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Research Article

ECO-FRIENDLY, OPERATOR-SAFE AND COST-EFFECTIVE RP-HPLC METHOD FOR STABILITY-INDICATING ASSAY OF PREDNISOLONE TABLETS USING ETHANOL: WATER AS MOBILE PHASE

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ABSTRACT

An RP-HPLC method was developed for the stability-indicating assay of prednisolone in tablet formulation by replacing more toxic and less eco-friendly methanol and acetonitrile with greener ethanol. The chromatographic analysis was achieved on the Phenomenex C18 column (150 mm x 4.6 mm, 5 µm) at 50 °C, using a mixture of ethanol:water (30:70, v/v) as the mobile phase with the flow rate of 0.8 mL min^{-1}. The detector wavelength was set at 254 nm. The peak of prednisolone well resolved from various degradation products as well as the tablet excipients at the retention time of about 10 min. The validation results demonstrated that the method showed an excellent linearity (r^2 = 0.99996), satisfactory precision (% relative standard deviation 0.52 – 0.57 % for the intra-day precision and 0.32% for the inter-day precision), accuracy (99.58 – 100.15% recovery) and robustness. In addition, student *t*-test analysis confirmed that the assay results obtained from the proposed method were not significantly different from those obtained from the British Pharmacopoeia method. Hence, the proposed method was proven an effective alternative assay in the aspect of both analytical performance and sustainable viewpoint.

Keywords: Eco-friendly, Ethanol, HPLC, Mobile Phase, Prednisolone

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) is considered as the technique of choice for drug assay and impurity analysis in the pharmaceutical industry. For these applications, polar mobile phases such as aqueous solutions containing organic modifiers are frequently used. At present, acetonitrile and methanol are by far the most popular modifiers because of their favorable characteristics including excellent miscibility with water, little interference in the short wavelength region in UV detection, relatively low viscosity [1], commercial availability at high purity grade and broad compatibility with the analytes, HPLC instrument and columns. However, these solvents also have notable disadvantages. In a case of acetonitrile which is a minor by-product in the manufacture of acrylonitrile polymers and cannot be considered as a renewable resource, a downturn in plastic production has occasionally resulted in global shortages and rising cost of this solvent [2]. Furthermore, acetonitrile is ranked by the U.S. Environmental Protection Agency (U.S. EPA) as a toxic chemical as liquid or vapor and waste has to be detoxified through special chemical treatment, leading to high to very high disposal cost [3]. Later, attempts have been made to switch the mobile phase from acetonitrile to methanol. However, methanol is still highly toxic to humans and causes adverse effects on aquatic life [3].

As a consequence of the aforementioned problems together with the current "green" movement to protect and promote the sustainable environment, LC community is considering greener replacement for acetonitrile and methanol with safer and more environmentally friendly solvents. Ethanol is acknowledged as green because of its biomass origin e.g. from agricultural feedstock and its biodegradability. Also, it is much less harmful to human health as well as the environment and requires less expensive and easier waste management, therefore making ethanol:water an alternative mobile phase suitable for RP-HPLC applications [4]. The tendency to use ethanol in place of acetonitrile or methanol has been evidenced by the gradually increasing number of publications. For example, Ribeiro et al. reported the efficient use of ethanol:water mobile phase for the separation of mixtures containing neutral and basic compounds [1]. Ethanol has been used as mobile phase in LC separations of cosmetic products e.g. for the determination of ascorbic acid and its derivatives [5], sunscreen agents [6] and phthalate [7]. In addition, Destandau et al. demonstrated that ethanol could be used with macroporous monolithic columns [8].

