaluated by analyzing the same solution over three consecutive days, using either six replicates per day; [b] n = 6; [c] n = 18.

The resulting data are illustrated in a Box-Whisker plot, providing a visual representation of measurement precision across the validation period (Fig. 4). As shown in Fig. 4, all measurement results for each day fall within the whiskers, which provides strong evidence of

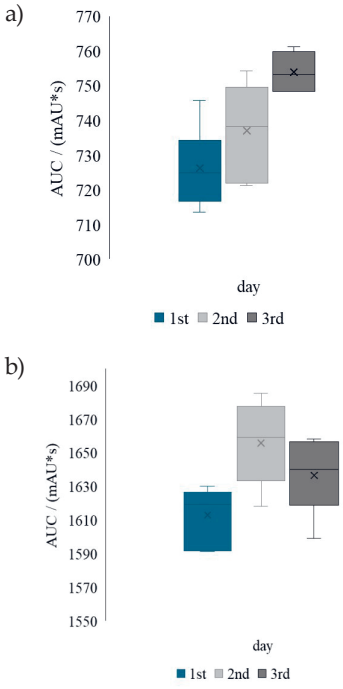

Fig. 4. Box-Whisker plot of measurement precision at the high concentration range (40 μg mL^{-1}) over three validation days; mean values (×); median (central line within the box): a) prednisolone disodium phosphate; b) prednisolone acetate.

excellent method stability and consistency. The close agreement between mean values and medians within each day further supports a symmetrical distribution of the results. Upon evaluating variability across the three days, the results from day 1 display the widest interquartile range (IQR), suggesting slightly greater intra-day variability compared to the subsequent days. The data from day 2 similarly exhibit a pronounced IQR, with the median shifted higher than that observed on day 1. Although the precision remains within the predefined ICH acceptance criteria, a moderate increase in variability is evident. Conversely, the results obtained on day 3 reveal the narrowest box and whiskers, the lowest variability and the highest level of precision. Both the median and mean values are positioned near the upper end of the scale, indicating a stable and highly reproducible outcome. Overall, the plot highlights excellent precision and repeatability of measurements at the high analyte concentration level over the three-day validation period.

Stability. – The stability of the standard solution was assessed under three different storage conditions to simulate potential scenarios during preparation and storage. The objective was to evaluate the preservation of analyte integrity and ensure the reliability of analytical results over time. The evaluated stability conditions included: benchtop stability, short-term stability, and long-term stability. Benchtop stability was assessed by storing the standard solution of prednisolone derivatives (2.4 μg mL^{-1}) at room temperature for 8 hours, simulating temporary exposure during laboratory handling or sample preparation. Short-term stability was evaluated by storing the standard solution (2.4 μg mL^{-1}) at 4 °C for 72 hours, a condition that mimics typical storage conditions recommended for preserving sensitive analytes. Long-term stability was investigated by storing the same standard solution at −20 °C for 7 days, allowing the assessment of analyte persistence under reduced temperature conditions. The standard solution was analyzed immediately after preparation and after each storage condition. The recovery percentage was calculated, and the analytical results are presented in Table V. Given that all obtained results fall within the acceptable range of 95–105 %, the stability criterion is considered met. These findings confirm that the standard solutions of both analytes remain stable, with no significant changes in concentration, despite varying storage conditions. This further supports the reliability and applicability of the proposed analytical method under real-world conditions.

Table V. Stability evaluation data[a]

Analyte	Benchtop stability (recovery, %)	Short-term stability (recovery, %)	Long-term stability (recovery, %)	Reference value (recovery, %) (17, 23)
Prednisolone acetate	99.6	100.2	99.1	95–105
Prednisolone disodium phosphate	99.7	97.6	99.5	95–105

[a] Stability of the standard solutions was conducted on the prepared working solution, which contained each prednisolone disodium phosphate and prednisolone acetate at a concentration of 2.4 μg mL^{-1}, under different conditions: benchtop stability (at room temperature for 8 hours), short-term stability (at 4 °C for 3 days), and long-term stability (at –20 °C for 7 days).

Robustness. – In the final phase of analytical method validation, the robustness of the method was evaluated, defined as its capacity to preserve stability and reliability in the presence of small, deliberately introduced variations in experimental conditions. The investigation focused on the effects of adjustments to the flow rate (± 0.05 mL min^{-1}), column temperature (± 2 °C), and mobile phase gradient (± 1 %). Triplicate measurements revealed that these controlled modifications produced predictable shifts in retention times (all RSD values for both analytes remaining below 1.6 %) while maintaining peak shape (peak symmetry was in the range from 1.1 to 1.2) and detector response within the predefined acceptance criteria for prednisolone acetate (RSD values were lower than 1.3 %) and prednisolone disodium phosphate (RSD values were lower than 2.7 %). Overall, the method exhibited satisfactory resilience to typical fluctuations in these critical parameters, with no appreciable degradation in system suitability performance.

Prednisolone disodium phosphate and acetate content in pharmaceutical samples

The primary objective of this study was to quantitatively determine the content of prednisolone disodium phosphate and prednisolone acetate in API samples. Those materials often exhibit slightly lower purity compared to certified reference standards, as they may contain impurities arising from residual reagents used in the manufacturing process or from degradation products of the active substance (24).

