IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 5, Issue 4 (April 2015), PP. 07-15

Application of High Performance Thin Layer Chromatography with Densitometry for Determination of Active Ingredients and Preservatives in Various Pharmaceutical Marketed Formulations

Vesna Kostik[1], Biljana Gjorgeska[2], Sofija Petkovska[2]
[1]*Institute of Public Health of Republic of Macedonia, 50 Divizija No. 6, 1000 Skopje, Republic of Macedonia Assistant professor,*
[2]*Medical Faculty, Department of Pharmacy, University "Goce Delchev", Krste Misirkov bb, Shtip, Republic of Macedonia*

Abstract: High performance thin layer chromatography(HPTLC) is a fast separation technique which is advantageous in many means as it is simple to handle and requires a short analysis time to analyze the complex or the crude samples. A new, simple, precise and sensitive analytical method based on HPTLC technique has been developed and validated for the estimation of eighteen active ingredients and preservatives in various marketed pharmaceutical formulations. The proposed method was accurate and precise (mean recovery in the range from 83.3% to 98.8%, precision with RSD<3.95%; LOQ < 26.4 μg/mL). This method was applied for the quantification of active ingredients and preservatives in: ophthalmic solutions, nasal solution, otic solution, syrups, shampoos, suppositories, ointments, dietary supplements, tablets and capsules.
Keywords – active ingredients, high performance thin layer chromatography (HPTLC), preservatives

I. INTRODUCTION

From the stages of drug development to marketing and post marketing, analytical techniques play a great role, especially for understanding the physical and chemical stability of the drug, that have impact on the selection and design of the dosage form, assessing the stability of the drug molecules, quantification and identification of the impurities and degradation products, which are above the established threshold essential to evaluate the toxicity profiles, as well as, the content of drug in the marketed products [1]. Various analytical techniques as: UV-vis spectrophotometry [2-6], high performance thin layer chromatography (HPTLC) [7-10], high performance liquid chromatography (HPLC) [11-21], micellar electro- kinetic chromatography [22], sequential injection chromatography [23], and their corresponding analytical methods have been used in the analysis of pharmaceuticals. Although an old technique thin layer chromatography finds a lot of applications in the field of pharmaceutical analysis. With the advancement of the technique, HPTLC emerged as an important instrument in drug analysis. HPTLC is a fast separation technique and flexible enough to analyze a wide variety of samples. This technique is advantageous in many means as it is simple to handle and requires a short analysis time to analyze the complex or the crude samples. HPTLC evaluates the entire chromatogram with a variety of parameters without time limits. Moreover, there is simultaneous but independent development of multiple samples and standards on each plate, leading to an increased reliability of results. HPTLC has been used to quantitate drugs as ethinyl estradiol and cyproterone in tablets [7], alfuzosin in tablets [8], pentazocine and tramadol in mixtures [9], timolol maleate and brimonidine tartrate in ophthalmic solution [10], etc. The aim of our study was to develop and validate analytical methods based on a HPTLC technique for rapid, precise and reliable determination of active ingredients and preservatives in several different marketed pharmaceutical formulations.

II. MATERIALS AND METHODS

2.1 Instrumentation

Application of the samples and standards on the TLC plates was performed by semiautomatic spotting device, Linomat IV, (Camag, Switzerland) with 100 μl syringe, (Hamilton, USA). Development of TLC plates was done in a horizontal chamber, 10 x10 cm (Camag, Switzerland). The spots were measured by TLC Scanner-3, (Camag, Switzerland). The densitometric evaluation of HPTLC chromatograms was performed by CATS-4 Software.

All weighing were done on electronic analytical balance (Sartorius, Quintix65-1S). Calibrated volumetric flasks class A were used for the preparation of the standards and samples. Ultrasonic homogenizer (Hielscher, Germany) was used for the preparation of suppository samples.

