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Research paper

Densitometric thin layer chromatographic analysis of tretinoin and erythromycin in lotions for topical use in acne treatment

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Abstract

A TLC-method was developed to analyse tretinoin and erythromycin in a lotion in the presence of several excipients. Erythromycin was separated on a silica gel plate and a mobile phase with dichloromethane, methanol and ammonia 25% (60:6:1 (v/v/v)), tretinoin on a C_{18} RP plate with acetonitrile and water (50:25 (v/v)) as mobile phase, adding 1 ml acetic acid for the separation of the excipients and erythromycin. The derivatization for both was done with a dipping reagent, consisting of anisaldehyde, sulphuric acid and acetic acid (respectively 1, 2 and 10% (v/v/v)) and dissolved in chloroform/alcohol 94% v/v (60:30 (v/v)) for erythromycin and alcohol 94%/water (50:40 (v/v)) for tretinoin. These TLC-systems were quantitatively evaluated in terms of stability of the colour, precision, accuracy and calibration, proving the utility in the analysis of the lotion.

Keywords: Erythromycin; Tretinoin; Acne; Topical treatment; Thin-layer chromatography

1. Introduction

Erythromycin is the most widely used of the macrolide antibiotics for a variety of infectious diseases [1]. It has also been a mainstay for keeping inflammatory acne under control by topical application. A topical used erythromycin in a concentration of 2% was found to be effective because of its reduction of free acids of the surface lipids in the skin. Tretinoin (vitamin A acid) was used against comedones by preventing horny cells in the skin from sticking together [2]. Since the modes of action of both compounds differ so completely, is seemed to be interesting to develop a stable solution with both compounds for dermatological application.

Concerning the stability of erythromycin, this compound is very unstable in aqueous solutions. The activity of erythromycin in a solution, containing propylene glycol/water/alcohol (25:15:10 (v/v/v)) decreased to 90% within 7.5 days, whereas a propylene glycol/alcohol (10:40 (v/v)) solution retained 90% of the original activity during more than 50 days at 45°C [3]. For this reason, a alcoholic lotion as vehicle seems to be convenient from different points of view. A higher physico-chemical stability of erythromycin in lotions can be estimated and alcohol itself has a positive pharmacological importance in the treatment of acne because of its cooling effect [4].

A method based on thin layer chromatography was developed for the analysis of erythromycin and tretinoin and their degradation products in presence of several excipients. This technique was selected because some advantages (outlined below) in comparison with HPLC, especially for erythromycin,

1. Erythromycin has a low absorptivity at conventionally used detection wavelength (>220 mn).
2. Measurements at wavelengths below 220 nm, where the molar absorptivity is considerably higher, are associated with additional problems, such as interfering species co-extracted from the sample [5]. More convential HPLC techniques for erythromycin apply derivatization techniques [6] or special detection methods, including electrochemical detection [7].
3. The TLC-technique can be used, in some cases, without pretreatment of the sample. Excipients, injected on the column, have to migrate from a HPLC-column. A total migration or on the contrary no migration of the excipients are still satisfactory in TLC.

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2. Experimental

2.1. Samples and reagents

Tretinoin and erythromycin were obtained from Alpha-Pharma (Zwevegem, Belgium). Excipients for the lotion were propylene glycol (Fravers Lab., Ghent, Belgium). Butylhydroxytoluene (Alpha-Pharma, Zwevegem, Belgium) was added to protect tretinoin from oxidative influence.

Standard solutions of 0.1% erythromycin and tretinoin (g/v) in alcohol were prepared. Working solutions of lower concentrations were freshly prepared by appropriate dilution of the standard solution or lotion in alcohol. A degraded solution of tretinoin was prepared by exposing it to sunlight in translucent bottles until degradation. A high concentrated solution of erythromycin (2% (g/v)) was prepared in water to develop degradation products, such as erythromycin A, its enol ether and pseudo-enol ether [1,3]. The vehicle of the lotion with 2% (g/v) erythromycin and 0.05% tretinoin contained the following compounds: propylene glycol/alcohol 94% (v/v), polyethylene glycol 400/alcohol 94% (v/v) and isopropanol/alcohol 94% (v/v), all in a ratio of 50:50 (v/v). Butylhydroxytoluene in a concentration of 0.05% (g/v), having antioxidative properties on the light-sensitive tretinoin was added. Standards of these compounds were prepared in alcohol. The solvents used during our experiments, are 4-methoxybenzaldehyde, sulphuric acid 98%, ammonia 25%, methanol, chloroform, dichloromethane, acetic acid 96%, obtained from Merck (Darmstadt, Germany), acetonitrile from Carlo Erba Reagenti (Milano, Italy), MQ-water and alcohol.