The assay procedure was commenced with the preparation of test solutions in an identical manner to that of the standard solutions. HPLC-DAD chromatogram is presented in Fig. 5. The chromatogram revealed two distinct peaks: a smaller peak eluted first, corresponding to an impurity, and a larger peak eluted afterwards, corresponding to prednisolone disodium phosphate. The impurity exhibited greater polarity than prednisolone disodium phosphate, resulting in a shorter retention time relative to the main analyte. Quantitative analysis determined the prednisolone disodium phosphate content in the tested API sample to be 95.36 % (RSD = 1.6 %, n = 3).

HPLC analysis of the prednisolone acetate API sample was performed in accordance with the validated method, with the resulting chromatogram shown in Fig. 5. The chromatogram revealed a single main peak corresponding to prednisolone acetate and three smaller peaks corresponding to impurities. Two impurity peaks were more polar than the analyte, resulting in shorter retention times and relative retention times less than 1. The third impurity, of lower polarity, eluted immediately after prednisolone acetate, with a relative retention time greater than 1.

The content of prednisolone acetate in the API powder was determined to be 101.79 % (RSD = 2.9 %, n = 3), which falls within the pharmacopoeial acceptance limits. This confirms that the tested prednisolone acetate API meets the pharmacopoeial quality requirements and complies with the specified content criteria.

The extraction of the analyte from the in-house prepared model sample was carried out under controlled conditions, followed by analysis of the test sample. The procedure involved systematic optimisation of solvent selection and extraction time, with an emphasis on maximising analyte recovery, minimising matrix interferences, and ensuring high accuracy and reproducibility of measurements. The optimised conditions yielded an analytical recovery exceeding 96.9 % (Fig. 5).

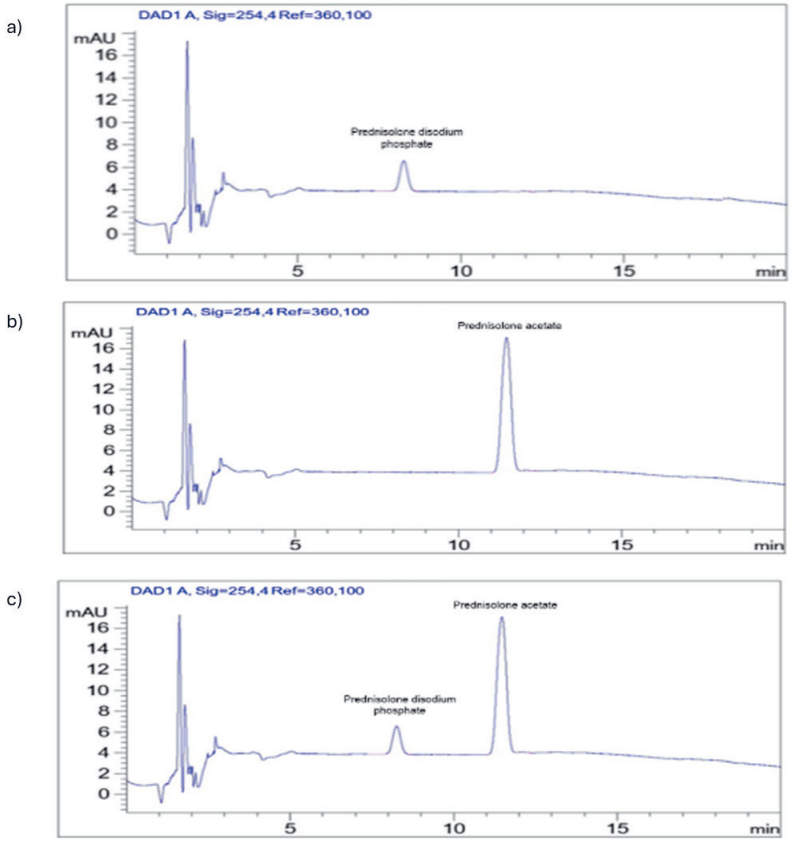

Fig. 5. Chromatogram: a) prednisolone disodium phosphate in API sample; b) prednisolone acetate in API sample; c) prednisolone disodium phosphate in in-house prepared model sample.

Sustainability of the proposed method

The environmentally friendliness of the developed chromatographic method was evaluated using the AGREE, AGREEprep, and ComplexMoGAPI tools (Table VI). These assessments considered critical parameters such as sample preparation procedures, sample throughput, energy consumption, waste production, environmental effects, and laboratory staff safety. The greenness scores, along with method attributes and corresponding hues, are detailed in Tables S1-S3 in the Supplementary materials.

The AGREE result for the proposed analytical procedure is displayed as a clock-shaped pictogram divided into twelve segments. The pictogram's central section showed a final score of 0.70 with a green colour, indicating the developed method's greenness. Moreover, the method exhibited a favourable profile, with none of the segments coloured red and seven out of twelve segments coloured green, with scores ranging from 0.65 to 1.00.

In our previous studies, we identified the sample preparation procedure as one of the most critical steps from the perspective of green analytical chemistry due to waste production and analyst safety (25, 26). The AGREEprep tool, which considers every aspect of the proposed sample preparation procedure, demonstrated its greenness with a pictogram consisting of ten trapezoidal bars. The procedure attained an AGREEprep score of 0.63 with light green hue, indicative of its high environmental friendliness due to the small sample size and high sample throughput (green segment scores were in the range from 0.64 to 1.00).

