Chemistry Central Journal

RESEARCH ARTICLE Open Access

Stress degradation studies and development of stability-indicating TLC-densitometry method for determination of prednisolone acetate and chloramphenicol in their individual and combined pharmaceutical formulations

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Abstract

A rapid and reproducible stability indicating TLC method was developed for the determination of prednisolone acetate and chloramphenicol in presence of their degraded products. Uniform degradation conditions were maintained by refluxing sixteen reaction mixtures for two hours at 80°C using parallel synthesizer including acidic, alkaline and neutral hydrolysis, oxidation and wet heating degradation. Oxidation at room temperature, photochemical and dry heating degradation studies were also carried out. Separation was done on TLC glass plates, pre-coated with silica gel 60F-254 using chloroform: methanol (14:1 v/v). Spots at R_f 0.21 ± 0.02 and R_f 0.41 ± 0.03 were recognized as chloramphenicol and prednisolone acetate, respectively. Quantitative analysis was done through densitometric measurements at multiwavelength (243 nm, λ_{max} of prednisolone acetate and 278 nm, λ_{max} of chloramphenicol), simultaneously. The developed method was optimized and validated as per ICH guidelines. Method was found linear over the concentration range of 200-6000 ng/spot with the correlation coefficient (r^2 ± S. D.) of 0.9976 ± 3.5 and 0.9920 ± 2.5 for prednisolone acetate and chloramphenicol, respectively. The developed TLC method can be applied for routine analysis of prednisolone acetate and chloramphenicol in presence of their degraded products in their individual and combined pharmaceutical formulations.

Keywords: Chloramphenicol, Prednisolone acetate, Stability-indicating TLC-densitometry, Stress degradation

Introduction

Prednisolone acetate (**1**), is a corticosteroid, used in polychemotherapy of cancer and also as an immunosuppressive to treat allergic disorders and hypersensitivity reactions [1,2]. Chloramphenicol (**2**), an antibiotic, possesses broad spectrum antibacterial activity and is used for the treatment of rickettsial and chlamydial diseases, gram + ve and gram -ve bacterial infections and topically for superficial conjunctivial infections [3]. A number of corticosteroid and antibiotic combinations are frequently used as antibacterial agents to cure infections particularly associated with the eye. These combinations are available in different formulations including eye ointment, eye drops and ophthalmic suspensions. Ophthalmic preparations of prednisolone acetate along with chloramphenicol are widely practiced for the treatment of superficial eye infections. However, this combination is not official with British Pharmacopoeia or US Pharmacopoeia. Many analytical methods have been reported for the determination of prednisolone acetate in cosmetics, Chinese medicinal preparations, human serum, urine and pharmaceutical preparations [4-6]. HPLC and UPLC methods are also reported for the estimation of chloramphenicol in aquatic products, pharmaceutical preparations, cosmetics comedo cream, blood, gastric contents, urine, tissues and cerebrospinal fluid [7-11].

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Simultaneous determination of prednisolone, chloramphenicol and a degraded product, 2- amino-1-(4-nitrophenyl)propane-1,3-diol in ophthalmic solutions through HPLC is also reported [12]. Few HPTLC methods have also been reported for the determination of chloramphenicol in combination with benzocaine and 2-amino-1-(4-nitrophenyl) propane-1,3-diol (ANPD), and for different corticosteroids including hydrocortisone, hydrocortisone acetate, prednisolone, betamethasone-17-valerate, prednisolone sodium phosphate, dexamethasone sodium phosphate and betamethasone sodium phosphate [13]. No report for the simultaneous determination of prednisolone acetate and chloramphenicol in the presence of their degraded products through TLC-densitometry has been found so far.

Intensive consideration and attention is being paid to the development of TLC-densitometry stability-indicating assay as it is fast, reliable and accurate and involves simultaneous analysis of many samples by using small quantity of mobile phase, thus minimizing analysis time and cost per analysis. Stress testing provides evidence that how the quality of a drug substance varies with time under the influence of various environmental factors (temperature, light, humidity, etc.) and helps to establish shelf life and recommended storage conditions for the drug [14].

Taking ICH guidelines Q1A in consideration, present study describes a simple and a validated TLC-densitometry method [15] for the simultaneous determination of prednisolone acetate and chloramphenicol in presence of their degraded products formed under the applied stress conditions. As all the pharmaceutical products are supposed to be assayed for potency, a validated TLC-densitometry method, demonstrating no interferences of degraded products with the drug active components can be useful in measuring these components in routine analysis.

Experimental

Standards, prednisolone acetate (**1**) and chloramphenicol (**2**), (Figure 1), were complementarily provided by Santa (Pvt.) Ltd, Karachi, Pakistan. Pharmaceutical products including prednisolone acetate eye drops (P1, Ophtha Pred, Schazoo; P2, Mildo Pred, Remington; P3, Predforte, Barrette Hodgson; P4, Pred +, Schazoo; P5, Prens, Vega), chloramphenicol eye drops (C1, spersinicol, Novartis; C2, chloroptic, Barrette Hodgson) and capsule (C3, chlormycetin, Pfizer), and ophthalmic suspensions containing prednisolone acetate and chloramphenicol in combination (PC1, prednisynth, Schazoo; PC2, prednicol, Remington) along with two expired pharmaceutical products (EC1, spersinicol, Novartis; EPC1, prednisynth, Schazoo) were purchased from local pharmacy shop, Karachi, Pakistan. Methanol and chloroform of analytical grade were purchased from the Fisher Scientific (UK). Deionized water was obtained from Millipore Milli Q Plus System (Bedford, USA). Sodium hydroxide was purchased from BioM Laboratories (Cerritos, USA) while hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2, 35% v/v) were obtained from Fisher Scientific (UK).

