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Research Article

Stability Indicating HPLC Method for the Simultaneous Estimation of Triamcinolone Acetonide and Benzyl Alcohol in Pure Form and Epirofen® Vial

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ABSTRACT

A rapid, sensitive, and accurate stability indicating HPLC method was developed for the simultaneous determination of triamcinolone acetonide and benzyl alcohol in pure form, degradation products and pharmaceutical preparation. The separation was carried out on RP BDS Hypersil® C₁₈ column (150 x 4.60 mm, 5µm) using an isocratic mobile phase composed of acetonitrile: 0.05 M phosphate buffer PH 3.50 (55 : 45). Both benzyl alcohol and triamcinolone acetonide quickly eluted at 1.67 min and 3.42 min, respectively, with a flow rate of 1.50 mL/min and UV detection at 254 nm. The linearity was in the range of 1 – 50 µg/mL for triamcinolone acetonide and 2 - 10 µg/mL for benzyl alcohol. The method has been validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification, robustness, and ruggedness as per the ICH guidelines. Finally, the method was compared statistically with reference methods indicating that there is no significant difference between them in respect of precision and accuracy.

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Introduction

Triamcinolone is chemically (1S,2S,4R,8S,9S,11S,12R,13S)-12-fluoro-11-hydroxy-8-(2-hydroxyacetyl)-6,6,9,13-tetramethyl-5,7-dioxapentacyclo[10.8.0.0.2,9.0.4,8.0.13,18]icosa-14,17-dien-16-one (Figure 1). Triamcinolone acetonide (TAM) is the acetonide salt form of triamcinolone, a synthetic glucocorticoid with an immunosuppressive and anti-inflammatory activity [1] (Link1). TAM binds to specific cytosolic glucocorticoid receptors and subsequently interacts with glucocorticoid receptor response element on DNA and alters gene expression [2]. This results in an induction of the synthesis of certain anti-inflammatory proteins while inhibiting the synthesis of certain inflammatory mediators [3]. Consequently, an overall reduction in chronic inflammation and autoimmune reactions are accomplished [4]. Few chromatographic methods have been reported also for

determination of TAM either alone or in combination with other drugs in different matrices [5-11].

On the other hand, benzyl alcohol (BZA) is chemically named as phenyl methanol (Figure 1) and it is an aqueous soluble preservative widely used in injectable pharmaceutical preparations as well as in cosmetic products [12]. Although toxic in neonates and infants, it is generally recognized as safe by the FDA at concentrations up to 5% in adults [13]. The effect of BZA present as preservative in bacteriostatic normal saline has recently come under investigation when administered via intra-articular injection. Patients who undergo MRI-based arthrography for standard clinical indications are commonly administered a contrast agent like gadolinium diluted in bacteriostatic saline (Link2) [14]. Few chromatographic methods have been reported also for determination of BZA in combination with other drugs [15-18].

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VI Preparation and Analysis of Pharmaceutical Preparations

2 ml of the well shaken Epirelefan® vial suspension (40 mg/mL TAM, 1 mg/mL BZA) was transferred into 100 mL volumetric flask, then the volume was completed to the mark with methanol. Working solutions were then prepared as in the above procedure and completed as previously mentioned under the general procedure.

Results and Discussion

I Optimization of the Chromatographic Conditions

This method was optimized after studying many variables. Many columns were used to obtain the optimum separation and shortest run time. RP BDS hypersil® C₁₈ column (150 x 4.60 mm, 5µm) (phenomenex, USA) was used. In order to obtain sharp, separated peaks without tailing or distortion, further optimization was needed. As such, different wavelengths and percentages of mobile phase contents were used. Mobile phase composed of acetonitrile and 0.05 M phosphate buffer PH 3.50 (55 : 45) respectively gave the best results with wavelength at 254 nm. Under these conditions, BZA and TAM in pure form can be separated and eluted at 1.67 and 3.42 min, respectively, as illustrated in (Figure 2A).

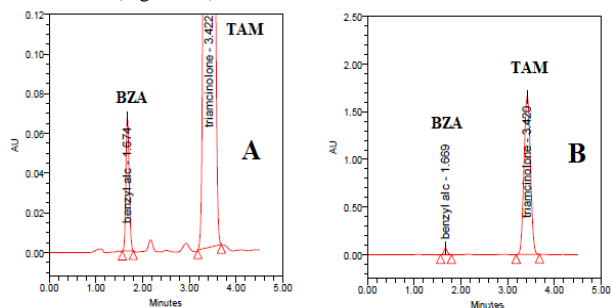


Figure 2: HPLC chromatogram of mixture of triamcinolone acetonide (TAM) and benzyl alcohol (BZA) in **A)** pure form and **B)** their tablets using RP BDS Hypersil® C₁₈ (150 x 4.60 mm, 5µm) and a mobile phase of acetonitrile : 0.05 M phosphate buffer pH 3.50 (55 : 45, v/v). Other chromatographic conditions are stated in (Table 1).