2.2. Spotting parameters
Band width: 5mm
Space between 2 bands: 5mm
Spraying rate: 10 μL/sec

2.3. Scanning parameters
Slit dimension: 4x.45 mm
Lamp: Deuterium, Tungsten
Measurement mode: Absorption

III. SAMPLES

Samples of marketed pharmaceutical formulations: ear drops oil solution (50 mg procaine hydrochloride and 50 mg phenazone per g), ophthalmic water solution (1 mg naphazoline hydrochloride and 1 mg diphenhydramine hydrochloride solution per mL), nasal drops water solution (0.1 mg dexamethasone sodium phosphate and 1 mg xylomethazoline hydrochloride per mL), ophthalmic water solution (sodium sulfacetamide 10%, W/V), ophthalmic water solution (0.5% timolol maleate, W/V), dietary supplement oil solution (retinyl palmitate 30,000 IU/mL; cholecalciferol 60,000 IU/mL), anti-parasitic shampoo water emulsion (1.2 mg pyrethrine I per mL and 1.8 mg pyrethrine II per mL), ointment (β- carotene 25%, W/W), suppository (paracetamol 120 mg each), oral water solution mixture (metoclopramide 0.1%, W/V and Nipasol P 0.1%, W/V), oral water suspension mixture (paracetamol 24 mg per mL; Nipasol P 0.04%, W/V and Nipasol M 0.08%, W/V), non-scored tablets (indapamide 2.5 mg per tablet) and capsules (nifedipine 5 mg per capsule) were obtained from the local pharmacy shops.

IV. STANDARDS AND REAGENTS

Analytical standards: procaine hydrochloride (purity ≥97%), phenazone (purity ≥98.5%), naphazoline hydrochloride (purity ≥99%), diphenhydramine hydrochloride (purity ≥98%), dexamethasone sodium phosphate (purity ≥98%), xylomethazoline hydrochloride (purity ≥98%), sulfacetamide sodium (purity ≥98%), timolol maleate (purity ≥98%), β-carotene (purity ≥97%), metoclopramide (purity ≥99.5%), indapamide (purity ≥99.5%), nifedipine (purity ≥99.5%), nipasol M (purity ≥99.5%), nipasol P (purity ≥99.5%), oil mixture of 25% (W/V) pyrethrine I and 25% pyrethrine II (W/V) were obtained from Sigma-Aldrich (Netherlands).
Analytical standard mixture consisted of retinyl palmitate (300,000 U/mL) and cholecalciferol (600,000 U/mL) was obtained from Alkaloid (Skopje, Macedonia).

Ethyl acetate, methanol, n-butanol, anhydrous acetic acid, benzene, acetone, n-hexane, acetic acid and ammonia (25%, V/V) with analytical grade of purity were obtained from Alkaloid (Skopje, Macedonia). Distilled water was obtained from glass apparatus.

4.1. Mobile phase systems
1. Ethyl acetate: methanol: distilled water: ammonia 25%, V/V (8.5:2.0:1.5:0.5)
2. N-butanol: anhydrous acetic acid: distilled water (6.0:2.0:2.0)
3. Benzene: ethyl acetate (8.5:1.5)
4. N-hexane: acetic acid (8.8:1.2)
5. Benzene: acetone (8.0:2.0)

4.2. Stationary phases
1. Precoated HPTLC Kiesel silica gel 60 F254 glass plates 10x10 cm, layer thickness 0.25mm were obtained from Merck, Darmstadt (Germany).
2. Precoated HPTLC RP8 F254 glass plates 10x10 cm, layer thickness 0.25mm were obtained from Merck, Darmstadt (Germany).

V. PREPARATION OF THE SAMPLES AND STANDARD SOLUTIONS

5.1. Oil solution (50 mg procaine hydrochloride per g and 50 mg phenazone per g)
a. Preparation of the sample
1g of the sample are transferred into a 100 mL volumetric flask and diluted with methanol up to the mark. 10 μL of the solution are applied onto the plate.
b. Preparation of the standard solutions
Stock solution: Procaine hydrochloride in methanol (1 mg/mL), phenazone in methanol (1 mg/mL). Working standard solution mixture: 5 mL of stock standard solution of procaine hydrochloride and phenazone are transferred into a 10 mL volumetric flask and diluted with methanol up to the mark (0.5 mg/L). 10 μL of the solution are applied onto the plate.

