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Development and validation of a stability-indicating RP-LC method for the simultaneous determination of otilonium bromide and its expected degradation product in bulk drug and pharmaceutical preparation

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ABSTRACT

A simple, precise, accurate and rapid stability indicating liquid chromatography method had been developed for the simultaneous determination of otilonium bromide (OB) and its expected degradation product; *p*-aminobenzoic acid (PABA) in bulk powder and pharmaceutical preparation. Chromatographic separation was carried out by isocratic elution on Waters Atlantis C18 column (4.6 × 150 mm, 5 µm) using 2 mM ammonium acetate buffer (pH = 2.35) containing 0.05% TFA: acetonitrile (30:70, *v*:*v*) as the mobile phase. The flow rate was 0.8 mL/min with UV detection at 290 nm. Linearity was obtained over a concentration range of 0.5-100 µg/mL with regression coefficient of 1 for OB, and over concentration range of 1-50 µg/mL with regression coefficient of 0.9998 for PABA. The values of LOD and LOQ were found to be 0.0665 and 0.2018 µg/mL for OB and 0.1974 and 0.598 µg/mL for PABA, respectively. The method was validated as per ICH guidelines. The method was successfully applied to the determination of the drug in bulk powder and pharmaceutical preparation.

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1. Introduction

Otilonium bromide is *N*, *N*-diethyl-*N*-methyl-2-[(4-{[2-(octyloxy)benzoyl]amino}benzoyl)oxy]ethanaminium bromide (Figure 1). OB is a member of the quaternary ammonium derivatives widely used for the treatment of hypermotility and hypersensitivity disorders of the intestine, and is used in particular for the treatment of Irritable Bowel Syndrome (IBS) [1]. Irritable bowel syndrome is a complex disease, in which the wide range of symptoms cannot be always attributed to the numerous pathological mechanisms hypothesized. That is the main reason of the lacking of an appropriate specific etiologic therapy [2,3]. OB shows specificity for the smooth muscle of the colon and rectum at therapeutic concentrations, having a local action with negligible systemic absorption [4-6]. Furthermore, OB does not cross the blood-brain barrier and is rapidly eliminated from major organs [7]. OB has been shown to be well tolerated, and it seems to be devoid of the side effects that are typical of the systemically acting anti-muscarinic spasmolytic agents [5,8].

There are few analytical methods reported in literatures for analysis of otilonium bromide either in dosage forms or biological fluids including LC with UV detection [9,10], LC-MS [6,11] and LC-MS/MS [12]. Besides, it has been determined in mixtures with benzodiazepines by capillary electrophoresis [13] and spectrophotometric methods [14-17].

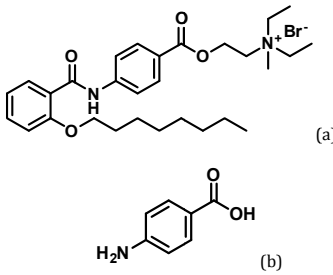

Figure 1. Chemical structures of OB (a) and its degraded product PABA (b).

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Otilonium bromide degraded at room temperature and readily underwent hydrolysis to yield 4-(2-(octyloxy) benzamido)benzoic acid through the ester group in the molecule [18], and the secondary amide group underwent hydrolysis to amine [19]. Therefore PABA is predicted to be one of the degraded products of OB from both hydrolysis. The present work aimed to develop a fast, robust and economic stability-indicating method for the simultaneous determination of OB and PABA (Figure 1) in bulk powder and pharmaceutical preparation.

2. Experimental

2.1. Instrumentation

An Agilent 1260 HPLC system (Germany), equipped with vacuum degasser, quaternary-pump, column oven and UV/VIS detector was used. Separation and quantitation were made on Waters Atlantis C18 column (4.6 × 150 mm, 5 µm). Ultrasonic processor model KBK 4200 (Germany) and SCILOGEX MX-S vortex mixer (Berlin) were used.

