STUDIA UBB CHEMIA, LVII, 2, 2012 (p. 83 - 93)

COMPARATIVE STUDY OF DIFFERENT TLC-IMAGE ANALYSIS METHODS FOR QUANTITATIVE EVALUATION OF PARABENS IN PHARMACEUTICAL SUSPENSIONS

IOANA ANAMARIA TUHUŢIU[a], DORINA CASONI[a,*], COSTEL SÂRBU[a]

ABSTRACT. A high-performance thin-layer chromatographic method combined with a sample preparation procedure and digital images processing has been developed for simultaneous determination of parabens in pharmaceutical suspensions. For the quantitative evaluation of the chromatographic spots, three different software that combines 2D (ImageDecipher-TLC and Sorbfil TLC) and respectively 3D (JustTLC) image analysis were investigated. The statistical parameters of the linear relation between the applied concentrations and both the peaks area and volume respectively, revealed no statistical significant differences in terms of the regression determination coefficient (R^2). The lowest limits of detection and quantification values were obtained for ethylparaben and butylparaben using the ImageDecipher-TLC software. Also, by using ImageDecipher-TLC software with conversion of color images of chromatographic plates into grey scale, the precision of the developed method increased in all cases. The results obtained for commercial samples showed that the proposed method, using new UV-Vis TLC scanner device with ImageDecipher-TLC software, is suitable for rapid routine analysis of parabens in pharmaceutical suspensions.

Keywords: *quantitative evaluation, parabens, HPTLC, digital processing of images, method validation*

INTRODUCTION

The esters of para-hydroxybenzoic acid are called parabens and they are a class of chemicals widely used as preservatives in the cosmetic, pharmaceutical and food industries. Common parabens include methylparaben, ethylparaben, propylparaben and butylparaben, and less common parabens include isobutylparaben, isopropylparaben and benzylparaben. Parabens are effective preservatives in many types of formulas, being used primarily for their antibacterial and antifungal properties, against molds and yeast. Their efficacy as preservatives, in combination with the long history of their use, their low cost, broad spectrum of activity, inertness, worldwide regulatory

[a] Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, Arany Janos Str. No 11, RO-400028 Cluj-Napoca, România, * casoni_dorina@yahoo.com

acceptance, biodegradability, and their excellent chemical stability in relation to pH and temperature [1], probably explains why parabens are so commonplace. However, they are becoming increasingly controversial, because they have been found in extremely low concentrations in breast cancer tumors [2]. Parabens have also displayed the ability to weakly mimic estrogen [2], however, no causal link between parabens and cancer has been established [3]. The most frequently used parabens in pharmaceutical products are methylparaben and propylparaben. Generally the first one is preferred because as the chain length of the ester group of the parabens increases, antimicrobial activity increases, but water solubility decreases [4]. Usually, the microbial replication occurs in the water phase and hence, the amount of paraben dissolved in the water phase determines the preservative ability [1].

Several methods such as gas chromatography (GC) [5, 6], high performance liquid chromatography (HPLC) [7–10], high performance thin-layer chromatography (HPTLC) [11, 12], micellar electrokinetic capillary chromatography [13, 14] and electrophoretic methods [15, 16] are presented in literature for the determination of parabens in pharmaceutical products. Among them, HPTLC is a widely accepted technique for its high accuracy, precision, reproducibility of results in addition to its low per sample operating cost, easy sample preparation, and short analysis time. The quantitative determination in HPTLC is usually performed in two ways: by slit-scanning or charge coupled (CCD) cameras devices. Standard slit-scanning densitometry measures the absorbance or fluorescence of the chosen tracks on the chromatogram. The main disadvantage of this method is unfavorable error propagation and low spatial resolution since slit-scanning operates by observing a small portion of light emanating from the chromatographic surface defined by the scanning slit [17, 18]. The CCD camera evaluates the TLC plates in several different modes like transmission [18, 19], reflectance [19] or fluorescence, and it has the advantage that the evaluation time is shorter than in slit-scanning densitometry [19]. Also, the comparison between CCD cameras and densitometry, presented in the literature, showed that the CCD cameras offer higher linear concentration ranges than densitometers [19]. In addition, new systems based on digital processing of images of chromatographic plates were recently reported in literature as important TLC methods for quantitative determination of various classes of compounds [20-23].

