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Original Article

Stability indicating methods for selective determination of Oxetacaine in the presence of its degradation products

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ABSTRACT

Two methods, HPLC and TLC were presented for the determination of Oxetacaine (OXT) in the existence of its different degradation products. HPLC method was based on the separation of OXT from its degradation products using reversed phase C18 column at room temperature and isocratic elution with mobile phase mixture of acetonitrile: 5 mM sodium dihydrogen orthophosphate dihydrate, pH was adjusted to 2.4 with orthophosphoric acid (50:50 v/v). Quantitation was based on peak area at 210 nm. The second TLC-densitometric method relies on the separation and quantitation of OXT from its degradation products on TLC silica gel 60 F_{254} plates, using 2-propanol: triethylamine (10:0.5 v/v) as a developing system and densitometric measurement of the developed bands at 210 nm. Validation of the proposed methods was performed according to the ICH guidelines and applied to evaluate the stability of OXT under different stress conditions.

1. Introduction

Oxetacaine (OXT) (Fig. 1) is 2,2′-(2-hydroxyethylimino) bis [N- (α, α-dimethylphenethyl) - N-methylacetamide. It is a local anaesthetic used in relief of gastritis and duodenal ulcers pain [1]. The British pharmacopeia analyzed OXT by a potentiometric method and titration with 0.1 M perchloric acid [2]. OXT was analysed by GC-MS method to detect urinary excretion of OXT metabolites; Mephentermine and Phentermine. The World Anti-Doping Agency forbidden these two metabolites in sports [3]. Slobodan Rendic developed GC-MS method to determine the structure of the parent drug and its metabolites in urine [4]. OXT metabolites were also separated by Unterhalt B. and Wenning C. using NP-HPLC method [5]. While Mei-Chich Hsu et al. analyzed the excretions of three volunteers who presented with positive results for mephentermine and/or phentermine after ingestion of OXT to ascertain that OXT is a source of mephentermine and phentermine in urine specimens of athletes [6].

OXT and its metabolites were also analysed by Soo-Yeun Lee et al. using liquid chromatography-tandem mass spectrometry in rat plasma after administration of OXT to investigate the relationship between phentermine production by OXT intake and the possibility of OXT dependence by comparison with results of phentermine itself after phentermine administration [7]. They also analysed OXT metabolites by LC-MS/MS in rat hair after OXT administration to study the possible detection of phentermine in hair of OXT abusers. Reversed phase chromatographic method was reported for the determination of Sucralfate and Oxetacaine [8].

The guidelines of the International Conference on Harmonization (ICH) on 'stability testing of new drug substances and products' enforced stress testing to be performed to predict the inherent stability characteristics of any active substance [9].

Two stability indicating HPLC methods were reported for OXT. The first method was for the determination of oxetacaine and sucralfate in suspension [10]. Another HPLC method for stability study of OXT was proposed by Veerabhadram et al. [11]. In both methods, forced degradation study was conducted using acid, alkaline, thermal, and photolytic degradation. The first developed method by S. Ahmed et al. used mild conditions to study the stability of OXT and sucralfate simultaneously in their combined sample solution in which no discrimination was done between the degradation products of sucralfate and oxetacaine and the provided chromatograms didn't show clearly any degradation products which is required in any claimed stability study [10]. While Veerabhadram et al. method didn't specify exactly the duration of exposure of OXT to the stated stress conditions and the method was less sensitive regarding the LOD and LOQ [11]. Both methods had narrow linearity range (20–60 μg/ml) for S. Ahmed et al. method and (2.5–15 μg/ml) for Veerabhadram et al. method and no trials were made to structurally elucidate any of the degradation

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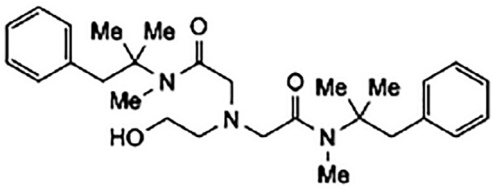


Fig. 1. Chemical structure of Oxetacaine.

products. So we aimed at this work to develop a more sensitive and rapid methods for the determination of OXT in the existence of its basic and oxidative degradation products using HPLC and TLC in its bulk powder and pharmaceutical formulation.