5.2. Water solution (1 mg naphazoline hydrochloride per mL and 1 mg diphenhydramine hydrochloride solution per mL)

a. Preparation of the sample

2 mL of the sample are transferred into a 10 mL volumetric flask and filled up with methanol up to the mark. 20 µL of the solution are applied onto the plate.

b. Preparation of the standard solutions

Stock solutions of naphazoline hydrochloride (1 mg/mL) and diphenhydramine hydrochloride (1 mg/mL) are prepared in methanol. Working standard solution mixture is prepared by transferring 2 mL of stock standard solutions of naphazoline hydrochloride and diphenhydramine hydrochloride into a 10 mL volumetric flask and dilution with distilled water up to the mark (0.2 mg/mL). 20 µL of the solution are applied onto the plate.

5.3. Water solution (0.1 mg dexamethasone sodium phosphate per mL and 1 mg xylomethazoline hydrochloride per mL)

a. Preparation of the sample

10 µL of the sample are directly applied onto the plate.

b. Preparation of the standard solutions

Stock standard solutions of dexamethasone sodium phosphate (1 mg/mL) and xylometazoline hydrochloride (1 mg/mL) are prepared in distilled water. Working standard solution mixture is prepared by transferring of 1 mL stock standard solution of dexamethasone sodium phosphate and 5 mL of stock standard solution of xylometazoline hydrochloride to a 10 mL volumetric flask and dilution with distilled water up to the mark (0.1 mg/L dexamethasone sodium phosphate, 0.5 mg/L xylometazoline hydrochloride).10 µL of the working standard solution are applied onto the plate.

5. 4. Sulfacetamide sodium water solution (10%)

a. Preparation of the sample

1 mL of the sample are transferred into a 50 mL volumetric flask and filled up with distilled water to the mark (i). 1 mL of the solution (i) is transferred into a 10 mL volumetric flask and diluted with distilled water up to the mark (ii). 5 µL of the solution (ii) is applied onto the plate.

b. Preparation of the standard solutions

Stock solution of sulfacetamide sodium (1 mg/mL) is prepared by dissolving 10 mg sulfacetamide in 10 mL distilled water (i). Working standard solution (0.2 mg/mL) is prepared by transferring 2 mL of stock standard solution (i) in a 10 mL volumetric flask and dilution with distilled water up to the mark (ii). 5 µL of the solution (ii) is applied onto the plate.

5. 5. Timolol maleate water solution (0.5%)

a. Preparation of the sample

2 mL solution of eye drops is transferred into a 10 mL volumetric flask and filled up with methanol to the mark. 10 µL of the solution is applied onto the plate.

b. Preparation of the standard solution

Stock solution of timolol maleate (1 mg/mL) was prepared by dissolving 10 mg timolol maleate in a 10 mL mixture methanol: distilled water (8:2). 10 µL of the stock solution is applied onto the plate.

5. 6. Retinyl palmitate and cholecalciferol oil solution (30,000 IU/mL retinyl palmitate and 60,000 IU/mL cholecalciferol)

a. Preparation of the sample

1 mL of the sample are transferred in a 50 mL and diluted with n-hexane up to the mark. 5 µL of the solution is applied onto the plate.

b. Preparation of the standard solutions

Working standard mixture (i): 1 mL of stock standard solution of retinyl palmitate (300,000 IU/mL) and 1 mL of stock standard solution of cholecalciferol (600,000 IU/mL) are transferred into a 50 mL volumetric flask and filled up with n-hexane up to the mark. Working standard mixture (ii): 1 mL of working standard mixture (i) is transferred into a 10 mL volumetric flask and filled with n-hexane up to the mark. 5 µL of the solution is applied onto the plate.

5. 7. Pyrethrine I and pyrethrine II water emulsion mixture (1.2 mg pyrethrine I per mL and 1.8 mg pyrethrine II per mL)

a. Preparation of the sample

1 mL of the solution is diluted with methanol up to 10 mL (b). 10 µL of the solution are applied onto the plate.

b. Preparation of the standard solutions
0.5 mL of standard mixture (25% pyrethrine I and 25% pyrethrine II) is diluted up to 100 mL with n-hexane (i).1 mL of solution (i) is diluted up to 10 mL wit n-hexane (ii). 10 μL of the solution (ii) are applied onto the plate.

5. 8. β-carotene ointment (25%)
a. Preparation of the sample
25 mg of the sample are dissolved in 10 mL chloroform and filled up to 25 mL with chloroform. 5 μL of the solution are applied onto the TLC plate.
b. Preparation of the standard solution
10 mg of the standard are dissolved in 50 mL chloroform to obtain concentration of 0.20 g/L. 6 μL of the solution are applied onto the TLC plate.

