Analytical Chemistry Insights

ORIGINAL RESEARCH

Utility of Experimental Design in Pre-Column Derivatization for the Analysis of Tobramycin by HPLC—Fluorescence Detection: Application to Ophthalmic Solution and Human Plasma

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Abstract: A novel, selective, and sensitive reversed phase high-performance liquid chromatography (HPLC) method coupled with fluorescence detection has been developed for the determination of tobramycin (TOB) in pure form, in ophthalmic solution and in spiked human plasma. Since TOB lacks UV absorbing chromophores and native fluorescence, pre-column derivatization of TOB was carried out using fluorescamine reagent (0.01%, 1.5 mL) and borate buffer (pH 8.5, 2 mL). Experimental design was applied for optimization of the derivatization step. The resulting highly fluorescent stable derivative was chromatographed on C_{18} column and eluted using methanol:water (60:40, v/v) at a flow rate of 1 mL min^{-1}. A fluorescence detector (λ_{ex} 390 and λ_{em} 480 nm) was used. The method was linear over the concentration range 20–200 ng mL^{-1}. The structure of the fluorescent product was proposed, the method was then validated and applied for the determination of TOB in human plasma. The results were statistically compared with the reference method, revealing no significant difference.

Keywords: tobramycin, experimental design, HPLC, derivatization, fluorescamine, plasma

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Introduction

Tobramycin (TOB, Fig. 1) is an aminoglycoside antibiotic produced by *Streptomyces tenebrarius*. It exhibits a broad spectrum activity against aerobic gram-negative bacteria, particularly *Pseudomonas aeruginosa,* which makes it the antibiotic of choice in the treatment of pulmonary infections.[1,2] The bactericidal activity of TOB is accomplished by inhibiting ribosomal function leading to interruption in bacterial protein synthesis.[3] It is used topically for treatment of eye infections, parenterally for treatment of serious bacterial infection, and also for local application in the oral cavity and stomach as part of selective decontamination of the digestive tract.[1,4] Like other aminoglycosides, the use of TOB can create potential dose-related side effects of ototoxicity and nephrotoxicity. Even though it is poorly absorbed, prolonged oral administration can produce such toxic effects.[3] Therefore, careful monitoring of the drug level in plasma is required for therapeutic and toxic control, especially when therapy is of long duration.[5]

Several analytical techniques were reported for the analysis of TOB in dosage forms and in biological fluids including spectrophotometry,[6–9] spectrofluorimetry,[6,7,10] capillary electrophoresis,[11] and TLC densitometry.[12,13] A number of high-performance liquid chromatography (HPLC) methods were described using specific detection modes such as evaporative light scattering detection,[14,15] pulsed electrochemical detector, and tandem mass spectrometry.[16–18] Chemically, TOB consists of amino sugars linked glycosidically with 1,3-diaminocyclohexane central ring.[19] Like most carbohydrates, TOB lacks UV absorbing chromophores and does not possess native fluorescence, leading to a major challenge in the analysis of such a compound due to problematic detection.[20] Therefore, derivatization with a suitable absorbance-enhancing or fluorescence-producing agent is required for the detection by chromatographic techniques. HPLC methods with fluorescence detection, after derivatization[21] or indirect fluorescence detection, based on ligand displacement[22] were previously employed. However, most of these techniques have various limitations, for example, the use of 2,4,6-trinitrobenzenesulfonic acid[23] and 1-fluro-2,4-dinitrobenzene[24] as pre-column derivatizing agents is undesirable due to their high toxicity. The main disadvantages of 2,4-dinitrofluorobenzene reagent, employed by USP,[25] and fluorescein isothiocyanate[21] were the length of time and the temperature required to achieve the reaction. O-phthalaldehyde, used in post-column derivatization, led to the formation of a derivative with poor stability.[26] Therefore, the objectives of this work were to employ a non toxic derivatizing agent and to enhance the formation of a more stable fluorescent derivative while maintaining high sensitivity.

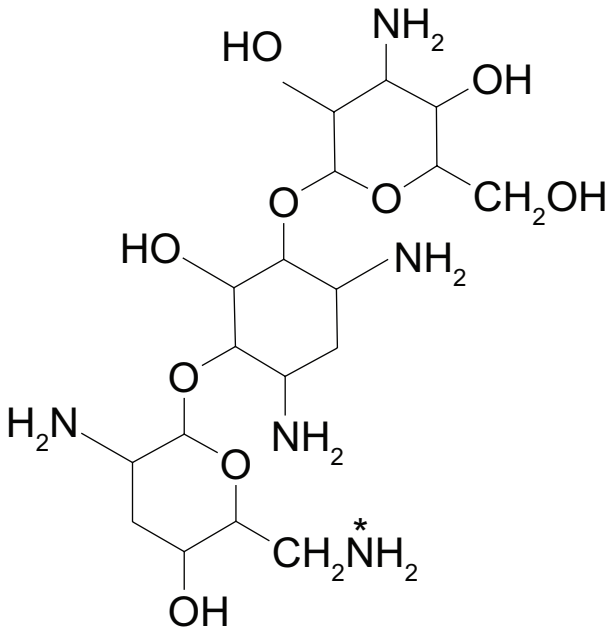

Figure 1. Chemical structure of TOB.