2. Experimental

2.1. Instrumentation

An HPLC system that consists of Agilent 1200 set control module by Agilent chemstation for HPLC equipped with a quaternary pump, injector with a 20 µL loop and a photodiode array detector (Minnesota, USA), Inertsil ODS-3 C18 RP column (150 × 4.6 mm, 5 µm particle size), Sonicator (R. Espinar S.L,Spain) , Digital PH meter (HANNA, USA), rotary evaporator (Shimdazu, Japan).

A Camag TLC Scanner (Switzerland) and Camag TLC software (WINCATS software) was used for data collections and analysis. Application of samples to the plates was done by Camag Linomat autosampler. TLC plates aluminum plates (20 × 20 cm^2) precoated with silica gel 60 F^{254}) from Merck (Buchs, Germany). The measurement experimental conditions were λ = 210 nm, mode: absorbance, slit dimensions 5 × 0.2 mm.

2.2. Materials and reagents

Oxetacaine working standard was a kind gift sample from EIPICO Company (Cairo, Egypt). Its potency was found to be 99.9% according to the BP official method [2].

Mucogel® suspension (Mucogel®, Batch No. 1200472, label claim: 0.2 gm/100 mL) manufactured by EIPICO, Egypt was purchased from local market.

Double distilled Water was used and filtered through 0.45-mm membrane filter for HPLC analysis. All reagents used were of HPLC grade and chemicals were of analytical grade. Acetonitrile HPLC (Scharlau, Barcelona, Spain), Methanol HPLC (Macron fine chemicals, Norway), Ortho-phosphoric acid 85% (Adwic, Egypt), Concentrated hydrochloric acid; Honeywell Burdick & Jackson, USA (1 N HCl was prepared in methanol), Sodium hydroxide; El Nasr company for chemicals (1 N NaOH was prepared in methanol), Hydrogen peroxide 30% (Panreac, Spain), Sodium dihydrogen phosphate (El Nasr company, Egypt), Propanol-2-ol (Scharlau Chemie, S.A., Spain), Triethyl amine (Qualikems, India)

Preparation of phosphate buffer was made by dissolving 0.78 g of sodium dihydrogen phosphate in one litter of distilled water and adjusting pH at 2.4 with orthophosphoric acid.

2.3. Standard solutions

For HPLC method an accurately weighed 10 mg of OXT was transferred into 100 mL volumetric flask and dissolved in 2 mL methanol then the volume was completed with mobile phase mixture and mixed well. This prepare a final stock concentration 100 µg/mL.

For TLC method an accurately weighed 250.00 mg of OXT was transferred into 50 mL volumetric flask and dissolved in 20.00 mL methanol then the volume was completed with methanol. This prepare a final stock concentration 5 mg/mL.

2.4. Preparation of the degradation products

All degradation stock solutions were derived from complete degradation of 1 mg/mL of OXT standard solution.

2.4.1. Preparation of the acid and base degradation products

Oxetacaine was dissolved in either 1 N methanolic HCl or 1 N methanolic NaOH, refluxed in 250-mL round-bottom flask for 10 and 6 h, respectively. Then each solution was cooled, neutralized with NaOH and HCl, respectively then filtered.

The stressed acid and base degradation process give the same degradation products as confirmed by HPLC but in longer time in case of acidic condition.