Prednisolone is a glucocorticoid and widely used as a potent anti-inflammatory or immunesuppressive drug. The literature survey reveals that some analytical methods such as UV spectrophotometry have been developed for the assay of commercial prednisolone preparations [9,10]. The pharmacopeial methods for the determination of prednisolone in tablet formulations are carried out by RP-HPLC in which methanol and water (42:58) is employed as mobile phase in British Pharmacopoeia[11] whereas The United States Pharmacopeia's eluent is a solution containing bytyl chloride, water-saturated butyl chloride, tetrahydrofuran, methanol and glacial acetic acid (95:95:14:7:6) [12]. For the chromatographic purity analysis, the mobile phase composed of the mixture of water and acetonitrile was employed [12]. A literature search from databases has not presented the evidence of using greener and less toxic ethanol for the HPLC analysis of prednisolone. Therefore, the aim of the present study was to assess the feasibility of using ethanol as a mobile phase modifier for the development of an eco-friendly, operator-safe and cost-effective RP-HPLC assay for prednisolone tablets. The parameters i.e. linearity and range, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision were validated to ascertain the analytical performance. Interestingly, the proposed method was proven to effectively resolve the drug from its degradation products; therefore demonstrating the stability-indicating capability.

MATERIALS AND METHODS

Instrumentation

HPLC analysis was performed on Aglient HPLC 1100 series equipped with quaternary pumps coupled to a diode array detector (Agilent Technology, California, USA). Data acquisition and the evaluation of peak purity were performed using ChemStation software. The reversed-phase column used was Phenomenex C18 Gemini-NX (5 µm, 150 x 4.6 mm i.d.).

Materials and Reagents

Methanol and ethanol were of HPLC grade (Merck, Germany) and water used throughout the experiment was obtained from a Milli-Q purification system (Millipore, Netherlands). All other chemicals were

of analytical grade (Sigma-Aldrich, USA). Standard prednisolone was supplied by Department of Medical Sciences, Ministry of Public Health, Thailand. Commercial prednisolone tablets were purchased from Thai market, containing prednisolone 5 mg per tablet.

Proposed method using ethanol:water mobile phase

Standard and sample solution preparation

The solutions were prepared by dissolving 25 mg standard prednisolone with the diluent (ethanol:water 30:70) in 25 mL volumetric flasks and the solution was further diluted using the same diluent to obtain a set of prednisolone solutions containing 50, 100, 200, 400, 600, 800 µg ml^{-1}.

For the preparation of sample solution, twenty tablets were weighed, finely powdered and homogenized. An accurately weighed portion of the powder equivalent to 10 mg of prednisolone was then taken into 50 mL volumetric flask. About 40 mL of diluent was added to this volumetric flask and sonicated in an ultrasonic bath for 10 min. This solution was then diluted up to the mark with diluent and mixed well. It was then filtered through 0.2 µm nylon syringe filter and the filtrate was collected after discarding first few milliliters.

HPLC conditions

Twenty microlitres of standard or sample solutions were injected into C18 column (5 µm, 150 x 4.6 mm i.d.). The mobile phase was ethanol:water 30:70 (v/v) which was previously filtered and degassed. The flow rate was 0.8 ml min^{-1}, the wavelength was at 254 nm, the column temperature was maintained at 50 °C, the run time was 12 min. The quantitation was performed using the regression equation between peak area counts and known standard concentrations.

Method validation

Linearity, LOD and LOQ

The linearity of the proposed method was assessed by assaying six calibration standard solutions of prednisolone covering 50 to 800 µg ml^{-1} (50, 100, 200, 400, 600, 800 µg ml^{-1}). Regression curve was obtained by plotting peak area versus concentration, using the least squares method. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the residual standard deviation of regression line (σ) of the calibration curve and its slope (S) in accordance with the equation LOD = $3.3(\sigma/S)$ and LOQ = $10(\sigma/S)$.

Precision

The precision of the assay was determined by the repeatability (intra-day) and intermediate precision (inter-day). The intra-day precision was calculated as the relative standard deviation (R.S.D.) of assay results from six samples during the same day. The inter-day precision was studied by comparing the assay results on three consecutive days and the R.S.D. was calculated and reported. For the satisfactory precision, the R.S.D. of less than 2.0% was recommended.