Fluorescamine reagent is a useful derivatizing reagent that reacts with primary amino group to form fluorescent pyrrolinone moieties.[27] Optimization of the pre-column derivatization step was performed using Design of Experiments (DOE) approach. The chemometric approach requires a relatively limited number of experiments to define the factors which affect the derivatization reaction and to obtain the optimum conditions for the formation of fluorescent derivative.[28,29]

This manuscript describes the development of a new HPLC method coupled with fluorescence detection for the analysis of TOB after pre-column derivatization. The validated method was applied for the determination of TOB in eye drops and in spiked human plasma.

Experimental

Instrumentation

All fluorescence measurements were carried out using a Shimadzu RF—1501 Spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan),

with excitation and emission band pass of 5 nm using 1 cm quartz cell. Experimental matrices, three dimensional (3D) surface plots, and contour curves were generated using Minitab (Version 15) statistical software (State College, Pennsylvania, USA). The chromatographic system was composed of a solvent delivery (LC-10AD, Shimadzu, Japan), a system controller model CBM-20A Communications BUS module and a spectrofluorometric detector (RF-551) with excitation and emission wavelengths set at 390 nm and 480 nm, respectively. Separation was achieved on Waters C_{18} column (250 × 4.6 mm, i.d.) packed with 5 µm particle size (USA). The mobile phase was composed of methanol:water (60:40, v/v) and pumped at 1 mL min^{-1} flow rate. The mobile phase was filtered through 0.45 µm membrane filter (Sartorius Stedim Biotech GmbH, Germany) and degassed before use. All the work was carried out at room temperature, at Center of Applied Research and Advanced Study (CARAS) in Faculty of Pharmacy, Cairo University.

Materials and reagents

All chemicals and solvents were of analytical reagent grade. TOB sulfate pure sample was kindly supplied by Sigma Chemical Co., Germany. Its purity was found to be 99.92 ± 0.56 according to the reference spectrophotometric method.[30] Pharmaceutical dosage form containing TOB sulfate was purchased from the local market. Tobrin® sterile ophthalmic solution (Batch No. 1202155) was labeled to contain 0.3% TOB base and was manufactured by Egyptian Int. Pharmaceutical Industries CO. (E.I.P.CO.), 10th of Ramadan City, Egypt. Fluorescamine was purchased from Sigma-Aldrich Chemie GmbH, Germany and a stock solution of 0.01% w/v was prepared in acetone (Chromasolv, Sigma—Aldrich Chemie GmbH, Germany). The solution was stable for at least 7 days, if kept in the refrigerator. Aqueous borate buffer solution (pH 8.5) was prepared by mixing appropriate volumes of 0.2 M boric acid/0.2 M potassium chloride with 0.1 M sodium hydroxide and adjusting the pH to 8.5 using pH meter.[31] Boric acid, potassium chloride and sodium hydroxide (El-Nasr Pharmaceutical Chemicals, Egypt) were of analytical reagent grade. Drug free human plasma was obtained from blood transfusion center, Cairo University, Kasr El-Aini hospital (Cairo, Egypt) and stored at –20 °C until use after gentle thawing to room temperature.

Preparation of the standard solutions

Stock solution of TOB sulfate was prepared by dissolving 10 mg of the drug in 100 mL of distilled water. This solution was further diluted with the same solvent in order to obtain a working standard solution of a final concentration of 1 µg mL^{-1} of TOB sulfate.

Experimental design for optimization of pre-column derivatization reaction

A three-level face centered composite (FCC) design with five center points was applied to evaluate main, interaction, and quadratic effects of the factors affecting the pre-column derivatization reaction. Buffer pH, volume of the buffer, and volume of fluorescamine reagent were investigated in three different levels of each. Table 1 shows the experimental planning proposed by DOE. A total of 20 experiments, including five central points, were conducted, each experiment corresponds to a particular combination of the different levels of factors. The corresponding fluorescence intensities were measured and the data were analyzed using Minitab (version 15) statistical software. The model obtained was described by the following general mathematical second-order equation:[32]

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

Where Y is the response; b_0 is the arithmetic mean response; b_1, b_2 and b_3 are the regression coefficients of the factors X_1, X_2 and X_3, respectively; b_{12}, b_{13} and b_{23} are interaction terms; and b_{11}, b_{22} and b_{33} are square regression coefficients terms. The terms b_1X_1, b_2X_2 and b_3X_3 are the individual effects of each factor. $b_{12}X_1X_2$, $b_{13}X_1X_3$ and $b_{23}X_2X_3$ indicate the interaction among the factors, and the terms $b_{11}X_1^2$, $b_{22}X_2^2$ and $b_{33}X_3^2$ are the quadratic terms of each factor.

Construction of calibration graph

Aliquots of TOB sulfate working standard solution containing a final drug concentration of 20–200 ng mL^{-1} were transferred into a series of 10 mL volumetric flasks. To each flask, 2 mL of borate buffer with pH 8.5 were added followed by 1.5 mL of FL reagent (0.01% w/v).