The greenness of the proposed method was further evaluated using the ComplexMoGAPI tool. This tool provided a significant advantage over AGREE and AGREEprep by offering a comprehensive and holistic assessment of method sustainability, considering a wider range of parameters, including activities carried out before, during and after sample preparation and analysis. The result, presented in a clear color-coded pictogram with five pentagrams and an additional hexagonal segment, showed that the proposed method is green with an excellent overall score of 85. This was further confirmed by achieving 13 green and 7 yellow fields out of a total of 26, indicating that the method is highly sustainable across various attributes.

The practicality of the proposed method, a very important parameter in routine analytical laboratories, was evaluated using the BAGI tool (Table S4 in the Supplementary materials). An asteroid pictogram, coloured in various shades of blue, demonstrated the method's practicality (Table VI). A BAGI score of 82.5 was attained for the chromatographic method, and the entire analytical protocol shows good potential for routine application.

The greenness and blueness assessment of the proposed method using the aforementioned tools demonstrated its superior sustainability compared to HPLC and UV-Vis assay methods included in USP and Ph. Eur. monographs for prednisolone acetate and prednisolone disodium phosphate (Table VI, Tables S5-S20 in the Supplementary materials). The AGREE score of these pharmacopeial methods was more than 0.14 units lower due to a large sample size, multiple sample preparation steps, and high waste generation. Accordingly, the AGREEprep scores of the three pharmacopeial methods were low, at 0.34, and coloured orange. A slightly higher AGREEprep score of 0.55 was observed for Ph. Eur. assay method for prednisolone disodium phosphate due to the use of water as a solvent. For this reason, the highest ComplexMoGAPI score was obtained for prednisolone disodium phosphate assay by the Ph. Eur., following the proposed method. The ComplexMoGAPI scores of the other pharmacopeial methods were 3 points lower than that of the proposed HPLC-DAD method due to a large sample volume. The BAGI score of the proposed method was 7.5 points higher than the score of the USP methods due to simultaneous analysis of prednisolone acetate and prednisolone disodium phosphate, as well as a low sample size. The BAGI scores of the Ph. Eur. methods were 17.5 points lower compared to the proposed method due to the aforementioned reasons, as well as the manual sample preparation and analysis process.

CONCLUSIONS

HPLC-DAD method intended for simultaneous determination of prednisolone acetate and prednisolone disodium phosphate was suggested and fully validated in accordance

Table VI. Sustainability results of five methods used for the determination of prednisolone acetate and/or prednisolone disodium phosphate

Sustainability tool	The developed method	Prednisolone acetate assay by the USP	Prednisolone disodium phosphate assay by the USP	Prednisolone acetate assay by the Ph. Eur.	Prednisolone sodium phosphate assay by the Ph. Eur.
AGREE[a]					
AGREEprep[b]					
Complex-MoGAPI[c]					
BAGI[d]					

[a] AGREE: sample procedure (1), sample size (2), sampling (3), sample preparation steps (4), automatization (5), derivatization (6), waste (7), analysis throughput (8), energy consumption (9), renewable reagents (10), toxicity of reagents (11) and operator' safety (12); [b] AGREEprep: sampling (1), hazardous materials (2), sustainability, renewability, and reusability of materials (3), waste (4), economy of sample (5), sample throughput (6), automatization (7), energy consumption (8), analytical instrumentation (9) and operator's safety (10); [c] ComplexMoGAPI: collection (1), preservation (2), transport (3), storage (4), type of method (5), scale of extraction (6), solvent/reagents used (7), additional treatments (8), amount (9), health hazard (10), safety hazard (11), energy (12), occupational hazard (13), waste (14), waste treatment (15); quantification (o), yield (i), temperature/time (ii), number of rules met (iii), health hazard (iv_a), safety hazard (iv_b), technical setup (v_a), energy (v_b), occupational hazard (v_c), workup and purification of the end product (vi_a) and purity (vi_b); [d] BAGI: type of analysis (1), multi- or single-element analysis (2), analytical technique (3), simultaneous sample preparation (4), sample preparation (5), samples per h (6), reagents and materials (7), preconcentration (8), degree of automatization (9) and amount of sample (10).

with relevant analytical guidelines. Although these glucocorticoid derivatives are typically used separately in different pharmaceutical products, the purpose of developing this method was to establish a unified and sustainable analytical procedure suitable for the control of formulations containing either analyte or their combination. This approach enhances laboratory efficiency and facilitates direct comparison of data related to purity, stability, and active substance content across different formulations.

The method was successfully applied to quantification of prednisolone derivatives in active pharmaceutical ingredients and in nasal dosage formulations. The method demonstrated excellent analytical performances, *e.g.*, resolution and precision, confirming its suitability for routine quality control of corticosteroid derivatives in complex pharmaceutical matrices. It also provides an economical and environmentally friendly alternative in such analyses. Its benefits were demonstrated through a comprehensive evaluation using various environmental sustainability metrics.

Abbreviations, acronyms, symbols. – AGREE – Analytical GREEnness metric approach and software; AGREEprep – Analytical GREEnness metric for sample preparation; API – active pharmaceutical ingredients; BAGI – Blue Applicability Grade Index; ComplexMoGAPI – Complementary Modified Green Analytical Procedure Index; FTIR – Fourier transform infrared spectroscopy; GC-MS – gas chromatography-mass spectrometry; HPLC – high-performance liquid chromatographic method; ICH – The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; LC-MS/MS – liquid chromatography coupled with tandem mass spectrometry; TLC – thin-layer chromatography; UV-Vis – ultraviolet-visible spectroscopy.