Accuracy

An accuracy study was performed by spiking known amounts of standard prednisolone into the sample solutions (containing 200 µg ml^{-1} prednisolone) prepared from the tablets. Recovery of the method was evaluated at three different prednisolone concentration levels i.e. 100, 200 and 300 µg ml^{-1} (corresponding to 50%, 100% and 150% of test preparation concentration). For each concentration level, the experiments were performed in triplicate. The actual and found concentrations were compared and the % recoveries with the R.S.D. values were calculated. The method was considered as accurate if the % mean recovery was in the range of 98-102% and the R.S.D. was not more than 2 %

Robustness

The robustness of the method was carried out to evaluate the influence of small but deliberate variations in the chromatographic conditions. This was done by injecting the same standard prednisolone solution (200 µg ml^{-1}) under varied operating conditions i.e. the flow rate (0.75. 0.80, 0.85 ml min^{-1}), ethanol:water composition in mobile phase (28:72, 30:70, 32:72) and column temperature (45, 50, 55 °C). The change of chromatographic parameters e.g. retention time, peak area, tailing factor and number of theoretical plates were recorded.

Solution stability

The stability of prednisolone solution which was prepared at the analytical concentration (200 µg ml^{-1}) in the mobile phase was studied by storing the sample solutions in light-resistant, tightly capped vials at ambient temperature (25 °C) and in the refrigerator (2-5 °C) for 48 h. The remaining drug concentrations were checked at the interval of 24 and 48 h.

Force degradation and stability-indicating studies

All force degradation experiments were performed at the drug concentration of 50 µg ml^{-1}. Acid hydrolysis was performed by heating the drug content in 0.1 M HCl at 80°C for 6 h and the mixture was neutralized. The study under alkali hydrolysis condition was carried out in 0.1 M NaOH at 50°C for 15 min and the mixture was neutralized. For the hydrolytic decomposition under neutral condition, drug solution was heated at 80 °C for 18 h. Oxidative study was carried out in 10% hydrogen peroxide at room temperature for 18 h. After the samples were subjected to the HPLC analysis, the resolution of prednisolone from its degradation products on the chromatogram was investigated. To ensure that no co-migrating species contributed to the peak response, the purity of peak was verified by using a diode array detector and ChemStation software.

RESULTS AND DISCUSSION

Development of optimized and stability-indicating method

In this study, a safer and eco-friendlier alternative mobile phase was formulated for the analysis of prednisolone in the tablets using green ethanol:water system. Additionally, the method was intended to have the stability-indicating capability. Although ethanol has some shared or similar characteristics to methanol and acetonitrile, some properties are different. By nature, ethanol has a low UV cut-off (210 nm) but has twice the viscosity of methanol, and almost 4 times the viscosity of acetonitrile. In addition, it has different strength of the solvents. Thus, the mobile phase composition as well as the operating conditions needed to be optimized. In the aspect of the interference from the absorption background of mobile phase, it was not a problem for the prednisolone assay because the drug itself well absorbed the light at 254 nm. By optimizing the proportion of ethanol:water and the flow rate at the ambient temperature (25°C), it was found that ethanol:water at the ratio of 30:70 running at 0.8 mL min^{-1} showed the satisfactory separation results since it could resolve prednisolone from all possible degradation products, albeit the drug was eluted at a rather long time of about 14 min. To shorten the analysis time, the separation was further tested at different elevated temperatures in order to increase the mass transfer and the results revealed that the higher temperature reduced the retention time of the eluted components without significant change in the resolution and selectivity. Consequently, 50 °C was chosen as the operating temperature and to compromise with the column life as recommended by the manufacturer. Fig. **1** (middle panel) shows the chromatograms obtained from the analysis of prednisolone in the presence of degradation products from various reactions. Obviously, ethanol:water 30:70 system carried out under the optimized condition gave the resolved peak of prednisolone at about 10 min. The verification by photo diode array detector confirmed that the peaks were pure, guaranteeing that no degradation products co-eluted with the parent drug. Although the retention time of the proposed system was longer than that of the BP method (about 5 min) which used 58:42 methanol:water eluent, it was clearly found that the latter pharmacopoeial method was not stability-indicating (Fig. **1**, right panel), and may give unreliable results if aged samples are assayed.