Table 1. Experimental matrix and experimental plan of the face centered composite design.

Number of experiments	Experimental variables					
	X_1	X_2	X_3	Buffer pH	Volume of buffer (mL)	Volume of fluorescamine (mL)
1	–1	–1	–1	7.5	1	0.5
2	0	1	0	8.5	3	1.0
3	0	0	1	8.5	2	1.5
4	0	–1	0	8.5	1	1.0
5	1	–1	–1	9.5	1	0.5
6	0	0	0	8.5	2	1.0
7	–1	1	1	7.5	3	1.5
8	0	0	0	8.5	2	1.0
9	0	0	0	8.5	2	1.0
10	1	–1	1	9.5	1	1.5
11	–1	0	0	7.5	2	1.0
12	1	1	1	9.5	3	1.5
13	–1	–1	1	7.5	1	1.5
14	0	0	0	8.5	2	1.0
15	–1	1	–1	7.5	3	0.5
16	1	0	0	9.5	2	1.0
17	–1	0	0	8.5	2	1.0
18	1	1	–1	9.5	3	0.5
19	0	0	–1	8.5	2	0.5
20	0	0	0	8.5	2	1.0

Each solution was shaken for 5 minutes and then filled to flask volume with distilled water. After standing for 15 minutes, 20 µL portions of each solution were injected in three replicates into the chromatograph. The eluents were detected by the fluorescence detector with the wavelength of excitation fixed at 390 nm and that of emission fixed at 480 nm. The signals emerging from the detector were integrated as peak area and a calibration graph was obtained by plotting the peaks areas against the corresponding concentrations of TOB sulfate and the regression equation was computed.

Analysis of pharmaceutical dosage form

Sample solution of TOB was prepared at a concentration of 1.2 µg mL^{-1} by diluting 1 mL of Tobrin® eye drops (equivalent to 3 mg TOB base) to 25 mL with distilled water, then diluting 1 mL of the resulting solution to 100 mL with the same solvent. Different aliquots of the obtained solution were analyzed using the procedure mentioned in the section "Construction of calibration graph."

Procedure for spiked human plasma

Aliquots of drug free human plasma (1 mL) were transferred into a series of centrifuge tubes and spiked with different concentrations of TOB sulfate standard solution. Acetonitrile (2 mL) was then added to each tube to precipitate the plasma proteins. The samples were mixed by vertical agitation (2 minutes) using vortex apparatus and centrifuged at 3000 rpm for 5 minutes. The resulting supernatant was transferred into a clean glass tube and evaporated to dryness in a water bath (40 °C). The residue obtained was reconstituted in distilled water (1 mL) and the obtained solutions were analyzed using the procedure mentioned in section "Construction of calibration graph." The area under the peak, arising at a retention time 3 ± 0.1 minutes, was recorded and the concentration of the drug in plasma was determined by using the regression equation.

Determination of the stoichiometry of the reaction

Based on the optimum experimental conditions, the stoichiometry of the reaction was studied by adopting the limiting logarithmic method.[33] Log of the molar concentration of fluorescamine was plotted versus log of the fluorescence intensity using constant concentration of TOB. Additionally, log of the molar concentration of TOB was plotted versus log of the fluorescence intensity using constant concentration

of fluorescamine. The slopes of the two straight lines obtained were measured.

Results and Discussion

TOB structure is characterized by the presence of saturated ring system that does not exhibit any significant absorption in the UV and visible region. The very low absorptivity of TOB does not permit its direct quantification at low concentrations, particularly if there is need to estimate the drug in plasma or in biological fluids.[20] This problem could be solved by derivatizing the compound by a suitable fluorogenic reagent. Fluorescamine offers enhanced fluorescence detection of primary amine.[27] It has no inherent fluorescent property and is quickly hydrolyzed in water. Additionally, degradation products are non-fluorescent. Therefore, the fluorescence of the solution containing amines and fluorescamine is proportional to the quantity of free amine groups present. Fluorescamine is widely used as a derivatizing reagent for the determination of many drugs, such as amoxicillin[34] and oseltamivir.[35] Since TOB contains primary aliphatic amino group, it reacted with fluorescamine in alkaline medium, at room temperature. The condensation reaction was complete in a few minutes and formed a highly fluorescent pyrrolinone derivative. The formed stable fluorescent derivative of TOB was quantified in ophthalmic solution and spiked human plasma using HPLC method coupled with fluorescence detection.

Optimization of the pre-column derivatization reaction conditions

The traditional method performed for optimization of reaction conditions was based on changing one variable at a time (OVAT approach) which did not depict the combined effects of all the variables involved in the reaction.[29] Therefore, experimental design using FCC design was applied to facilitate method development by varying all the factors together. FCC design has distinct advantages, such as the use of minimum number of experiments and feasibility of generating data that can be analyzed statistically to provide valuable information on the interactions among experimental parameters.