2.2. Chromatographic procedure

Chromatography was performed mainly on ready-made silica gel plates from Merck, Darmstadt, Germany namely Silica gel 60 F (with gypsum) 254 and RP-18 F 254 S. Some experiments were carried out on Sil G-25 (organic binder) 5 μm and Sil G-25 HR (with gypsum) 5 μm and Sil G-25 5 μm ready-made silica gel plates from Macherey-Nagel (Düren, Germany). Aliquots (10 μl) of the working solutions were applied in bands of 1 cm (1 μl/mm) on the plate by using a 100 μl syringe and automatically spotted with a Linomat IV (Camag, Lot, Belgium) and dried under a nitrogen flow during 10 s/μl. The plates were developed in a TLC tank, containing overnight saturated mobile phases at 25°C (elution zone is +/ 6 cm). After drying for at least 15 min, the plate was dipped in a freshly prepared derivatization solution (+/−200 ml) using a dipping tank from Camag (Lot, Belgium). The solution can be preserved at 4°C. The plate was heated on a plate heater (Camag, Lot, Belgium) at 110°C during 5 min for qualitative evaluation and longer for quantitative analysis. The spots were qualitatively observed in daylight or at 366 nm. For quantitative analysis, the scanner used in this study was a PMQ3 Spectrophotometer (Zeiss, Oberkochen, Germany) with an integrated program for data storage, developed by Unda (Harzé, Belgium). Measurements were performed in VIS in transmission mode (wavelength 520 and 565 nm for tretinoin and erythromycin respectively). The scanning speed was 50 mm/min. 200 points were registered when scanning the spot over a distance of 5 cm. Absorption curves were obtained, calculated from transmission scan of a blank and the analyte, following the principles of Lambert and Beer. The peak areas were determined, utilising the Peakfit Software from Jandel (Erkrath, Germany), specialised in fitting and calculation of peak area of 'no linear curves'.

3. Results

3.1. Qualitative results

3.1.1. Selection of the mobile and stationary phase for erythromycin

Initial tests were done, following the method of Cachet [8] and Kibwage [9]. Some problems were observed: the elution front was not right, presumably due to an unsaturated mobile phase; some brands of thin layer plates did not withstand the mobile phase. Therefore, other mobile phases, containing different ratios of ethanol/chloroform/ammonia 25% (50:50:0.5 (v/v)), (75:25:0.5 (v/v)), (25:75:0.5 (v/v)) were applied but didn't fulfill. Erythromycin migrated in some of them but the same separation efficiency of different degradation products, as it was achieved in the study of Cachet [8] and Kibwage [9], could not be reached. The best brand of thin layer plates that can be used in these investigations, are these from Merck (Darmstadt, Germany). The TLC-plate with organic binder, the Sil G-25 from Macherey-Nagel (Düren, Germany), did not withstand the mobile phase. As could be estimated, the organic mobile phase possibly dissolved the binder, which caused cracking of the thin layer. The one with inorganic binder, Sil G-25 HR (with gypsum) from Macherey-Nagel (Düren, Germany) resisted the mobile phase but not really the derivatization reagent.

Using the TLC-plates from Merck (Darmstadt, Germany) as a stationary phase and a mobile phase in a lower volume than presented by Cachet [8], namely dichloromethane-methanol-ammonia 25% (60:6:1 (v/v)) instead of (90:9:1.5 (v/v)), in standardised conditions (overnight saturation at exactly 25°C), resulted in an equivalent separation efficiency, however without destruction of the thin layer, which is necessary for quantitative work.

3.1.2. Selection of the mobile and stationary phase for tretinoin

In earlier studies of tretinoin on HPLC [10], it seemed to be possible to separate tretinoin from its degradation products with a reversed system. Based on this information, we adapted the HPLC-technique to a thin layer chromato-

Table 1
Rf-values of tretinoin and erythromycin and their degradation products and the used excipients in the different TLC-systems.