2.2. Reagents and reference samples

All chemicals and reagents used were of analytical or HPLC grade. OB (>99%) was kindly provided by Minapharm Co, Egypt. *p*-aminobenzoic acid (99%) and ammonium acetate buffer analytical grade were purchased from Merck, Germany. Acetonitrile (HPLC grade) was purchased from Fisher Scientific, UK. Spasmomen® 40 mg tablet nominally containing 40 mg OB was purchased from a local pharmacy. Deionized water was produced in-house by Barnstead diamond RO, U.S.A.

2.3. Chromatographic conditions

Chromatographic separation was achieved on Waters Atlantis C18 column (4.6 × 150 mm, 5 µm) by applying isocratic elution based on a mobile phase consisting of 2 mM ammonium acetate buffer (pH = 2.35) contain 0.05% TFA: acetonitrile (30:70, *v*:*v*). The mobile phase was pumped through the column at a flow rate of 0.8 mL/min. Analysis was performed at controlled column temperature 25 °C and detection was carried out at 290 nm. The injection volume was 2 µL.

2.4. Preparation of OB standard stock solution

Accurately weight 25 mg of OB was transferred into a 25 mL volumetric flask and dissolved in and completed to the volume with acetonitrile (1 mg/mL).

2.4.1. Preparation of OB working solution

Five mL of OB stock solution was transferred to a 50 mL volumetric flask; the volume was completed to mark with acetonitrile (100 µg/mL).

2.4.2. Preparation of PABA stock solution

Accurately weight 25 mg of PABA was transferred into a 25 mL volumetric flask and dissolved in and completed to the volume with acetonitrile (1 mg/mL).

2.4.3. Preparation of PABA working solution

Five mL of PABA stock solution was transferred to a 50 mL volumetric flask; the volume was completed to mark with acetonitrile (100 µg/mL).

2.5. Sample solution preparation

Ten Spasmomen® 40 mg tablets were accurately weighted, crushed then mixed well. Accurately weighed 128.1 mg of the powder tablets equivalent to 50 mg otilonium bromide, were transferred to 50 mL volumetric flask to which about 30 mL acetonitrile were added. The solution was sonicated for 15 min with intermediate shaking. Finally, the volume was completed to the mark with acetonitrile. The solution was filtered discarding first 10 mL and the filtrate was used as the sample solution (1 mg/mL).

2.6. Procedure

2.6.1. Construction of calibration curves

Different aliquots from OB working solution were transferred into a series of 10 mL volumetric flasks, the volumes were completed with the mobile phase to give solutions containing 0.5-100 µg/mL OB. Alternatively, different aliquots from PABA working solution were transferred into another series of 10 mL volumetric flasks, and completed to volumes with mobile phase to give solutions containing 1-50 µg/mL PABA. A volume of 2 µL of each solution was injected in triplicates into the chromatograph. The conditions including the mobile phase at flow rate 0.8 mL/min and detection at 290 nm were adjusted. Calibration curves were obtained by plotting area under the peak (AUP) against concentration (C).

2.6.2. Assay of OB and PABA in bulk

The procedure mentioned in Section 2.6.1 was repeated using concentrations equivalent to 3-70 µg/mL OB in bulk and 5-45 µg/mL PABA in bulk, respectively.

2.6.3. Assay of OB in laboratory prepared mixtures with PABA

The procedure mentioned in Section 2.6.1 was repeated for simultaneous determination of OB and PABA in laboratory prepared mixtures using concentrations equivalent to 10-96 and 1.2-3.5 µg/mL of OB and PABA, respectively, and completed to the mark by mobile phase (Figure 2). Recovered concentrations of OB and PABA were separately calculated each from its calibration equation.