Scanning of the excitation and emission spectra of the reaction product of fluorescamine with TOB showed that optimal excitation and emission wavelengths were 390 nm and 480 nm, respectively (Fig. 2). Distilled water was the solvent of choice for the drug as alcohols were found to react with fluorescamine to form additional products that could drastically reduce the reactivity of the reagent toward primary amines.[36] Borate buffer was found to be suitable as it contains no primary amines. The factors selected for consideration were buffer pH (X_1), volume of borate buffer (X_2), and volume of fluorescamine reagent (X_3).

The FCC design was applied and twenty experiments were conducted using the levels described in Table 1. The coefficients of the second—order polynomial model were computed and the following equation was deduced:

$$Y = -4725.49 + 1112.3X_1 + 51.39X_2 + 539.12X_3 - 60.86X_1^2 + 1.28X_2^2 - 129.08X_3^2 - 13.19X_1X_2 - 19.67X_1X_3 + 37.51X_2X_3 \quad (2)$$

Where Y is the fluorescence intensity; and X_1, X_2 and X_3 are buffer pH, volume of buffer and volume of fluorescamine reagent, respectively.

From the values obtained for the parameters in the FCC design (Table 2), it could be established that the fluorescence intensity is directly related to all the three factors. Two of the factors have significant influence on the response, buffer pH, and volume of fluorescamine reagent ($P < 0.05$) which significantly increases the fluorescence intensity when they are increased. However, the significant quadratic term ($X_1^2, P = 0.024$) indicates non-linear correlation between the factor and the response, as revealed in the interaction plots (Fig. 3). The individual effects of buffer pH and volume of fluorescamine are positive while their quadratic effects are negative, thus

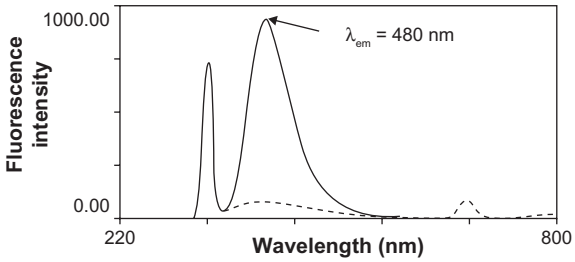

Figure 2. Excitation and emission spectra of the derivatized TOB produced by reaction between 200 ng mL^{-1} of TOB sulfate and fluorescamine (____) and reagent blank (------) (λ_{ex} = 390 nm, λ_{em} = 480 nm).

Table 2. Estimated regression coefficients and associated probability values (*P*-value) for fluorescence intensity.

Term	Coefficients	*P*
Constant	–4725.49	0.000
Buffer pH	1112.30	0.025
Volume of buffer	51.39	0.162
Volume of fluorescamine	539.12	0.000
Buffer pH * Buffer pH	–60.86	0.024
Volume of buffer * volume of buffer	1.28	0.957
Volume of fluorescamine * volume of fluorescamine	–129.08	0.188
Buffer pH * volume of buffer	–13.19	0.348
Buffer pH * volume of fluorescamine	–19.67	0.479
Volume of Buffer * volume of fluorescamine	37.51	0.192

indicating that the fluorescence intensity increases with increase of the factor up to a critical threshold after which a further increase results in a decrease in the response (level 0 of X_1 and level 1 of X_3 were chosen). In addition, the interaction between the three factors is not significant ($P > 0.05$).[37]

Pareto charts (Fig. 4) reveal that the factors which were statistically significant ($P < 0.05$) are buffer pH and volume of fluorescamine, confirming the results deduced from the polynomial equation (2).[37]

Graphical evaluation, residual and statistical analysis of FCC design

Three dimensional response surface plots and two dimensional contour plots, keeping one of the variables at the central point, are presented in Figure 5. From these plots, optimal conditions for the pre-column

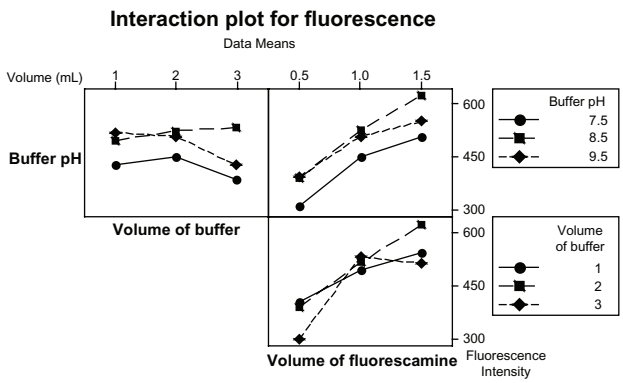

Figure 3. Interaction plots for the effects of buffer pH, volume of buffer and volume of fluorescamine on fluorescence intensity.

