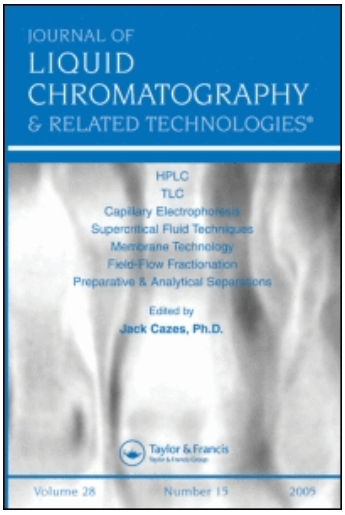

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Method Development and Validation of 9α-Fluoro-16β-Methyl-Prednisolone-17-Valerate by HPLC

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Abstract: In this study, the development and validation of an analytical method for the assay of 9α-fluoro-16β-methyl-prednisolone-17-valerate (FMPV) using reversed-phase high performance liquid chromatography (HPLC) is reported. The chromatographic separation is achieved with methanol-water (63:37, v/v) as mobile phase, a C_{18} column, and UV detection at 240 nm. The method was critically validated to demonstrate its selectivity, linearity, precision, accuracy, specificity, limit of detection and quantitation. The calibration curve showed good linearity ($r^2 = 0.9999$) over the concentration range 20 to 150 μg/mL. The mean percent relative standard deviation values for precision studies were less than 0.37%. Mean recoveries were 99.73–100.02%. The limit of detection was 3.0 μg/mL and limit of quantitation was 20.0 μg/mL. This method represents a useful protocol for routine testing of 9α-fluoro-16β-methyl-prednisolone-17-valerate drug substance.

Keywords: 9α-Fluoro-16β-methyl-prednisolone-17-valerate, Reversed-phase chromatography, Method development, Method validation

INTRODUCTION

9α-Fluoro-16β-methyl-prednisolone-17-valerate (FMPV, Figure 1) is a steroidal anti-inflammatory drug used in medicinal formulations. FMPV is

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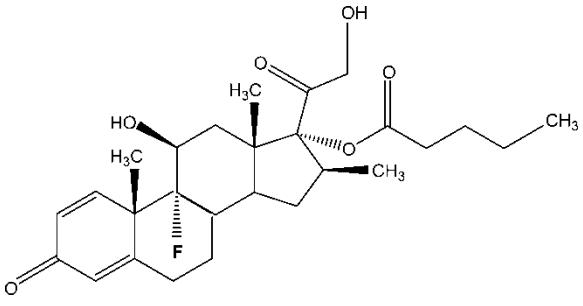

Figure 1. Chemical structure of 9α-fluoro-16β-methyl-prednisolone-17-valerate.

an active topical corticosteroid that produces a rapid response in those inflammatory dermatoses that are normally responsive to topical corticosteroid therapy, and is often effective in the less responsive conditions such as psoriasis. It is used to treat many conditions including dermatitis, arthritis, inflammatory bowel disease, reactive airways disease, and respiratory distress syndrome in preterm infants, and pruritus in corticosteroid responsive dermatoses. FMPV is an example of a synthetic drug. It is designed to be a substitute for cortisone in relieving the symptoms of rheumatoid arthritis with fewer undesirable side effects.[1] FMPV is also chemically known as 9-fluoro-11-β,17,21-trihydroxy-16-β-methylpregna-1,4-diene- 3,20-dione-17-valerate. In the present study, a simple reversed-phase high performance liquid chromatographic (HPLC) assay for FMPV was developed and extensively validated. HPLC is a widespread separation technique that occupies the leading position in routine pharmaceutical analysis.