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Authors contributions. – Conceptualization, D.A.K. and A.M.; methodology, D.A.K. and A.M.; analysis D.A.K., J.K., I.K., P.B., L.P., and E.K.; investigation, D.A.K., J.K., I.K., P.B, L.N.N., and A.M.; visualization, D.A.K., I.K., and P.B.; writing, original draft preparation D.A.K., I. K., L.N.N., and A.M.; writing, review and editing, D.A.K., J.K., I.K., P.B, L.P., L.N.N., and A.M.; supervision, D.A.K., I.K., and A.M.; funding acquisition, D.A.K, I.K., L.N.N., and A.M.; project administration, D.A.K. and A.M. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

Table S1. AGREE data for the proposed analytical method.

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling procedure	Off-line analysis	2	0.48	yellow
2	Amount of sample (g or mL)	1	2	0.65	light green
3	Positioning of the analytical advice	At-line	2	0.33	orange
4	Sample preparation steps	3 or fewer	2	1.00	dark green
5	Integration and automatization	Semi-automatic; none or miniaturized	2	0.75	green
6	Derivatization agents	None	2	1.00	dark green
7	Amount of waste (g or mL)	20	2	0.29	dark orange
8	Sample throughput	Analytes determined in a single run: 2; Samples analysed per hour: 3	2	0.38	light orange
9	Energy consumption	LC; the power consumption of a single analysis: 0.11	2	0.99	dark green
10	Reagents	Some reagents are bio-based	2	0.50	yellow
11	Toxic reagents and solvents	Yes; 1 mL	2	0.80	green
12	Threats	Highly flammable	2	1.00	dark green
TOTAL SCORE				**0.70**	**green**

Table S2. AGREEprep data for the proposed sample preparation procedure.

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling preparation and placement	On-site	1	0.33	orange
2	Hazardous materials (g or mL)	0	5	1.00	dark green
3	Sustainability, renewability, and reusability of materials	50-75% of reagents and materials are sustainable or renew, but can be used ONCE	2	0.50	yellow
4	Waste (g or mL)	5	3	0.37	light orange
5	Size economy of the sample (g or mL)	10	2	0.33	orange
6	Sample throughput	50	3	0.92	green
7	Integration and automatization	3 steps; semi-automated systems	2	0.38	light orange
8	Energy consumption (Wh)	40	4	0.64	light green
9	Post-sample preparation configuration for analysis	Liquid chromatography, gas chromatography with quadropole detection, etc.	2	0.25	dark green
10	Operator's safety	1 hazard	3	0.75	green
TOTAL SCORE				**0.63**	**light green**

Table S3. ComplexMoGAPI data for the proposed analytical method.

Numeric notation	Criterion	Input data	Score*	Colour
Sample preparation and analysis				
Sample preparation				
1	Collection	On-line or at-line	2	yellow
2	Preservation	None	3	green
3	Transport	None	3	green
4	Storage	None	3	green
5	Type of method	Simple procedure	2	yellow
6	Scale of extraction	Not applicable	0	white
7	Solvent/reagents used	Non-green solvents/reagents	1	red
8	Additional treatments	None	3	green
Reagents and solvents				
9	Amount	< 10 mL (< 10 g)	3	green
10	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
11	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
12	Energy	≤ 0.1 kWh per sample	3	green
13	Occupational hazard	Hermetic sealing of analytical procedure	3	green
14	Waste	> 10 ml (> 10 g)	1	red
15	Waste treatment	Degradation, passivation	2	yellow
O	Quantification	Yes	6	not applicable
Pre-analysis processes				
Yield and conditions				
I	Yield	Not applicable	0	white
II	Temperature/time	Not applicable	0	white
Relation to green economy				
III	Number of rules met	5-6	3	green
Reagents and solvents				
IV_a	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
IV_b	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
V_a	Technical setup	Common setup	3	green
V_b	Energy	≤ 0.1 kWh per sample	3	green
V_c	Occupational hazard	Hermitization of analytical process	3	green

Workup and purification				
VI_a	Workup and purification of the end product	None or simple process	3	green
VI_b	Purity	> 98 %	3	green
TOTAL SCORE			**85**	**green**

* This scoring system considers the range of choices within each category. The total points are aggregated and divided by the maximum achievable points to determine the percentage score.

Table S4. BAGI data for the proposed analytical method.

Numeric notation	Criterion	Input data	Score	Colour
1	Type of analysis	Quantitative and confirmatory	10	dark blue
2	Multi- or single-element analysis	Multi-element analysis for 2-5 compounds of the same chemical class	5	light blue
3	Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID, etc.)	7.5	blue
4	Simultaneous sample preparation	13-95	7.5	blue
5	Sample preparation	Not required or on-site sample preparation if required	10	dark blue
6	Samples per h	2-4	5	light blue
7	Reagents and materials	Common commercially available reagents (methanol, acetonitrile, HNO_3, nitrogen or other common gases, etc.)	10	dark blue
8	Preconcentration	No preconcentration required. Required sensitivity and / or legislation criteria are met directly.	10	dark blue
9	Degree of automatization	Semi-automated with common devices (e.g. HPLC autosampler)	7.5	blue
10	Amount of sample	< 100 µg (or mg) bioanalytical samples; < 10 mL (or g) food / environmental	10	dark blue
TOTAL SCORE			**82.5**	**blue**