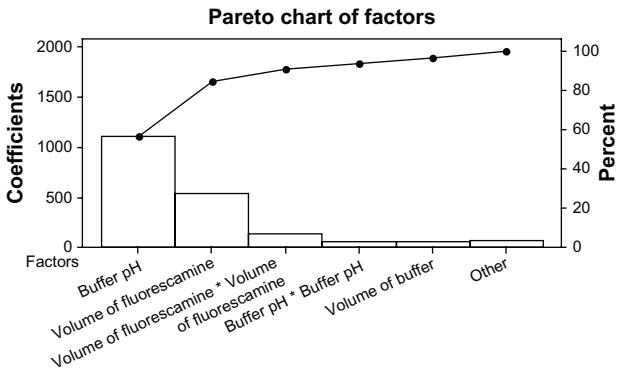

Figure 4. Pareto chart showing the influence of studied factors on the fluorescence intensity.

derivatization reaction were derived. The highest fluorescence intensity was obtained upon using 2 mL of borate buffer pH 8.5 and 1.5 mL of fluorescamine (0.01% w/v in acetone). Contour plots showed curvature, indicating the non-linear effects of these factors on fluorescence intensity.[28]

Close inspection of Figure 6A reveals that the residuals fall on a straight line, indicating that the errors are normally distributed. Histogram of the residuals is bell-shaped indicating the absence of skewness and outliers (Fig. 6B). The plots of residuals versus fits and versus order (Fig. 6C and D) show that no obvious pattern appeared. An almost equal scatter above and below the X-axis can be observed, implying the absence of non-constant variance and that the proposed model is adequate.[29]

ANOVA analysis of the model reveals that the model is significant, that is, at least one of the terms in the regression equation (linear and quadratic terms) makes a significant impact on the mean response (Table 3). A non significant lack of fit ($P = 0.067$) indicates that the model fits the data well and can be used to predict the fluorescence intensity of TOB within the limits of the experiment.[29]

Finally, the reaction was complete after shaking for 15 minutes and the product remained stable for at least 3 hours.

Stoichiometry and mechanism of the reaction

By applying the liming logarithm method,[33] two straight lines were obtained with slope values of 0.41 and 0.45 (Fig. 7A). By dividing the slopes of the two lines, a value of 1.09 was obtained. It was therefore

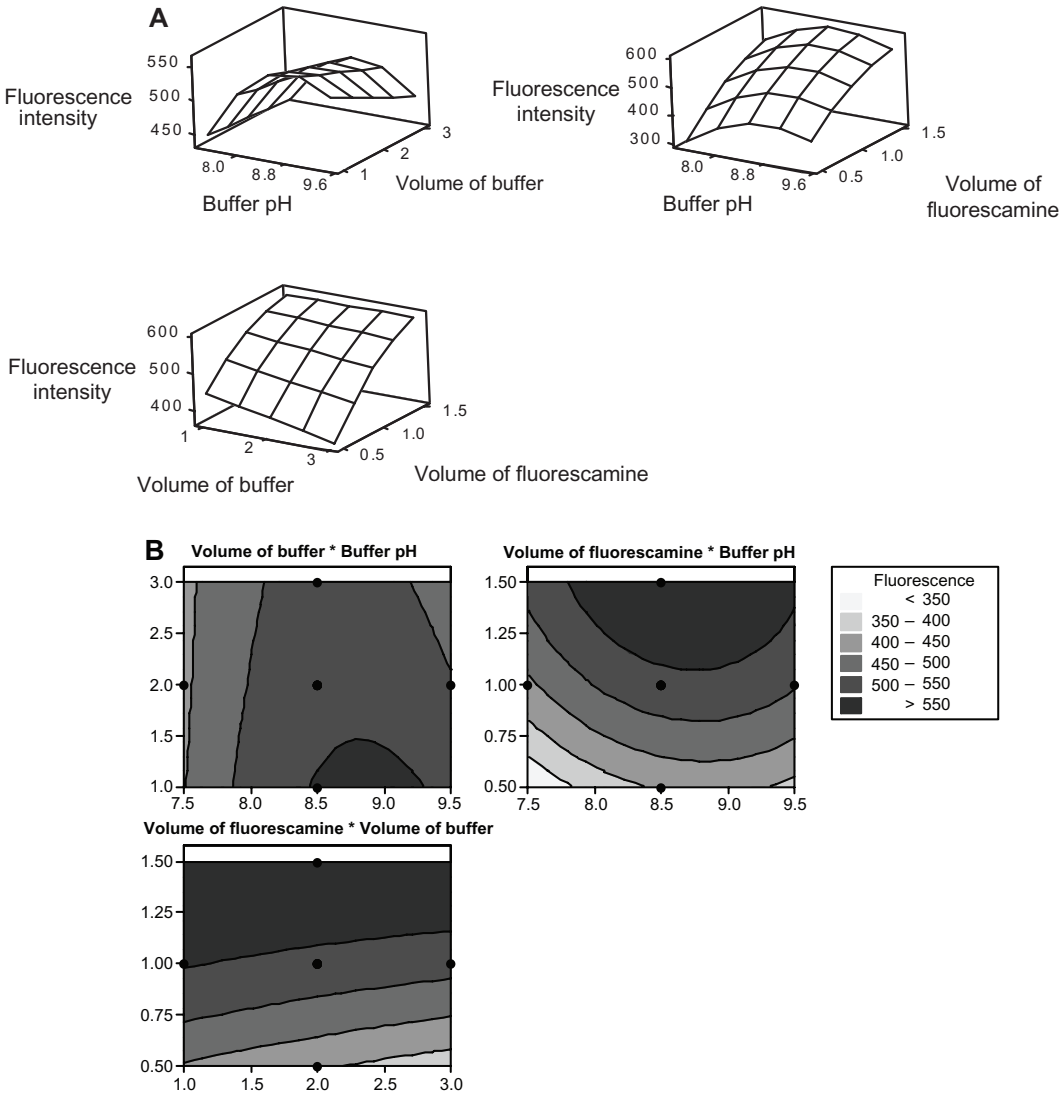

Figure 5. Response surface plots (**A**) and contour plots (**B**) showing the influence of studied factors on fluorescence intensity.