Therefore the aim of this work was to develop a simple, fast, precise, accurate and sensitive HPTLC method, in fluorescence quenching mode, for the quantitative determination of parabens in pharmaceuticals, using a UV scanner equipped with a CCD camera and specialized software for digital processing of images.

RESULTS AND DISCUSSIONS

Image analysis and chromatograms processing

The new UV scanner device for TLC analysis was used in this study for a quantitative evaluation of chromatographic plates. This device can detect visible and also weak fluorescent spots under UV light at 254nm or 365nm. The scanner captures the visible fluorescence or reflected light using a Charge Coupled Device (CCD) that turns the light into a proportionally electrical signal. Further the electric signal is transformed into digital information and the computer shows the information in an image. The brightness or grey degree is proportional with the concentration of the substance on the TLC plate. A good separation of compounds and a good scanning resolution of the chromatographic plate are very important for an accurate quantitative evaluation. The chosen HPTLC conditions have yielded to a good separation of the investigated parabens ($R_{F(Ethylparaben)}$ = 0.54, $R_{F(Propylparaben)}$ = 0.41, $R_{F(Butylparaben)}$ = 0.30) which appeared as dark spots on the chromatographic plates in UV light (λ = 254 nm). Examples of chromatograms obtained with three different software that combine 2D (ImageDecipher-TLC and Sorbfil TLC) and respectively 3D (JustTLC) image analysis, are presented in Figure 1.

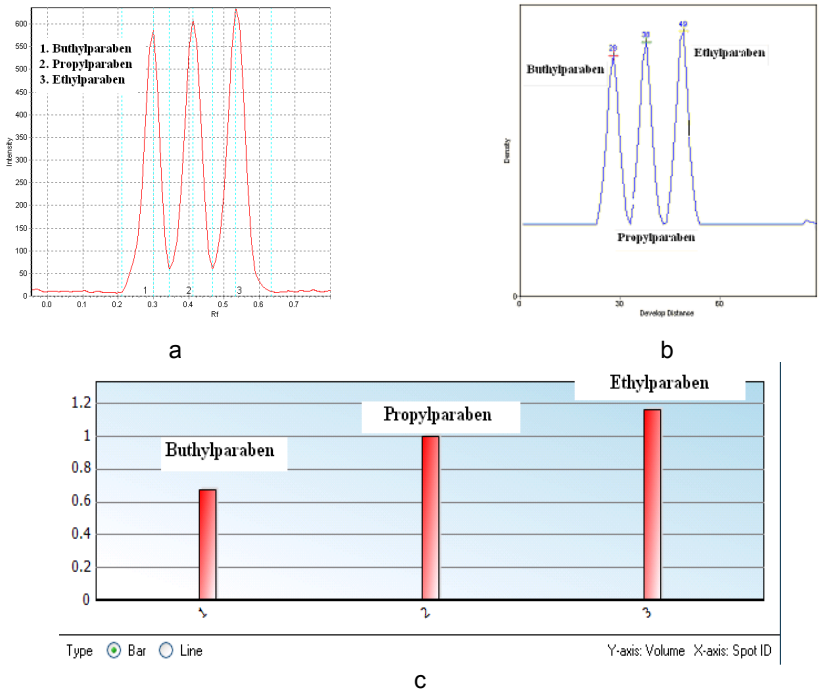

Figure 1. (a) Chromatogram obtained with Sorbfil TLC Videodensitometer software; (b) Chromatogram obtained with ImageDecipher-TLC software; (c) Chromatogram obtained with JustTLC software

Linearity, precision and accuracy of the method

The linear domain of the investigated parabens was studied using six different concentrations of parabens (applied in duplicates) by three different software for digital processing of images of chromatographic plates. The investigated linearity domain was in range 0.300 – 0.800 µg/spot for ethylparaben, propylparaben and butylparaben respectively. The statistical parameters of the linear relation between the applied concentrations and both the peaks area and volume respectively (Table 1), revealed no significant differences in terms of the regression determination coefficient (R^2). By a careful statistical investigation of the results (Table 1) we can observe slightly lower R^2 values in case of the software (JustTLC) that use the intergrated volume for the quantitative evaluation of the chromatographic spots.