5. 9. Paracetamol suppository (120 mg)
a. Preparation of the sample
1 suppository is dissolved in 50 mL methanol and placed on the water bath at 50 ^{0}C until complete dissolution. After the cooling the solution is filtered through the milipore membrane filter 0.45 μm into a 200 mL volumetric flask and filled up to the mark with methanol (i). 5 mL of the solution are transferred into a 50 mL volumetric flask and filled up with methanol to the mark (ii).10 μL of the solution (ii) are applied onto the plate.
b. Preparation of the standard solutions
Stock standard solution of paracetamol (0.6 mg/mL) was prepared in methanol (i). 1 mL of stock standard solution (i) was transferred into a 10 mL volumetric flask and filled up with methanol to the mark (ii).10 μL of the working standard solution (ii) were applied onto the plate.

5. 10. Metoclopramide water solution (0.1% metoclopramide per mL and 0.1% Nipasol P per mL)
a. Preparation of the sample
1 mL of the sample is transferred into a 10 mL flask and diluted with methanol up to the mark. 10 μL of the solution are applied onto the plate.
b. Preparation of the standard solutions
Stock solution: 10 mg metoclopramide is diluted with methanol in 10 mL volumetric flask to obtain concentration of 1 mg/mL (i). 10 mg nipasol P is diluted with methanol in 10 mL volumetric flask to obtain concentration of 1 mg/mL (ii). 1 ml of the stock solution (i) and 1 mL of the stock solution (ii) are transferred in a 10 mL volumetric flask and filled up with methanol to the mark to obtain concentration of 0.1 g/L (iii). 10 μL of the working standard solution (iii) are applied onto the plate.

5. 11. Paracetamol water suspension (24 mg paracetamol/mL, 0.04% nipasol P, 0.08% nipasol M)
a. Preparation of the sample
1a. Preparation of the sample and working standard solution for the determination of paracetamol
1 mL of the sample is transferred into a 250 mL volumetric flask and diluted with methanol up to the mark. 10 μL of the solution is applied onto the plate.
Preparation of the standard solutions
10 mg paracetamol are dissolved with methanol in a 10 mL volumetric flask and diluted with methanol to obtain concentration of 1 mg/mL (i). 1 mL of stock solution (i) is transferred into a 10 mL volumetric to obtain concentration of 0.1 g/L (ii). 10 μL of the working standard (ii) is applied onto the plate.
1b. Preparation of the sample for determination of Nipasol P and Nipasol M
1 mL of the sample is transferred into a 10 mL volumetric flask. 5 μL of the solution are applied onto the plate.
Preparation of the working standard solution
4 mL of stock standard solutions of Nipasol P (1 mg/mL) and 8 mL of Nipasol M (1 mg/mL) prepared in methanol are transferred into a 100 mL volumetric flask and diluted with methanol up to the mark (40 mg/L Nipasol P, 80 mg/L Nipasol M). 5 μL of the working standard solution mixture are applied onto the plate.

5. 12. Indapamide tablets (2.5 mg)
a. Preparation of the samples
2 tablets are dissolved in 20 mL methanol. After sonication, the solution is filtered through the membrane milipore filter 0.45 μm in a 50 mL volumetric flask and filled up with methanol to the mark. 10 μL of the solution are applied onto the plate.
b. Preparation of the standards

Stock solution of indapamide (1 mg/mL) is prepared in methanol. 1 mL of the stock solution is transferred to a 10 mL volumetric flask and diluted with methanol up to the mark (0.1 mg/mL).10 μL of working standard solution are applied onto the plate.

5. 13. Nifedipine capsules (5 mg)

a. Preparation of the sample

2 capsules are extracted with 20 mL methanol. After sonication, the solution is filtered through the milipore membrane filter into a 50 mL volumetric flask. 10 μL of the solution is applied onto the plate.

b. Preparation of the standard solutions

Nifedipine stock solution (1 mg/mL) is prepared in methanol. 2 mL of stock solution are transferred into a 10 mL volumetric flask and filled with methanol up to the mark (0.2 mg/mL). 10 μL of the working solution are applied onto the plate.