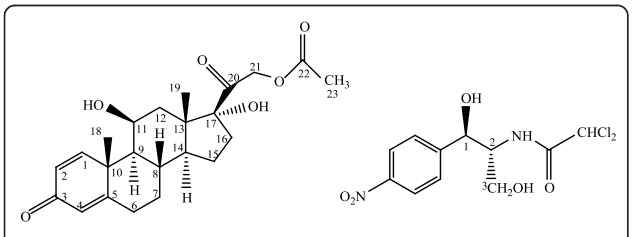

Figure 1 Structures of prednisolone acetate (1) and chloramphenicol (2).

Instrumentation and chromatographic system

Stress degradation studies were performed using parallel synthesizer (Smart Start Synthesizer, Chem Speed Ltd., Switzerland) with sixteen reaction vessels. Planar chromatography was performed by spotting the sample on TLC glass plate, pre-coated with silica gel 60F-254 (20 × 10 cm) with the aid of CAMAG microliter sample syringe using CAMAG automatic TLC Linomat V applicator (Muntenz, Switzerland). A constant sample application rate of 0.1 μL/s was adopted and the distance between the two bands was 6 mm. 15 mL of mobile phase (chloroform: methanol, 14:1 v/v) was used for linear ascending development and chromatogram was allowed to move to a distance of 8 cm, in twin trough glass chamber (CAMAG). The chamber saturation time for mobile phase was 8 minutes at 25 ± 2°C with relative humidity 42 ± 5%. The developed TLC plate was dried with the help of air dryer for 4 min. Densitometric scanning was performed on CAMAG Reprostar scanner III in the reflectance absorbance mode at multiwavelength (λ_{max}, 243 nm for prednisolone acetate and λ_{max}, 278 nm for chloramphenicol) by utilizing deuterium lamp as the source of radiation. Quantitative evaluation was performed via peak areas by WinCats software (version 1.2.3). Densitometric scanning parameters were as follows: bandwidth: 10 mm, slit width: 0.45 mm, slit length: 5 mm, scanning speed: 10 mm/s.

Preparation of standard solutions and pharmaceutical samples

Three stock solutions were prepared by dissolving 100 mg of each prednisolone acetate and chloramphenicol in 100 mL methanol, individually and in combination. Working standard solutions were prepared by dilution

of stock solution with methanol to give solutions in concentration range of 30-1000 μg/mL for calibration curve. Six-point calibration curve was formed by spotting 6 μL of each standard solution in concentration range of 200-6000 ng/spot containing both components, each concentration was spotted thrice on six replicate plates. For sample preparation, 1 mL ophthalmic samples (P1, P2, P3 and P4) containing 10 mg of prednisolone acetate were diluted in 20 mL volumetric flasks with methanol, separately. Similarly, 1 mL of spersinicol and chloroptic containing 0.5% chloramphenicol was diluted in 10 mL volumetric flasks with methanol. Chloramphenicol capsules, equivalent to 2500 mg (chlormycetin) were placed in 500 mL of volumetric flask and after addition of 100 mL water, heated on steam bath till the capsules weredisintegrated. After further addition of 300 mL water, it was again heated on steam bath with mixing. After cooling to room temperature, it was diluted to volume with water. 5 mL of the resulting solution was transferred to 100 mL volumetric flask and diluted with methanol [16,17]. 1 mL ophthalmic samples (prednisynth and prednicol) containing 5 mg of prednisolone acetate and 2 mg chloramphenicol were diluted in 10 mL volumetric flasks with methanol [4,12]. In similar manner, solutions for expired spersinicol and prednisynth were also prepared (1 mL in 10 mL volumetric flask). 6 μL of each sample was applied on TLC plate for chromatographic analysis.

Method validation

The developed method was validated as per the requirements of the ICH guidelines. Linearity was evaluated by determining six standard working solutions at a concentration 200-6000 ng/spot. Peak area and concentration was subjected to the least square linear regression equation to calculate the regression data and correlation coefficients. In order to calculate S/N ratio for LOD and LOQ, the formulae used were 3.3 δ/S and 10 δ/S, respectively where δ is the residual error and S stands for slope of calibration curve. In order to check the robustness, following parameters were deliberately changed within the range of ± 5% at three different concentration levels (200, 400 and 800 ng); amount of mobile phase, mobile phase composition, time from spotting to chromatography, time from chromatography to scanning and chamber saturation time. Intra-day and inter-day precisions were determined with the standards and degraded reaction mixtures. For method repeatability, assay at three different concentration levels (200, 400 and 800 ng) was repeatedly performed six times on the same day (intra-day). For reproducibility, same samples at three concentration levels (200, 400 and 800 ng) were analyzed in different days (inter-day) and results were statistically evaluated in terms of % R.S.D. For recovery studies, pre analyzed pharmaceutical drugs containing prednisolone acetate (P1), chloramphenicol (C1) and both in combination (PC1) were spiked with extra 25, 50 and 75% of prednisolone acetate and chloramphenicol. The specificity of the proposed method was analyzed by overlapping the densitogram of the standard and samples and comparing it at peak start, peak apex and peak end positions.