The limit of detection (LOD) and quantification (LOQ) were calculated based on confidence bands generated from calibration experiments using ordinary least squares method and the results are presented in Table 1. The lowest LOD and LOQ values were obtained for ethylparaben and butylparaben using the ImageDecipher-TLC software.

The precision of the method was determined on five identical spots at three concentration levels (0.400 µg/spot, 0.600 µg/spot and 0.800 µg/spot) for all of the investigated parabens. The developed chromatographic plates were processed as described before, the precision of the method being estimated in terms of relative standard deviation in all cases. As we can see from the obtained results (Table 2), the best precision (for a quantitative evaluation of the chromatographic spots of parabens) seems to be provided by using ImageDecipher-TLC software. Also, by conversion of color images of chromatographic plates into grey scale, the precision of the developed method increased in all cases.

The accuracy of the proposed method, expressed in terms of recovery, was evaluated at two levels of concentration (0.400 µg/spot and 0.600 µg/spot) using the standard addition method. There have been analyzed solutions with no initial added concentration and solutions with known added concentration of parabens. The results (Table 3) showed no significant differences between recovery values estimated using the investigated software in case of butylparaben and slightly high differences in case of ethylparaben and propylparaben respectively.

Analysis of parabens in pharmaceutical suspensions

On account of the good results obtained for linearity, precision and accuracy of the proposed method, its applicability was assessed for pharmaceutical suspensions analysis (Maalox suspension, Theraplix France). Because the pharmaceutical suspension has a low content of parabens, a sample concentration and purification step was done before TLC analysis.

Table 1. Linearity range, linear regression equations and some statistical parameters for the proposed method

Compounds	Linearity range (µg/spot)	Software	Scale	Regression equation	R^2	LOD (µg/spot)	LOQ (µg/spot)
Ethylparaben	0.300 - 0.800	ImageDecipher-TLC	grey	y = 1506.9x + 225.40	0.9964	0.062	0.117
			red	y = 4562.6x + 753.59	0.9968	0.065	0.122
		Sorbfil	grey	y = 4666.0x + 958.37	0.9935	0.080	0.149
			red	y = 4885.7x + 946.19	0.9962	0.076	0.143
		JustTLC	grey	y = 1.9771x - 0.0158	0.9959	0.058	0.105
Propylparaben	0.300 - 0.800	ImageDecipher-TLC	grey	y = 1201.7x + 146.06	0.9918	0.070	0.131
			red	y = 3680.7x + 460.36	0.9923	0.088	0.164
		Sorbfil	grey	y = 4208.0x + 563.60	0.9964	0.073	0.138
			red	y = 4287.4x + 592.75	0.9937	0.076	0.143
		JustTLC	grey	y = 1.7157x - 0.0245	0.9901	0.093	0.163
Butylparaben	0.300 - 0.800	ImageDecipher-TLC	grey	y = 1391.9x - 18.271	0.9956	0.066	0.125
			red	y = 3973.7x + 146.96	0.9909	0.062	0.118
		Sorbfil	grey	y = 4221.4x + 241.88	0.9947	0.077	0.144
			red	y = 4277.1x + 274.90	0.9958	0.054	0.103
		JustTLC	grey	y = 1.4186x + 0.0573	0.9922	0.071	0.134

