Stability-Indicating RP-HPLC Assay for Simultaneous Determination of Chlorpheniramine Maleate and Prednisolone in Veterinary Injection

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The present work described a simple, rapid, sensitive, accurate, and precise method for simultaneous determination of chlorpheniramine maleate (CHRM) and prednisolone acetate (PRED) in injection samples by high-performance liquid chromatography (HPLC) coupled with UV-Vis detection. Chromatographic separation was accomplished, employing isocratic mode and a mobile phase comprised of acetonitrile and a phosphate buffer (50:50, v/v, 30 °C), adjusted to pH 3.0. The flow rate used was 1.0 mL/min on a Thermo Hypersil ODS C18 column (5 µm, 4.6 × 250 mm), and the injection volume of sample was 20 µL. Analysis of CHRM and PRED was performed at a wavelength of 254 nm. The runtime for analysis was 12.5 min, and the retention times of CHRM and PRED were found to be 2.81 and 5.07 min, respectively. The calibration graph showed linearity over the concentration range 10–70 µg/mL for CHRM and 20–140 µg/mL for PRED with a coefficient of determination (R^2) ≥ 0.9986 . Repeatability and reproducibility (expressed as % RSD) were lower than 1.72 and 1.47%, respectively. The proposed HPLC method was demonstrated to be simple and rapid for the determination of CHRM and PRED in injection formulation, providing recoveries between 101.6–102.3%, whereas complete separation of degradation products, from analyte under investigation, provided the specificity of the proposed HPLC method.

Keywords: antihistamines, corticosteroid, HPLC, validation, stability-indicating method

1. Introduction

Chlorpheniramine maleate (CHRM, Figure 1a) is chemically 3-(4-chlorophenyl)-N,N-dimethyl-3-pyridin-2-ylpropan-1-amine and an antihistamine drug commonly used to treat common cold, hay-fever, and allergic conditions, such as urticaria and rhinitis. CHRM is one of the most widely used antihistamines for small animal veterinary practices. CHRM is combined with narcotic or aminophylline for treatment of cough and upper respiratory symptoms [1–3]. Prednisolone (PRED, Figure 1b), chemically 11β, 17α, 21-trihydroxy-pregna-1,4-diene-3,20-dione 21-acetate, is a corticosteroid widely used in inflammatory conditions and as an immunosuppressive drug [4–5]. From a literature survey, it is revealed that different analytical methods were available for the estimation of prednisolone acetate and chlorpheniramine maleate individually or in fixed-dose combinations with other drugs. The analytical methods existed for PRED and CHRM, in combination with other drugs, including micellar electrokinetic chromatography [6–7], mass spectrophotometry [8–9], high-performance liquid chromatography (HPLC) [10–12], FT-Raman spectroscopy [13], and thin-layer chromatography densitometry [14]. To the best of our knowledge, the simultaneous determination of chlorpheniramine maleate and prednisolone acetate in pharmaceutical formulations by HPLC with a diode array detector (HPLC–DAD) has not been reported yet. HPLC–DAD is mostly used due to many advantages, such as

high sensitivity, rapid analysis, and high selectivity. According to the requirements of the International Conference on Harmonization (ICH), stress testing during method development is a part of developmental strategy. Stress studies are usually carried out under severe conditions rather than accelerated conditions and provide information about drug's inherent stability. Stability study approach is being extended to fixed-dose pharmaceutical combinations to enable precise and accurate estimation of drugs in the presence of degradation products [15–16]. To develop the stability-indicating methods, force degradation is used to demonstrate specificity. That's why it should be performed before implementation of stability studies to make certain that the developed analytical method is the stability-indicating one. Therefore, there is a challenge to develop stability-indicating reversed-phase (RP)-HPLC method for the separation and determination of CHRM and PRED from each other and degradation products. Hence, an attempt has been made to develop a precise, linear, accurate, repeatable, reproducible, robust, and specific HPLC method for the determination of CHRM and PRED in the presence of their degradation products.

The objective of the present research work was to develop a simple, specific, precise and accurate new stability-indicating liquid chromatographic (HPLC) method for the simultaneous determination of CHRM and PRED in injection samples for veterinary uses. The developed HPLC method was validated according to the ICH guidelines [17] and successfully applied to determine CHRM and PRED in fixed-dose pharmaceutical combinations. The results showed that the developed method could be useful for quality control and stability studies.