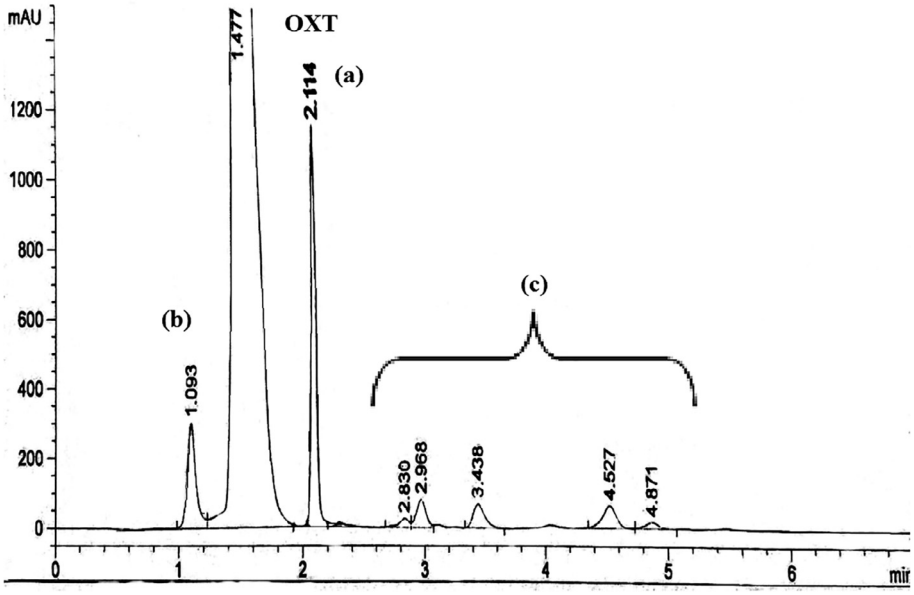


Fig. 2. HPLC chromatogram of resolved mixture of (a) OXT (Rt = 2.113), (b) OXTs' hydrolytic degradation products (Rt = 1.093 ,1.572) and c) oxidative degradation products (Rt = 2.83, 2.968, 3.438,4.527,4.871) on a C18 column, mobile phase consists of acetonitrile: phosphate buffer pH 2.4 (50:50 by volume).

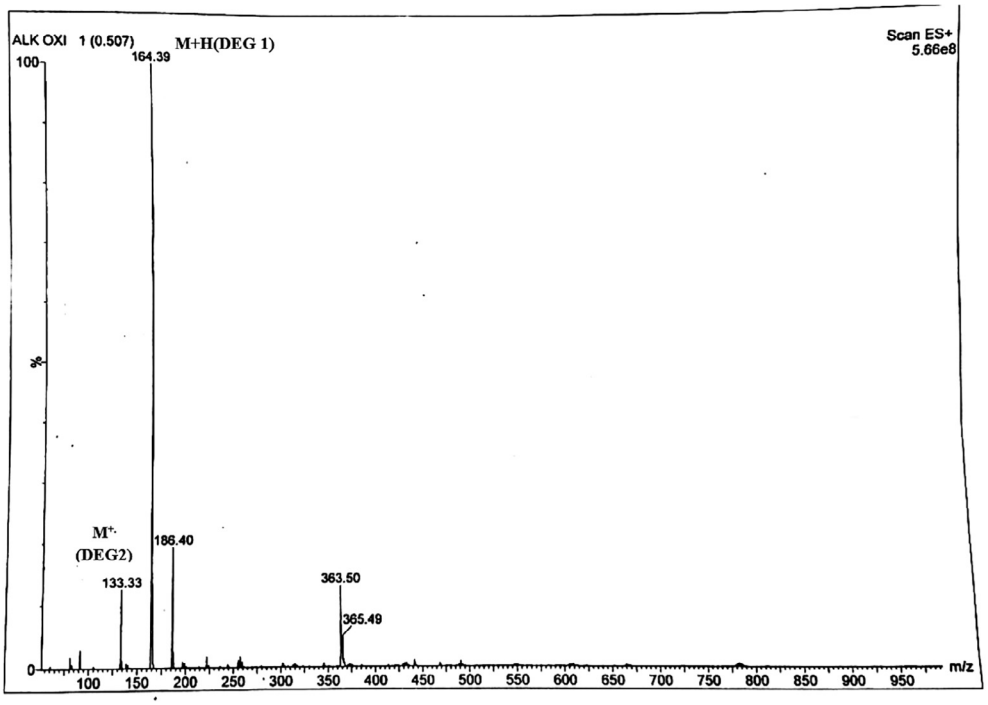
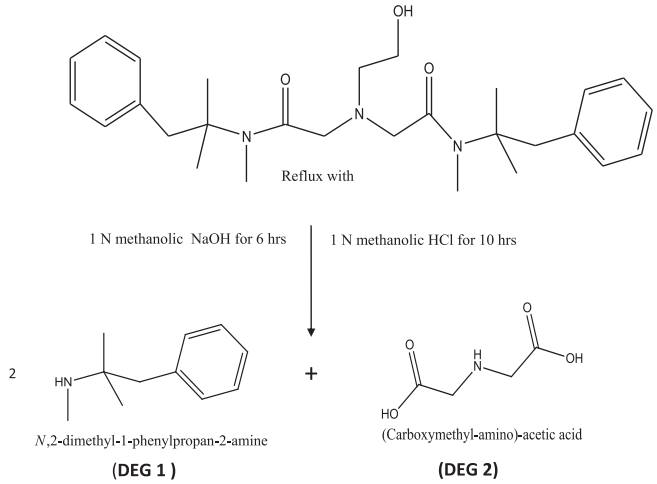


Fig. 3. Mass spectrum of hydrolytic products of oxetacaine.