Analytes and degradation products	Rf in 'tretinoin' system[c]	Rf in 'erythromycin' system[d]
1. Tretinoin[a]	0.08	0.00
2. Isotretinoin[b]	0.14	
3. Erythromycin[a]	Without acetic acid, 0.11 and overlaps tretinoin because of tailing Adding acetic acid 1 ml, migrates above the tretinoin spot	0.37
4. Erythromycin[b]		0.29
5. Erythromycin[b]		0.52
Excipients		
6. Butylhydroxytoluene	0.14	Separated but not visible after dilution
7. Isopropanol	Not visible (volatile)	Not visible (volatile)
8. Propylene glycol	0.87	Not visible
9. Polyethylene glycol	0.79	Not visible

[c] Stationary phase, RP C18; mobile phase, acetonitrile/water (50:25 (v/v)).
[d] Stationary phase, silica gel F 60; mobile phase, dichloromethane/methanol/ammonia 25% (60:6:1 (v/v/v)).
[a] Analytes.
[b] Degradation products.

graphic method: a mobile phase, consisted of acetonitrile and water (50:25 (v/v)), and the precoated reversed phase plates RP-18 254S as stationary phase. This system gave a good separation of the active component tretinoin from its degradation products.

3.1.3. Analysis of a lotion of 2% (g/v) erythromycin and 0.05% (g/v) tretinoin and several excipients with the individual TLC-systems.

The spots of a lotion, after a five times dilution for tretinoin and 20 times for erythromycin, were compared with a working solution containing both compounds.

Concerning erythromycin, the separation of tretinoin was obtained without any change to the TLC-method. The spots of tretinoin could not be detected because of the high dilution of the lotion. Although, the spot of tretinoin did not migrate ($Rf = 0$).

In the reversed phase system of tretinoin, the spot of erythromycin appears as a tailing and covers the spot of tretinoin totally. This problem was resolved by adding 1 ml acetic acid to the mobile phase, containing acetonitrile/water (50:25 (v/v)). Erythromycin migrated totally to the front line. A possible explanation is the development of a more polar derivative, which likes to migrate with the polar mobile phase.

The following excipients, used in the lotion need to be separated from the analytes,

1. To reduce the drying effect of pure alcohol on the skin, isopropanol, propylene glycol and polyethylene glycol 400 were used in a concentration of 50% (v/v) in the vehicle of the lotion. No interference was detected. Isopropanol evaporates completely under the nitrogen flow, used for drying the spots, the other ones were too diluted to be visualised in case of erythromycin or migrated completely in the TLC system of tretinoin.
2. Butylhydroxytoluene 0.05% (g/v) was added for its stabilising effect on tretinoin (antioxidant) [11]. The separation of butylhydroxytoluene was obtained in each TLC-system. Concerning erythromycin, the butylhydroxytoluene-spot could not be visualised. In the analysis of tretinoin, butylhydroxytoluene appeared as a blue coloured spot after derivatization with anisaldehyde ($Rf_{\text{butylhydroxytoluene}} > Rf_{\text{tretinoin}}$).

Table 1 gives the Rf-values of each active compound with their degradation products and the used excipients.

3.1.4. Selection of the derivatization reagent

The spraying reagent of anisaldehyde was used in the study of Cachet [8] and Kibwage [9], sulfuric acid 96% and methanol (1:2:9) was used to visualise erythromycin. As mentioned by Touchstone [12], they do not fulfill the requirements for quantitative analysis because of the fact that uniform spraying is practically impossible. High acidic solution can crack the thin layer. Dipping solutions are in generally less concentrated than the respective spraying agents. Both reasons explained why a dipping solution was developed. Concerning the choice of an anisaldehyde solution, this derivatization agent was already used for erythromycin and as mentioned by Touchstone [12], the reaction is not well known but it reacts with many compounds, containing keto-, amino-functions and others. As it is seen from our results, it was worth to test it on tretinoin, which is characterised by carboxyl functions. Earlier studies in our laboratory suggested that a solution of 1% (v/v) anisaldehyde, 2% (v/v) sulfuric acid and 10% (v/v) acetic acid in alcohol fulfilled the requirements as a