Preparation of forced degradation products

Methanolic stock solutions (1 mg/mL) of both prednisolone acetate (set 1) and chloramphenicol (set 2) were prepared, separately and in combination (set 3) to perform forced degradation studies in parallel synthesizer by refluxing the reaction mixtures for two hours at 80° C. After the reactions were completed, all the solutions were preserved at -80°C till analysis. Average peak areas of active components were analyzed after triplicate analysis.

For acidic hydrolysis, 1N and 5N HCl were used, for alkaline hydrolysis, 0.1N, 1N and 5N NaOH were used while for neutral hydrolysis Milli Q water was used. 3 mL of each concentration of acidic and alkaline solutions and Milli Q water were added into 3 mL (1 mg/mL) stock solutions of all three sets. To study wet heating degradation, 3 mL (1 mg/mL) of stock solution of each set 1, set 2 and set 3 was subjected to degradation. Oxidation was carried out by adding 1 mL of H_2O_2 (35% v/v) in 3 mL stock solution of each set. All the resultant solutions were refluxed for two hours at 80°C in parallel synthesizer. 1 μL (500 ng/spot) of 1N HCl, 0.5 μL (250 ng/spot) of 5N HCl, 4 μL (2000 ng/spot) of 0.1N, 1N and 5N NaOH treated solutions, 4 μL (2000 ng/spot) of neutral hydrolysis, 6 μL (6000 ng/spot) from wet heat degradation mixture and 4 μL (3000 ng/spot) from oxidation mixture were applied on TLC plate in triplicate for chromatographic analysis.

Dry heat degradation was conducted by taking standard prednisolone acetate and chloramphenicol and heated in oven at 90°C for 4 hrs. 1 mg of each treated standard was dissolved in 1 mL of methanol and 4 μL (4000 ng/spot) of resultant solution of each, prednisolone acetate, chloramphenicol and both in combination was applied on TLC plate in triplicate for chromatographic analysis. For oxidation reaction at room temperature, 3 mL stock solution of each set was added with 1 mL of H_2O_2 (35% v/v) and the resultant solutions were kept for 24 hours at room temperature. 4 μL (3000 ng/spot) of each treated solution was applied on TLC plate in triplicate for chromatographic analysis. In order to evaluate photochemical degradation of prednisolone acetate, chloramphenicol and both in combination, stock solution of each set was directly exposed to the sunlight for three days from 8 to 18 hrs at 30 ± 2°C. 6

μL (6000 ng/spot) of each treated solution was applied on TLC plate in triplicate for chromatographic analysis.

Results and discussion

Optimization of TLC system and method validation

With the aim to develop a reliable stability indicating method, solvent system was optimized with standards, samples and degraded products. Different solvent systems of 2-propanol, toluene, ether, chloroform, methanol, glacial acetic acid and acetone were tried in varying ratios. Solvent systems and resulting R_f values of both standards are summarized in Table S1 [see Additional file 1]. Most of the solvent systems showed diffused spots of prednisolone acetate. Suitable separation with best resolution was achieved with chloroform: methanol (14: 1 v/v) which showed sharp bands with R_f value of chloramphenicol at 0.21 ± 0.02 and of prednisolone acetate at 0.41 ± 0.03 (Figure 2).

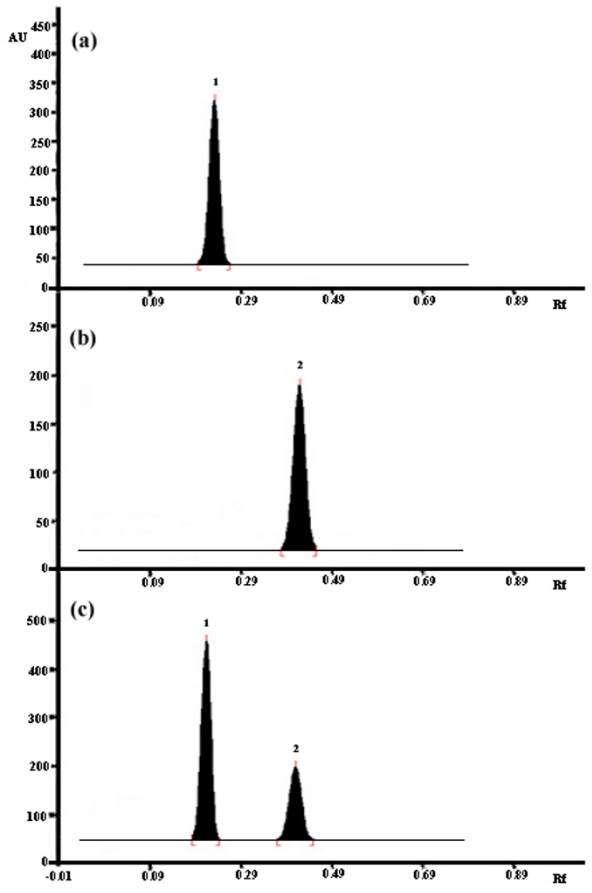

Figure 2 Chromatogram of (a) standard chloramphenicol at λ_{max} 278 nm (b) standard prednisolone acetate at λ_{max} 243 nm (c) standard prednisolone acetate and chloramphenicol in combination at λ 278 nm.