VI. RESULTS AND DISCUSSION

6.1. Method optimization

In order to obtain the best separation for all tested components, a series of preliminary investigations using different mobile and stationary phases were tested. The optimum separation of components of interest was achieved with mobile phases and stationary phases shown in Table 1.

Table 1. Experimental (chromatographic) conditions for the determination of the active and preservatives in various pharmaceutical formulations

Pharmaceutical dosage form	Active component	Stationary phase	Mobile phase	*HRf (%)	Wavelength (nm)
Ear drops (oil solution)	Procaine hydrochloride	1	1	67	254
	Phenazone	1	1	37	254
Eye drops (water solution)	Naphazoline hydrochloride	1	1	5	254
	Diphenhydramine hydrochloride	1	1	35	254
Nasal drops (water solution)	Dexamethasone sodium phosphate	1	Development with mobile phase 1, after drying development with mobile phase 2	54	254
	Xylomethazoline hydrochloride	1		82	254
Eye drops (water solution)	Sulfacetamide sodium	1	1	86	254
Eye drops (water solution)	Timolol maleate	1	1	30	254
Dietary supplement (oil solution)	Retinyl palmitate	2	1	53	254
	Cholecalciferol	2	1	73	254
Ointment	β carotene	2	1	70	430
Tablets	Indapamide	1	5	47	254
Capsules	Nifedipine	1	1	93	254
Shampoo (water emulsion)	Pyrethrine II	1	3	76	254
	Pyrethrine I	1	3	87	254
Syrup (water solution)	Metoclopramide	1	1	26	254
	Nipasol P	2	4	62	270
Syrup (water suspension)	Paracetamol	1	1	81	254
	Nipasol M	2	4	43	270
	Nipasol P	2	4	63	270

*Hrf – retention factor (Rf) •100

Chromatograms of some of the analyzed components are shown in Fig. 1.

