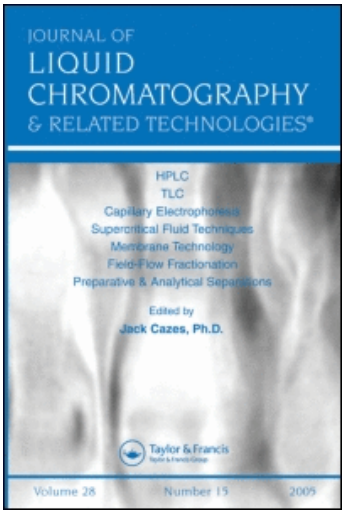

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hubicka, Urszula , Krzek, Jan and Szczyrbowska, Olga(2009) 'TLC-UV and -VIS Densitometric Detection Method for Determination of Oxyphenonium Bromide and its Degradation Products in Tablets', Journal of Liquid Chromatography & Related Technologies, 32: 9, 1196 — 1209
To link to this Article: DOI: 10.1080/10826070902854813
URL: http://dx.doi.org/10.1080/10826070902854813

Journal of Liquid Chromatography & Related Technologies®, 32: 1196–1209, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902854813

TLC-UV and -VIS Densitometric Detection Method for Determination of Oxyphenonium Bromide and its Degradation Products in Tablets

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Abstract: A chromatographic-densitometric method was developed for determination of oxyhyphenonium bromide and its degradation products under UV/VIS spectra. Silica gel TLC_{F254} plates were used as stationary phase and butanol-1–glacial acetic acid–water (12.8:2:2.8 v/v/v) as mobile phase. Densitometric measurements were done at $\lambda \approx 225$ nm and $\lambda \approx 530$ nm. Degradation products α-cyclohexyl-α-hydroxybenzeneacetic acid and *N,N*-diethyl-*N*-methylaminethanol were determined by ^{1}HNMR method. The chromatographic-densitometric method is specific for studied compounds. Besides, it is shown that this method is very sensitive, precise, and has a broad range of linearity.

Keywords: Degradation products, Densitometry, Drug analysis, Oxyphenonium bromide, TLC

INTRODUCTION

Oxyphenonium bromide (BO), 2-[(cyclohexylhydroxyphenylacetyl)oxy]-*N,N*-diethyl-*N*-methylethanaminium bromide (Figure 1) belongs to the class of anticholinergic agents that have an effect on the peripheral

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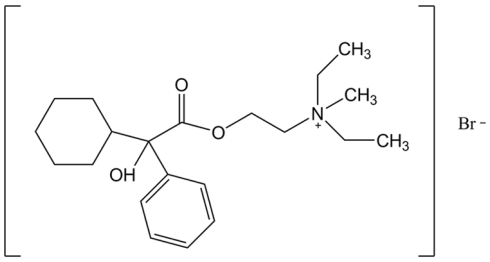

Figure 1. Chemical structure of oxyphenonium bromide.

nervous system. Anticholinergic agents are irreversible acetylcholine esterase inhibitors that work on muscarinic receptors.[1]

Oxyphenonium bromide is an active compound of drug products such as Spasmophen, Antrenyl, Helkamon, and Spastrex. It is administered as a relaxing agent in the therapeutic treatment of digestive tract contraction.[2,3] Oxyphenonium bromide is a representative of chiral compounds whose enantiomers differ in pharmacological and toxicity activities.[4]

Ester constitution of oxyphenonium bromide makes it prone to degradation. No therapeutic activity was observed for the degradation products.[5]

A number of various methods were published regarding determination of oxyphenonium bromide in pharmaceutical or biological materials.

Some of the most popular methods are liquid chromatographic methods, e.g., reverse-phase ion-pair, using two counter ions in the mobile phase.[6,7]

Spectrophotometric[5] and high performance liquid chromatographic (HPLC)[8] techniques have been used for analysis of oxyphenonium bromide and its degradation products.

A radioreceptor assay (RRA) method was used for pharmacokinetic analysis of oxyphenonium bromide. The RRA method is very sensitive (2 ng/mL) and specific.[9–11] Gas chromatography with derivatisation and electron capture detection (ECD) provided good results for oxyphenonium bromide in urine and serum.[12,13]

Serum samples containing oxyphenonium bromide were analyzed by an LC-TSI-MS method.[14] However, LC-MS/MS, in the positive electrospray ionisation mode (ESI), was used for screening of cholinolytic compounds (doping agents) in the urine of race horses.[15,16]

Capillary electrophoresis-mass spectrometry (CE-MS) is a quicker method with the same application as LC-ESI-MS/MS. This method allows for fast detection (12 min) of up to 8 drugs at a concentration of 1 ng/mL each, simultaneously.[17]

In this study, the authors have tried to develop a novel chromatographic-densitometric method for simultaneous detection and identification of oxyphenonium bromide (BO) and its degradation products α-cyclohexyl-α- hydroxybenzeneacetic acid (CHBO) and *N,N*-diethyl-*N*-methylaminethanol (DMA).