Standard calibration curve for both prednisolone acetate and chloramphenicol in the concentration range of 200-6000 ng was found linear with r^2 ± S.D. 0.9966 ± 3.5 and 0.9920 ± 2.5, respectively. For Intra-day and inter-day precision, % R.S.D. observed for prednisolone acetate was 1.32 and 3.90, respectively while for chloramphenicol, 1.09 and 1.68, respectively. For prednisolone acetate and chloramphenicol, LODs were found to be 4.77 and 3.00 ng/μL, respectively while LOQs were found to be 14.46 and 9.12 ng/μL, respectively. For robustness analysis, the S.D. of peak area of standard levels (200, 400 and 800 ng) was estimated for each parameter. S.D. was 1.07 and 3.9 for changing the amount of mobile phase, 2.48 and 2.70 for varying in mobile phase composition, 0.95 and 2.96 for varying time from spotting to chromatography, 0.56 and 1.09 for varying time from chromatography to scanning and 1.53 and 1.32 for varying chamber saturation time for prednisolone acetate and chloramphenicol, respectively. SD for peak areas were calculated and summarized in Table S2 [see Additional file 1]. Spiking studies showed recovery of prednisolone acetate (98-104%) and chloramphenicol (93-101%) from their pharmaceutical products (Table S3 [see Additional file 1]). Peak purity was estimated by comparing the peak positions of both prednisolone acetate and chloramphenicol in standard spectra with those in reaction solutions. Good correlation, r^2 (start, middle) = 0.999 and r^2 (middle, end) = 0.9999 was observed by comparing the spectra of standard and samples, in both cases. The linear regression data and the method validation results are summarized in Table 1.

Stability indicating property in prednisolone acetate

76.2% of prednisolone acetate was decomposed in 1N HCl and 100% in 5N HCl. Three additional peaks at R_f 0.01,

Table 1 Linear regression data and validation parameters of prednisolone acetate (1) and chloramphenicol (2).

Parameters	Prednisolone acetate (at λ_{max} 243 nm)	Chloramphenicol (at λ_{max} 278 nm)
Linearity range	200-6000 ng/spot	200-6000 ng/spot
Correlation coefficient, r^2 ± SD	0.9966 ± 3.5	0.9920 ± 2.5
Slope ± SD	2.42 ± 0.5	2.74 ± 0.34
Intercept ± SD	1106 ± 0.48	4183 ± 2.15
Y = mx + c	2.42x + 1106	2.738x + 4183
Intra-day (n = 3), % RSD	1.32	1.09
Inter-day (n = 3), % RSD	3.90	1.68
Limit of detection	4.77 ng/μL	3.0 ng/μL
Limit of quantification	14.46 ng/μL	9.12 ng/μL
Robustness	Robust	Robust
Specificity	Specific	Specific

0.03, and 0.29 were commonly generated in both acidic reaction mixtures. In addition to those peaks, three additional peaks at R_f 0.17, 0.19 and 0.73 were only generated in 1N HCl reaction mixture while four additional peaks at R_f 0.02, 0.58, 0.78 and 0.80 were only obtained under strong acidic condition (5N HCl). Moreover, 100% degradation was observed under alkaline conditions. Degraded peaks were observed at R_f 0.01, 0.02 and 0.50. In addition to common peaks, some additional peaks were also observed. Three peaks were at R_f 0.17, 0.36 and 0.78 in 0.1N NaOH, and peaks at R_f 0.03 and 0.29 in 1N and 5N NaOH treated solutions, respectively. Prednisolone acetate showed 77.3% degradation by neutral hydrolysis with four additional peaks at R_f 0.01, 0.02, 0.17 and 0.29. Prednisolone acetate degradations under wet and dry heating were 95.8 and 18.5%, respectively. Both conditions showed common degraded peaks at R_f 0.01, 0.17, 0.26 and 0.47, while additional peaks were observed at R_f 0.29, 0.46 and 0.80 for wet heating and R_f 0.78 for dry heating conditions. Both oxidation mixtures, refluxed for 2 hours at 80°C and 24 hours at room temperature, showed 21.05% and 5.47% degradation, respectively with three common peaks at R_f 0.05, 0.14, and 0.29. Under photochemical conditions, 100% degradation was observed with the degraded peaks at R_f 0.01, 0.29, 0.33, 0.47, 0.58, and 0.73.