Scheme 1. Suggested degradation pathway of Oxetacaine.

2.4.2. Preparation of oxidative degradation product

Oxetacaine was dissolved in methanol, refluxed in 250-mL round-bottom flask with 5 mL 30% H_2O_2 for 9 h, evaporated by rotary evaporator then the residue was dissolved in methanol.

3. Procedures

3.1. Experimental conditions

For HPLC method mobile phase mixture of acetonitrile: phosphate buffer pH 2.4 in a ratio of (50:50 v/v) was prepared, filtered through 0.45 μm membrane filter and degassed for 15 min in an ultrasonic water bath before use. The UV wavelength of detection was 210 nm with flow rate 1 mL/min at room temperature. Samples were filtered through a 0.45 μm syringe filter, and 20 μL were injected into the system by microsyringe. The column was conditioned for at least 30 min or until stable baseline.

For TLC method different aliquots of each dilution were applied as bands onto TLC plates (bandwidth: 6 mm; spacing: 15 mm; 15 mm from bottom edge of the plate) using a CAMAG linomat applicator. Development was performed in a formerly saturated chromatographic tank with the developing system consisting of propan-2-ol: triethyl amine (10: 0.5 v/v) at the room temperature. The developed plates were oven dried and scanned.

3.2. Construction of calibration curves

For HPLC method into a set of 10-mL volumetric flasks, portions equivalent to 25–800 μg from OXT standard stock solution were transferred, and the volume to the mark was completed with mobile phase mixture. Twenty microliters of each dilution were injected in triplicates under the formerly stated chromatographic conditions. The peak areas in the recorded chromatograms were determined, the calibration curve between peak areas to the corresponding concentrations of OXT was constructed and the regression equation was calculated.

For TLC method into a set of 10-mL volumetric flasks, portions equivalent to 5.00–40 mg from OXT standard stock solution were transferred, and the volume to the mark was completed with methanol. Ten microliters were separately applied in triplicates as bands onto TLC plates using Camag autosampler, and the formerly stated conditions were applied. The plates were scanned and the calibration curve of the peak areas to the corresponding concentrations of the drug was plotted. The regression equation was computed.

3.3. Analysis of OXT in mucogel® suspension

For HPLC method Mucogel® suspension bottle was shaken well then 5 mL was accurately transferred into 100 mL volumetric flask, 10 mL of methanol was added to solubilize OXT, sonicated well for 30 min and the volume was completed with mobile phase mixture and filtered through 0.45 syringe filter. Aliquots equivalent to 150–250–350–550–750 μg of OXT were transferred into a set of 10-mL volumetric flasks. The volume was completed to the mark with mobile phase mixture and analyzed under the formerly stated conditions. The concentrations of OXT were calculated from the corresponding regression equation.