EXPERIMENTAL

Apparatus

Densitometer: Camag (Muthenz, Switzerland) TLC Scanner 3 with winCats 1.3.4 software; Sample applicator: Linomat IV manufactured by Camag (Muthenz, Switzerland); NMR spectrophotometer: Mercury VX 300 MHz; Varian (USA)

TLC plates: 10.5 × 10 cm, (cut from 20 × 20 cm precoated TLC sheets of silica gel 60 F_{254} on aluminium; Art. 1.05554; Merck Darmstadt, Germany); Chromatographic chamber: 18 × 9x18 cm (Sigma–Aldrich, St. Louis, MO, USA, Cat. No. Z20, 415–3).

Materials and Methods

Standard Substances and Standard Solutions

Oxyphenonium bromide (LOT OB/008/2006. Resonance, India) was used as a chemical reference substance (CRS). Pollutant substances, DMA and CHBO, were obtained by the method described in the section, "Identification of Degradation products."

Methanol Solutions

BO–0.1 mg/mL and 0.5 mg/mL, DMA–0.25 mg/mL, CHBO–0.25 mg/mL. BO solution for alkaline hydrolysis was prepared. Approximately 0.05 g BO was placed inside a 50.0 mL flask. Then, 50 mL 0.5-M NaOH was added. The flask was capped and incubated in a water bath for 2 hours at 80°C. After cooling to room temperature (RT), 1.0 mL of solution was diluted to 10.0 mL with methanol.

Studied Formulations and Solutions

The Spasmophen formulation: tablets containing 5 mg of oxyphenonium bromide (Lot 20806, Pharmaceutical Works Polfa in Pabianice, Poland) were studied.

For purity studies, the powdered mass of 20 tablets, corresponding 0.2 g BO, was weighed. Then, 2.0 mL water was added to the sample. The sample was shaken for 2 min and 8.0-mL methanol was then added and the whole solution was shaken for another 10 min. The suspension was centrifuged at 3,000 rpm. The resultant solution at a concentration of 20 mg/mL BO was used to perform analysis of DMA and CHBO. For the analysis of BO in tablets, the solution was diluted to a concentration of 0.5 mg/mL.

Reagents

Glacial acetic acid, butanol-1, methanol, sodium hydroxide, hydrochloric acid. All reagents were analytically pure and manufactured by Merck (Germany).

Chromatographic Analysis

Determination conditions for BO were established by spreading degradation products BO solutions (1–30 μL) at concentration of 0.1 mg/mL over thin layer chromatography (TLC) plates, before and after alkaline hydrolysis. Chromatograms were developed in various mobile phases and dried in atmospheric air. Densitometric measurements were carried out in the UV range and VIS range after sprinkling chromatograms with Dragendorff's reagent.

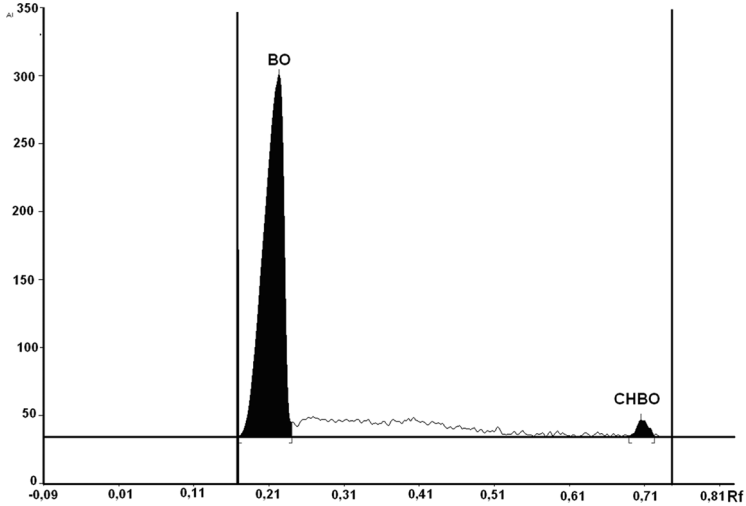

Figure 2. Densitogram of 0.1 mg/mL of oxyphenonium bromide solution recorded under UV.