Table S5. AGREE data for the prednisolone acetate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling procedure	Off-line analysis	2	0.48	yellow
2	Amount of sample (g or mL)	140	2	0	red
3	Positioning of the analytical advice	At-line	2	0.33	orange
4	Sample preparation steps	5	2	0.60	light green
5	Integration and automatization	Semi-automatic; not miniaturized	2	0.25	orange
6	Derivatization agents	None	2	1.00	dark green
7	Amount of waste (g or mL)	260	2	0	red
8	Sample throughput	Analytes determined in a single run: 2; Samples analysed per hour: 3	2	0.34	orange
9	Energy consumption	LC; the power consumption of a single analysis: 0.11	2	0.99	dark green
10	Reagents	Some reagents are bio-based	2	0.50	yellow
11	Toxic reagents and solvents	Yes; 100 mL	2	0	red
12	Threats	Highly flammable	2	0.80	green
TOTAL SCORE				**0.44**	**light green**

Table S6. AGREEprep data for the prednisolone acetate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling preparation and placement	On-site	1	0.33	orange
2	Hazardous materials (g or mL)	6	5	0.07	red
3	Sustainability, renewability, and reusability of materials	50-75% of reagents and materials are sustainable or renew, but can be used ONCE	2	0.50	yellow
4	Waste (g or mL)	260	3	0	red
5	Size economy of the sample (g or mL)	100	2	0	red
6	Sample throughput	50	3	0.92	dark green
7	Integration and automatization	4 steps; semi-automated systems	2	0.25	dark orange
8	Energy consumption (Wh)	40	4	0.64	light green
9	Post-sample preparation configuration for analysis	Liquid chromatography, gas chromatography with quadropole detection, etc.	2	0.25	dark orange
10	Operator's safety	2 hazards	3	0.50	yellow
TOTAL SCORE				**0.34**	**orange**

Table S7. ComplexMoGAPI data for the prednisolone acetate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Score*	Colour
Sample preparation and analysis				
Sample preparation				
1	Collection	On-line or at-line	2	yellow
2	Preservation	None	3	green
3	Transport	None	3	green
4	Storage	None	3	green
5	Type of method	Simple procedure	2	yellow
6	Scale of extraction	Not applicable	0	white
7	Solvent/reagents used	Non-green solvents/reagents	1	red
8	Additional treatments	None	3	green
Reagents and solvents				
9	Amount	> 100 mL (> 100 g)	1	red
10	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
11	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
12	Energy	≤ 0.1 kWh per sample	3	green
13	Occupational hazard	Hermetic sealing of analytical procedure	3	green
14	Waste	> 10 ml (> 10 g)	1	red
15	Waste treatment	Degradation, passivation	2	yellow
O	Quantification	Yes	6	not applicable
Pre-analysis processes				
Yield and conditions				
I	Yield	Not applicable	0	white
II	Temperature/time	Not applicable	0	white
Relation to green economy				
III	Number of rules met	5-6	3	green
Reagents and solvents				
IV_a	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
IV_b	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
V_a	Technical setup	Common setup	3	green
V_b	Energy	≤ 0.1 kWh per sample	3	green

V_c	Occupational hazard	Hermitization of analytical process	3	green
Workup and purification				
VI_a	Workup and purification of the end product	None or simple process	3	green
VI_b	Purity	> 98 %	3	green
TOTAL SCORE			**82**	**green**

* This scoring system considers the range of choices within each category. The total points are aggregated and divided by the maximum achievable points to determine the percentage score.

Table S8. BAGI data for the prednisolone acetate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Score	Colour
1	Type of analysis	Quantitative and confirmatory	10	dark blue
2	Multi- or single-element analysis	Single element	2.5	white
3	Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID, etc.)	7.5	blue
4	Simultaneous sample preparation	13-95	7.5	blue
5	Sample preparation	Not required or on-site sample preparation if required	10	dark blue
6	Samples per h	5-10	7.5	blue
7	Reagents and materials	Common commercially available reagents (methanol, acetonitrile HNO_3, nitrogen or other common gases, etc.)	10	dark blue
8	Preconcentration	No preconcentration required. Required sensitivity and / or legislation criteria are met directly.	10	dark blue
9	Degree of automatization	Semi-automated with common devices (e.g. HPLC autosampler)	7.5	blue
10	Amount of sample	> 1000 µg (or mg) bioanalytical samples; > 100 mL (or g) food / environmental	2.5	white
TOTAL SCORE			**75.0**	**blue**

Table S9. AGREE data for the prednisolone sodium phosphate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling procedure	Off-line analysis	2	0.48	yellow
2	Amount of sample (g or mL)	100	2	0	red
3	Positioning of the analytical advice	At-line	2	0.33	orange
4	Sample preparation steps	3 or fewer	2	1.00	dark green
5	Integration and automatization	Semi-automatic; not miniaturized	2	0.25	dark orange
6	Derivatization agents	None	2	1.00	dark green
7	Amount of waste (g or mL)	200	2	0	red
8	Sample throughput	Analytes determined in a single run: 1; Samples analysed per hour: 2	2	0.12	red
9	Energy consumption	LC; the power consumption of a single analysis: 0.11	2	0.99	dark green
10	Reagents	Some reagents are bio-based	2	0.50	yellow
11	Toxic reagents and solvents	Yes; 11 g	2	0.19	dark orange
12	Threats	Highly flammable	2	0.60	light green
TOTAL SCORE				**0.46**	**yellow**