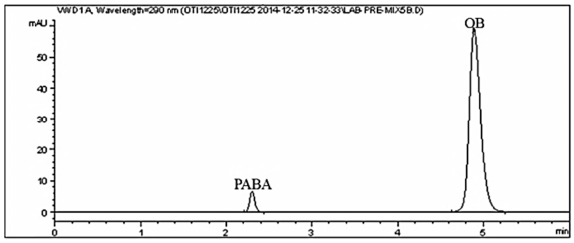

Figure 2. A typical chromatogram of 75 µg/mL OB and 1.5 µg/mL PABA in laboratory prepared mixture.

2.6.4. Assay of OB in pharmaceutical preparations

The sample solution prepared in Section 2.5 was serially diluted, completed with mobile phase and then injected in triplicates (Figure 3). The concentrations of OB were calculated using its calibration equation.

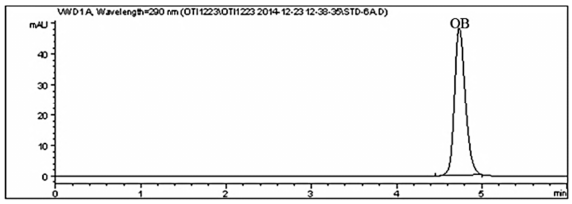

Figure 3. A typical LC chromatogram of 50 µg/mL of otilonium bromide sample.

Table 1. System suitability tests for the proposed LC method.

Item	Data for OB	Data for PABA
N, No of theoretical plates per column	6320	6674
T, Tailing factor	1.16	1.07
%R.S.D. of six injections peak area	0.170	0.101
% R.S.D of six injection of retention time (min)	0.25	0.08
R, Resolution factor	14.63	14.63

%R.S.D: % Relative standard deviation.

Table 2. Results obtained by the proposed LC method for the determination of OB and PABA.

Item	OB	PABA
Retention time	4.97±0.012	2.03±0.002
Wavelength of detection, nm	290	290
Linearity range, µg/mL	0.5-100	1-50
Regression equation	y = 7.0898 x - 1.2818	y = 18.591 x - 0.4337
Regression coefficient (r^2)	1.000	0.9998
S_a, Standard deviation of intercept	1.0000	3.8277
S_b, Standard deviation of slope	0.0200	0.1264
Confidence limit for the slope	7.0898±0.0541	18.548±0.3514
Confidence limit for the intercept	-1.2817±2.821	-0.4337±10.65
Limit of quantification, LOQ, µg/mL	0.2018	0.5980
Limit of detection, LOD, µg/mL	0.0665	0.1974
Drug in bulk	100.31±2.142	99.96±1.615
Recovery for Standard added	100.07±0.422	-
Recovery for drug in laboratory prepared mixtures	101.35±0.759	101.57±0.816

3. Results and discussion

3.1. Method development

The core intention of this paper was to develop a stability indicating assay for OB with a short run time to allow for the high capacity of quality control laboratories. Thus, the conditions affecting the chromatographic performance were carefully studied in order to recognize the most suitable chromatographic system for determination of OB in presence of its degradation product, PABA. The choice was based on the highest number of theoretical plates and the best resolution between the two peaks. The selection of the mobile phase is of prime importance in the development of a chromatographic technique for proper elution, resolution, symmetrical peak shapes and reproducibility of the analytes [20]. Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers. Methanol was tried as organic modifier; broad peaks with insufficient separation were obtained. Triethylamine and 1-octane sulfonic acid sodium salt monohydrate as ion pairing reagents in the aqueous phase were tried to control the tailing but this did not give a satisfactory peaks tailing factor. The separation was investigated using mobile phases containing (30 and 40%) of acetonitrile with phosphate buffer with different pH's (3.0, 4.5, and 6.5). It was found that peak skewing occurred with increasing the ratio of acetonitrile in the mobile phase while it had slight decreasing effect on the retention time. A mobile phase containing 70% acetonitrile was enough to give sharp symmetric peaks within a short runtime with reasonable resolution and sensitivity. The separation was investigated using mobile phases containing (50, 60, and 70 %) of phosphate buffer (pH = 3.0). It was found increasing the ratio of the buffer lead to longer run times with broad peaks, as phosphate buffer lead to inadequate resolution between OB and its degradation product, finally when acetate buffer was tried (30 %) pH = 2.35 with acetonitrile (70%), it gave sharp peak with shorter run time. The mobile phase was pumped at a flow rate of 0.8 mL/min which was suitable for good separation within a reasonable time.