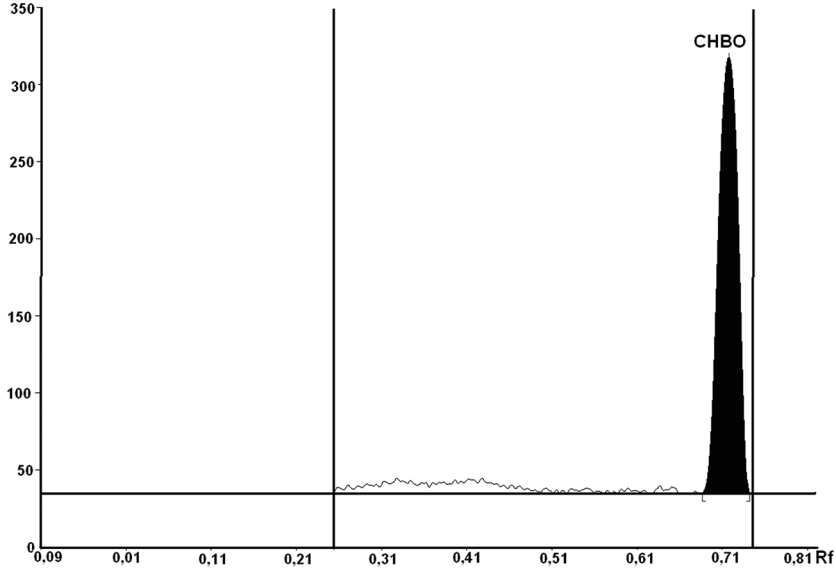

Figure 3. Densitogram of BO solution after alkaline hydrolysis, recorded under UV.

Mobile phase, butanol-1–glacial acetic acid–water (12.8:2:2.8 v/v/v) was chosen to perform analyses on the basis of previous results. A substance-derived main spot ($R_F \approx 0.20$) and an extra small spot ($R_F \approx 0.70$) were detected on chromatograms recorded under UV for the BO

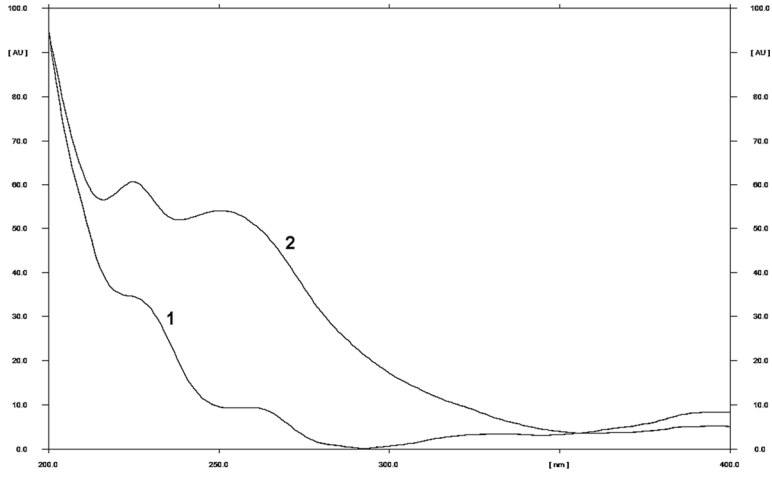

Figure 4. Absorption spectra recorded under UV: 1–BO, 2–CHBO.

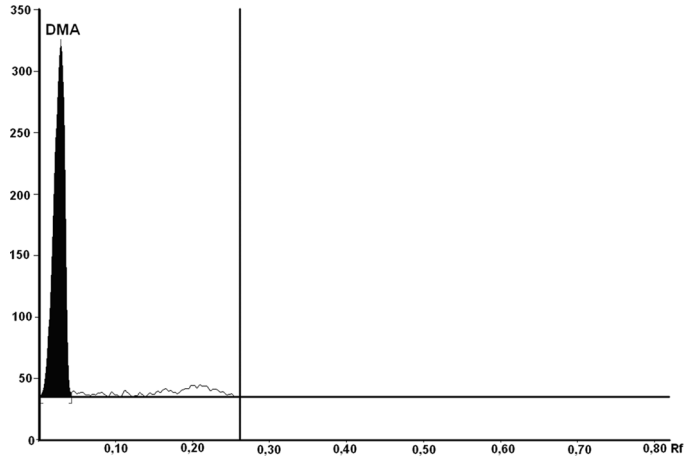

Figure 5. Densitogram of BO solution after alkaline hydrolysis and sprinkling of Dragendorff's reagent.

standard solution (Figure 2). Only one spot ($R_F \approx 0.70$) was observed on the evaluated chromatogram of BO solution after alkaline hydrolysis (Figure 3).