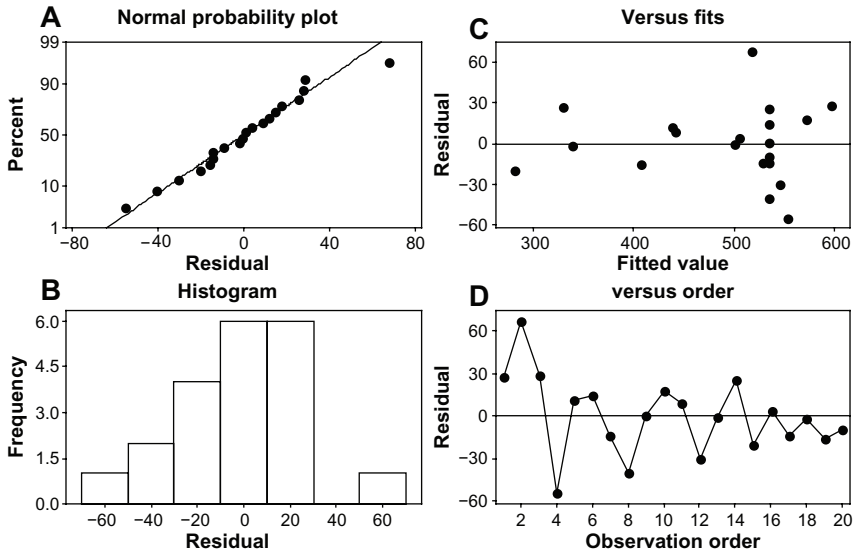

Figure 6. Residual plots for fluorescence intensity: Normal probability plot (**A**), histogram (**B**), residuals versus fits (**C**), and residuals versus order (**D**).

Table 3. Analysis of variance (ANOVA) results for fluorescence intensity.

Source	*P*
Regression	0.000
Linear	0.000
Square	0.005
Interaction	0.374
Residual error	
Lack-of-fit	0.067

concluded that the reaction proceeds in a molar ratio of 1:1. This could be attributed to the decreased basicity of the amino group by its vicinal hydroxyl groups, leaving only one amino group (NH_2*) with enough basicity to react with fluorescamine.[38]

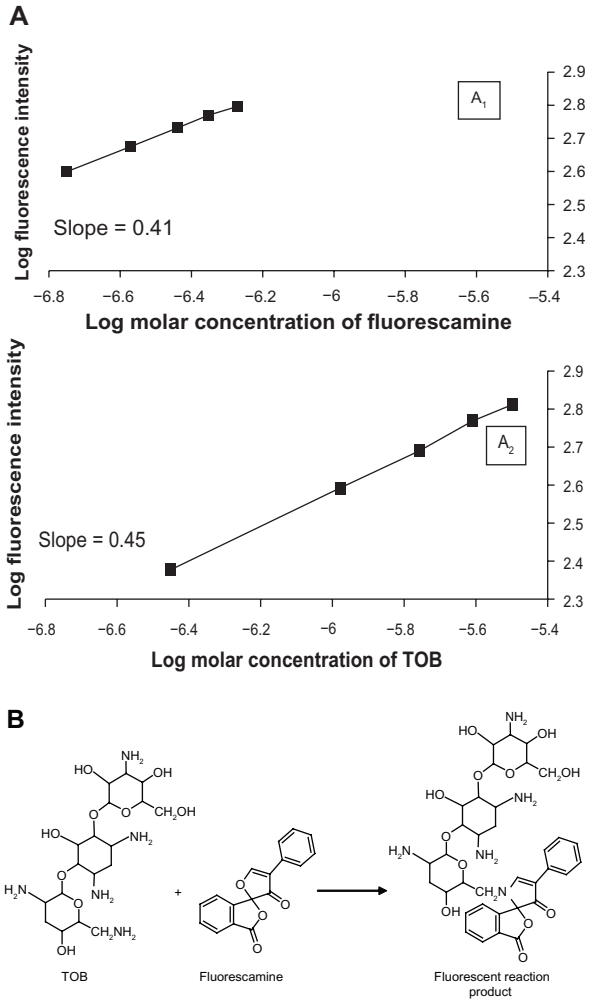

Figure 7. (**A**) Stoichiometry of the reaction between TOB sulfate and fluorescamine reagent by adopting the limiting logarithmic method, variable fluorescamine concentrations and constant TOB concentration (A_1), variable TOB concentrations and constant fluorescamine concentration (A_2). (**B**) Suggested pathway for the reaction between TOB and fluorescamine reagent.