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Figure 1. Chemical structures of CHRm (a) and PRED (b)

2. Experimental

2.1. Chemicals and Reagents. CHRm and PRED reference standards were obtained from Schazoo Pharmaceutical Laboratories (Lahore, Pakistan). Diammonium hydrogen phosphate $((\text{NH}_4)_2\text{HPO}_4)$, phosphoric acid (H_3PO_4 85%) and sodium hydroxide were obtained from Sigma Aldrich (St. Louis, USA). Acetonitrile (ACN) and methanol (MeOH) were of HPLC-grade and supplied by Merck (Darmstadt, Germany). Double distilled water used throughout the experiment was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). Other chemicals and solvents used were of analytical reagent grade. Injections containing CHRm (4.0 mg/mL) and PRED (10.0 mg/mL) were collected from a local market.

2.2. Chromatographic Conditions. For HPLC analysis, a PG LC200, high-performance liquid chromatographic system with a LC210 Pump, a PC220 UV/Vis detector, a LC250 column oven, a LC240 vacuum degasser, and LC Win 1.0 Software was used. The best separation of CHRm, PRED, and degradation products was achieved on a Thermo Hypersil ODS C18 column (5 μm , 4.6×250 mm). The mobile phase was comprised of ACN and a phosphate buffer pumped in a ratio of 50:50 (v/v) at pH 3.0 adjusted by dilute NaOH (pH meter, Orion 5 Star, Thermo Scientific, UK). The detection was carried out at a column temperature 30 $^\circ\text{C}$ by injecting 20 μL of the sample, while the flow rate and wavelength were set at 1.0 mL/min and 254 nm, respectively. All the solutions including standards, samples, and mobile phase were filtered by a 0.45- μm nylon filter (Sartorius, Germany) before injection into the LC system. Peak integration and quantification were performed by using LC Win 1.0 software.

2.3. Standard and Sample Solutions Preparation. Accurately weighed CHRm (20 mg) and PRED (50 mg) were dissolved in methanol and diluted to a final volume of 100 mL. A portion (10.0 mL) of the above prepared stock solutions was diluted to 100 mL with methanol to prepare working standard solutions with a final concentration of 20.0 $\mu\text{g/mL}$ (CHRm) and 50.0 $\mu\text{g/mL}$ (PRED). A small volume (5.0 mL) of the sample solution, from injection vial containing CHRm and PRED, was taken in a 100-mL flask, dissolved in methanol and made the volume up to the mark with methanol. The working sample solution was prepared by diluting 10 mL of the above prepared sample solution to 100.0 mL with methanol to achieve a final concentration of 20.0 $\mu\text{g/mL}$ (CHRm) and 50.0 $\mu\text{g/mL}$ (PRED).

2.4. System Suitability Testing. The working standard solution of CHRm (20 $\mu\text{g/mL}$) and PRED (50 $\mu\text{g/mL}$) was injected 6 times on HPLC, on 3 separate days, and conformity of chromatographic parameters was done as explained in United States Pharmacopeia (USP) including retention time ($<2\%$ relative standard deviation [RSD]), peak area ($<2\%$ RSD), tailing factor (<2), selectivity factor (>1), resolution (>2), and theoretical plate count (>1000) [18–19].

2.5. Validation Studies. Validation studies were performed according to the ICH guidelines [20] in terms of specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ).

2.5.1. Linearity. For LC-based methods (HPLC), the linear dynamic range was selected within 10–70 $\mu\text{g/mL}$ for CHRm and 20–140 $\mu\text{g/mL}$ for PRED. A linear calibration curve in the form of $y = ax + b$ was obtained by plotting the peak area (y) of the drugs CHRm and PRED in triplicate against the nominal concentration (x) of 7 concentrations of CHRm and PRED, whereas a is the slope of the calibration curve and b is the intercept. Linear regression equation was demonstrated, and the necessary parameters were tabulated. The resultant parameters of the linear regression, including the standard deviation (SD) of the response based on the slope and intercept, were used to determine the LOD and LOQ. The LOD and LOQ were defined as $3.3\sigma/S$ and $10\sigma/S$, respectively [11, 21–25], where σ is standard deviation, and S is slope of the regression line.