Reversed-phase chromatography is probably the most commonly used separation mechanism in liquid chromatography and consists of a non-polar stationary phase (normally octadecyl, C_{18} or octyl C_8 chains) bonded to a solid support that is generally micro particulate silica gel (non-polar). The mobile phase is polar and, therefore, the sample compounds are partitioned between the mobile and the stationary phases. The separation is normally performed using aqueous mobile phase containing different percentages of organic modifiers (e.g., methanol, ethanol, acetonitrile, or THF) to increase the selectivity between species. Solute retention is also influenced by eluent pH, which affects the dissociation level of the analyte and, therefore, its partition between the mobile and stationary phases. Most of the analytical techniques for FMPV described in the literature are based on the liquid chromatographic determination of this drug in topical dosage forms, creams, lotions, and ointment formulations involve a liquid-liquid extraction, photochemical dramatization procedures,[2–7] alcoholic extraction,[8] or silica gel column separation[9] prior to chromatography, which are expensive and time consuming. The aim of the present work was to develop a cost

effective, simple, fast, precise, and accurate HPLC method to be applied to the quantitative analysis of incoming raw materials of FMPV used in pharmaceutical formulations. The method was fully validated using step-by-step protocol[10] as a best practice,[11] and guidelines.[12–15]

EXPERIMENTAL

Chemicals and Reagents

Methanol (HPLC-grade) was obtained from Merck (Darmstadt, Germany). 9α-Fluoro-16β-methyl-prednisolone-17-valerate (FMPV, 98% pure), sodium dihydrogen phosphate (NaH_2PO_4), and orthophosphoric acid (H_3PO_4) were purchased from Sigma chemicals (St. Louis, MO, USA). De-ionised distilled water was used throughout the experiment. All other reagents were of analytical grade.

HPLC System and Analytical Conditions

A PerkinElmer (Norwalk, CT) HPLC system equipped with a module LC 235C diode array detector (DAD), series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven were used in this work. The data were acquired via PE TurboChrom Workstation data acquisition software using PE Nelson series 600 LINK interfaces. A mixture of methanol-water (63:37, v/v) was used as mobile phase at a flow rate of 0.8 mL/min. The mobile phase was filtered through a 0.45 μm membrane filter and continuously degassed on-line. The injection volume was 10 μL and the detection wavelength was set at 240 nm. The chromatographic separation was achieved using a 100 × 3 mm, C_{18} ChromSpher polymeric octadecylsilane (ODS)-encapsulated spherical silica column with a 5 μm particle size obtained from Varian (Palo Alto, CA, USA). The separation was carried out at temperature, 25 ± 0.5°C.

Preparation of the Standard and Sample Solutions

An accurately weighed amount (50 μg) of 9α-fluoro-16β-methyl-prednisolone-17-valerate, standard was placed in a 100 mL volumetric flask and dissolved in methanol (stock). Pipette a 10 mL aliquot of stock solution to a second 100 mL volumetric flask, add 53 mL methanol and make up to volume with 25 mM phosphate buffer (adjusted to pH 3.0 using orthophosphoric acid). A linearity experiment was performed by preparing the drug substance (9α-fluoro-16β-methyl-prednisolone-17-valerate) in the range of 20–150 μg/mL.

RESULTS AND DISCUSSION

Chromatography

To obtain the best chromatographic conditions, different columns and mobile phases consisting of acetonitrile-water or methanol-water were tested to provide sufficient selectivity and sensitivity in a short separation time. The best signal was achieved using methanol-water (63:37, v/v) with a flow rate of 0.8 mL/min in a C_{18} analytical column. The low flow rate and the short run time resulted, comparatively, in lower consumption of the mobile phase solvents with a better cost effective relation. Solutions of 10 μL were injected automatically into the column. The optimal wavelength for FMPV detection was established using two UV absorbance scans over the range of 190 to 400 nm, one scan of the mobile phase, and the second of the analyte in the mobile phase. It was shown that 240 nm is the optimal wavelength to maximize the signal. A typical chromatogram obtained by the proposed HPLC method, is shown in Figure 2, confirming specificity with respect to FMPV. The chromatogram of the blank is displayed in Figure 3. The low retention time of 4.41 min allows a rapid determination of the drug, which is an important advantage for the routine analysis.