Prednisolone acetate showed greater degradation susceptibility to acidic, alkaline and neutral hydrolyses, wet heating and photochemical conditions. Total eighteen degraded products were observed under various stress conditions with some common and different peaks. Peaks with higher R_f values indicated less polar nature than prednisolone acetate. A common degraded product with R_f 0.01 was attributed to all the stress conditions except oxidation reaction. Degraded product with R_f 0.02 was only generated by acidic (5N HCl), alkaline (0.1, 1 and 5N NaOH) and neutral hydrolysis. Peak at R_f 0.03 was generated under acidic (1 and 5N HCl) and alkaline (1N NaOH) hydrolysis while peaks at R_f 0.05 and 0.14 were only obtained by oxidation reaction. Degraded product at R_f 0.17 was commonly generated by acidic (1N HCl), alkaline (0.1N NaOH) and neutral hydrolysis and wet and dry heating degradation, while peak at R_f 0.26 was only found in wet and dry heating conditions. Peak at R_f 0.29 was found in various stress conditions including acidic (1 and 5N HCl), alkaline (1 and 5N NaOH) and neutral hydrolysis, wet heating, oxidation and photo degradation. Peaks at R_f 0.33 and 0.36 are the characteristic peaks of photo degradation and alkaline hydrolysis (0.1 N NaOH), respectively. Similarly peak at R_f 0.46 was only generated in wet heat degradation reaction, while a peak with R_f 0.47 was observed in wet and dry heating and photo degradation conditions. Degraded product at R_f 0.58 was generated by acidic hydrolysis (5N HCl) and photo degradation. Peak with R_f 0.73 was found in acidic hydrolysis (1N HCl) and photo degradation. Degraded peak at R_f 0.78 was commonly generated by acidic hydrolysis (5N HCl), alkaline hydrolysis (0.1N NaOH) and dry heating. Degraded product at R_f 0.80 was generated through acidic hydrolysis (5N HCl) and wet heating degradation. Stress degradation study of prednisolone acetate is summarized in Table 2. Chromatograms of stress degraded products obtained from prednisolone acetate are shown in Figures 3 and 4 while video densitogram pictures are shown in Figure 5.

Stability indicating property in chloramphenicol

Chloramphenicol was 23.7% degraded in 1N HCl and 69.3% in 5N HCl. Common degraded peaks were observed at R_f 0.01 and 0.04, while in strong acidic medium an additional peak generated at R_f 0.81. Under alkaline conditions, chloramphenicol was 100% degraded and a common degraded peak at R_f 0.01 was also generated. Under all alkaline conditions, additional degraded products were observed at R_f 0.44 and 0.79, while peaks at R_f 0.81 were found in 1N NaOH treated solution. Three additional peaks at R_f 0.02, 0.04 and 0.48 were found in 5N NaOH treated solution. A single degraded product was found under neutral hydrolysis at R_f 0.01. In wet and dry heat degradation conditions, two common additional peaks were found at R_f 0.01 and 0.79. Under oxidation conditions, three additional peaks at R_f 0.01, 0.12 and 0.31 were generated, while seven

Table 2 Summary of stress degradation studies of prednisolone acetate (1)

Degradation conditions	% Degradation	R_f of degraded products
Acidic hydrolysis[a]		
1N HCl	76.26	0.01, 0.03,0.17, 0.19, 0.29, 0.73
5N HCl	100	0.01, 0.02, 0.03, 0.29, 0.58,0.78,0.80
Basic hydrolysis[a]		
0.1N NaOH	100	0.01, 0.02, 0.17, 0.36, 0.50,0.78
1N NaOH	100	0.01, 0.02, 0.03, 0.29, 0.50
5N NaOH	100	0.01, 0.02, 0.29, 0.50
Neutral hydrolysis[a]		
H_2O	77.26	0.01, 0.02, 0.17, 0.29
Wet heating[a]	95.86	0.01, 0.17, 0.26, 0.29, 0.46, 0.47, 0.80
Dry heating	18.566	0.01, 0.17, 0.26, 0.47, 0.78
Photochemical	100	0.01, 0.29, 0.33, 0.47, 0.58, 0.73
Oxidation		
35%v/v H_2O_2 [a]	21.05	0.05, 0.14, 0.29
Oxidation at room temp	5.47	0.05, 0.14, 0.29

[a]Reflux in parallel synthesizer for two hours at 80°C

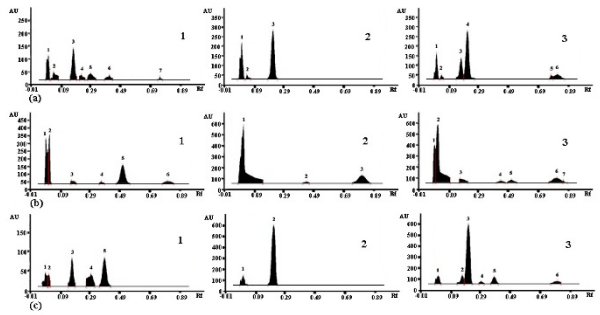

Figure 3 Chromatogram of prednisolone acetate at λ_{max} 243 nm (1), chloramphenicol at λ_{max} 278 nm (2) and prednisolone acetate and chloramphenicol at λ 278 nm in combination (3) subjected to (a) acidic hydrolysis (1N HCl) (b) alkaline hydrolysis (0.1N NaOH) (c) neutral hydrolysis.

additional peaks at R_f 0.01, 0.09, 0.15, 0.27, 0.44, 0.81 and 0.85 were found by photochemical degradation.