2.5.2. Accuracy. The accuracy of the method was determined, by spiking in triplicate a known amount of pure drug to the pre-analyzed injections at 50, 100, and 150% of analyte in the dosage formulation. The resulting final concentrations of 30.0, 40.0, and 50.0 $\mu\text{g/mL}$ for CHRm and 75, 100.0, and 125.0 $\mu\text{g/mL}$ for PRED were obtained.

2.5.3. Precision. Precision were determined at different concentrations (30.0, 40.0, and 50.0 $\mu\text{g/mL}$ for CHRm and 75, 100.0, and 125.0 $\mu\text{g/mL}$ for PRED), represented by mean recovery and %RSD. The intra-day precision (repeatability) was evaluated by 5 replicates on one day, whereas the inter-day precision (reproducibility) was determined over 3 consecutive days.

2.6. Forced Degradation Studies. Forced degradation studies for stability indicating assay, by the proposed HPLC method, were performed under acidic, basic, oxidative, photolytic, and thermal conditions at a final concentration of 20 $\mu\text{g/mL}$ for CHRm and 50 $\mu\text{g/mL}$ for PRED from their commercial product under investigation. The stock solution was separately treated with HCl (0.1 M) and NaOH (0.1 M) for acidic and basic hydrolysis, whereas H_2O_2 (3.0% v/v) was employed for oxidative stress (1 h, 75% RH/40 $^\circ\text{C}$). Photolytic stress was performed after exposing the solid form for 6 h under UV (254 nm). For thermal stress, the solid form was kept in an oven for 6 h at 100 $^\circ\text{C}$. After the described time, the stressed compound was dissolved in methanol, and 10.0 mL of these stock solutions were then diluted to 100 mL using the mobile phase.

3. Results and Discussion

The main goal of the developed HPLC method is to separate closely eluting degradation products from active pharmaceutical ingredients (APIs). During method development, different chromatographic conditions, such as stationary phase and mobile phase compositions, were employed and optimized by running degradation samples.

3.1. Optimization of Chromatographic Conditions. An HPLC method was developed for simultaneous determination of CHRm and PRED in liquid dosage form. To achieve sufficient resolution of the target compound, the presence of interfering substances, including excipients and matrix effect, is vital in developing the liquid chromatographic (LC) method. The present method was based on optimizing one-factor-at-a-time strategy. In the first step, a UV absorption spectrum was taken in the range of 200–400 nm, resulting in a maximum absorbance at 254 nm for both analytes. Different columns with various stationary phases like Thermo Hypersil

Table 1. Results of tested stationary phase

Column	Analyte	R_s	T	k'	N
Thermo Hypersil ODS, C18 (250 × 4.6 mm, 5 μm)	CHRM	-7.84	1.02	2.96	4665
	PRED		1.12	6.14	5123
Venusil XBP C18 (250 × 4.6 mm, 5 μm)	CHRM	-6.81	1.32	2.80	3451
	PRED		1.39	6.01	3654
Purospher® RP-18, C18 (250 × 4.6 mm, 5 μm)	CHRM	-6.13	1.85	2.73	3857
	PRED		1.73	5.69	3078

R_s : Resolution; T : Tailing Factor; k' : Capacity factor; N : Theoretical plates. Acceptance criteria ($R_s > 2.00$; $T < 2.00$; $k' > 1.00$; $N > 1000$).

Working conditions: mobile phase 50:50 (ACN–phosphate buffer; pH = 3.0) at 254 nm, flow rate: 1.0 mL/min; column temperature: 30 °C.

ODS, C18 (250 × 4.6 mm, 5 μm), Venusil XBP C18 (250 × 4.6 mm, 5 μm), and Purospher® RP-18, C18 (250 × 4.6 mm, 5 μm) were employed for the separation of CHRM, PRED, and their degradation products (Table 1). The optimum separation was achieved using Thermo Hypersil ODS, C18 (250 × 4.6 mm, 5 μm), having the advantages of separating 2 drugs from their degradation products with good retention time.