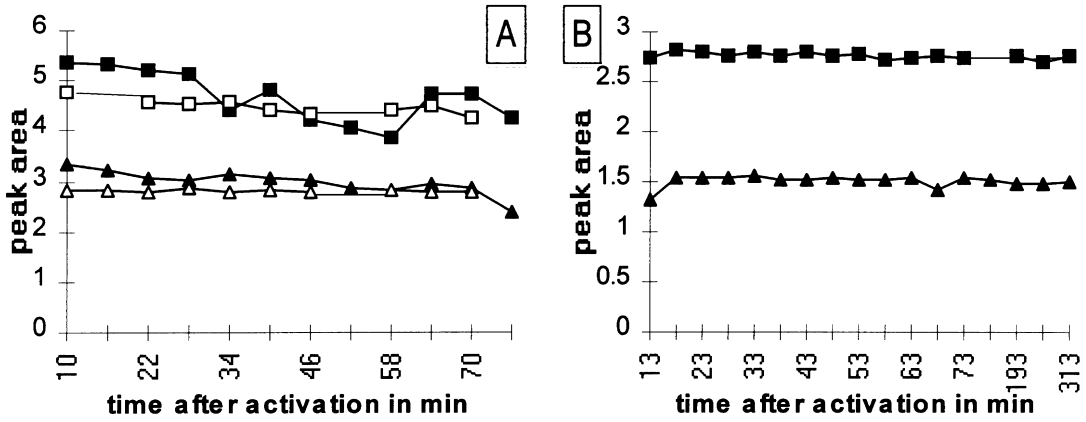

Fig. 1. Stability of the developed colour for erythromycin 5 and 2.75 μg after 8 and 10 minutes (A) and tretinoin (B) 2.5 μg and 1.5 μg after 10 min. ((i) erythromycin (A): 5 μg, □ 10 min and ■ 8 min; 2.75 μg, ▲ 10 min and △ 8min; (ii) tretinoin (B): 10 min, ▲ 1.5 μg; ■ = 2.5 μg).

dipping reagent. Some other parameters have to be taken into account for its usefulness in quantitative analysis: the thin layer has to maintain intact after dipping; the drying time has to be as short as possible and the spots have to be in fine bands without any disturbance.

Based on the activation conditions for an anisaldehyde spraying reagent namely 110°C, the first tests were performed with 100% alcohol as the solvent in the dipping reagent. Whereas it did not really fulfill, other solvents were tested in the dipping solution for erythromycin and tretinoin namely water/alcohol 94% (v/v) (40:50 and 60:30 (v/v)) and chloroform/alcohol 94% (v/v) (60:30 and 40:50 (v/v)). Whereas tretinoin was visualised as flew and speckled spots after derivatization with a chloroform containing solution, an aqueous based solution was selected. The opposite was observed for erythromycin, which means that a chloroform/ethanol solution performed the best spots. The following dipping solutions, containing 1% (v/v) anisaldehyde, 2% (v/v) sulfuric acid, and 10% (v/v) acetic acid, and respectively methanol/water (50:40) (v/v) and methanol/chloroform (50:40) (v/v) were preferred. They gave blue and red, well formed spots in day light and a bright orange colour in UV light (366 nm) for each compound and their degradation products.

3.2. Quantitative results

3.2.1. Stability of the developed colour after derivatization

Before executing some quantitative work on the analytes, the stability of the developed colour was investigated. The plate was heated under the mentioned conditions during 8 and 10 min.

The experiment was performed on a working standard of erythromycin (5 and 2.75 μg were spotted), after 8 and 10 min heating. A best colour stability was observed after 10 min.

Concerning tretinoin, a 10 min heating of tretinoin 1.5 and 2.5 μg resulted in a stable colour for more than 5 h.

The RSD and VC in the intervals are respectively 0.0233 and 0.83% for erythromycin ($n = 9$) and 0.1067 and 2.40 % for tretinoin ($n = 16$).

Fig. 1 gives the stability curves of erythromycin and tretinoin after 8 and 10 min.

3.2.2. Precision

Precision was examined at several levels: in order to control the measurements with the Zeiss-spectrophotometer, one spot was analyzed several times, the precision of the automatic spotter and derivatization technique was investigated by measuring different spots of the same aliquot of the analyte. Results are given in Table 2.

The precision for each analyte, as determined at several levels, was consistently less than 5%.

3.2.3. Accuracy, robustness and repeatability of the method

The accuracy was tested on the lotion with alcohol 94% (v/v)/isopropanol as vehicle. Several lotions were prepared with varying pH from 5 to 9 and bytylhydroxytoluene's content and analyzed on different days, using several plates and developed in every day freshly prepared mobile phases.