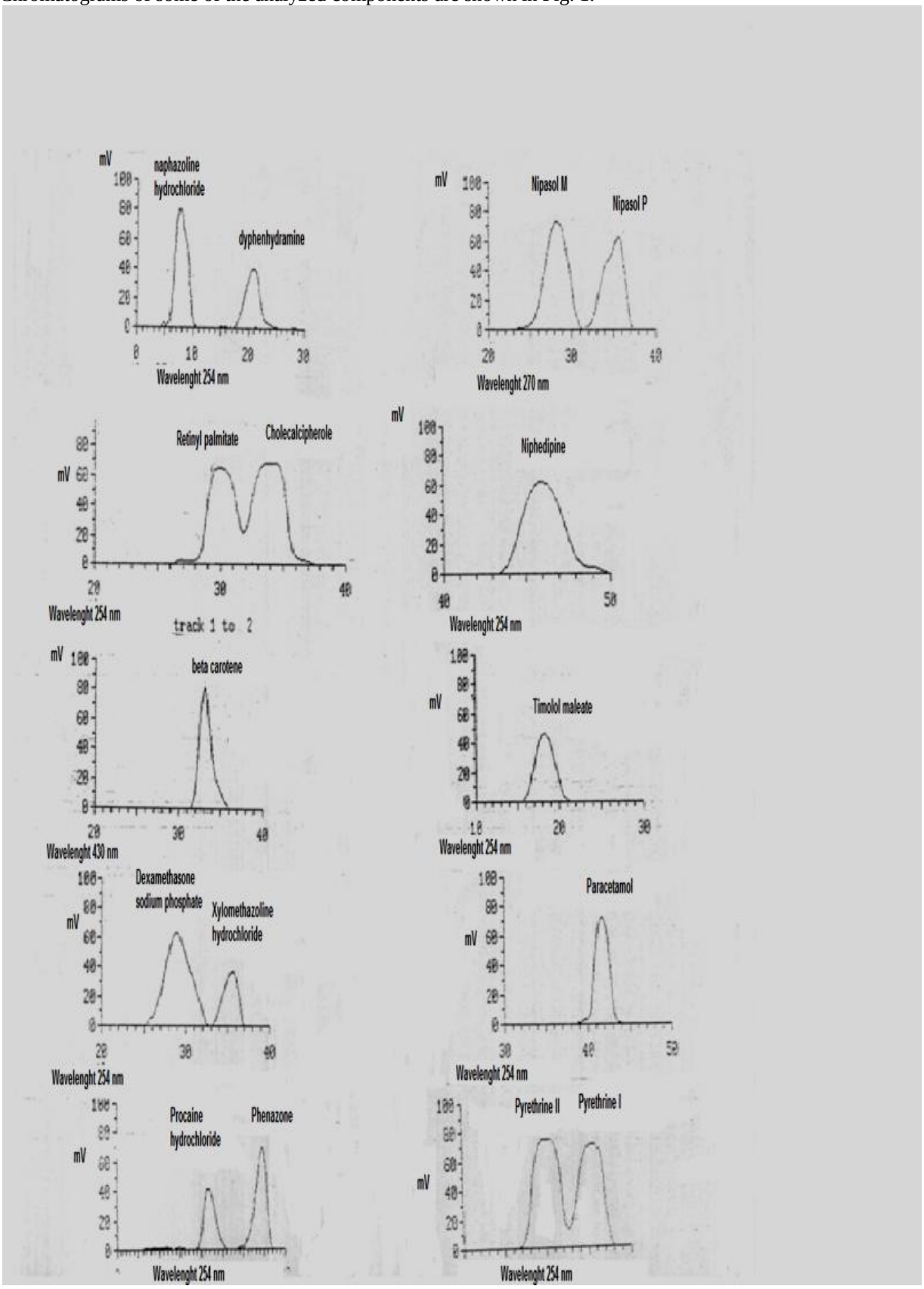

Figure. 1 Chromatograms of some of the tested components

6.2. Method validation

6.2.1. Recovery, precision and linearity

Recovery, precision and linearity of all the tested components were determined using fortified samples of pharmaceutical formulations which were previously tested on the presence of active ingredients and preservatives. In each case, 5 replicates each at 5 levels were fortified into the samples. The obtained results for recovery (R) and precision (RSD) are shown in Table 2.

Table 2. Recovery data obtained with the proposed methods

Component	Concentration present (μg/mL)	Concentration added (μg/mL)	Concentration found (μg/mL)	Recovery (%)	RSD (%), n=3
Procaine hydrochloride	500	200	680	97.1	2.32
Phenazone	500	200	692	98.8	2.45
Naphazoline hydrochloride	200	100	285	95.5	1.98
Diphenhydramine hydrochloride	200	100	276	92.0	2.87
Dexamethasone sodium phosphate	100	50	139	92.7	2.05
Xylomethazoline hydrochloride	500	200	673	96.1	1.97
Sulfacetamide sodium	200	150	332	94.9	3.05
Timolol maleate	500	200	635	90.7	3.12
*Retinyl palmitate	330	200	490	92.4	3.23
**Cholecalciferol	30	20	42	84.0	3.95
Pyrethrine I	700	300	930	93.0	2.15
Pyrethrine II	700	300	894	89.4	2.55
β-carotene	200	150	305	87.1	2.90
Paracetamol (suppository)	60	30	82		
Paracetamol (syrup)	96	50	135	92.5	1.85
***Nipasol P	40	20	50	83.3	3.15
Nipasol M	80	40	108	90.0	2.51
Metoclopramide	100	50	139	92.3	2.90
****Nipasol P	100	50	141	94.0	1.85
Indapamide	100	50	144	96.0	1.55
Nifedipine	200	100	283	94.3	2.35

*Retinyl palmitate 1IU=0.55 μg

**Cholecalciferol 1IU=0.025 μg

***Nipasol P in Paracetamol syrup formulation

** **Nipasol P in Metoclopramide water solution formulation

High analytical recoveries ranging from 83.3% to 98.8% were obtained for all the tested components. From the obtained values for relative standard deviation (RSD), ranging from 1.55% to 3.95% indicated that the proposed method is precise enough and therefore suitable for the determination of tested compounds in pharmaceutical formulations.

The obtained values for multiple correlation coefficients (Table 3), ranged from 0.990 to 0.998, revealed that the method has a good linearity for all the tested components, and are in line with methods performed by methods based on HPLC technique [11-21].