Absorption spectra of spots ($R_F \approx 0.70$) recorded directly from chromatograms had maximum absorption at 225 nm and 252 nm, like the BO spectrum. The only difference between them was in their shape (Figure 4).

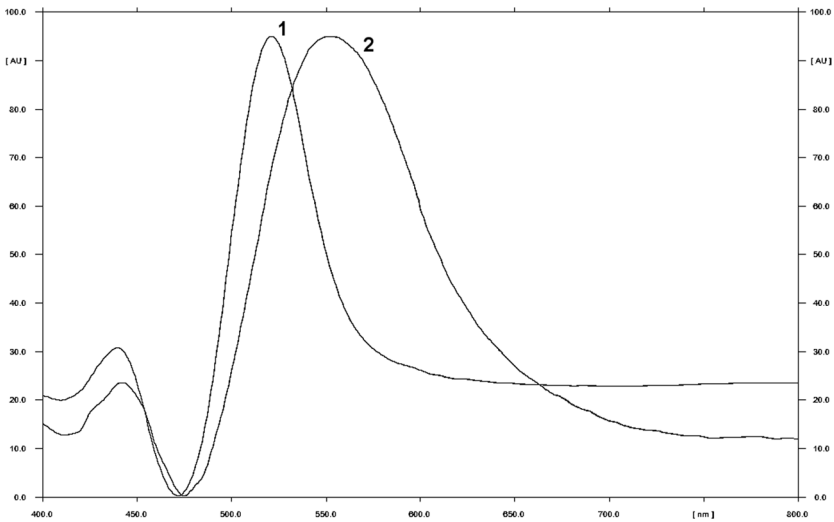

Figure 6. Absorption spectra recorded under VIS: 1–BO, 2–DMA.

Thus, it can be concluded that the analysed spots came from two different compounds.

The chromatograms were evaluated after sprinkling of Dragendorff's reagent in the second stage of study. The studied substance-derived orange spot ($R_F \approx 0.20$) and dark pink smaller spot ($R_F \approx 0.04$) were detected on the chromatogram developed for the BO reference solution. Only one spot ($R_F \approx 0.04$) was detected on the chromatogram of the solution after alkaline hydrolysis (Figure 5). Absorption spectrum of BO recorded from chromatograms had absorption maximum at $\lambda \approx 520$ nm. The absorption maximum for the spots with $R_F \approx 0.04$ were detected at 560 nm. An analytical wavelength for BO and its degradation product was chosen at 530 nm (Figure 6).

Identification of Degradation Products

Preparation of Degradation Products for Studies

Alkaline-hydrolysed BO solution was acidified with 0.1 mol/L HCl to pH $\approx$ 2. During this acidification, some sediment precipitation was observed. The solution was extracted twice with 20 mL of ethyl ether. The two separated phases (water: ether) were distilled off. CHBO and DMA were obtained from the ether and water phases, respectively. Both substances were dissolved in methanol. Both the dissolved solutions at 0.25 mg/mL concentration were spread over TLC plates. One spot ($R_F \approx 0.70$) was observed on the chromatograms of the CHBO solution measured at 225 nm. However, a violet spot ($R_F \approx 0.04$) on a yellow background was observed for the DMA solution after sprinkling of Dragendorff's reagent. Recorded absorption spectra were consistent with spectra obtained for products of hydrolysis of oxyphenonium bromide. In the next step, the substances were analysed by a ^{1}HNMR method.

^{1}HNMR Analysis

^{1}HNMR analyses were done in dimethyl sulfoxide (DMSO). Characteristic signals of phenyl ring protons at δ 7.56 ppm (d, 2H, J = 7.5 Hz)–H_A, δ 7.30 ppm (t, 2H, J = 7.5 Hz)–H_B, and δ 7.21 ppm (t, 1H, J = 7.5 Hz)–H_C were observed in the spectrum of CHBO (Figure 7). Moreover, signals of the cyclohexyl group at δ 2.13 ppm (m, 1H)–H_D and from broad and complex multiplet coming from the rest ring protons at δ 1.8–0.8 ppm (m, 10H) were observed. The hypothesis that CHBO is α-cyclohexyl-α-hydroxybenzeneacetic acid was confirmed on the basis of obtained results.