The 2 organic solvents (acetonitrile and methanol) have been tried for the optimization of the HPLC method. Acetonitrile was selected initially due to greater reported elution strength [26]. The different ratios of water and acetonitrile were used, but the presence of phosphate buffer improved the separation of components. The effect of pH on the separation of components was studied from pH range 2.0 to 7.0. It was observed that by the increase in pH, the retention time of CHRM increased, while that of PRED slightly decreased. Also, higher pH values caused distortion of peak shape and decreased sensitivity. Thus, pH 3.0 was selected for the best separation. Actually, the presence of phosphoric acid (buffer solution) affects the charge on the analyte and ultimately on their retention times. So, it is essential to optimize the pH and the ratio of buffer solution in the mobile phase for the best separation.

The chromatograms have broad peaks and long retention times, as observed by using low ACN and higher buffer concentrations. The analysis time reduced, when the ACN concentration was increased and the buffer concentration was decreased, and the peaks were sharp and more symmetrical. In the end, the mobile phase consisting of ACN and phosphate buffer in a ratio of 50:50 (v/v) with the pH adjusted to 3.0 was selected. For the best separation, the column temperature was

maintained at 30 °C, and the flow rate was kept at 1.0 mL/min. Sharp and symmetric peaks were obtained by using a Thermo Hypersil ODS C18 column (5 μm, 4.6 × 250 mm) for HPLC analysis, at a detection wavelength of 254 nm (Figure 2.). The placebo solution (sodium metabisulphite, Tween-80) was also used in HPLC to assess any interfering peaks. The detection of components was done by injecting 20 μL of the sample. The retention times for CHRM and PRED were 2.81 and 5.07 min, respectively by HPLC as shown in Figure 2. It was found that presence of excipients made no effect on the separation of both the drugs. Thus, the method would be applied for the determination of CHRM and PRED in their pharmaceutical formulations.

3.2. Validation Studies. The system suitability parameters were assessed for the chromatographic system, as they are conformity of chromatographic parameters that ensures the suitability and performance of the analytical system. The system suitability parameters including tailing factor (T), resolution (R_s), capacity factor (k'), theoretical plates (N), and asymmetry (A_f) are presented in Table 2. All critical parameters, as defined above, met the acceptance criteria and indicate good specificity of the analytical method for the assessment of stability of CHRM and PRED. The resolution factors were greater than 2 for PRED from the nearest CHRM peak, indicating the efficient resolution. The results in Table 2 show good resolution, good peak shapes, and reasonable testing time, under the optimized chromatographic conditions for the separation of CHRM and PRED.

The calibration curves were plotted based on the regression analysis of detector response versus concentration of the two

Table 2. System suitability data of reference solution of CHRM and PRED

ICH Criteria	Specification	CHRM			PRED		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Retention time (t_R in min)	%RSD < 2	0.23	0.15	0.32	0.11	0.12	0.72
Tailing factor (T)	<2.0	1.07	1.19	1.06	1.16	1.10	1.06
Resolution (R_s)	>2.0	—	—	—	7.86	7.94	7.92
Capacity factor (k')	>1.0	2.94	2.96	2.93	6.19	6.11	6.13
Theoretical plates (N)/meter	>1000	4546	4603	4531	5134	5225	5235
Asymmetry (A_f)	0.8–1.2	0.94	0.95	0.97	1.12	1.11	1.15
Area	%RSD < 2	0.47	0.21	0.18	0.31	0.42	0.34

Figure 2. Typical HPLC chromatograms of the standard (a), sample (b), mobile phase (c), and placebo (d) (working conditions: mobile phase 50:50 [ACN–phosphate buffer, pH = 3.0] at 254 nm, flow rate 1.0 mL/min, Column temp. 30 °C)

Table 3. Regression data of CHRM and PRED by HPLC

Parameters	Standards	
	CHRM	PRED
Linearity range (µg/mL)	10–70	20–140
Slope	466,156,514	96,876,450
Intercept	750,735	2,542,680
Standard error of slope	303,168	2,309,835
Standard error of intercept	13,667,870	195,996
Coefficient of determination (R^2)	0.9986	0.9994
LOD (µg/mL)	0.012	0.112
LOQ (µg/mL)	0.037	0.374