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the formulas LOD = 3.3 · SD/slope and LOQ = 10 · SD/slope [24]. The obtained results are shown in Table 3.

Table 3. Statistical data for limit of detection (LOD), limit of quantification (LOQ) and correlation coefficient (R2)

Component	Range (μg/mL)	R2	LOD (μg/mL)	LOQ (μg/mL)
Procaine hydrochloride	50-500	0.992	6	19.8
Phenazone	50-500	0.995	7	23.1
Naphazoline hydrochloride	20-200	0.991	5	16.5
Diphenhydramine hydrochloride	20-200	0.996	5	16.5
Dexamethasone sodium phosphate	10-100	0.995	2	6.6
Xylomethazoline hydrochloride	50-500	0.998	5	16.5
Sulfacetamide sodium	20-200	0.995	6	19.8
Timolol maleate	50-500	0.992	8	26.4
Retinyl palmitate	30-300	0.990	7	23.1
Cholecalciferol	5-30	0.991	1	3.3
Pyrethrine I	100-700	0.994	8	26.4
Pyrethrine II	100-700	0.990	7	23.1
β-carotene	20-200	0.991	5	16.5
Paracetamol	10-100	0.997	2	6.6
Nipasol P	4 - 40	0.995	1	3.3
Nipasol M	8 - 80	0.993	2	6.6
Metoclopramide	10-100	0.997	2	6.6
Indapamide	10-100	0.998	3	9.9
Nifedipine	20-200	0.995	3	9.9

VII. CONCLUSIONS

Analytical methods based on a HPTLC technique were developed for rapid, precise and reliable determination of active ingredients and preservatives in 13 different pharmaceutical formulations taken from the market. From the validation parameters obtained it can be concluded that this methods are useful for routine analyses in determining the quality of the marketed pharmaceutical formulations. The proposed method is precise enough with corresponding methods based on the HPLC technique.

REFERENCES

[1] M Raza Siddiqui., Z.A AlOthman and N. Rahman, Analytical techniques in pharmaceutical analysis: A review, Arabian Journal of chemistry, 2013, http://dx.doi.org/10.1016/j.arabjc.2013.04.016.

[2] G. Santoni, P. Mura, S. Pinzauti and E. Lombardo, Simultaneous UV spectrophotometric determination of procaine hydrochloride and phenazone in an otic formulation, International Journal of Pharmaceutics, 10, 1990, (doi: 10.1016/0378-5173(90)90274-8).

[3] M.A. Korany, M.M. Bedair and A. El-Gindy, Analysis of Diphenhydramine Hydrochloride and Naphazoline Hydrochloride in Presence of Methylene Blue in Eye Drops by Second Derivative Spectrophotometry, Drug Development and Industrial Pharmacy, 16(9),1555-1564 (doi:10.3109/03639049009074383).

[4] L.S. Ramesh., R. Ahmed, R.S. Supriya and D..R. Sheetal, Spectrophotometric estimation of paracetamol and promethazine in tablet dosage forms, Der Pharma Chemica, 4(2), 2012, 714-719.

[5] S Nabeel, H.S. Othman, A. Mahmood and N. Khaleel, Spectrophotometric and High Performance Liquid Chromatographic Methods for Determination of Metoclopramide in Pharmaceutical Preparations, Raf. J. Sci., 22(3), 2011, 39- 56.

[6] N. Rahman, S. Najmul and H. Azmi, New spectrophotometric methods for the determination of nifedipine in pharmaceutical formulations, Acta Biochimica Polonica, 52(4), 2005, 915–922.

[7] K Pavic, O. Cudina,, D Agbaba, S.Vladimirov, , Quantitative analysis of cyproterone acetate and ethinylestradiol in tablets by use of planar chromatography, J. Planar Chromatgr.-Mod. TLC, 16, 2003, 45–47.

[8] A.S Fayed,., M.A-A Shehata, N.Y Hassan., and S.A., El-Weshahy, Thin layer chromatography in drug analysis, J. Sep. Sci, 29, 2006, 2716–2724.

[9] Z.A.J Ebrahim, D Balalau, D.L Baconi,, C.M. Gutu, and M Ilie, HPTLC method for the assay of tramadol and pentazocine from mixture, Farmacia , 59, 2011, 381–387.