The signal pattern of two chemically equal ethyl groups were observed in the DMA spectrum at δ 3.36 ppm q, 4H, 7.4 Hz coming from H_C protons

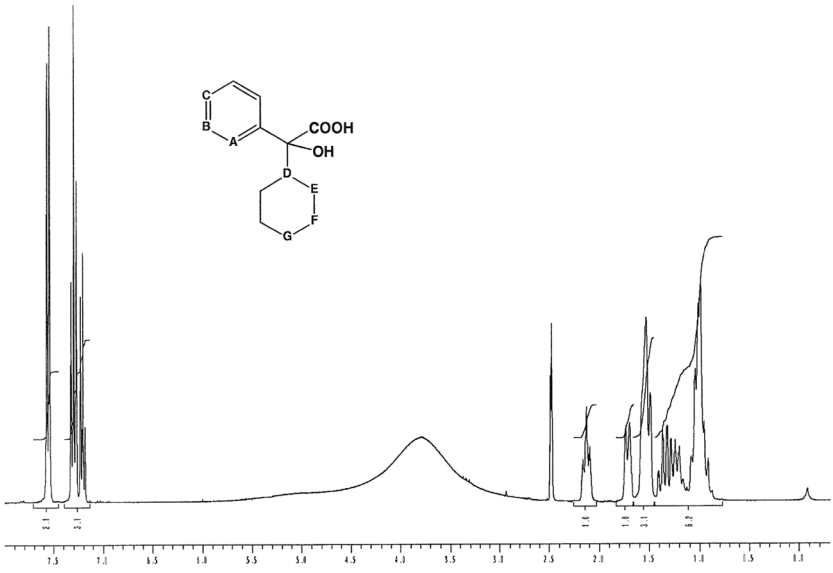

Figure 7. ^{1}HNMR spectrum of degradation product with $R_F \approx 0.70$.

and at δ 1.19 ppm (t, 6H, 7.4 Hz) from H_D. (Figure 8). However, the singlet reflecting protons of the methyl group H_E was detected at δ 2.29 ppm (s, 3H). The signal coming from the CH_2 group (H_B protons) at δ 3.76 ppm (m, 2H) and split signals of H_A protons at δ 5.36 ppm (t, 1H, J = 5.3 Hz) and at δ 3.32 ppm (m, 1H), because of their unequal chemical surrounding (probably caused by rotation stopped), were also observed in the analysed spectrum. The hypothesis that DMA is *N,N*-diethyl-*N*-methylaminethanol was confirmed on the basis of obtained results.

The protocol for determination of BO and its degradation products was established on the basis of chromatographic results. The degradation products may occur in a therapeutic agent during its production or storage.

Carrying out the Analysis

20 μL of reference solutions were applied to TLC plates (10.5 × 10 cm), using a Linomat V applicator, as 0.8 cm width bands with distance of 1 cm from the plate bottom, 0.8 cm from the edge of the plate to the edge of the spot and 0.8 cm between the edges of the spots. The chromatograms were developed as far as 8.5 cm from the start using a mobile phase consisting of butanol-1–glacial acetic acid–water (12.8:2:2.8 v/v/v).

The chromatograms were dried in air (RT). Densitometric measurements in UV were carried out at $\lambda \approx 225$ nm for BO and CHBO. After

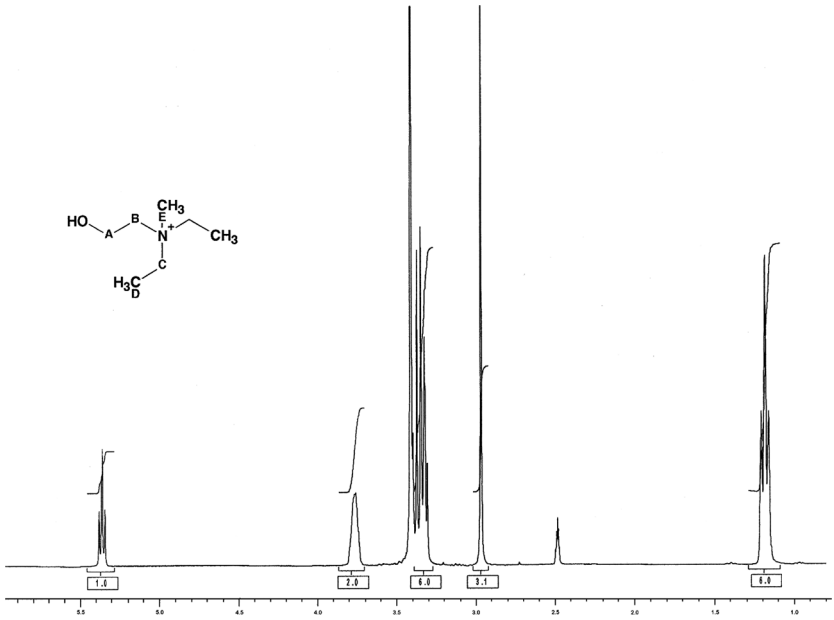

Figure 8. ^{1}HNMR spectrum of degradation product with $R_F \approx 0.03$.

spraying with Dragendorff's reagent, scanning was done at $\lambda \approx 530$ nm for BO and DMA.