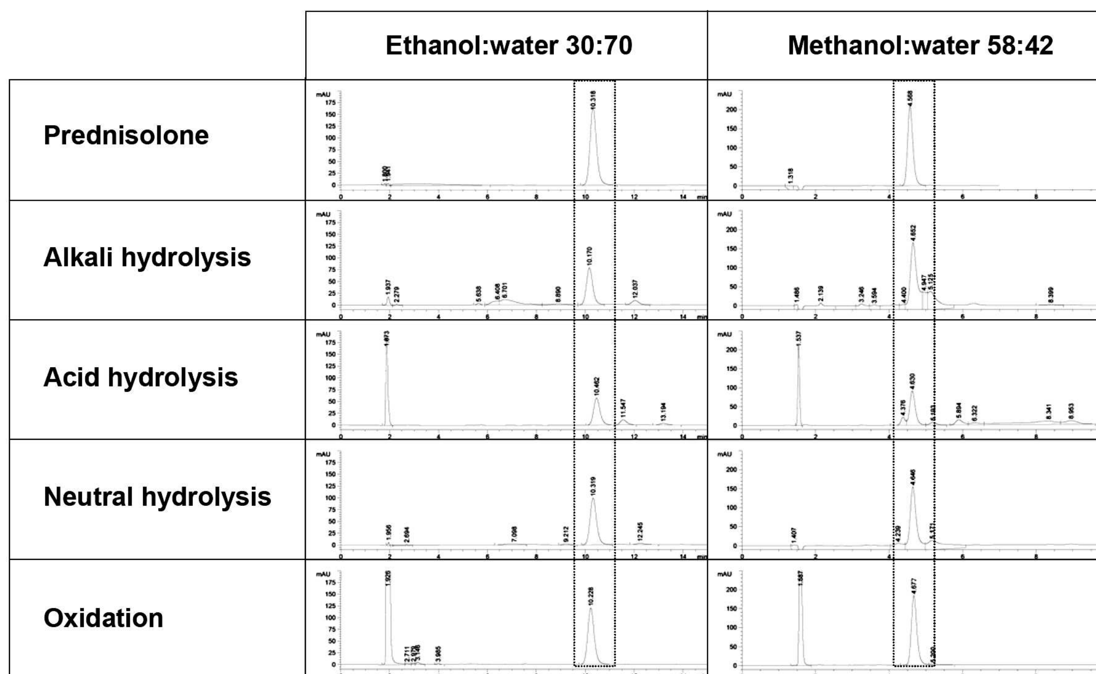

Fig. 1: HPLC chromatograms of prednisolone with its degradation products formed under various forced conditions, as analyzed by the proposed method (ethanol:water 30:70) and the British Pharmacopoeia method (methanol: water 58:42).

Another advantage of using ethanol as organic modifier was attributed to a high elution strength of ethanol which allowed a decrease of the organic solvent proportion in the mobile phase and thus lowered the solvent consumption. Despite of a higher viscosity of ethanol:water mobile phase compared to methanol:water mixtures, the lower ethanol content in the mobile phase (30%) used in this study produced a back pressure of about 70-80 bars which was acceptable by common RP-HPLC columns.

Validation of optimized method

The HPLC condition of the optimized method was evaluated for its linearity, LOD, LOQ, precision, accuracy and robustness. It was found that the calibration curve constructed by plotting the peak area of the main peak versus drug concentration showed an excellent linearity over a wide range (50 - 800 µg ml^{-1}) with a correlation coefficient of 0.99996 (Table **1** and Fig. **2**). The regression line equation obtained from the experimental results was y = 55.9399x – 120.1780. From these calibration data, LOD and LOQ of prednisolone were determined and found to be 6.93 and 16.61 µg ml^{-1}, respectively.

Table 1: Linearity, LOD and LOQ of the proposed method

Validation parameters	
Linearity and range	
Calibration range (µg mL^{-1})	50 - 800
Calibration points	6
Regression equation	y = 55.9399x – 120.1780
Correlation coefficient (r^2)	0.99996
Limit of detection (µg mL^{-1})	6.93
Limit of quantitation (µg mL^{-1})	16.61

In term of precision, the method was tested for the variability of the results from repeated analyses of the same homogeneous sample under identical experimental conditions. The intra- and inter-day precision data are summarized in Table **2**. All R.S.D. values for the repeatability and intermediate precision studies were less than 2%, indicating that the method was adequately precise.