[10] S.K. Mehta and D.G. Maheshwari, Analytical method development and validation for simultaneous estimation of timolol maleate and brimonidine tartrate in bulk and marketed ophthalmic formulation, Journal of Pharmaceutical Science and Bioscientific Research, 4(6), 2014, 351-356.

[11] T. Huang, N. Chen, D. Wang, Y. Lai and Z. Cao, A validated stability-indicating HPLC method for the simultaneous determination of pheniramine maleate and naphazoline hydrochloride in pharmaceutical formulation, Chem Cent J., 8(7), 2014. (doi: 10.1186/1752-153X-8-7).

[12] Z. Milojevic, D. Agbaba, S Eric, D. Boberic-Borojevic. P Ristic and M Solujic High-performance liquid chromatographic method for the assay of dexamethasone and xylometazoline in nasal drops containing methyl p-hydroxybenzoate, J. Chromatogr A, 949(1-2), 2002 , 79-82.

[13] M. C. C. Urban , R. M. Mainardes, M. P. D. Gremião, Development and validation of HPLC method for analysis of dexamethasone acetate in micro emulsions, Brazilian Journal of Pharmaceutical Sciences, 45(1), 2009, 87-92.

[14] N Erk, Rapid and sensitive HPLC method for the simultaneous determination of dorzolamide hydrochloride and timolol maleate in eye drops with diode-array and UV detection, Pharmazie, 58(7), 2003, 491-493.

[15] A. Kwiecień , U. Hubicka , and J. Krzek, Determination of retinyl palmitate in ointment by HPLC with diode array detection, Acta Pol Pharm, 67(5), 2010, 475-479.

[16] D. Šatínský. D. Kameníčková and P. Chocholouš, Fast HPLC Method for Determination of Fenoxycarb and Permethrin in Antiparasitic Veterinary Shampoo Using Fused-Core Column, Chromatographia , 11, 2013; (doi: 10.1007/s10337-013-2464-0).

[17] J. Schierle, B. Pietsch, A. Ceresa, and C. Fizet, Method for the Determination of β-Carotene in Supplements and Raw Materials by Reversed-Phase Liquid Chromatography: Single Laboratory Validation, J AOAC Int.,87(5) 2004, 1070–1082.

[18] E Kalmár, B Kormányos, G Szakonyi and G. Dombi, Validated HPLC determination of 4-dimethylaminoantipyrine in different suppository bases, Indian Journal of Pharmaceutical Sciences, 76(1), 2014, 31-37.

[19] S. Kumar, S. Mathkar, C. Romero, and A.M. Rustum, Development and Validation of a Single RP-HPLC Assay Method for Analysis of Bulk Raw Material Batches of Four Parabens that are Widely Used as Preservatives in Pharmaceutical and Cosmetic Products, Journal of Chromatographic Science, 49, May/June 2011.

[20] H. Kaur, H Pannu, M., P. Mahajan and S. D. Sawant, Validated RP-HPLC Method for the Determination of Indapamide in Bulk and Tablet Dosage Form, Der Pharma Chemica, 4(3), 2012, , 996-1002.

[21] K. Sankaa, R. Gullapellia , N. Patila , P. Rao and P.V Divan, Development and validation of RP-HPLC method for nifedipine and its application for a novel proniosomal formulation analysis and dissolution study, Der Pharma Chemica, 6(1),2014, 279-289.

[22] J.M. Lemus Gallego, J. Pe´rez Arroyo, Determination of prednisolone acetate, sulfacetamide and phenylefrine in local pharmaceutical preparations by micellar electrokinetic chromatography, Journal of Pharmaceutical and Biomedical Analysis, 31, 2003. 873 - 884.

[23] H. Sklenářová Petra Koblová, P. Chocholouš, D. Šatínský, L. Krčmová, M. Kašparová, D. Solichová and P. Solich, Separation of Vitamins Retinol Acetate, Ergocalciferol, or Cholecalciferol and Tocopherol Acetate Using Sequential Injection Chromatography, Analytical Letters, 44, (1-3), 2011.
(doi: 10.1080/00032719.2010.500784).

[24] J.C. Miller. and J. N. Miller, Statistic for analytical chemistry, 3rd Ed., Ellis Horwood Ptr. Prentice Hall, 1993, 104 – 141.