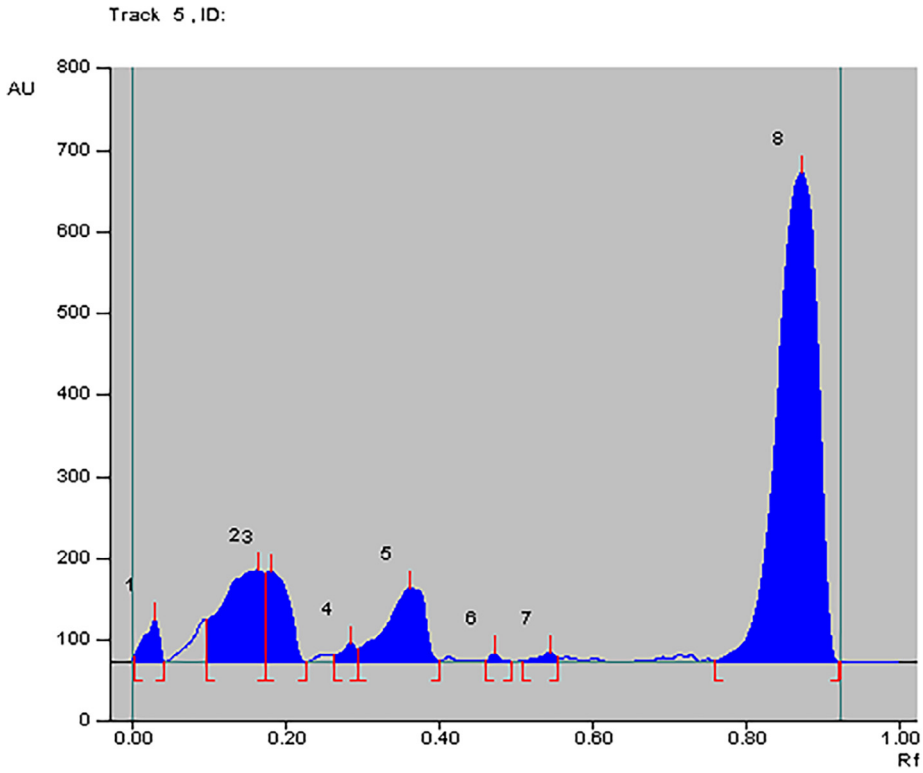


Fig. 4. TL chromatogram of resoluted peaks of (a) OXT hydrolytic degradation products (Rf = 0.03 & 0.36) peaks 1,4,5 (b) Oxetacaine (Rf = 0.87) peak 8 (c) Oxidative degradation products (Rf = 0.16, 0.18, 0.47, 0.54) peaks 2,3,6,7 using mobile phase consists of propan-2-ol: triethyl amine (10: 0.5 by volume) at 210 nm.

Table 1
Parameters required for system suitability testing of HPLC and TLC method for determination of OXT.

Parameter	Obtained value for HPLC method	Obtained value for TLC method	Reference value [13,14]
Rs "Resolution"	2.06	3.3	Rs ≥ 2
α "Selectivity"	1.37	5.67	NLT 1
K′ "Capacity factor"	3.2	0.15	K′ > 2 for HPLC The higher the capacity factor, the shorter the Rf for TLC
N "Column efficiency"	3329	473.06	N > 2000 for HPLC For TLC Conventional TLC is capable of producing up to 600 theoretical plates and The higher the value, the higher the column efficiency in both HPLC and TLC
HETP[a]	4.5×10^{-2}		The smaller the value, the higher the column efficiency
T "Tailing factor"	1.25	0.89	T ≤ 2

[a] Height equivalent to theoretical plate (cm/plate).

For TLC method Mucogel® suspension bottle was shaken well then 100 mL were accurately transfered into 500 mL separating funnel, extracted five times each with 50 mL dichloromethane, the collected organic layer was filtered through 0.45 μm membrane filter then the filtrate was evaporated. The residue was then dissolved in 50 mL methanol. Aliquots equivalent to 5, 10, 20, 36, 40 mg were transferred to 10 mL volumetric flasks completed to volume with methanol. Ten microliters were spotted on TLC plates, developed and scanned under the formerly mentioned chromatographic conditions. The concentrations of OXT were calculated from the corresponding regression equation.

4. Results and discussion

4.1. Method development and optimization

For HPLC method: several mobile phases were tried to resolve OXT from its degradation products such as methanol: water (50:50, 30:70, and 70:30 by volume), methanol: phosphate buffer with different ratios and pHs but all tried systems failed to achieve satisfactory resolution between the hydrolytic, oxidative degradation products and the intact OXT. Then phosphate buffer: acetonitrile mixture in different ratios was finally tried. By decreasing the pH of the phosphate buffer to 2.4 by orthophosphoric acid and using ratio of phosphate buffer: acetonitrile 50:50 v/v, good peak symmetry and satisfactory resolution of OXT from its degradation products was finally obtained. Best separation was obtained on C18 column (150 × 4.6 mm, 5 μm particle size) with the system operated at flow rate 1 mL/min. Maximum wavelength sensitivity was obtained at 210 nm using PDA screening at the whole UV spectrum. Good separation was obtained using the optimized conditions, where the retention time (Rt) values were 2.113 ± 0.1, 1.093, 1.572 for OXT, its two hydrolytic degradation products formed upon reflux in either acidic or basic medium and 2.83, 2.968, 3.438, 4.527, 4.871 for oxidative degradation products. In addition to a large signal at Rt 1.477 corresponding to hydrogen peroxide which obscures the peak of one of the hydrolytic degradation products at Rt 1.572 (Fig. 2). The hydrolytic degradation products were identified by LC-MS (Fig. 3) and the suggested pathway for the hydrolytic cleavage is shown in Scheme 1.

