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Current Pharmaceutical Analysis, **2018,** *14,* **000-000**

RESEARCH ARTICLE

Three Different Spectrophotometric Methods Exploiting Ratio Spectra for the Selective Determination of Iohexol in the Presence of its Acidic Degradate

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Abstract: ***Background:*** Non-ionic X-ray contrast agents constitute a very important class of pharmaceutical compounds produced in large quantities. Iohexol is an important example of such compounds.

Objective: Three simple and selective stability indicating spectrophotometric methods utilizing ratio spectra were proposed for the determination of the widely used X-ray contrast medium, iohexol in the presence of its acidic degradate and in its pharmaceutical formulation.

Methods: The first method is the first derivative of ratio spectra method (DD^1), the second is the ratio difference method (RD), and the last one is the mean centering method (MC).

Results: The three proposed methods showed a good linearity over the concentration range of 4-40 $\mu g.mL^{-1}$. The selectivity of the three developed methods was evaluated by analyzing different laboratory-prepared mixtures and satisfactory results were obtained.

Conclusion: Iohexol has been successfully determined in its pure form and pharmaceutical formulation (Omnipaque® vials) utilizing the proposed methods with no interference from the present additives. The results obtained by each of the proposed methods were statistically compared to the official United States pharmacopeial method and non-significant difference was obtained regarding accuracy or precision.

ARTICLE HISTORY

Received: June 20, 2017
Revised: September 27, 2017
Accepted: October 08, 2017

DOI:
10.2174/1573412913666171016163544

Keywords: Iohexol, derivative ratio, ratio difference, mean centering, and stability indicating methods.

1. INTRODUCTION

Iohexol is used widely as a non-ionic contrast agent for several medical diagnostic purposes. Unlike the ionic contrast media, iohexol has lower osmolality that reduces or eliminates virtually all the hemodynamic alterations due to osmotic property (osmotoxicity) [1]. Iohexol was evidenced to have lower contrast-induced nephrotoxicity which is a known risk to the use of ionic high osmolar contrast media for many years [2].

Reviewing literature in hand showed that there is no a stability indicating spectrophotometric method for the assay of iohexol in the presence of its acidic degradate. Iohexol was determined in human and animal plasma by HPLC and capillary electrophoresis techniques [3-9]. Additionally, iohexol is officially assayed in the USP using a precipitimetric titration method depending on the presence of iodide in the structure of the drug [10].

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A Stability Indicating Method (SIM) is defined by FDA as a validated analytical method which can measure the active ingredients (pure substance or drug product) without any interference from potential impurities, excipients and degradation products [11]. The ideal SIM should be designed and evaluated with common sense and chemical information, considering the manufacturing process, the synthetic precursors, and the nature of the final drug product. The stability profile needs to be established for drug product to assure patient safety, drug efficacy, and quality [12, 13].

In this research, our goal is to develop three validated and simple UV-spectrophotometric methods that depend mainly on manipulation of the ratio spectra of iohexol namely, derivative ratio, ratio difference, and mean centering method. The proposed methods provide a sensitive and applicable way for routine analysis and quality control of iohexol without the need to hazardous organic solvents or sophisticated apparatus or software.

2. EXPERIMENTAL

2.1. Apparatus

SHIMADZU double-beam UV-Vis spectrophotometer (Kyoto, Japan), model UV-1650. A desktop computer con-

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nected to a LaserJet HP1020 printer. The software used was SHIMADZU UV-Probe, version 2.21 personal spectroscopy software. The spectral band was 2 nm and scanning rate was 2800 nm/min and 0.1 nm interval.

2.2. Reference Samples

Iohexol reference standard was obtained from Sigma-Aldrich Company (USA), and its purity was labelled as ≥ 99.99%.

2.3. Pharmaceutical Formulation

Omnipaque® vial produced by GE healthcare (Cork, Ireland), batch number 12062934 was labeled to contain iohexol with a concentration of 0.647 $g.mL^{-1}$.