Table 2. Precision of the proposed method for three levels of concentration

Compound	Concentration (µg/spot)	Scale	Peak area/volume mean			Standard error of mean			RSD (%)		
			1	2	3	1	2	3	1	2	3
Ethylparaben	0.400	grey	366.80	6125.00	1.55	6.09	417.15	0.07	1.66	6.81	4.44
		red	953.68	15925.00		18.89	1168.02		1.98	7.33	
	0.600	grey	485.80	7696.80	1.99	1.77	170.96	0.02	0.37	2.22	1.19
		red	1263.08	20011.68		5.49	529.97		0.44	2.65	
	0.800	grey	649.20	9928.80	2.30	17.52	227.26	0.11	2.70	2.29	4.86
		red	1687.92	25814.88		54.32	704.49		3.22	2.73	
Propylparaben	0.400	grey	289.60	5710.40	1.38	7.03	364.16	0.04	2.43	6.38	3.00
		red	752.96	14847.04		21.78	1128.88		2.89	7.60	
	0.600	grey	397.60	7424.80	1.84	7.50	126.56	0.08	1.89	1.71	4.07
		red	1033.76	19304.48		23.25	392.34		2.25	2.03	
	0.800	grey	489.60	8920.40	2.13	17.72	297.48	0.06	3.62	3.34	2.70
		red	1272.96	23193.04		54.94	922.19		4.32	3.98	
Butylparaben	0.400	grey	304.80	5472.00	1.02	8.36	231.64	0.05	2.74	4.23	4.96
		red	792.48	14227.20		25.91	718.08		3.27	5.05	
	0.600	grey	414.20	6806.20	1.42	16.47	132.12	0.06	3.98	1.94	3.98
		red	1076.92	17696.12		51.06	409.56		4.74	2.31	
	0.800	grey	489.60	8081.00	1.75	18.68	273.42	0.06	3.81	3.38	3.22
		red	1272.96	21010.60		57.89	847.61		4.55	4.03	

1 – ImageDecipher-TLC; 2 – Sorbfil; 3 – JustTLC

Table 3. Recovery studies carried out for two levels of concentration

Compound	Concentration (µg/spot)	Scale	Found concentration (µg/spot)*			Recovery (%)*		
			1	2	3	1	2	3
Ethylparaben	0.400	grey	0.341	0.293	0.311	85.13	73.36	77.87
		red	0.330	0.297		82.54	74.21	
	0.600	grey	0.585	0.581	0.540	97.58	96.83	89.96
		red	0.557	0.562		92.82	93.61	
Propylparaben	0.400	grey	0.359	0.366	0.368	89.79	91.56	91.97
		red	0.360	0.367		89.93	91.83	
	0.600	grey	0.549	0.577	0.579	91.44	96.19	96.52
		red	0.555	0.576		92.49	96.16	
Butylparaben	0.400	grey	0.432	0.417	0.421	108.03	104.18	105.33
		red	0.441	0.413		110.18	103.15	
	0.600	grey	0.598	0.601	0.578	99.73	100.13	96.39
		red	0.610	0.588		101.67	97.95	

* data are mean of five replicate spots
1 – ImageDecipher-TLC; 2 – SorbfilTLC; 3 – JustTLC

Table 4. Quantitative evaluation of parabens in pharmaceutical suspension

Compound	Added concentration (mg/100 mL)	Scale	Observed concentration (mg/100 mL)			Obtained concentration (mg/100 mL)		
			1	2	3	1	2	3
Propylparaben	0.000	grey	52.410	46.479	48.162	52.410	46.479	48.162
		red	53.508	47.128		53.508	47.128	
	10.000	grey	63.811	54.683	57.176	53.811	44.683	47.176
		red	63.015	56.324		53.015	46.324	

1 – ImageDecipher-TLC; 2 – Sorbfil; 3 – JustTLC

The pharmaceutical suspension labeled with propylparaben content in no specified concentration was analyzed after a sample preparation step (including centrifugation) developed as described in the experimental part. The results obtained for the unspiked and spiked samples of pharmaceutical suspension are presented in Table 4. As it is shown, no statistical significant differences were obtained between values of propylparaben concentration using both the spiked and unspiked samples with ImageDecipher-TLC and JustTLC software respectively.