For TLC method: Different developing systems were attempted to improve the separation of drug from its degradation products. Initially toluene and ethylacetate combination (8: 2 v/v) was used but this

Table 2
Validation parameters of the proposed methods for determination of OXT.

Parameter	Value in case of HPLC method	Value in case of TLC method
Wavelength	210 nm	210 nm
Linearity range	2.5–80 μg/ml	5–40 μg/band
Intercept	−8.5	(0, 4566.69)
Slope	69.85	
Correlation coefficient	1	1
Accuracy (Mean ± RSD)	99.35 ± 0.7	99.85 ± 1.41
Accuracy for determination of OXT in Mucogel® suspension (Mean ± RSD)	100.08 ± 1.36	99.77 ± 1.39
Selectivity	**Purity factor** 997.957	100.03 ± 1.01
Repeatability[a]	0.44	0.57
Intermediate precision[b]	0.67	0.49
LOD[c]	0.266 μg/ml	1.5 μg/band
LOQ[c]	0.81 μg/ml	2.2 μg/band

[a] The intra-day (n = 9) average of three different concentrations (2.5, 40, 80 μg mL^{-1}) for HPLC method and (5, 20, 40 μg $band^{-1}$) for TLC method repeated three times within 1 day.
[b] The inter-day (n = 9) average of three different concentrations (2.5, 40 and 80 μg mL^{-1}) for HPLC method and (5, 20, 40 μg $band^{-1}$) for TLC method repeated three times in 3 successive days
[c] Were calculated per ICH guidelines based on a signal-to-noise ratio 3:1 of the response for estimating the detection limit and 10:1 for The quantitation limit.

combination didn't achieve any separation. Increasing the percentage of ethyl acetate in the mixture did not improve the separation between OXT and its degradation products. So different mobile phase was tried consisting of propan-2-ol: triethyl amine (10: 0.5 v/v) to give finally sharp and symmetric peak of OXT and a good separation between the drug and its degradation products with sufficient difference in their Rf (Fig. 4). Well resoluted bands were obtained when the chromatographic tank was formerly saturated with the developing system for 30 min at ambient temperature.

4.2. Method validation

The ICH guidelines scheme was applied for validation of the proposed methods [12].

Table 4
Robustness of the proposed TLC densitometric method.

Robustness parameter		T[a]	K[a]	Rs[b]	% Assay
Varying proportion of propanol-2-ol in mobile phase mixture	50–5 mL	0.88	0.18	2.83	99.6
	50 + 5 mL	0.86	0.13	2.95	98.36
Duration of saturation of the chromatographic tank	30–5	0.84	0.16	3.1	100.25
	30 + 5	0.87	0.14	3.17	101.6

% Assay calculated from regression equation.
[a] Tailing factor and capacity factor determined for individual peaks.
[b] Resolution factor determined between OXT's peak and the previous one.

4.2.1. System suitability

The system suitability parameters for the HPLC and TLC are shown in (Table 1). All parameters passed the recommended criteria of the ICH validation guidelines of chromatographic methods.

4.2.2. Linearity and limits of detection and quantitation

The linearity of the proposed methods was tested by analysing a set of different concentrations of OXT. The linearities of the developed methods were calculated, and the linear regression data for the calibration curves shown perfect linearity (r = 1) over the concentration range of 2.5–80 μg/mL in case of HPLC method and polynomial linearity (r = 1) over concentration range of 5–40 μg/band in case of TLC method.

The regression equations were computed and found to be:

$$PA_{OXT} = 69.85\ C_{OXT} - 8.5 \qquad \text{for HPLC method}$$

$$PA_{OXT} = -17.81\ C_{OXT}^2 + 1492.75\ C_{OXT} + 4566.69 \qquad \text{for TLC method}$$

where PA is the peak area at 210 nm, C is the corresponding concentration in μg/mL in case of HPLC method and μg/band in case of TLC method

Limits of detection and quantitation for OXT were found to be 0.266 μg/mL and 0.81 μg/mL, respectively in case of HPLC method and 1.5 μg/band and 2.2 μg/band, respectively in case of TLC method (Table 2).

Table 3
Robustness of the proposed HPLC method.