To identify the components on the chromatograms, the R_F coefficients and absorption spectra were used. However, for quantity analysis, the size of the spot areas of reference and studied solutions were used.

Validation of the Method

To evaluate the reliability of the developed method according to the requirements of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH),[18] some features such as specificity, linearity, accuracy, limit of detection, and recovery were studied. Densitometric measurements were carried out at 225 nm and 530 nm after sprinkling chromatograms with Dragendorff's reagent.

Specificity of the Method

Studies were done for BO, CHBO and DMA substances with or without placebo. Only spots coming from studied substances with characteristic R_F and absorption values were observed on the chromatograms. This

finding rules out mutually interference of these substances. The peak of DMA was not recorded under UV. Similarly, the peak of CHBO was not also observed under VIS on chromatograms after sprinkling of Dragendorff's reagent. No impact of placebo components was observed in places where spots of studied components occurred.

Based on the obtained results, it can be said that the method used by the authors is specific towards studied analyte.

Linearity

The study of linearity was done by applying freshly prepared reference solutions of 0.50 mg/mL BO (12, 20, 28, 36, 44, 52 μL), 0.25 mg/mL CHBO (2, 4, 6, 8, 10, 12, 14 μL) and 0.25 mg/mL DMA (15, 20, 25, 30, 35, 40 μL) to TLC plates. Developed chromatograms were dried at RT. Then, they were scanned at $\lambda \approx 225$ nm and at $\lambda \approx 530$ nm after sprinkling of Dragendorff's reagent and peak areas were recorded. Linearity was determined as the relationship between the peak areas and the amount of studied substance applied to the spot (μg/spot). A rectilinear run was observed for curves describing this relationship. The correlation coefficient was close to unity. Table 1 depicts all details.

Limit of Detection and Quantitation

Limit of Detection (LOD) and Quantitation (LOQ) values were measured on the basis of curve values obtained for BO, CHBO, and DMA (correlation of spot area with the amount of spread substance over the spot). Calculation was made on the basis of following formulae: $LOD = 3.3\ S_e/a$ and $LOQ = 10\ S_e/a$; where S_e–standard estimation error, a–directional coefficient, slope of a straight line coefficient of area field of peaks on concentrations. Table 1 summarizes the study results.

Precision

Intra-day and inter-day precision methods were determined on the basis of conformity of recorded peak area fields for BO (0.5 mg/mL), DMA (0.25 mg/mL) and CHBO (0.25 mg/mL) solutions. Five analyses were done by applying 20-μL BO, 25-μL DMA and 6-μL CHBO to plates. Table 1 shows results in the UV/VIS range with statistical estimation.

Recovery

Recovery percentage was measured at three concentration levels, i.e., 80%, 100%, and 120% for model solutions where definite amounts of

Table 1. Validation of the method

parameter	BO 225 nm	BO 530 nm	CHBO 225 nm	DMA 530 nm
Limit of detection, μg/spot	2.1	1.8	0.3	0.9
Limit of quantitation μg/spot	6.4	5.6	0.8	2.8
Linearity range, μg/spot	7.0–26.0	6.0–22.0	1.0–3.5	3.0–10.0
Regression coefficients	$a = 357.58$	$a = 799.78$	$a = 2850.61$	$a = 504.94$
$P = ac + b \pm S_e$[a]	$b = 699.23 \pm 227.79$	$b = 1647.64 \pm 446.19$	$b = 341.56 \pm 221.85$	$b = -1046.0 \pm 130.50$
Standard deviation of	$S_a = 13.22$	$S_a = 28.22$	$S_a = 83.85$	$S_a = 24.96$
the regression coefficients	$S_b = 234.10$	$S_b = 405.28$	$S_b = 187.5$	$S_b = 179.65$
Correlation coefficients, r	$r = 0.99728$	$r = 0.99814$	$r = 0.99784$	$r = 0.99515$
Inter-day precision	$x_{sr} = 4372.9$	$x_{sr} = 9792.7$	$x_{sr} = 4638.4$	$x_{sr} = 2236.7$
$n = 5$	$S_d = 122.05$	$S_d = 204.46$	$S_d = 113.80$	$S_d = 54.45$
	RSD = 2.79%	RSD = 2.09%	RSD = 2.45%	RSD = 2.43%
Intraday precision	$x_{sr} = 4601.9$	$x_{sr} = 11397.0$	$x_{sr} = 4826.9$	$x_{sr} = 2415.5$
$n = 5$	$S_d = 80.53$	$S_d = 284.17$	$S_d = 72.90$	$S_d = 72.07$
	RSD = 1.75%	RSD = 2.49%	RSD = 1.51%	RSD = 2.98%
Recovery, %	$x_{sr} = 101.57$	$x_{sr} = 99.77$	$x_{sr} = 101.25$	$x_{sr} = 99.80$
	$S_d = 1.95$	$S_d = 2.79$	$S_d = 2.33$	$S_d = 2.89$
	RSD = 1.92%	RSD = 2.80%	RSD = 2.30%	RSD = 2.90%