CONCLUSIONS

In this study a new chromatographic method based on image analysis of TLC plates was developed for simultaneous determination of parabens in pharmaceutical suspensions. For the quantitative evaluation of the chromatographic spots three diffrent software that combines 2D and respectively 3D image analysis were investigated. The obtained results indicated the new ImageDecipher-TLC software based on 2D image analysis as being the most appropriate for simultaneous determination of parabens. Also, the results obtained working in grey scale, proved to be more precise and accurate, comparing to those obtained working in red scale. The proposed sample preparation methodology and the new UV-Vis scanner device for TLC analysis with ImageDecipher-TLC software proved to be a valuable alternative for rapid routine analysis of parabens in pharmaceutical suspensions. The new developed method offer several advantages regarding the effective cost and comparative short analysis time made in reliable and easy reproducible mode.

EXPERIMENTAL SECTION

Reagents

The analytical purity ethyl, propyl and butylparaben, used in this study, were obtained from Sigma-Aldrich (Steinheim, Germany). The analytical grade methanol was obtained from Chemical Company (Iaşi, Romania).

Equipment and software

The standard and sample spots were applied using a semi-automatic sample applicator for qualitative and quantitative TLC analysis (Linomat 5, Camag). The quantitative evaluation of the chromatographic plates was made using BioDit Thin Layer Chromatography (TLC) Scanner (the second-generation instrument for quantitative measurements in TLC) equipped with high qualified Micortek® 3-linear color CCD. ImageDecipher-TLC version 2.0 (BioDit Technology, Co. www.biodoit.com), Sorbfil TLC Videodensitometer (Sorbpolymer,

Krasnodar, Russia) and JustTLC (Sweday, Sweden, www.sweday.com) software were used for digital processing of images and quantification of parabens on the TLC plates. The limit of detection and the limit of quantification (LOD and LOQ) were calculated using SMAC (Statistical Methods in Analytical Chemistry) and Statistica 8.0 software package was used for statistical data treatment.

Standard and Sample Preparation

The stock solution, mixture of ethyl, propyl and butylparaben was prepared by dissolving 0.200 g from each standard in 100 mL ethanol. Six different volumes (with a concentration between 0.300 – 0.800 µg/spot for each of the parabens) of standard stock solution were spotted on the chromatographic plates in duplicate. For the isolation and concentration of the parabens from a pharmaceutical suspension (Maalox suspension, Theraplix France) a centrifugation step was done. 5 mL sample of pharmaceutical suspension was centrifuged 3 times with 5 mL of methanol, at 4000 rpm. After each centrifugation the liquid phase was collected in a flask and filled with methanol to 25 mL. This solution was next used for the TLC analysis.

HPTLC procedure

HPTLC was performed using RP-18WF_{254S} chromatographic plates (20cm x 10cm, Merck, Darmstadt, Germany) and mixture of methanol-water as mobile phase. For a good separation of the parabens, the plates were developed twice: firstly the plates were developed using 60% methanol in mobile phase composition. Then the plates were dried at room temperature for 30 min to eliminate any trace of water, and they were developed again, in the same direction, using 30% methanol in mobile phase composition. In both cases the ascending technique (in a developing chamber saturated for 15 minutes with vapors of mobile phase) and a developing distance of 8 cm were used. After the second elution, the plates were dried at room temperature for 30 min and prepared for scanning process.

Image Analysis

The chromatographic plates were scanned using the BioDit TLC Scanner under UV light at 254 nm and an optical resolution of 300 dpi in order to obtain images of chromatographic plates (bmp file format). The image of the TLC plate was imported directly from the scanner using ImageDecipher-TLC software and the evaluation of the plates was performed by digitalization of images, after their conversion into grey and red scale. For a comparative analysis, the images in grey and red scale, bmp files, were then converted in 'jpg' format and processed by Sorbfil TLC Videodensitometer software in order

to calculate the spots area. Also the images were processed by JustTLC, an advanced digital image analysis software packed with features for editing, quantifying and comparing spots by their automatically detection, only in grey scale. Unlike to the first two investigated software that evaluate the chromatograms in two dimensions (by spot area), the new one truly compare chromatograms in three dimensions performing quantitative analysis based on the spot volumes.