Robustness parameter		Concentration (μg/ml)	Tailing factor (T)	Capacity factor (k′)	Rt
% of acetonitrile in mobile phase	48		1.44	3.30	2.31
	50	5.00	1.13	3.23	2.02
	52		1.19	3.10	1.76
		Mean ± SD	1.25 ± 0.16	3.21 ± 0.10	2.03 ± 0.28
	48		1.21	3.30	2.26
	50	40.00	1.17	3.19	2.16
	52		1.13	3.15	1.74
		Mean ± SD	1.17 ± 0.04	3.21 ± 0.08	2.05 ± 0.28
	48		1.26	3.31	2.26
	50	80.00	1.20	3.24	2.11
	52		1.17	3.14	1.75
		Mean ± SD	1.21 ± 0.05	3.23 ± 0.09	2.04 ± 0.26
pH of mobile phase	2.2		1.13	3.30	2.02
	2.4	5.00	1.13	3.23	2.02
	2.6		1.23	3.25	1.93
		Mean ± SD	1.16 ± 0.068	3.26 ± 0.046	1.99 ± 0.05
	2.2		1.17	3.24	1.93
	2.4	40.00	1.17	3.19	2.16
	2.6		1.12	3.29	1.92
		Mean ± SD	1.15 ± 0.03	3.24 ± 0.05	2.00 ± 0.14
	2.2		1.21	3.57	1.92
	2.4	80.00	1.20	3.24	2.11
	2.6		1.16	3.61	1.97
		Mean ± SD	1.19 ± 0.03	3.47 ± 0.2	2.00 ± 0.10