Table 4. Recovery studies of CHRM and PRED by HPLC

Analyte	Concentration after spiking ^a (µg/mL)	Concentration found ^b (µg/mL) ± SEM; RSD	(%) Recovery [BIAS]
CHRM	30.0	29.84 ± 0.39; 1.12	99.84 [0.16]
	40.0	40.12 ± 0.18; 1.02	100.12 [0.12]
	50.0	49.74 ± 0.17; 0.72	99.48 [0.52]
PRED	75.0	74.87 ± 0.49; 1.27	99.83 [0.17]
	100.0	100.22 ± 0.38; 0.72	100.22 [0.22]
	125.0	124.84 ± 0.17; 0.22	99.87 [0.13]

^aActual concentration each of CHRM = 20 µg/mL, PRED = 50 µg/mL.

^bAll measurements were made in replicate of five.

SEM: standard error of mean.

RSD: relative standard deviation.

Acceptance criteria (RSD < 2.00).

analytes by preparing standard solutions of CHRM and PRED at 7 different concentration levels. The calibration graphs showed good linearity within concentration range of 10–70 µg/mL for CHRM and 20–140 µg/mL for PRED. The linearity was validated by the linear correlation coefficient values (>0.998) with small intercept values.

The LOD and LOQ for CHRM and PRED were determined based on the signal-to-noise ratio.

LOD and LOQ determined by the proposed LC method were 0.012 and 0.037 µg/mL for CHRM while 0.112 and 0.374 µg/mL for PRED, respectively, as shown in Table 3.

Accuracy of the method was determined by evaluating the recovery studies after spiking the known amount of standard drugs in commercial products. The recovery results were obtained between the ranges 99.48–100.12% and 99.83–100.22% (Table 4) for CHRM and PRED by proposed HPLC

method, respectively, which justified the suitability of the method for their intended applications.

Precision of the assay was determined in terms of repeatability (intra-day) and reproducibility (inter-day). Intra-day precision was determined by injecting 3 different concentrations (50, 100, and 150% level of analyte under investigation) of standard solutions of CHRM and PRED ($n = 5$) on the same day. Meanwhile, the inter-day precision was determined by injecting the same concentration of standard solutions for 3 consecutive days. The relative standard deviation (RSD, %) values for repeatability and reproducibility obtained were less than 1.72 and 1.47, respectively, for CHRM while below 1.32 for repeatability and 1.42 for reproducibility assays of PRED (Table 5).

3.3. Forced Degradation Studies. Force degradation studies under stress conditions (Table 6) were performed for presenting the stability-indicating capacity of the proposed HPLC method.

The chromatograms (Figure 3) after forced degradation are presented, while the amount remaining and the extent of degradation are tabulated after each treatment. No degradations were observed for CHRM under acidic and for PRED under thermal stress. Thus, the extent of degradation was declared as none.

In contrast, significant degradation was observed under basic and acidic stress, and the remaining concentration of PRED was found to be 9.58 and 7.38, respectively. Two impurity peaks at unique retention times $t_R = 3.85$ min and $t_R = 4.58$ min (AP-2 and AP-3, Figure 3b) were observed after basic degradation, while 3 impurity peaks at $t_R = 3.0$ min, $t_R = 4.25$, and $t_R = 4.72$ min (DP-1, DP-2, and DP-3, Figure 3c) were observed under acidic stress. PRED was slightly degraded under photolytic conditions with unique impurity peak at $t_R = 3.56$ min (PP2) but not significantly degraded under oxidative stress, the remaining amount was 87.54, and the impurity peak was observed at $t_R = 3.45$ (OP2). CHRM was slightly degraded under oxidative, photolytic, and thermal stress conditions with impurity peaks at $t_R = 1.98$ (OP1), $t_R = 3.45$ (PP1), and $t_R = 2.0$ (TP1), respectively, were observed. As a result of these experiments, the proposed HPLC method was found to be specific for the analyzed active pharmaceutical ingredients.