Table S10. AGREEprep data for the prednisolone sodium phosphate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling preparation and placement	On-site	1	0.33	orange
2	Hazardous materials (g or mL)	6.6 g	5	0.06	red
3	Sustainability, renewability, and reusability of materials	50-75% of reagents and materials are sustainable or renew, but can be used ONCE	2	0.50	yellow
4	Waste (g or mL)	200	3	0	red
5	Size economy of the sample (g or mL)	100	2	0	red
6	Sample throughput	50	3	0.92	dark green
7	Integration and automatization	4 steps; semi-automated systems	2	0.25	dark orange
8	Energy consumption (Wh)	40	4	0.64	light green
9	Post-sample preparation configuration for analysis	Liquid chromatography, gas chromatography with quadropole detection, etc.	2	0.25	dark orange
10	Operator's safety	2 hazards	3	0.50	yellow
TOTAL SCORE				**0.34**	**orange**

Table S11. ComplexMoGAPI data for the prednisolone sodium phosphate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Score*	Colour
Sample preparation and analysis				
Sample preparation				
1	Collection	On-line or at-line	2	yellow
2	Preservation	None	3	green
3	Transport	None	3	green
4	Storage	None	3	green
5	Type of method	Simple procedure	2	yellow
6	Scale of extraction	Not applicable	0	white
7	Solvent/reagents used	Non-green solvents/reagents	2	yellow
8	Additional treatments	None	3	green
Reagents and solvents				
9	Amount	> 100 mL (> 100 g)	1	red
10	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
11	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
12	Energy	≤ 0.1 kWh per sample	3	green
13	Occupational hazard	Hermetic sealing of analytical procedure	3	green
14	Waste	> 10 ml (> 10 g)	1	red
15	Waste treatment	Degradation, passivation	2	yellow
O	Quantification	Yes	6	not applicable
Pre-analysis processes				
Yield and conditions				
I	Yield	Not applicable	0	white
II	Temperature/time	Not applicable	0	white
Relation to green economy				
III	Number of rules met	5-6	3	green
Reagents and solvents				
IV_a	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
IV_b	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
V_a	Technical setup	Common setup	3	green
V_b	Energy	≤ 0.1 kWh per sample	3	green
V_c	Occupational hazard	Hermitization of analytical process	3	green

Workup and purification				
VI_a	Workup and purification of the end product	None or simple process	3	green
VI_b	Purity	> 98 %	3	green
TOTAL SCORE			**82**	**green**

* This scoring system considers the range of choices within each category. The total points are aggregated and divided by the maximum achievable points to determine the percentage score.

Table S12. BAGI data for the prednisolone sodium phosphate assay by the United States Pharmacopoeia (USP 48 – NF 43).

Numeric notation	Criterion	Input data	Score	Colour
1	Type of analysis	Quantitative and confirmatory	10	dark blue
2	Multi- or single-element analysis	Single element	2.5	white
3	Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID, etc.)	7.5	blue
4	Simultaneous sample preparation	13-95	7.5	blue
5	Sample preparation	Not required or on-site sample preparation if required	10	dark blue
6	Samples per h	5-10	7.5	blue
7	Reagents and materials	Common commercially available reagents (methanol, acetonitrile HNO_3, nitrogen or other common gases, etc.)	10	dark blue
8	Preconcentration	No preconcentration required. Required sensitivity and / or legislation criteria are met directly.	10	dark blue
9	Degree of automatization	Semi-automated with common devices (e.g. HPLC autosampler)	7.5	blue
10	Amount of sample	> 1000 µg (or mg) bioanalytical samples; > 100 mL (or g) food / environmental	2.5	white
TOTAL SCORE			**75.0**	**blue**

Table S13. AGREE data for the prednisolone acetate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling procedure	Off-line analysis	2	0.48	yellow
2	Amount of sample (g or mL)	200	2	0	red
3	Positioning of the analytical advice	Off-line	2	0	red
4	Sample preparation steps	3 or fewer	2	1.00	dark green
5	Integration and automatization	Manual; not miniaturized	2	0	red
6	Derivatization agents	None	2	1.0	dark green
7	Amount of waste (g or mL)	200	2	0	red
8	Sample throughput	Analytes determined in a single run: 1; Samples analysed per hour: 4	2	0.29	dark orange
9	Energy consumption	UV-Vis Spectrometry; the power consumption of a single analysis: 0.05	2	1.00	dark green
10	Reagents	All reagents are bio-based	2	1.00	dark green
11	Toxic reagents and solvents	Yes; 200 mL	2	0	red
12	Threats	Toxic to aquatic life, highly flammable and oxidizable	2	0.60	light green
TOTAL SCORE				**0.45**	yellow

Table S14. AGREEprep data for the prednisolone acetate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling preparation and placement	On-site	1	0.33	orange
2	Hazardous materials (g or mL)	200	5	0	red
3	Sustainability, renewability, and reusability of materials	75% of reagents and materials are sustainable or renew	2	0.75	green
4	Waste (g or mL)	200	3	0	red
5	Size economy of the sample (g or mL)	200	2	0	red
6	Sample throughput	4	3	0.33	orange
7	Integration and automatization	3 steps; manual systems	2	0.19	dark orange
8	Energy consumption (Wh)	40	4	0.64	light green
9	Post-sample preparation configuration for analysis	Spectrophotometry, surface analysis techniques, voltammetry, potentiometry etc.	2	0.75	green
10	Operator's safety	1 hazard	3	0.75	green
TOTAL SCORE				**0.34**	**orange**