To evaluate the quantitative nature of the analytical method, a series of samples with different amounts of FMPV were run to investigate the best assay concentration. Using a C_{18} column, the best concentration was assessed by injecting six standards of the drug in the range of 10 to 200 μg/mL. The integrated peak areas (μVs) were plotted versus amount injected. The calibration curve was found to be linear from the concentration range 20 to 150 μg/mL with correlation coefficient of 0.9999. On the bases of this data, the best concentration (50 μg/mL) was chosen as a working concentration for the assay.

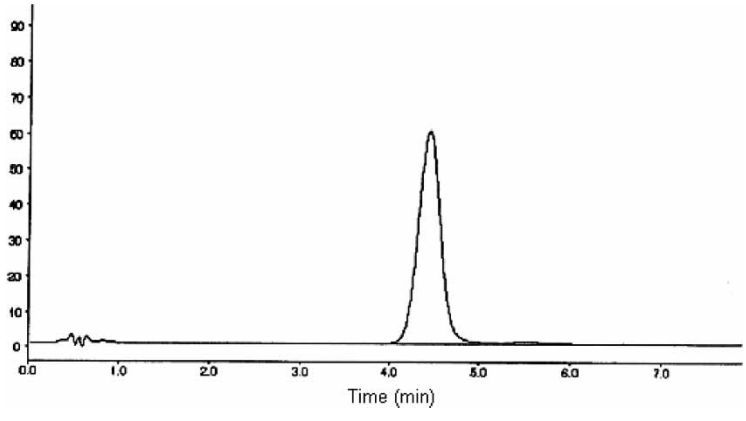

Figure 2. Chromatogram obtained for a 9α-fluoro-16β-methyl-prednisolone-17-valerate standard solution.

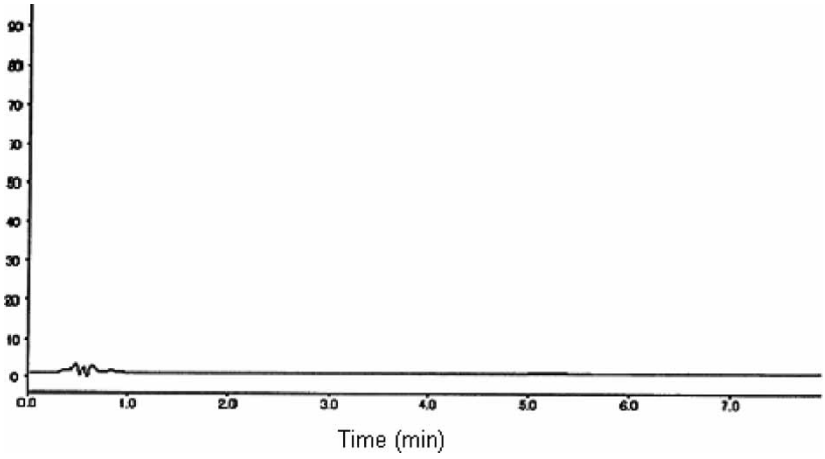

Figure 3. HPLC chromatogram of blank obtained without adding 9α-fluoro-16β-methy-lprednisolone-17-valerate standard.

System suitability testing was performed to determine the accuracy and precision of the system from six replicate injections of a solution containing 50 μg FMPV/mL. The percent relative standard deviation (%RSD) of the retention time (min) and peak area were found to be less than 0.29%. The retention factor (also called capacity factor, k) was calculated using the equation $k = (t_r/t_0) - 1$, where t_r is the retention time of the analyte and t_0 is the retention time of an unretained compound; in this study, t_0 was calculated from the first disturbance of the baseline after injection and capacity factor value was obtained 9.32 for FMPV peak. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted peaks, respectively. The separation factor for the FMPV peak obtained was 2.18. The plate number (also known as column efficiency, N) was calculated as $N = 5.54\ (t_r/w_{0.5})^2$ where $w_{0.5}$ is the peak width at half peak height. In this study, the theoretical plate number was 2812. Resolution is calculated from the equation $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are retention times of the first and second eluted peaks, respectively, and t_{w1} and t_{w2} are the peak widths. The resolution for FMPV peak was >2.0. The asymmetry factor (A_s) was calculated using the US Pharmacopoeia (USP) method. The peak asymmetry value for each FMPV peak was 1.07.