Table 2
Precision of measurements with the Zeiss-spectrofotometer and spot volume of the automatic spotter

Product	Precision	Peak areas: mean ± SD	V.C. (%)
Erythromycin 1.75 μg	1 spot (n = 6)	2.409 ± 0.023	0.98
	5 spots	1.996 ± 0.086	4.32
Tretinoin 1.15 μg	1 spot (n = 6)	1.296 ± 0.018	1.43
	5 spots	1.190 ± 0.052	4.42

Table 3
Comparison of the analytical parameters of two chromatographic methods applied on tretinoin and erythromycin: TLC and HPLC

Parameter	Tretinoin		Erythromycin	
	HPLC[a]	TLC	HPLC[b]	TLC
Detection wave length	350 nm	520 nm (colour)	215 nm	565 nm (color)
Sample volume	20 μl	10 μl or more	20 μl	10 μl or more
Linearity range	0.02–0.24 μg	0.5–5 μg	1–10 μg	1–8 μg
Elution time	16 min/sample	30 min for max 10 spots	15 min per sample	30 min for max. 10 spots
Resolution	11.6	3.0	5.6	13.1
Number of theoretical plates	10 816	3080	10816	312

[a] HPLC method for tretinoin: stationary phase is a Lichrospher RP18 (5 μm, 25 cm) from Merck and the mobile phase contains acetonitrile, water, acetic acid 96% in a ratio of 80:20:1 (v/v/v).

[b] HPLC method for erythromycin: stationary phase is a Lichrospher RP18 (5 μm, 25 cm) from Merck and the mobile phase contains acetonitrile, ammoniumphosphate-buffer (0.2 M, pH 4), tertamethylammoniumhydroxide (0.2 M, pH 4) and water in a ratio of 40:5:20:40 (v/v/v/v).

The concentration of each active compound in the lotions was investigated.

Measuring seven solutions of 0.05% tretinoin, the RSD is 0.02327 and VC is 7.26%.

6 solutions of 2% erythromycin were investigated, giving the following results, RSD = 0.00393 and VC = 4.68%.

3.2.4. Calibration

Adequate aliquots of working solutions of erythromycin and tretinoin were spotted.

Linear calibration curves were obtained between 1 μg and 8 μg for erythromycicin. (($y = 0.800(x) + 0.803$ with y = area under the peak and x = quantity of the analyte in μg and correlation of 0.997 ($n = 8$)).

Higher concentrations were measurable with higher concentrations of anisaldehyde and sulfuric acid but destroyed the thin layer. Concerning tretinoin, a higher sensitivity was observed; higher concentrations than 0.05% can not be measured because of a strong broading of the spots. Linear calibration curves were obtained between 0.5 μg and 5 μg ($y = 1.151(x) - 0.079$ with y = area under the peak and x = quantity of the analyte in μg and correlation of 0.998 ($n = 8$)).

As can be concluded from the results, the limit of quantification of tretinoin is smaller than for erythromycin. And so does the detection limit, which is respectively 0.25 and 0.5 μg.

3.2.5. Comparison of the TLC method for erythromycin and tretinoin with HPLC methods

Results of HPLC dosage analysis of erythromycin and tretinoin, used for stability investigation in our lab [10,13] are compared with the two presented developed TLC methods. The results are given in Table 3.

The results are obtained directly by measurement of the developed spots on the thin layer plate [14].

Concerning sensitivity of the methods, lower concentrations of tretinoin can be analyzed by using UV-spectrophotometrical detection in HPLC. In case of TLC, the sample volume can be changed under certain conditions, taking into account that the solvent evaporates easily. In HPLC, higher sample volumes can influence the peak of the analyte. More samples can be analyzed in a shorter time, which is very convenient for our stability investigation purposes.

Looking to the values of resolution, for both products in both techniques, much higher values than one were obtained, which proves the utility of all methods. Despite some loss of efficiency for tretinoin in TLC compared to HPLC, a great improvement, as for sensitivity, resolution as for theoretical plates, was found for erythromycin. Values for the calculated parameters are in the range values generally obtained for HPTLC [14].

Finally, we can conclude that especially for erythromycin a TLC-technique, using derivatization is more convenient than the HPLC-technique at 215 nm. Much better values for resolution as well as for the number of theoretical plates, which is calculated on the actual length of the stationary phase, were obtained.

4. Conclusion

This study represents a good alternative to analyse quantitatively tretinoin and especially erythromycin separately and is a good technique to analyse them together in the suggested lotion. This technique will be used for the evaluation of the chemical stability of these compounds in lotions with different solvent combinations.

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