Chloramphenicol showed more degradation to alkaline, wet heating and photochemical stress conditions. Degraded product with R_f 0.01 was commonly formed under all stress conditions while degraded peak with R_f 0.02 was observed only under alkaline hydrolysis (5N NaOH). Peak with R_f 0.04 was generated by acidic (1 and 5N HCl) and alkaline hydrolysis (1 and 5N NaOH), while peak at R_f 0.48 was observed only in alkaline hydrolysis (5N NaOH). Degraded products with R_f 0.12 and 0.15 were found under oxidation and photo degradation conditions, respectively, while peak with R_f 0.31 was formed only in oxidation reaction. Peak with R_f 0.44 was generated by alkaline hydrolysis (0.1N NaOH) and photo degradation reactions. Degraded peak with R_f 0.79 was generated by alkaline hydrolysis (0.1N NaOH) and wet and dry heat degradations. Degraded product with R_f 0.81 was generated by acidic hydrolysis (5N HCl), alkaline hydrolysis (1N NaOH) and photo degradation

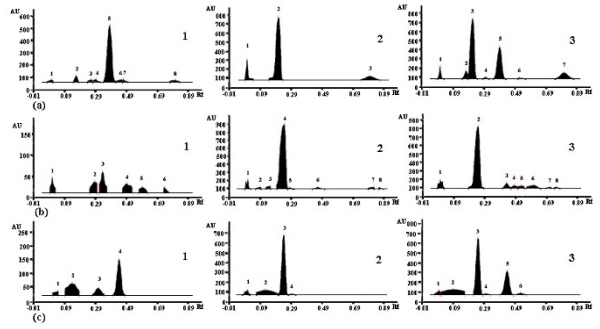

Figure 4 Chromatogram of prednisolone acetate at λ_{max} 243 nm (1), chloramphenicol at λ_{max} 278 nm (2) and prednisolone acetate and chloramphenicol at λ 278 nm in combination (3) subjected to (a) wet heat (b) photodegradation (c) oxidation at room temperature.

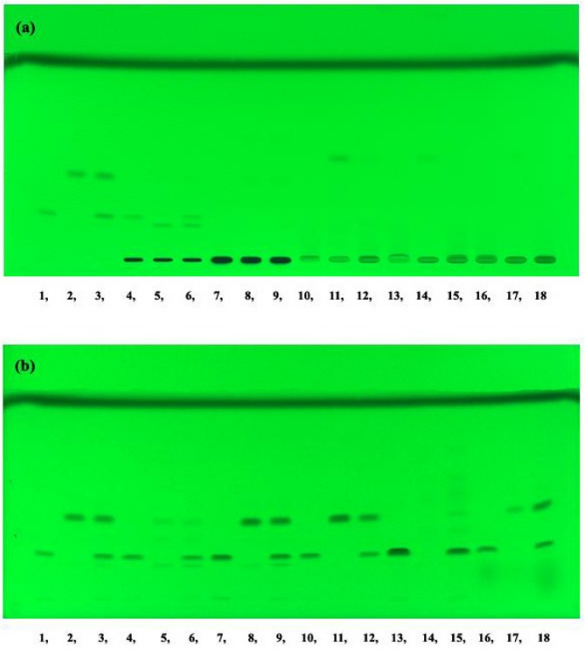

Figure 5 TLC desitometric pictures of degradation products of chloramphenicol (1), prednisolone acetate (2), chloramphenicol and prednisolone acetate in combination (3). (a) under 1N HCl (4, 5, 6), 5N HCl (7, 8, 9), 0.1N NaOH (10, 11, 12), 1N NaOH (13, 14, 15), 5N NaOH (16, 17, 18), **(b)** under neutral hydrolysis (4, 5, 6), wet heating (7, 8, 9), dry heating (10, 11, 12), photo degradation (13, 14, 15) and oxidation at room temperature (16, 17, 18).

conditions. Similarly peaks with R_f 0.09, 0.27 and 0.85 were only generated through photo degradation reaction. Stress degradation study of chloramphenicol is summarized in Table 3. Chromatograms of chloramphenicol stress degraded products are shown in Figures 3 and 4 while video densitogram pictures are shown in Figure 5.

Stability indicating property of prednisolone acetate and chloramphenicol in combination

In comparison with the individual standard solutions, a combined standards solution showed two additional peaks under acidic (1N HCl) condition at R_f 0.78 and 0.81 while three degraded products of prednisolone acetate at R_f 0.03, 0.19 and 0.73 were missing. 5N HCl stress condition showed two additional peaks at R_f 0.17 and 0.38. Peak at R_f 0.81 corresponding to the degraded product of chloramphenicol while degraded products of prednisolone acetate with R_f 0.03 and 0.80 were missing. Moreover, under both acidic conditions, prednisolone acetate was 100% degraded.