Table S15. ComplexMoGAPI data for the prednisolone acetate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Score*	Colour
Sample preparation and analysis				
Sample preparation				
1	Collection	Off-line	1	red
2	Preservation	None	3	green
3	Transport	None	3	green
4	Storage	None	3	green
5	Type of method	Simple procedure	2	yellow
6	Scale of extraction	Not applicable	0	white
7	Solvent/reagents used	Green solvents/reagents	2	yellow
8	Additional treatments	None	3	green
Reagents and solvents				
9	Amount	> 100 mL (> 100 g)	1	red
10	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
11	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
12	Energy	≤ 0.1 kWh per sample	3	green
13	Occupational hazard	Hermetic sealing of analytical procedure	3	green
14	Waste	> 10 ml (> 10 g)	1	red
15	Waste treatment	Degradation, passivation	2	yellow
O	Quantification	Yes	6	not applicable
Pre-analysis processes				
Yield and conditions				
I	Yield	Not applicable	0	white
II	Temperature/time	Not applicable	0	white
Relation to green economy				
III	Number of rules met	5-6	3	green
Reagents and solvents				
IV_a	Health hazard	Moderately toxic; could cause temporary incapacitation, NFPA = 2 or 3	2	yellow
IV_b	Safety hazard	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.	2	yellow
Instrumentation				
V_a	Technical setup	Common setup	3	green
V_b	Energy	≤ 0.1 kWh per sample	3	green

V_c	Occupational hazard	Hermitization of analytical process	3	green
Workup and purification				
VI_a	Workup and purification of the end product	None or simple process	3	green
VI_b	Purity	> 98 %	3	green
TOTAL SCORE			**82**	**green**

* This scoring system considers the range of choices within each category. The total points are aggregated and divided by the maximum achievable points to determine the percentage score.

Table S16. BAGI data for the of the prednisolone acetate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Score	Colour
1	Type of analysis	Quantitative and confirmatory	10	dark blue
2	Multi- or single-element analysis	Single element	2.5	white
3	Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID, etc.)	7.5	blue
4	Simultaneous sample preparation	2-12	5	light blue
5	Sample preparation	Not required or on-site sample preparation if required	10	dark blue
6	Samples per h	2-4	5	light blue
7	Reagents and materials	Common commercially available reagents (methanol, acetonitrile HNO_3, nitrogen or other common gases, etc.)	10	dark blue
8	Preconcentration	No preconcentration required. Required sensitivity and / or legislation criteria are met directly.	10	dark blue
9	Degree of automatization	Manual treatment and analysis	2.5	white
10	Amount of sample	> 1000 µg (or mg) bioanalytical samples; > 100 mL (or g) food / environmental	2.5	white
TOTAL SCORE			**65.0**	**blue**

Table S17. AGREE data for the prednisolone sodium phosphate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Weight	Score	Colour
1	Sampling procedure	Off-line analysis	2	0.48	yellow
2	Amount of sample (g or mL)	350	2	0	red
3	Positioning of the analytical advice	Off-line	2	0	red
4	Sample preparation steps	3 or fewer	2	1.00	dark green
5	Integration and automatization	Manual; not miniaturized	2	0	red
6	Derivatization agents	None	2	1.00	dark green
7	Amount of waste (g or mL)	350	2	0	red
8	Sample throughput	Analytes determined in a single run: 1; Samples analysed per hour: 4	2	0.29	dark green
9	Energy consumption	UV-Vis Spectrometry; the power consumption of a single analysis: 0.05	2	1.00	dark green
10	Reagents	All reagents are bio-based	2	1.00	dark green
11	Toxic reagents and solvents	No	2	1.00	dark green
12	Threats	No	2	1.00	dark green
TOTAL SCORE				**0.56**	**light green**

Table S18. AGREEprep data for the prednisolone sodium phosphate assay by the European Pharmacopeia 11th edition.

Code	Criterion	Input data	Weight	Score	Colour
1	Sampling preparation and placement	On-site	1	0.33	orange
2	Hazardous materials (g or mL)	0	5	1.00	dark green
3	Sustainability, renewability, and reusability of materials	75% of reagents and materials are sustainable or renew	2	0.75	green
4	Waste (g or mL)	350	3	0	red
5	Size economy of the sample (g or mL)	350	2	0	red
6	Sample throughput	4	3	0.33	orange
7	Integration and automatization	3 steps; manual systems	2	0.19	dark orange
8	Energy consumption (Wh)	40	4	0.64	light green
9	Post-sample preparation configuration for analysis	Spectrophotometry, surface analysis techniques, voltammetry, potentiometry etc.	2	0.75	green
10	Operator's safety	No hazards	3	1.00	dark green
TOTAL SCORE				**0.55**	**light green**

Table S19. ComplexMoGAPI data for the prednisolone sodium phosphate assay by the European Pharmacopeia 11th edition.