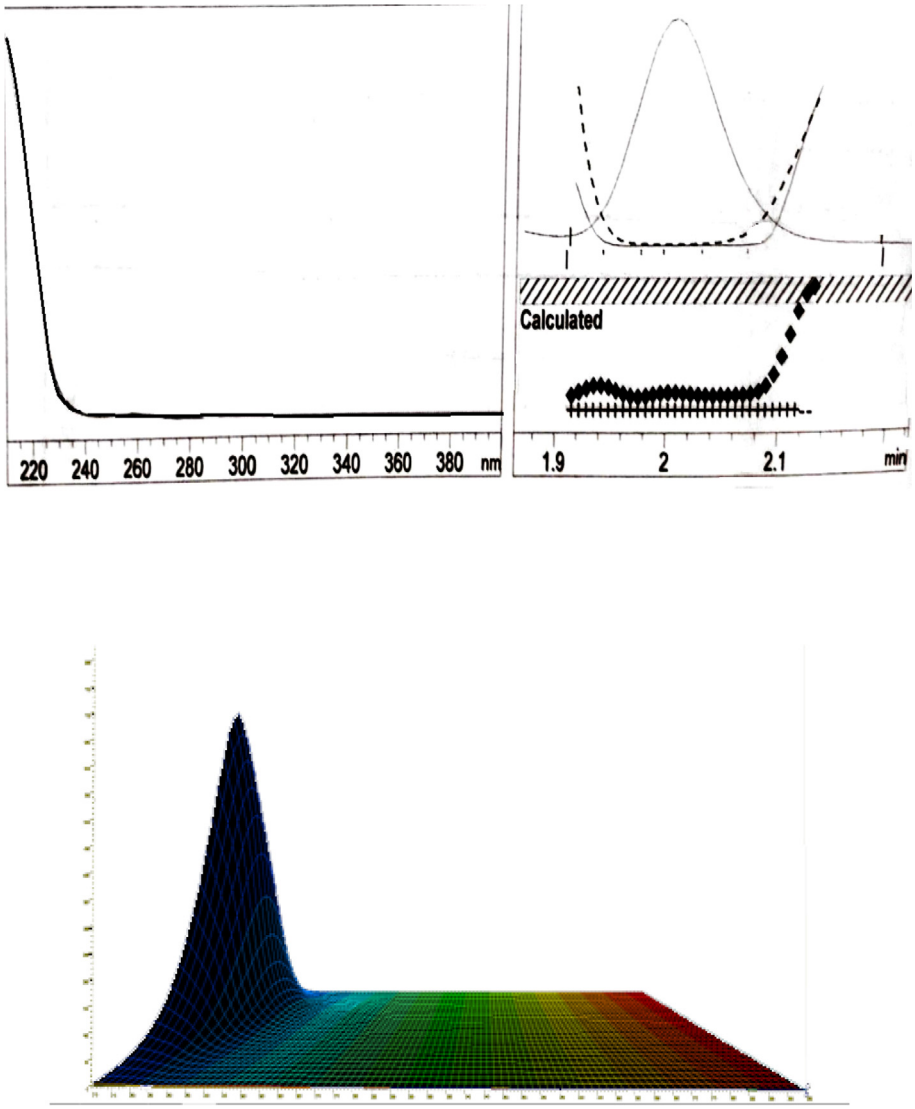


Fig. 5. 3D-display of the peak purity of Oxetacaine in the synthetic mixture of the standard spiked with its degradation products using PDA detector.

Table 5
Statistical comparison of the results obtained by applying the proposed methods and the reported official potentiometric method for the determination of Oxetacaine in its pure form.

Statistical Term	Reference method**	HPLC method	TLC method
Mean	100.12	100.08	99.77
S.D	0.89	1.36	1.39
S.E	0.40	0.61	0.62
%RSD	0.89	1.36	1.39
n	5.00	5.00	5.00
V	0.79	1.85	1.93
Student't test		0.06 **(2.306)***	0.47 **(2.306)***
F-value		2.34 **(6.39)***	2.44 **(6.39)***

* Figures in parentheses are the theoretical t and F values at (p = 0.05).
** Potentiometric method by titration with 0.1 M perchloric acid and end point detected potentiometrically [2].

4.2.3. Accuracy

Accuracy of the methods was studied by recovery experiments of three different concentrations covering the linearity range. The results of percentage recoveries of OXT in pure drug solutions are presented in (Table2) indicating that the proposed methods are accurate.

4.2.4. Precision

Pure samples of OXT were determined over different days to get inter-days (intermediate precision, n = 3 for each concentration) and within the same day to get intra-day precision (repeatability, n = 3 for each concentration), then the RSDs % values were computed. The results of repeatability and intermediate precision experiments are presented in (Table 2). The RSD% was < 2% indicating the good precision of the proposed methods.

4.2.5. Robustness

Robustness of the methods was ascertained by checking the effect of minor deliberate changes in the experimental conditions on the parameters of system suitability.

For assessment of robustness of the HPLC method, two parameters were chosen including the percentage of acetonitrile (48, 50, 52%) and the pH (2.2–2.4 and 2.6) of mobile phase. Then the effect on the chromatographic parameters (retention time, tailing factor, capacity factor) was investigated (Table3).

In TLC method, robustness was checked by varying proportion of propanol-2-ol in mobile phase mixture propan-2-ol: triethyl amine (50 ± 5: 2.5 v/v) and duration of saturation of the chromatographic tank (30.00 ± 5 min). Then the effect on the chromatographic parameters (retention time, tailing factor, capacity factor) was investigated

(Table 4).

4.2.6. Specificity

For HPLC method the specificity of the proposed method was checked by testing the peak purity of OXT in synthetic drug mixture of the standard spiked with different degradation products using photodiode array detector (PDA) which by comparison of each acquired spectrum over wide wavelength range during peak elution can determine peak purity [15].

The peak is pure if acquired spectra during peak migration is close to perfect 100% match expressed as purity or similarity factor as shown in Fig. 5.

For TLC method, specificity was tested by analysis of different laboratory prepared mixtures of the drug, its hydrolytic and oxidative degradation products where satisfactory recovery results were obtained with good resolution and selectivity between the drug and its degradation products (Table 2).

4.3. Analysis of OXT in mucogel® suspension

The proposed methods could be successfully applied for the quantitative assay of OXT in its pharmaceutical formulation with good percentage recoveries. Results are shown in (Table 2).

The results were compared with the official method [2] showing no significant difference. Results of the statistical comparison are shown in Table 5.

5. Conclusion

The proposed HPLC and TLC methods provide simple, precise and accurate quantitative determination of OXT in its pure form, its pharmaceutical suspension and in the existence of its hydrolytic and oxidative degradation products. The excipients didn't cause any interference in OXT analysis. The TLC method was the first reported one for the stability study of OXT taking the advantage of TLC simplicity and using less volumes of solvents, compared to the HPLC method, but the HPLC method has higher sensitivity. The two suggested methods were fully validated and can be used for routine quality control analysis.

Conflict of interest

None.

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