Under alkaline conditions, chloramphenicol and prednisolone acetate were completely degraded. Two common additional peaks were generated under all alkaline conditions at R_f 0.01 and 0.02. Under 0.1N NaOH stress

Table 3 Summary of stress degradation studies of chloramphenicol (2).

Degradation conditions	% Degradation	R_f of degraded products
Acidic hydrolysis[a]		
1N HCl	23.75	0.01, 0.04
5N HCl	69.36	0.01, 0.04, 0.81
Basic hydrolysis[a]		
0.1N NaOH	100	0.01, 0.44, 0.79
1N NaOH	100	0.01, 0.04, 0.81
5N NaOH	100	0.01, 0.02, 0.04, 0.48
Neutral hydrolysis[a]		
H_2O	14.35	0.01
Wet heating[a]	28.44	0.01, 0.79
Dry heating	24	0.01, 0.79
Photochemical	26.029	0.01,0.09,0.15, 0.27,0.44,0.81,0.85
Oxidation		
35%v/v H_2O_2 [a]	4.129	0.01, 0.12, 0.31
Oxidation at room temp	9.58	0.01, 0.12, 0.31

[a]Reflux in parallel synthesizer for two hours at 80°C

condition, two additional peaks were found at R_f 0.29 and 0.85 while chloramphenicol degraded peak at R_f 0.44 and prednisolone acetate degraded peaks at R_f 0.36 and 0.78 were missing. Moreover, under 1N NaOH stress condition, an additional peak was observed at R_f 0.44 while chloramphenicol degraded products at R_f 0.04 and 0.81 and prednisolone acetate degraded product at R_f 0.50 were missing. In 5N NaOH treated solution, comparison showed an additional peak at R_f 0.31 while chloramphenicol degraded products at R_f 0.04 and 0.48 and prednisolone acetate degraded products at R_f 0.29 and 0.50 were missing. In neutral hydrolysis reaction mixture, an additional peak was generated at R_f 0.79, while peak for prednisolone acetate at R_f 0.02 was missing.

Similarly an additional degraded product at 0.50 was observed in combined sample under wet heat degradation while degraded products of prednisolone acetate at R_f 0.47 and 0.80 were missing. Under dry heat degradation condition, comparison showed an additional peak at R_f 0.73 while prednisolone acetate degraded peaks at R_f 0.17 and 0.78 were missing. Under wet heat degradation condition, prednisolone acetate was 17% degraded while chloramphenicol was degraded up to 36.9%.

For oxidation reaction, two conditions were applied, under oxidation condition 1 (refluxed for two hours at 80°C), additional peak at R_f 0.55 while two additional peaks at R_f 0.23 and 0.55 were observed under oxidation condition 2 (reaction mixture kept for 24 hours at room temperature). Prednisolone acetate degraded peaks at R_f 0.05, 0.14 and 0.29 were missing.

Under photochemical degradation condition, additional degraded products at R_f 0.49, 0.50 and 0.69 were generated only in combined sample while degraded products of prednisolone acetate at R_f 0.29 and 0.33 and those of chloramphenicol at R_f 0.09, 0.15, 0.28, 0.44, 0.81 and 0.85 were missing. Stress degradation study of prednisolone acetate and chloramphenicol in combined sample is summarized in Table 4 while video densitogram pictures are shown in Figures 3 and 4.

Table 4 Summary of stress degradation studies of prednisolone acetate and chloramphenicol in combination

Degradation conditions	% Degradation of 1	% Degradation of 2	R_f of degraded products
Acidic hydrolysis[a]			
1N HCl	100	31.8	0.01, 0.04, 0.17, 0.29,0.78, 0.81
5N HCl	100	82.7	0.01, 0.02, 0.04, 0.17, 0.29, 0.38,0.58, 0.78
Basic hydrolysis[a]			
0.1 N NaOH	100	100	0.01, 0.02, 0.17,0.29, 0.50, 0.79,0.85
1N NaOH	100	100	0.01, 0.02, 0.03, 0.29,0.44
5N NaOH	100	100	0.01, 0.02, 0.31
Neutral hydrolysis[a]			
H_2O	74.52	17.9	0.01, 0.17, 0.29, 0.79
Wet heating[a]	17	36.9	0.01, 0.17, 0.29, 0.50,0.79
Dry heating	0	0	0.01, 0.26, 0.47,0.73,0.79
Photochemical	85.808	17.1	0.01, 0.47, 0.49,0.50,0.58, 0.69,0.73
Oxidation			
35%v/v H_2O_2 [a]	30.56	7.20	0.01, 0.12, 0.31, 0.55
At room temp	9.029	13.4	0.01, 0.12, 0.23,0.31, 0.55