Table 5. Precision studies of CHRM and PRED by HPLC

Analyte	Repeatability ($n = 5$)		Reproducibility ($n = 5$)		
	Concentration (µg/mL)	Concentration found (µg/mL) ± SEM; RSD	Concentration found (µg/mL) ± SEM; RSD		
			Day 1	Day 2	Day 3
CHRM	30.0	29.81 ± 0.23; 1.43	29.95 ± 0.42; 0.98	29.68 ± 0.87; 0.84	29.74 ± 1.09; 1.08
	40.0	40.31 ± 0.18; 1.02	40.09 ± 0.12; 1.12	40.11 ± 0.47; 1.47	39.98 ± 0.22; 1.11
	50.0	49.89 ± 1.17; 1.72	49.87 ± 1.54; 1.22	49.85 ± 1.27; 1.45	50.15 ± 1.52; 1.44
PRED	75.0	74.34 ± 0.22; 1.11	75.34 ± 0.22; 0.65	75.11 ± 0.32; 1.13	75.21 ± 0.32; 1.42
	100.0	99.23 ± 0.43; 1.04	100.21 ± 0.33; 0.43	99.83 ± 0.21; 1.12	100.11 ± 0.21; 1.32
	125.0	125.16 ± 0.42; 1.32	124.32 ± 0.41; 0.63	125.08 ± 1.53; 1.32	125.21 ± 1.23; 1.11

SEM: standard error of mean; RSD: relative standard deviation.

Acceptance criteria (RSD < 2.00).

Table 6. Forced degradation results of CHRM and PRED by HPLC

Nature of stress	Amount remaining, mean ± SEM (%) ^a		Extent of degradation	
	CHRM	PRED	CHRM	PRED
0.1 M HCl (1 h)	99.43 ± 0.21	9.58 ± 0.84	None	Significant
0.1 M NaOH (1 h)	86.32 ± 0.23	7.38 ± 1.09	Non-significant	Significant
3% H ₂ O ₂ (1 h)	96.64 ± 0.18	87.54 ± 0.43	Slight	Non-significant
6 h at 100 °C	92.86 ± 0.11	98.12 ± 0.12	Slight	None
6 h under UV-254 nm	94.32 ± 0.18	93.41 ± 0.21	Slight	Slight

^aAll measurements were made in triplicate.

SEM: Standard error of mean.

Figure 3. Chromatograms of CHRM and PRED under non-stressed conditions (a), acidic stress (b), basic stress (c), oxidative stress (d), photolytic stress (e), and thermal stress (f)

Table 7. Assay result of CHRM and PRED by HPLC in commercial formulation

Product	Analyte	Label claim (mg/mL)	Concentration found (mean $\pm$ SEM, mg; RSD, %) ^a	Recovery (%)
Solomin	CHRM	4.0	4.09 $\pm$ 0.34; 0.54	102.3
Injection	PRED	10.0	10.16 $\pm$ 0.51; 0.11	101.6

^aResults are expressed as average of three measurements.

Acceptance criteria (RSD < 2.00).

3.4. Analysis of Commercial Formulation. The applicability of the proposed HPLC method was evaluated by examining the commercial injection (Solomin Injection[®]), with the reported concentration of 4 mg/mL and 10 mg/mL for CHRM and PRED, respectively. The analysis was performed after dilution with mobile phase, and the percentage recoveries of CHRM and PRED were assessed in triplicate as mentioned in the label claim. It was concluded that the proposed HPLC method was sufficiently accurate and precise (Table 7) with recovery, and the RSD found ranged between 101.6–102.3% and 0.11–0.54%, respectively.

4. Conclusion

A Sensitive and selective HPLC method was validated for the determination of CHRM and PRED in pharmaceutical formulations. The developed isocratic chromatographic method enabled unified quantification of CHRM and PRED with convincingly good precision and accuracy in pharmaceutical dosage forms. The behavior of chlorpheniramine and prednisolone was studied under different stressed conditions according to the ICH guidelines. The results of stability studies revealed that the degradation products formed under all

stressed conditions. The total run time of analysis was found to be less than 7 min. The recovery results were obtained between the ranges 99.48–100.12% and 99.83–100.22% for CHRM and PRED, respectively. The RSD values of repeatability and reproducibility for CHRM and PRED were found to be less than 2. The results of validation parameters show that the proposed method is simple, precise, accurate, and selective for the routine analysis of CHRM and PRED in pharmaceutical industries, where time and economy are essentially required.

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