[a]P = peak area; c = concentration; a and b = regression coefficients, S_e = standard error of the estimate, S_a = standard deviation of the regression coefficient a, S_b = standard deviation of the regression coefficient b, RSD = Relative standard deviation.

Table 2. The results of the analysis of the contents of BO and also DMA and CHBO contaminations in Spasmophen formulation

Content of BO [mg/tabl]		Percentage contents of degradation products	
225 nm	530 nm	DMA	CHBO
5.17	4.84	0.40	0.12
5.01	5.05	0.41	0.11
5.00	5.23	0.35	0.12
4.97	4.98	0.38	0.11
5.20	4.95	0.40	0.11
5.12	4.88	0.35	0.12
$x_{sr} = 5.08$	$x_{sr} = 4.99$	$x_{sr} = 0.38$	$x_{sr} = 0.12$
$S_d = 0.097$	$S_d = 0.14$	$S_d = 0.026$	$S_d = 0.005$
RSD = 1.91%	RSD = 2.80%	RSD = 6.92%	RSD = 4.76%

suitable components were added. Table 1 shows results; their statistical estimation is an average value for $n = 6$.

Determination of the Concentration of Oxyphenonium Bromide

Studied solutions (20 μL, 0.5 mg/mL) and the same amount of comparative substance were applied to the TLC plates to determine the level of BO in a Spasmophen formulation. The chromatograms were estimated in the UV/VIS range. Quantity analysis of active substance in the formulation was made on the basis of peak areas received for reference and studied solutions. Table 2 shows study results.

Determination of the Content of Degradation Product

The purity of the Spasmophen formulation was studied under the developed conditions. A methanol solution (20 mg/mL, 30 μL) was applied to the TLC plates. Then, the chromatograms were UV/VIS densitometrically analysed. Table 2 shows all study results.

RESULTS AND DISCUSSION

The developed chromatographic–densitometric method allows for analysis of BO and its two degradation products in the UV/VIS range. There are no published data about the usage of TLC in this type of measurement, in spite of the fact that TLC could be an alternative for very expensive and time-consuming methods (HPLC and GC).[8,12,13]

Very good separation of studied substances was obtained by the use of TLC_{F254} plates covered by silica gel (stationary phase) and use of butanol-1–glacial acetic acid–water (12.8:2:2.8 v/v/v, mobile phase). The retention coefficient for the studied substances was as follows: DMA–$R_F \approx 0.04$, BO–$R_F \approx 0.20$, CHBO–$R_F \approx 0.70$. The determination of BO and CHBO can be performed under UV at 225 nm. However, DMA chromatograms have to be sprinkled with Dragengorff's reagent. A spot of BO was also observed on the chromatograms, close to DMA. Both components were densitometrically analysed at $\lambda \approx 530$ nm.

Using an ^{1}HNMR method it was possible to determine the structures of the degradation products (α-cyclohexyl-α-hydroxybenzeneacetic acid (CHBO) and *N,N*-diethyl-*N*-methylaminethanol (DMA)).

It was shown that this method is specific for BO, CHBO, and DMA according to the requirements of ICH Q2. Moreover, detection (LOD) and quantification (LOQ) limits prove that this method is very sensitive. The linearity range is broad and correlation coefficients are almost identical. Values of relative standard deviation show that the precision is good (the values are 1.51%–2.79% under UV and 2.09%–2.98% under VIS). High recovery was obtained. Average values in the UV range was 101.57% (BO) and 101.25% (CHBO), and in the VIS, 99.77% (BO) and 99.80% (DMA).