Numeric notation	Criterion	Input data	Score*	Colour
Sample preparation and analysis				
Sample preparation				
1	Collection	Off-line	1	red
2	Preservation	None	3	green
3	Transport	None	3	green
4	Storage	None	3	green
5	Type of method	Simple procedure	2	yellow
6	Scale of extraction	Not applicable	0	white
7	Solvent/reagents used	Green solvents/reagents	2	yellow
8	Additional treatments	None	3	green
Reagents and solvents				
9	Amount	> 100 mL (> 100 g)	1	red
10	Health hazard	Slightly toxic; slightly irritant, NFPA = 0 or 1	3	green
11	Safety hazard	Highest NFPA flammability or instability score = 0 or 1. No special hazard is used.	3	green
Instrumentation				
12	Energy	≤ 0.1 kWh per sample	3	green
13	Occupational hazard	Hermetic sealing of analytical procedure	3	green
14	Waste	> 10 ml (> 10 g)	1	red
15	Waste treatment	Degradation, passivation	2	yellow
O	Quantification	Yes	6	not applicable
Pre-analysis processes				
Yield and conditions				
I	Yield	Not applicable	0	white
II	Temperature/time	Not applicable	0	white
Relation to green economy				
III	Number of rules met	5-6	3	green
Reagents and solvents				
IV_a	Health hazard	Slightly toxic; slightly irritant, NFPA = 0 or 1	3	green
IV_b	Safety hazard	Highest NFPA flammability or instability score = 0 or 1. No special hazard is used.	3	green
Instrumentation				
V_a	Technical setup	Common setup	3	green
V_b	Energy	≤ 0.1 kWh per sample	3	green

V_c	Occupational hazard	Hermitization of analytical process	3	green
Workup and purification				
VI_a	Workup and purification of the end product	None or simple process	3	green
VI_b	Purity	> 98 %	3	green
TOTAL SCORE			**87**	**green**

Table S20. BAGI data for the prednisolone sodium phosphate assay by the European Pharmacopoeia 11th edition.

Numeric notation	Criterion	Input data	Score	Colour
1	Type of analysis	Quantitative and confirmatory	10	dark blue
2	Multi- or single-element analysis	Single element	2.5	white
3	Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID, etc.)	7.5	blue
4	Simultaneous sample preparation	2-12	5	light blue
5	Sample preparation	Not required or on-site sample preparation if required	10	dark blue
6	Samples per h	2-4	5	light blue
7	Reagents and materials	Common commercially available reagents (methanol, acetonitrile HNO_3, nitrogen or other common gases, etc.)	10	dark blue
8	Preconcentration	No preconcentration required. Required sensitivity and / or legislation criteria are met directly.	10	dark blue
9	Degree of automatization	Manual treatment and analysis	2.5	white
10	Amount of sample	> 1000 µg (or mg) bioanalytical samples; > 100 mL (or g) food / environmental	2.5	white
TOTAL SCORE			**65.0**	**blue**

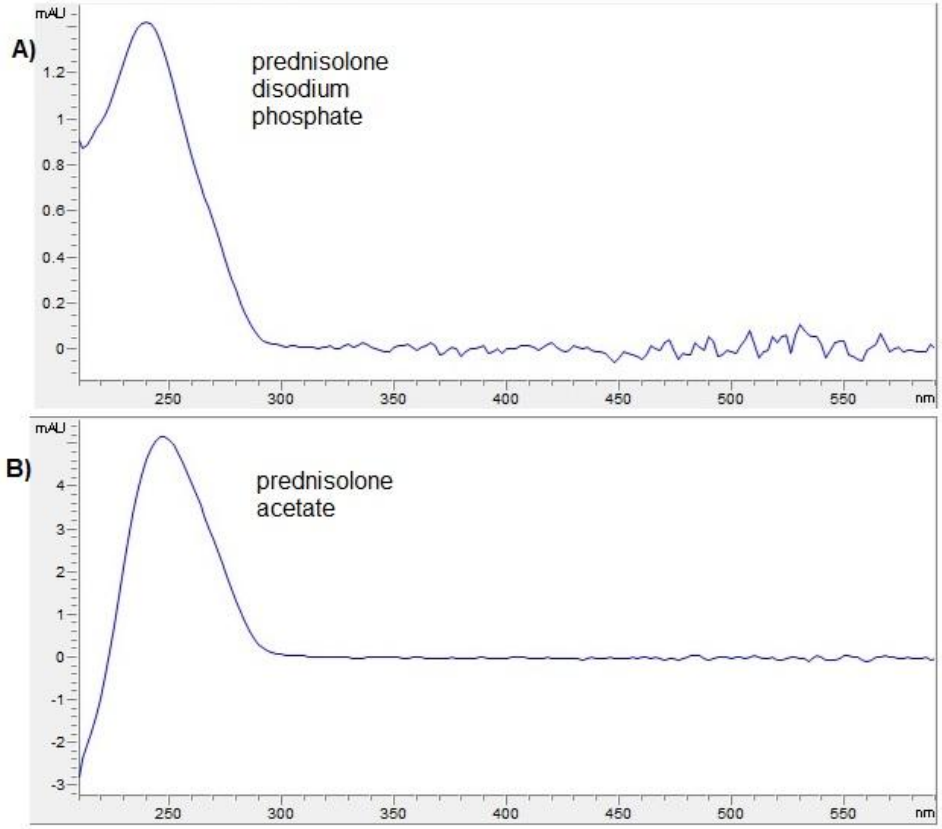

Fig. S1. Uv-Vis spectra: A) prednisolone disodium phosphate and B) prednisolone acetate.