Based upon these facts and the previous reported studies,[27] the reaction pathway between TOB and fluorescamine could be represented as shown in Figure 7B.

Development of the chromatographic method

A variety of mobile phases were investigated in the development of the HPLC-fluorescence detection method for the analysis of TOB. The suitability of mobile phase was decided on the basis of assay sensitivity, suitable retention time, and peak shape. A mobile phase consisting of methanol:water in different ratios was first tried. It was found that increasing the volume of water helped to sharpen the peak of TOB. Replacing methanol with acetonitrile distorted the peak shape with no significant change in retention time. A mobile phase composed of methanol:water (60:40, v/v) at a flow rate of 1 mL min^{-1} gave sharp peak of TOB at a retention time of 3 ± 0.1 minutes (Fig. 8A).

Upon optimization of the factors affecting the fluorescence intensity in the pre-column derivatiza-

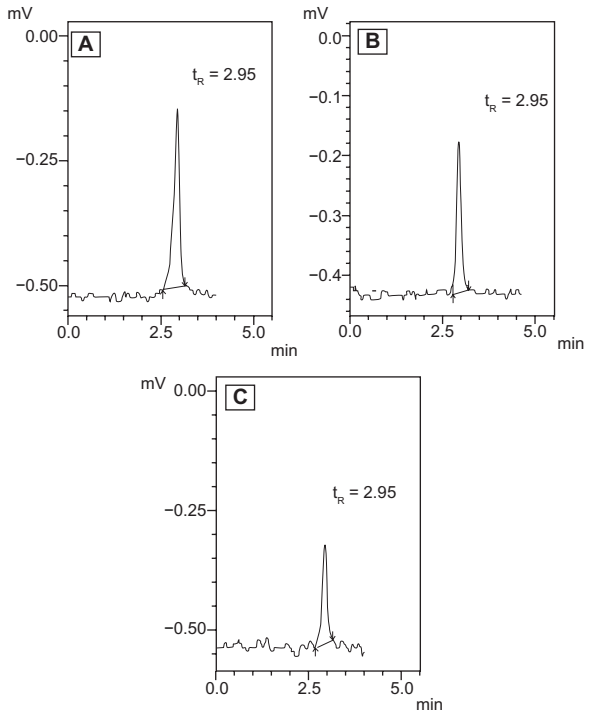

Figure 8. HPLC chromatograms of: TOB sulfate standard solution (120 ng mL^{-1}) after pre-column derivatization with fluorescamine reagent (**A**), plasma spiked with 70 ng mL^{-1} of TOB sulfate after derivatization with fluorescamine reagent (**B**) and Tobrin® eye drops (equivalent to 30 ng mL^{-1} TOB base) (**C**).

Table 4. Assay parameters and method validation obtained by applying HPLC-fluorescence detection method for the determination of TOB.

Parameter	TOB
Excitation wavelength	390 nm
Emission wavelength	480 nm
Retention time (min)	3 ± 0.1
Tailing factor	0.9
Number of theoretical plates	2130.462
Height equivalent to theoretical plate (HETP)	0.0117
Range of linearity	20–200 ng mL^{-1}
Regression equation	y = 0.0243 x + 1.0652
Correlation coefficient (r)	0.9990
S_b	0.001
S_a	0.061
Confidence limit of the slope	0.0243 ± 0.003
Confidence limit of the intercept	1.0652 ± 0.194
Standard error of the estimation	0.069
LOD[a]	5.34 ng mL^{-1}
LOQ[a]	16.30 ng mL^{-1}
Intraday[b] % RSD	0.800–0.773–0.576
Interday[c] % RSD	0.789–0.773–0.331

Notes: [a]Limits of detection and quantification are determined via calculations:[39] LOD = 3.3 × SD/slope, LOQ = 10 × SD/slope, where SD is standard deviation of response; [b]the intraday (n = 3), average of three concentrations of TOB (40,100, 180 ng mL^{-1}), repeated three times within the day; [c]the interday (n = 3), average of three concentrations of TOB (40,100, 180 ng mL^{-1}), repeated three times in three successive days.

tion step and that of the chromatographic procedure, the suggested method was applied successfully to the determination of TOB in pure form, both in eye drops and in spiked human plasma.

Method validation

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guidelines.[39]

Linearity and range

Linearity was evaluated by linear regression analysis. A linear relationship between the peak areas and the corresponding concentrations was demonstrated by a good correlation coefficient obtained for the regression line (0.9990), across the concentration range 20–200 ng mL^{-1}. Data from the regression line was used to provide mathematical estimation of the degree of linearity. Parameters for the analytical performance of the proposed method and descriptive statistics of the regression line were revealed in Table 4.

Accuracy

Accuracy of the suggested method was evaluated by replicate analysis and recovery determination of pure samples of TOB covering the linearity range (Table 5). The results obtained were compared with those obtained using the reference spectrophotometric method.[30] Statistical analysis, using student's t-test and variance ratio F-test, revealed no significant difference between the proposed method and the reported one with respect to accuracy and precision (Table 6).