In all cases, the obtained results were based to the fact that both area and volume of the chromatographic spots are proportional with the amount of compound applied on the TLC plate.

Method Validation

For the calibration procedure, six different volumes of stock solution were used and the calibration curve was constructed for each of the parabens, by plotting the measured peaks area or volume versus applied amount of compound. The linearity was characterized by the linear range, the regression equation, and the coefficient of determination value (R^2).

The precision of the method, expressed as relative standard deviation (RSD), was determined at three concentration levels by analyzing five replicate spots for each concentration.

The accuracy of the method, expressed as recovery, was investigated at two concentration levels for five replicate spots using the standard addition method. Known amounts of paraben standards were added to the sample matrix and the sample was processed and analyzed as described above.

REFERENCES

1. M.G. Soni, I.G. Carabin, G.A. Burdock, *Food and Chemical Toxicology*, **2005**, *43*, 985.
2. P.W. Harvey, D.J. Everett, *Journal of Applied Toxicology*, **2004**, *24,* 1.
3. R. Golden, J. Gandy, G. Vollmer, *Critical Reviews in Toxicology*, **2005**, *35*, 435.
4. R.L. Elder, *Journal of the American College of Toxicology*, **1984**, *3*, 147.
5. P. Majlát, E. Barthos, *Journal of Chromatography A*, **1984**, *294*, 431.
6. P. Canosa, I. Rodriguez, E. Rubi, M.H. Bollain, R. Cela, *Journal of Chromatography A*, **2006**, *1124*, 3.
7. L.E. Koundourellis, E.T. Malliou, T.A. Broussali, *Journal of Pharmaceutical and Biomedical Analysis,* **2000**, *23,* 469.

8. R. Hajkova, P. Solich, J. Dvorak, J. Sicha, *Journal of Pharmaceutical and Biomedical Analysis,* **2003**, *32*, 921.
9. G.A. Shabir, *Journal of Pharmaceutical and Biomedical Analysis*, **2004**, *34*, 207.
10. E. Marengo, V. Gianotti, S. Angioi, M.C. Gennaro, *Journal of Chromatography A*, **2004**, *1029*, 57.
11. M. Thomassin, E. Cavalli, Y. Guillaume, *Journal of Pharmaceutical and Biomedical Analysis*, **1997**, *15*, 831.
12. D. Casoni, I.A. Tuhuţiu, C. Sârbu, *Journal of Liquid Chromatography & Related Technologies*, **2011**, *34*, 805.
13. R. Hamoudová, M. Pospisilová, A. Kavalirová, P. Solich, J. Sicha, *Journal of Pharmaceutical and Biomedical Analysis*, **2006**, *40*, 215.
14. S. He, Y. Zhao, Z. Zhu, H. Liu, M. Li, Y. Shao, Q. Zhuang, *Talanta*, **2006**, *69*, 166.
15. B. Baalbaki, M. Blanchin, H. Fabre, *Analytica Chimica Acta*, **2002**, *463*, 15.
16. H. Huang, Y. Lai, C. Chiu, J. Yeh, *Journal of Chromatography A*, **2003**, *993*, 153.
17. L. Zhang, X. Lin, *Journal of Chromatography A*, **2006**, *1109*, 273.
18. I. Vovk, M. Prošek, *Journal of Chromatography A*, **1997**, *779*, 329.
19. C.F. Poole, *Journal of Chromatography A*, **1999**, *856*, 39.
20. F. Soponar, A.C. Moţ, C. Sârbu, *Journal of Chromatography A*, **2008**, *1188*, 295.
21. L. Cieśla, M. Waksmundzka-Hajnos, *Journal of Chromatography A*, **2009**, *1216*, 1035.
22. F. Soponar, A.C. Moţ, C. Sârbu, *Chromatographia*, **2009**, *69*, 151.
23. F. Soponar, A.C. Moţ, C. Sârbu, *Journal of AOAC International*, **2010**, *93*, 804.