II Method Validation

Method Validation of the developed method was carried out according to ICH guidelines for its linearity, accuracy, precision, specificity, robustness, ruggedness, limit of detection and limit of quantification [19].

i System Suitability Parameters

The column efficiency, resolution, and peak symmetry were calculated for the standard solutions (n=6). The values obtained demonstrated the suitability of the system for the analysis of this drug combination (Table 2). The optimum mobile phase showed symmetrical peaks ($1.03 < T < 1.14$), capacity factor ($1 < k' < 10$), resolution > 2 and theoretical plates > 2000 as per required by the center for drug evaluation and research [20].

Table 2: System suitability parameters for triamcinolone acetonide (TAM) and benzyl alcohol (BZA) in their pure form.

Parameters	BZA	TAM	Reference values [20]
Retention time, tr	1.67	3.42	
Capacity factor, k'	1.09	3.27	Accepted k' value (1-10)
Peak asymmetry (Tailing factor, T)	1.14	1.03	Accepted T value ≤ 2
Theoretical plates, N	2726	2869	Accepted N value > 2000
Resolution, Rs	-----	7.61	Accepted value > 2
Selectivity (Separation factor, α)	-----	3.00	

ii Linearity

Linearity of this method was studied by preparing five different concentrations of the standard solutions of TAM and BZA. Area under peak of these solutions were collected and the calibration curves were obtained by plotting absorbance vs. concentration of the drugs showing linearity in the concentration range of 1-50 µg/mL for TAM and 2-10 µg/mL for BZA (Table 3). Linear regression equations were found to be $y = 8.545x + 0.3018$, and $y = 1615420x + 1269$, while the regression coefficient values (r) were 1 and 0.9999 for TAM and BZA, respectively, indicating a high degree of linearity.

Table 3: Results of analysis for triamcinolone acetonide (TAM) and benzyl alcohol (BZA) in pure form using the proposed method.

	TAM			BZA		
	Taken µg/mL	Found µg/mL	Recovery %	Taken µg/mL	Found µg/mL	Recovery %
	1	0.99	99.09	2	1.99	99.73
	5	4.98	99.53	4	4.03	100.63
	10	10.05	100.53	6	6.01	100.08
	20	19.98	99.91	8	7.94	99.19
	50	49.84	99.67	10	10.04	100.40
Mean			99.75			100.01
±SD			0.53			0.02
±RSD			0.53			0.02
±SE			0.24			0.019
Variance			0.28			0.32
LOD (µg/mL)			0.19			0.15
LOQ (µg/mL)			0.62			0.46

iii Accuracy

Accuracy was determined by using concentrations in the calibrations range from the vial sample. These concentrations represent the lower part of calibration curve (5 µg/mL of TAM and 4 µg/mL of BZA), the

middle part (10 & 20 µg/mL of TAM and 6 & 8 µg/mL of BZA), and the higher concentration part (50 µg/mL of TAM and 10 µg/mL of BZA). Mean recoveries were reported to be 99.83 and 100.85 while RSD were calculated to be 0.51 and 0.87 for TAM and BZA, respectively, indicating a high degree of the method accuracy (Table 4).

Table 4: Results of accuracy study (recovery).

Sample No.	TAM			BZA		
	Nominal conc. (µg/mL)	Found conc. (µg/mL)	Recovery %	Nominal conc. (µg/mL)	Found conc. (µg/mL)	Recovery %
1	5	4.96	99.30	4	4.04	101.13
2	10	10.05	100.49	6	6.03	100.57
3	20	19.98	99.88	8	8.15	101.89
4	50	49.83	99.6528	10	9.98	99.81
Mean			99.83			100.85
SD			0.50			0.88
RSD			0.51			0.87

iv Precision

Precision was calculated in terms of method precision and intermediate precision. Method precision (% repeatability) of the proposed HPLC method was determined by 6 replicate injections of different homogenous samples of TAM and BZA in the same day, while

intermediate precision (day to day precision) was determined by 3 replicate injections of TAM and BZA per day table. However, the % recovery was within the acceptable range of 98 to 102 % and RSD of these injections does not exceed 2% as reported in (Table 5), indicating that the developed method is highly precise and reproducible.

Table 5: Results of intra-day and inter-day precision of triamcinolone acetonide (TAM) and benzyl alcohol (BZA).

Drug	conc. µg/mL	Intra-day		Inter-day	
		Mean ± SD	RSD	Mean ± SD	RSD
TAM	10	100.95 ± 0.083	0.0824	101.02 ± 0.078	0.077
BZA	4	101.44 ± 0.17	0.17	101.86 ± 0.12	0.12

v Limit of Detection and Limit of Quantification

The limit of detection (LOD) was calculated from the linearity curve using the formula $LOD = 3.3 \times SD/Slope$ while the limit of Quantification (LOQ) was calculated from the linearity curve using the formula $LOQ = 10 \times SD/Slope$. The LOD for TAM was confirmed to be 0.19 µg/mL and that for BZA was 0.15 µg/mL. On the other hand, The LOQ for TAM was calculated to be 0.62 µg/mL and 0.46 µg/mL for BZA (Table 3). These results indeed show that the proposed method is

highly sensitive and applicable for pharmaceutical studies even if detection of small concentrations in the nanogram range is required.

vi Specificity

The specificity of the analytical method was checked in whereas, the chromatograms of the vial or the degradation products under different drastic conditions illustrate that there is no interference of the peaks of excipients or the degradation products in the determination of TAM and BZA as seen in (Figures 2B & 3).