Robustness studies were also performed in the method development phase, applying the experimental design as shown in Table 1. A sample of FMPV was prepared at working concentration (50 μg/mL) and assayed using the experimental design with eight test combinations for seven different chromatographic parameters as shown in Table 2. For each parameter, four combinations of (AAAA) and four combinations of (aaaa) were studied. The actual value of each parameter ($V_A - V_G$) (Table 2)

Table 1. Experimental design for robustness study

Test parameter	1	2	3	4	5	6	7	8
A/a	A	A	A	A	a	A	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	c	c	C	C	C	c
D/d	D	D	d	d	d	D	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	F	f	F
G/g	G	g	g	G	g	G	G	g
Results	s	t	u	v	w	X	y	z

shows which parameter has a dominant influence on the developed analytical method. In all cases, good separation of FMPV was always achieved, indicating that the analytical method remained selective for the FMPV drug substance under the optimized conditions.

Validation of the Method

Linearity

Linearity was studied using six solutions in the concentration range 20–150 μg/mL and each one injected in duplicate. The regression equation was found by plotting the peak area (y) versus the FMPV concentration (x)

Table 2. Chromatographic parameter for robustness study

Parameter	Test conditions 1	Test conditions 2	Differences
Analytical column	A = column C-18	a = column C-18	$V_A = (1/4)(s + t + u + v) - (1/4)(w + x + y + z) = A - a$
Sample solvent	B = Buffer/ methanol	b = Mobile phase	$V_B = (1/4)(s + t + w + x) - (1/4)(u + v + y + z) = B - b$
Temperature	C = 20°C	c = 30°C	$V_C = (1/4)(s + u + w + y) - (1/4)(t + v + x + z) = C - c$
Flow rate	D = 0.6 mL/ min	d = 1.0 mL/ min	$V_D = (1/4)(s + t + y + z) - (1/4)(u + v + w + x) = D - d$
Wavelength	E = 238 nm	e = 242 nm	$V_E = (1/4)(s + u + x + z) - (1/4)(t + v + w + y) = E - e$
Mobile phase	F = 61% methanol	f = 65% methanol	$V_F = (1/4)(s + v + w + z) - (1/4)(t + u + x + y) = F = f$
Solubility stability	G = 24 h	g = 48 h	$V_G = (1/4)(s + v + x + y) - (1/4)(t + u + w + z) = G - g$

expressed in μg/mL. The correlation coefficient (0.9999) obtained for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of FMPV (Table 3).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for drug product). In the present study, a number of different solutions were prepared with a known added amount of drug substance and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as can be seen in Table 3, and it is evident that the method is accurate within the desired range.

Precision Studies

The precision of the analytical method, reported as %RSD, was estimated by measuring repeatability (intra-day precision) on ten replicate injections at 100% test concentration.

Table 3. Method validation results

Validation step	Concentration as % of 50 μg/mL	Results
Linearity ($k = 6$, $n = 2$)	20–150	y = 26679x – 115361 ($r^2 = 0.9999$)
Accuracy		
(%Recovery, %RSD, $n = 3$)	40	100.02 (±0.03)
	80	99.88 (±0.10)
	150	99.73 (±0.10)
Repeatability		
(Peak area, %RSD, $n = 10$)	50	0.27
Intermediate precision ($n = 3$)		
(Day 1, %RSD)	40	0.31
	60	0.34
	80	0.29
(Day 2, %RSD)	40	0.28
	60	0.36
	80	0.33
LOD		(s/n = 3.2), 3 μg/mL
LOQ ($n = 6$)		(s/n = 10.2), 20 μg/mL
Stability		
(%Change in response factors)	50	0.15

Intermediate precision (inter-day variation) was demonstrated by two analysts using two HPLC systems over two consecutive days (Figure 4), and evaluating the relative peak area percent data across the two HPLC systems at three concentration levels (40, 60, and 80%). The %RSD values presented in Table 3 were less than 0.37% in all cases, and illustrated the good precision of the chromatographic method.