[a]Reflux in parallel synthesizer for two hours at 80°C

Analysis of marketed samples

In prednisolone acetate eye drops, prednisolone acetate was found 97% (P1), 100% (P2), 93% (P3), 93% (P4) and 81% (P5) of the label claim. In chloramphenicol eye drops and capsule, the %s of chloramphenicol were found 98 (C1), 95 (C2) and 99 (C3). In eye drops, containing prednisolone acetate and chloramphenicol in combination, the %s of chloramphenicol were 96 (PC1) and 95 (PC2) while prednisolone acetate was found 100% in both samples. Two expired pharmaceutical products were also analyzed. In a expired sample (spersinicol), peak was not found for chloramphenicol at R_f 0.21 (100% degradation) but six degraded peaks were found at R_f 0.01, 0.09, 0.19, 0.15, 0.38 and 0.58. Stress degradation study data showed that peak with R_f 0.01 was commonly formed under all stress conditions. Degraded products with R_f 0.09 and 0.15 were formed by photo-degrdation Degraded products with R_f 0.38 and 0.58 were not found under any stress condition applied to chloramphenicol alone. Degraded product with R_f 0.38 was generated in combined sample of prednisolone acetate and chloramphenicol under acidic hydrolysis (5N HCl). In expired prednisynth product (light yellow in color), peak for chloramphenicol was found but with 59% degradation and prednisolone acetate was degraded up to 72.2%. Five additional peaks were also found at R_f 0.01, 0.15, 0.26, 0.38 and 0.58 (Figure 6). Comparison showed that degraded product at R_f 0.01 was a common product in both, individual and in combination. Degraded products at R_f 0.15 and 0.38 were generated by the degradation of chloramphenicol. Degraded products with R_f 0.26 (found under dry and wet heat degradation conditions) and 0.58 (produced under acidic hydrolysis and photo degradation) were associated with the degradation of prednisolone acetate (Table 5).

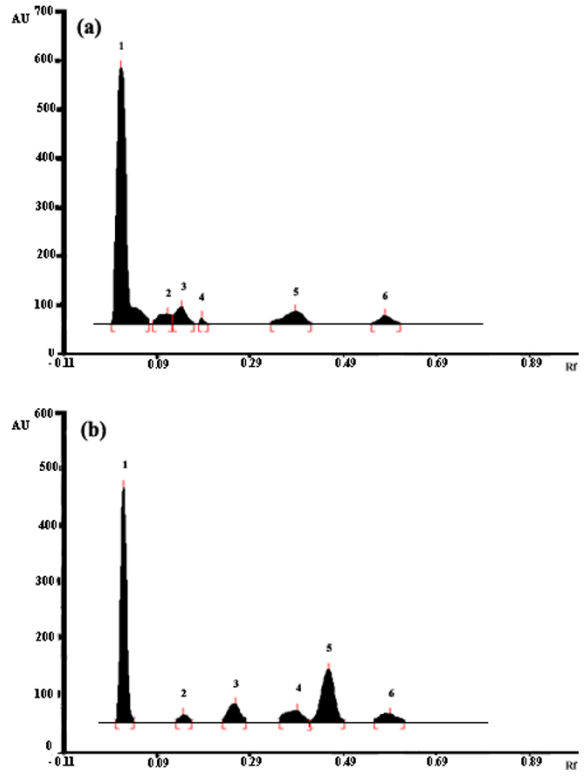

Figure 6 Chromatogram of (a) expired spersinicol and (b) expired prednisynth at 278 nm.

Conclusions

A validated TLC method for routine analysis to determine the stability of prednisolone acetate and chloramphenicol in pharmaceutical dosage forms has been developed. Current study demonstrates the degradation susceptibility of drugs to different stress conditions and thus helps in determining the changes in chemical, physical and microbiological properties of the drug samples with time. It also helps in understanding the mechanism and pathway of degraded products formation and in developing a profile reflecting the changes in identity, purity and potency of the product. The comparison of stress degradation in individual and combined formulations reveals the effect on % degradation and the number of degraded products. The developed stability indicating TLC method is simple, reproducible and can be used for the simultaneous analysis of two active components (chloramphenicol and prednisolone acetate). It appears suitable for routine analysis of these components selectively in presence of their degraded products in individual and in combined pharmaceutical formulations.

Table 5 Analysis of pharmaceutical products.

Product	Prednisolone acetate		Chloramphenicol	
	Conc. (in ng)	% Degradation	Conc. (in ng)	% Degradation
P1	2909.94	3.002	-	-
P2	3000	0	-	-
P3	2778.6	7.38	-	-
P4	2803.05	6.565	-	-
P5	2440.5	18.65	-	-
C1	-	-	2956.5	1.45
C2	-	-	2850.9	4.97
C3	-	-	2984.4	0.52
PC1	3000	0	1158.72	3.44
PC2	3000	0	1144.44	4.63
EC1	-	-	0	100
EPC1	833.55	72.215	490.05	59.15

Additional material

Additional file 1: Supplementary Material. Additional file contains the supplementary data regarding recovery studies, robustness and solvent optimization.

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Authors' contributions
SGM: Participated in the experimental designing and method optimization. UF: Performed the experiments. RS: Involved in the useful discussion and participated in designing experiment.

Competing interests
The authors declare that they have no competing interests.

Received: 5 November 2011 Accepted: 22 January 2012
Published: 22 January 2012

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doi:10.1186/1752-153X-6-7
Cite this article as: Musharraf *et al.*: Stress degradation studies and development of stability-indicating TLC-densitometry method for determination of prednisolone acetate and chloramphenicol in their individual and combined pharmaceutical formulations. *Chemistry Central Journal* 2012 **6**:7.