Table 2: Intra- and inter-day assay precision of the proposed method

	Day1	Day 2	Day 3	Inter-day
Mean % labeled amount of prednisolone in tablets	100.03	100.44	99.81	100.10
R.S.D. (%)	0.56	0.52	0.57	0.32
n	6	6	6	3

The accuracy of the method was evaluated by recovery studies at three concentration levels by standard addition method. The results presented in Table **3** refer to the mean percentage recoveries of three assays for each concentration, ranging from 99.58 to 100.15% (Table 3). The results were in good agreement with acceptable values for the validation of an analytical procedure (98 – 102%) and the method was thus proven to be accurate in estimating the amount of prednisolone in tablet formulation.

Table 3: Accuracy of the proposed method as determined by the spike recovery analysis (n = 3)

Added (µg mL^{-1})	Recovered (µg mL^{-1})	Recovery (%)	R.S.D. (%)
100	99.58	99.58	0.41
200	199.44	99.72	0.26
300	300.45	100.15	0.11

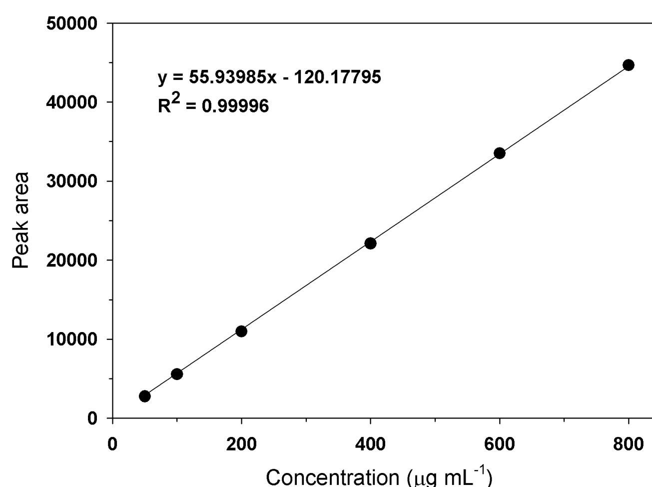

Fig. 2: Calibration curve representing the linear relationship between prednisolone concentration and peak area.

Table 4: Robustness of the proposed method

Parameter	Retention time (min)	Peak area	Tailing factor	Number of theoretical plates (plates/m)
Flow rate (mL min^{-1})				
0.75	10.92	10926	1.145	50427
0.80	10.29	10956	1.164	48893
0.85	9.86	10894	1.132	46420
Ethanol:water (v/v)				
28:72	12.05	11152	1.168	46520
30:70	10.29	10956	1.164	48893
32:68	9.57	10883	1.142	46467
Column temperature (ºC)				
45	11.15	10935	1.181	46053
50	10.29	10956	1.164	48893
55	9.46	10825	1.123	50727

Application of the validated method for determination of prednisolone in tablets

The validated method was applied to the quantitation of prednisolone in commercial tablets (5 mg prednisolone per tablet). It was found that the excipients present in the tablets did not interfere with the prednisolone peak as verified by the peak purity. Using the samples of the same batch, the mean percentage labeled amount as determined by the proposed method and BP method were 100.03 ± 0.56% and 100.12 ± 0.51% (n=6), respectively. These results were in accordance with the BP and USP criteria, which demands a value between 90.0 and 110.0% [11,12]. In addition, statistical analysis (student *t*-test) revealed that the results obtained from these two methods were not significantly different at 95% confidence level.

CONCLUSION

Considered in both analytical and sustainable viewpoints, the mixture of ethanol and water was an effective alternative mobile phase for the development of RP-HPLC method used for the assay of prednisolone in tablet formulation. The method established in this work was simple, precise, accurate and stability-indicating. Furthermore, it was safe for operators, environmentally friendly and economical in terms of cost of chemicals and waste management. Thus, this method not only suits for routine pharmaceutical quality control analysis work, but also presents a prototype for the development and utilization of greener analytical procedures in which an environmental impact is concerned.

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