Usability of this method for determination of BO was confirmed by the analysis of the contents of BO and also DMA and CHBO contaminations in Spasmophen formulation. The obtained results for BO level in the pharmaceutical formulation does not differ much from the declared level of component. However, the level of identified contaminations were 0.12% (CHBO) and 0.38% (DMA).

REFERENCES

1. Reynolds, J.E.F.; Parfitt, K.; Parsons, A. *Martindale the Extra Pharmacopoeia*; The Royal Pharmaceutical Society, 1996.
2. Podlewski, J.K.; Chwalibogowska-Podlewska, A. *Leki Współczesnej Terapii*; Wydawnictwo Fundacji PB Büchnera, 2001.
3. Zając, M.; Pawełczyk, E. *Chemia Leków dla Studentów Farmacji i Farmaceutów*; Wyd. Naukowe AM: Poznań, 2006.
4. Feitsma, K.G.; Postma, D.S.; Koëter, G.H.; Nossent, G.D.; Brenth, B.F.; De Zeeuw, R.A. Comparative study of The bronchodilating effects of (−)- and (+)-oxyphenonium bromide. Brit. J. Clin. Pharmacol. **1988**, *25* (6), 683–687.
5. Grabowska, J. Studies on resolution of oxyphenonium bromide and products of its hydrolysis. Acta Poloniae Pharmaceut. **1971**, *28* (1), 49–53.
6. De Schutter, J.A.; De Moerloose, P. Analysis of Propantheline bromide and related quaternary ammonium drugs by reversed-phase, ion-pair liquid

chromatography with two counterions in the eluent. Chromatogr. **1987**, *23* (9), 667–674.
7. De Schutter J.A.; De Moerloose, P. Reversed-phase ion-pair liquid chromatography of some quaternary ammonium drugs. J. Pharm. Biomed. Anal. **1988**, *6* (6–8), 879–885.
8. El-Gindy, A.; Emara, S.; Shaaban, H. Stability –Indicating method for determination of oxyphenonium bromide and Its degradation product by high-performance liquid chromatography. J. AOAC Intl. **2007**, *90* (5), 1250–1257.
9. Ensing, K.; De Zeeuw, R.A. Radioreceptor assay for oxyphenonium. Eur. J. Respir. Suppl. **1984**, *135*, 143–146.
10. Ensing, K.; Kluivingh, F.; Gording, K.; de Zeeuw, R.A. Development of a sensitive radioreceptor assay for oxyphenonium in plasma and urine. J. Pharm. Pharmacol. **1984**, *36*, 235–239.
11. Lisalo, E.; Kaila, T.; Lauren, L. Radioreceptor assay of anticholinergic drugs. Ann. Clin. Res. **1988**, *20* (5), 367–372.
12. Greving, J.E.; Jonkman, J.H.; Fiks, F.; de Zeeuw, R.A.; van Bork, L.E.; Orie, N.G. Determination of oxyphenonium bromide in plasma and urine by means of ion-pair extraction, derivatization and gas chromatography-electron-capture detection. J. Chromatogr. **1977**, *142* (11), 611–619.
13. Greving, J.E.; Jonkman, J.H.; Westenberg, H.G.M.; De Zeeuw, R.A. Novel Chromatographic methods for monitoring quaternary ammonium compounds in biological fluids. Pharm. World Sci. **1980**, *2* (1), 833–839.
14. Van der Hoeven, R.A.M.; Reeuwijk, H.J.E.M.; Tjaden, U.R.; van der Greef, J. Analysis of quaternary ammonium drugs by thermospray liquid chromatography–mass spectrometry using a resin – based stationary phase. J. Chromatogr. A. **1996**, *741*, 75–84.
15. Ariffin, M.M.; Anderson, R.A. LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood. J. Chromatogr. B. **2006**, *842* (2), 91–97.
16. Yiu, K.C.H.; Ho, E.N.M.; Wan, T.S.M. Detection of quaternary ammonium drugs in equine urine by liquid chromatography–mass spectrometry. Chromatographia (Suppl.). **2004**, *59*, 45–50.
17. Tang, F.; Leung, G.; Wan, T. Analyses of quaternary ammonium drugs in horse urine capillary electrophoresis–mass spectrometry. Electrophoresis. **2001**, *22*, 2201–2209.
18. ICH-Q2 (R1) Validation and Analytical Procedures: Text and Methodology, Int. Conf. Harmoniz., Geneva, November, 2005.

Received January 6, 2009
Accepted January 23, 2009
Manuscript 6469K