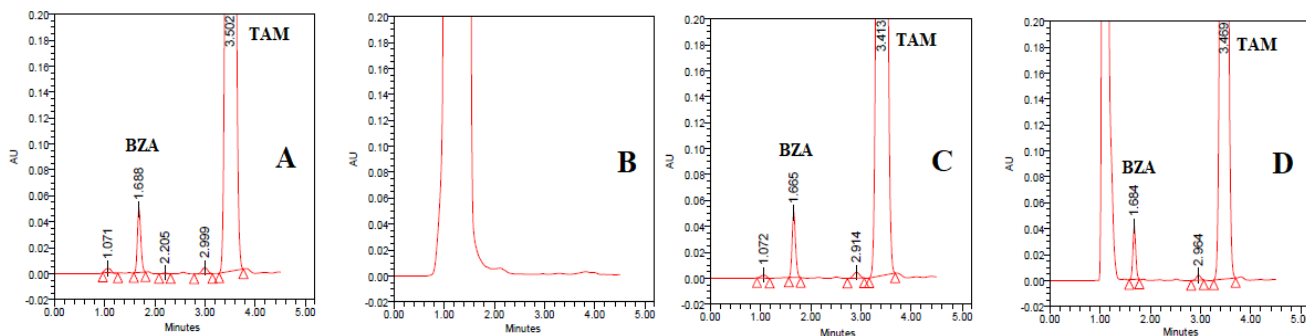


Figure 3: HPLC chromatogram of mixture of triamcinolone acetonide (TAM) and benzyl alcohol (BZA) in presence of **A**) acid degradation products, **B**) alkaline degradation products, **C**) heat degradation products, and **D**) H_2O_2 degradation products using RP BDS Hypersil® C_{18} (150 x 4.60 mm, 5µm) and a mobile phase of acetonitrile : 0.05 M phosphate buffer pH 3.50 (55 : 45, v/v). Other chromatographic conditions are stated in (Table 1).

vii Ruggedness and Robustness

Ruggedness of the method was determined by carrying out the experiment on by different operators using the same chromatographic conditions. Robustness of the method was determined by subjecting the

method to slight changes in the chromatographic conditions. No significant changes in the chromatograms were observed, where all SD values were less than 2 (Table 6), proving that the developed method is highly rugged and robust.

Table 6: Results of robustness.

Drug	TAM	BZA
	Mean ± SD	Mean ± SD
Flow rate 1.50	99.90 ± 0.08	101.44 ± 0.17
Flow rate 1.55	101.86 ± 0.12	99.65 ± 0.10
Mobile phase 55:45	99.90 ± 0.08	101.44 ± 0.17
Mobile phase 54:46	105.31 ± 0.57	106.45 ± 1.00

viii Analysis of Vial

EPIREFELAN® vial which contains 40 mg/mL TAM and 1 mg/mL BZA has been successfully analysed by the proposed RP-HPLC method. Excipients and impurities did not show interference indicating high specificity of the method. Results obtained were compared to those

obtained by applying reference methods where Student's t-test and F-test were performed for comparison [5, 15]. Results shown in (Table 7) indicated that calculated t and F values were less than tabulated ones for TAM and BZA which in turn indicate that there is no significant difference between proposed method and reference ones relative to precision and accuracy.

Table 7: Statistical analysis of results obtained by the proposed HPLC method applied on pharmaceutical formulation compared with reference methods.

DRUG	Recovery ± SD		Reference		Student t- values	F- Values
	Proposed Method	N	Reference Method	N		
TAM	100.70 ± 0.14	4	100.60 ± 0.32	4	0.08 (1.94) ^a	5.22 (9.28) ^b
BZA	100.90 ± 0.44	4	99.18 ± 0.75	4	1.92 (1.94) ^a	2.92 (9.28) ^b

^a and ^b are the Theoretical Student t-values and F-ratios at p=0.05.

Conclusion

In this study, an accurate, rapid, simple and sensitive RP-HPLC method with UV detection was developed for the assay of triamcinolone acetonide and benzyl alcohol in pure form, in presence of degradation products under different drastic conditions and in vial form. The complete separation of the analytes was achieved within short chromatographic run time (4 min) with no interfering peaks where there is no need for traditional HPLC methods with complex mobile phase mixtures, long chromatographic run times and more solvent consumed methods. The method was compared statistically with reference methods indicating that there is no significant difference between them in respect of precision and accuracy.

Conflicts of Interest

None.

Ethical Approval

This manuscript does not include any studies on human or animals.

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