Furthermore, the validity of the suggested method was evaluated by applying the standard addition technique. Recovery results suggest that the method was unaffected by the presence of formulation excipients and confirm high accuracy (Table 7).

Precision

Table 4 summarizes the results of the determination of intraday and interday assay precision. The intraday assay precision of the method, based on within

Table 5. Application of the proposed HPLC-fluorescence detection method for the determination of TOB in pure samples and in spiked human plasma.

Claimed taken (ng mL^{-1})	Pure samples		Spiked human plasma	
	Claimed found (ng mL^{-1})	% recovery[a]	Claimed found (ng mL^{-1})	% recovery[a]
40	39.868	99.67	39.333	98.33
100	100.486	100.49	100.156	100.16
140	141.391	100.99	138.181	98.70
170	168.840	99.32	172.955	101.74
Mean		100.12		99.73
± SD		0.761		1.554

Note: [a]Average of three determinations.

Table 6. Statistical analysis of the results obtained by applying the proposed HPLC-fluorescence detection method and the reference method.

TOB	Item	HPLC-fluorescence detection method	Reference method[30]
Pure form	Mean	100.12	99.92
	SD	0.761	0.56
	n	4	4
	Variance	0.579	0.314
	t-value	0.423 (2.447)*	
	F-value	1.847 (9.28)*	

Note: *Figures in parentheses are the corresponding theoretical *t*- and *F*-values at $P = 0.05$.

day repeatability, was performed by replicate analysis (n = 3) of three different concentrations of TOB standard solution covering three levels (low, medium and high) on the same day. The interday assay precision (intermediate precision) of the method was established by triplicate determination of the same three concentrations over a period of three successive days. The measured concentrations had relative standard deviation (RSD) values less than 2, indicating that the suggested method was precise.

Specificity

The method was developed to demonstrate the discrimination of the analyte in the presence of excipients in a pharmaceutical product and in human plasma. Specificity was indicated by the absence of any interference at the retention time of the peak of interest as evaluated by comparing the chromatograms of TOB pure sample, tobrin® eye drops, and plasma spiked with TOB. Figure 8 indicates that the suggested method was highly specific for the analysis of TOB.

Limit of detection and limit of quantification

According to ICH Q2B, the limit of detection (LOD) and limit of quantification (LOQ) were determined by establishing the minimum level at which the analyte can be reliably detected and the lowest concentration that can be measured, respectively. LOD and LOQ were calculated as $3.3 \times SD/slope$ and $10 \times SD/slope$, respectively, where SD is the standard deviation of the response (Table 4).

System suitability

The system suitability test was performed according to the USP[25] in order to check parameters such as tailing factor, number of theoretical plates and height equivalent to theoretical plate (HETP) (Table 4).

Analysis of TOB in spiked human plasma

The high sensitivity of the proposed method allowed the determination of TOB in human plasma. To avoid loss of analyte due to clean-up procedures and increase its recovery, the number of clean-up steps in a sample preparation procedure should be kept to a minimum. Protein precipitation is commonly used for fast sample clean-up and disrupting protein—drug binding. In the present method, acetonitrile was chosen as the protein precipitant as it resulted in good signal intensities, high extraction recoveries for TOB, and relatively clean chromatograms under fluorescence detection[40] (Fig. 8b). Satisfactory results were obtained (Table 5).

Table 7. Application of the proposed HPLC-fluorescence detection method for the determination of TOB in pharmaceutical dosage form with application of standard addition technique.

Pharmaceutical dosage form	Claimed[a]		Pure TOB added	
	(ng mL^{-1})	% recovery[b]	(ng mL^{-1})	% recovery[b]
Tobrin® eye drops	60	101.48	50	98.93
			60	99.11
			70	100.53
	120	98.89	50	99.45
			60	99.53
			70	101.13
	180	100.95	–	–
Mean		100.44		99.78
± SD		1.368		0.863

Notes: [a]Claimed taken equivalent to TOB base; [b]average of three different determinations.

Conclusion

A sensitive, precise, and accurate method based on HPLC coupled with fluorescence detection has been developed for the analysis of TOB in pure sample, eye drops, and spiked human plasma. Experimental design was successfully applied for optimization of the pre-column derivatization reaction, saving time, and cost. The method was successfully validated for linearity, accuracy, precision, and specificity. It offers several advantages including the use of non toxic derivatizing reagent and high sensitivity. In addition, specificity was evident from the analysis of TOB in pharmaceutical dosage form and in spiked human plasma with no interference from excipients. The extraction procedure, using deproteinization of plasma samples, was simple and the recovery was more than 95%. Therefore, the proposed method was found to be suitable for routine analysis of the drug in quality control laboratories.

Author Contributions

Conceived and designed the experiments: AAZ and MAM. Analysed the data: AAZ and MAM. Wrote the first draft of the manuscript: MAM. Contributed to the writing of the manuscript: AAZ. Agree with manuscript results and conclusions: AAZ and MAM. Jointly developed the structure and arguments for the paper: AAZ and MAM. Made critical revisions and approved final version: AAZ and MAM. All authors reviewed and approved of the final manuscript.

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Competing Interests

Authors disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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