Specificity

Forced degradation studies were performed to evaluate the specificity of FMPV under four stress conditions (heat, UV light, acid, base). Solutions of FMPV were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1 M hydrochloric acid) for 24 h, and base (1 M sodium hydroxide) for 4 h. A summary data of the stress results is shown in Table 4, which showed no changes in retention times of each FMPV by peak purity analysis on a DAD UV detector and, therefore, confirms the specificity of the method.

Limits of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure were performed on samples containing very low concentration of analyte. LOD is defined as the lowest concentration of analyte in a sample that can be detected above baseline noise. It is expressed as a concentration at a specified signal-to-noise (s/n) ratio, typically, three times the noise level. LOQ is defined as the lowest concentration of analyte in a sample that can be reproducibly quantitated above the baseline noise that gives

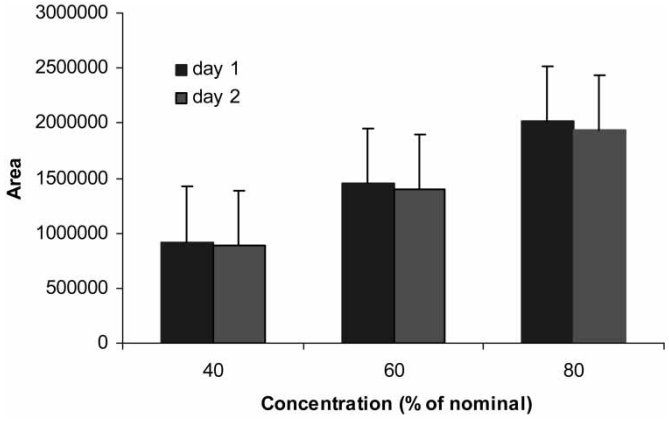

Figure 4. Typical graph obtained after analysis of FMPV to demonstrate intermediate precision variation studied over 2 consecutive days.

Table 4. Validation results obtained for the assay of FMPV under stress conditions

Stress conditions	Sample treatment	Retention time (min)	Recovery (%)	Peak area (μVs)
Reference	Fresh solution	4.41	99.99	1068056
Acid	1 M HCl for 24 hour	4.40	99.97	1056255
Base	1 M NaOH for 4 hour	4.41	99.98	1042723
Heat	50°C for 1 hour	4.41	99.96	1038726
Light	UV Light for 24 hour	4.40	99.97	1066276

s/n > 10. The LOD for FMPV was 3.0 μg/mL and s/n was >3.2. The LOQ was (s/n 10.2) 20.0 μg/mL and %RSD for six injections was 0.31% (Table 3).

Stability of Analytical Solutions

Three standard solutions (50 μg/mL) were chromatographed immediately after preparation and then reassayed after storage at room temperature (22°C ± 1°C) for 48 h. The results given in Table 3 show that there was no significant change (<1% response factor) in FMPV concentration over this period.

CONCLUSION

A new, fast, simple, cost effective, and accurate reversed-phase HPLC method for the determination of FMPV drug substance has been developed and extensively validated. The results showed that the method is selective; no significant interference peak was detected; accurate, with the FMPV recoveries of 99.73–100.02%, robust and reproducible with the %RSD less than 0.37% in all cases. The method was sensitive; as little as 3.0 μg/mL could be detected with the LOQ of 20.0 μg/mL. The suggested technique can be used in quality control for release of incoming raw material of 9α-fluoro-16β-methyl-prednisolone-17-valerate drug substance used in pharmaceutical products.

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