The chromatographic separation was tried using Discovery cyano column (Supelco) with dimension (15 cm × 4.6 mm, 5 µm) and Hypersil BDS C18 (15 cm × 4.6 mm, 5 µm). However, serious problems occurred in the determination of OB, in particular excessively tailed peaks, due to its quaternary ammonium nature. Inadequate resolution, broad peak and long retention time were obtained with the previous columns. But using Waters Atlantis C18 column (4.6 × 150 mm, 5 µm); less hydrophobic column showed better peak shapes within reasonable run time and satisfactory separation hence it became the column of choice for this study.

Detection was tried at 224, 254 and 290 nm but finally 290 nm was chosen as it was found optimum for measurements.

3.2. System suitability tests

The system suitability tests were performed to ensure that the proposed LC method was suitable to the analysis intended. The parameters of these tests are column efficiency (number of theoretical plates), tailing of chromatographic peak, repeatability as %R.S.D of peak area for six injections and reproducibility of retention as %R.S.D of retention time of a solution of a 50 µg/mL of OB (100% concentration) and 25 µg/mL of its degradant. The results of these tests for the proposed method are listed in Table 1.

3.3. Method validation

3.3.1. Linearity

In this study, eight concentrations were chosen for OB and six concentrations were chosen for PABA. Each concentration was analyzed three times and linearity was studied for both drugs. A linear relationship between area under the peak (AUP) and concentrations (C) was obtained and the regression equation for OB and PABA was computed, Table 2. The linearity of the calibration curve was validated by the high value of correlation coefficient.

3.3.2. Accuracy

Accuracy of the results was calculated by % recovery of 6 concentrations (injected in triplicates) of OB and PABA in bulk powder and in mixtures, and also by standard addition technique applied to Spasmomen® 40 mg. The results obtained are displayed in Table 2.

3.3.3. Precision

The repeatability (intra-day) was assessed by three determinations for each concentration (40, 50 and 60 µg/mL) for OB and (20, 25 and 30 µg/mL) for PABA representing 80, 100 and 120%, respectively.

Table 3. Results for determination of repeatability of OB in powder and in presence of PABA by proposed LC method.

Sample	OB in powder			OB in presence of PABA		
Concentration	40 µg/mL 80%	50 µg/mL 100%	60 µg/mL 120%	40 µg/mL 80%	50 µg/mL 100%	60 µg/mL 120%
Mean	40.8473	51.0867	61.0862	40.8230	50.7244	59.6314
%Recovery	102.12	102.17	101.81	102.06	101.45	99.39
S.D.	0.1294	0.2262	0.2895	0.1141	0.2547	0.6080
%R.S.D	0.3168	0.4428	0.4739	0.2795	0.5021	1.0196

Table 4. Results for determination of repeatability of PABA in bulk powder and in presence of OB by proposed LC method.

Sample	PABA in powder			PABA in presence of OB		
Concentration	20 µg/mL 80%	25 µg/mL 100%	30 µg/mL 120%	20 µg/mL 80%	25 µg/mL 100%	30 µg/mL 120%
Mean	19.7837	24.7078	29.6433	19.8655	24.7974	29.4563
%Recovery	98.92	98.83	98.81	99.32	99.19	98.19
S.D.	0.0876	0.2096	0.2019	0.0706	0.1302	0.2587
%R.S.D	0.4429	0.8481	0.6809	0.3556	0.5251	0.8782

Table 5. Results for determination of inter-day assay for OB in bulk powder and in presence of PABA by proposed LC method.

Sample	Inter-day in bulk powder			Inter-day in presence of PABA		
Concentration	40 µg/mL 80%	50 µg/mL 100%	60 µg/mL 120%	40 µg/mL 80%	50 µg/mL 100%	60 µg/mL 120%
Mean	40.49	50.99	60.39	40.74	50.30	59.76
%Recovery	101.23	101.98	100.65	101.85	100.60	99.60
S.D.	0.37	0.34	0.73	0.24	0.43	0.53
%R.S.D.	0.91	0.67	1.21	0.58	0.85	0.89

Table 6. Results for determination of inter-day assay for PABA in bulk powder and in presence of OB by proposed LC method.

Sample	Inter-day assay for PABA in bulk powder			Inter-day assay for PABA in presence of OB		
Concentration	20 µg/mL 80%	25 µg/mL 100%	30 µg/mL 120%	20 µg/mL 80%	25 µg/mL 100%	30 µg/mL 120%
Mean	19.89	24.96	29.92	20.04	24.99	29.86
%Recovery	99.45	99.84	99.73	100.2	99.96	99.53
S.D.	0.13	0.20	0.38	0.20	0.21	0.41
%R.S.D	0.65	0.82	1.26	0.99	0.83	1.38

Table 7. Influence of mobile phase flow rate on resolution of OB in presence of PABA.

Flow rate	0.7 mL/min	0.8 mL/min	0.9 mL/min
R, resolution factor	14.81	14.63	14.08

Table 8. Influence of acetonitrile strength of mobile phase on resolution of OB from PABA.

% of acetonitrile	68%	70%	72%
R, resolution factor	14.25	14.63	14.24

Table 9. Influence of change of pH of mobile phase on resolution of OB.

pH	2.25	2.35	2.45
R, resolution factor	14.45	14.63	14.66

The values of precision (%R.S.D.) of repeatability for both OB and PABA were found to be less than 1% in the three concentrations, (Table 3 and 4). Inter-day precision was evaluated through replicate analysis of 40, 50 and 60 µg/mL and 20, 25 and 30 µg/mL for OB and PABA for three successive days. The values of precision (%R.S.D.) for OB and PABA were found to be less than 2% (Table 5 and 6).

3.3.4. Selectivity

Selectivity is the ability of the analytical method to measure the analytes in the presence of interferences including degradation products or impurities, related substance and matrix components. In the present work, OB was determined and no chromatographic interference from any degradation products or excipients was found at the retention time of examined drug (Figure 3). In addition, the chromatogram of the sample solution was found identical to the chromatogram received by OB standard solution. These results demonstrate the absence of interference from other materials in the pharmaceutical formulation and therefore confirm the selectivity of proposed method.

3.3.5. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using following equation as per ICH guidelines [21].

$$LOD = 3.3 \times \sigma / S \quad (1)$$

$$LOQ = 10 \times \sigma / S \quad (2)$$

where σ is the standard deviation of response and S is the slope of the calibration curve, the results are shown in Table 2.

3.3.6. Robustness

Robustness was performed by deliberately changing the chromatographic conditions. Only one factor was changed at a time while the other kept constant. The factor measured after each change was the resolution factor between the two peaks. Changing the flow rate of mobile phase by ±0.1, acetonitrile ratio by ±2%, Varying the pH of the mobile phase by ±0.1 didn't have significant effect on chromatographic resolution by the proposed LC method for OB and its degradation product, indicating good robustness of the proposed method as shown in Tables 7-9.

4. Conclusion

The proposed LC method has the advantage of simplicity, precision, accuracy and convenience of separation and quantification of OB alone or in the presence of its expected degradation product in a reasonable analysis time. Moreover the proposed method is capable of simultaneous determination OB and it's degradant in laboratory prepared mixture and pharmaceutical formulation. Hence, the proposed LC method can be used for quality control of cited drug.

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