2.4. Materials and Reagents

Analytical grade chemicals and solvents used were sodium hydroxide, hydrochloric acid, ammonium hydroxide, and distilled water (Adwic, Cairo, Egypt). Methanol, acetone and 2-propanol were from Prolabo, West Chester, PA, USA.

2.5. Degraded Samples

In a 250-mL glass-stoppered flask, 10 mL of 1 mol/L HCl solution was added to 500 mg of pure iohexol and boiled under reflux for 5 hours. Complete degradation was confirmed by TLC using acetone: 2-propanol: methanol: ammonia (4:2.8:1.6:1.6 by volume) as a developing system. Only one spot was observed not corresponding to iohexol. The degraded solution was then neutralized using 1 mol/L NaOH till pH reached 7 and evaporated using a small flame. Finally, the degradate was dissolved in methyl alcohol, filtered, and left to evaporate at ambient temperature. The structure elucidation of the isolated degradate was performed using mass and IR spectrometry.

2.6. Standard Solutions

A stock standard solution of iohexol having a concentration of 0.2 $mg.mL^{-1}$ in distilled water was prepared in a 100-mL volumetric flask by dissolving 20 mg of pure iohexol in distilled water. Degradate stock solution having a concentration of 0.2 $mg.mL^{-1}$ was also prepared in the same solvent.

2.7. Procedures

2.7.1. Construction of Calibration Graphs

Accurate volumes (0.2-2 mL) of iohexol stock solution (0.2 mg/mL) were transferred into a series of 10-mL measuring flasks, and filled upto the mark using distilled water. The zero order spectra were recorded using distilled water as a blank, and then the recorded zero order spectra of the prepared solutions was divided by the spectrum of 20 µg/mL degradate.

2.7.2. Derivative Ratio Method

The first derivative of the obtained ratio spectra (DD_1) was obtained using scaling factor 10 and $\Delta\lambda$=8 nm. The peak amplitudes of the first derivative of the ratio spectra were measured at 224.6 nm. Calibration graph relating the peak amplitudes of the DD^1 curves at 224.6 nm to the corresponding concentrations of iohexol was constructed, and the corresponding regression equation was computed.

2.7.3. Ratio Difference Method

Calibration curve for iohexol was plotted between the difference in the peak amplitudes of the obtained ratio spectra at 255 and 265 nm versus the corresponding concentrations in µg/mL, and the regression equation was computed.

2.7.4. Mean Centering Method

The obtained ratio spectra were transferred to MATLAB® software. Then mean centering of these spectra with respect to wavelength was done. The maxima of the mean centered ratio spectra at 234 nm was measured, plotted against the corresponding concentrations and the regression parameters were computed.

2.7.5. Application of the Proposed Methods to the Analysis of Iohexol in Pharmaceutical Preparation

Omnipaque® vial was labeled to contain 0.647 gm/mL of iohexol. A stock solution with a concentration equal to 1 mg/mL was prepared by transfer of 0.15 mL of the solution to a 100-mL volumetric flask and the volume was completed using distilled water; from this stock, solution 0.1 mL was transferred into a 10-mL volumetric flask and the volume was completed with distilled water to get a concentration equal to 10 µg/mL. The procedure was then completed as described under (Construction of Calibration Graphs).

2.7.6. Analysis of Artificial Mixtures

Laboratory prepared mixtures containing iohexol and different percentages of its degradate were prepared and analyzed using the same procedure as described under the section of (Construction of Calibration Graphs).

2.7.8. Validation of the Proposed Methods

Validation of the proposed methods was performed according to USP-guidelines [10].

2.7.8.1. Specificity

The specificity of the proposed methods was assessed by analysis of different laboratory prepared mixtures containing different ratios of the degradation product ranging from 10 to 90 %.

2.7.8.2. Linearity and Range

The linearity of the proposed methods was evaluated by analyzing different concentrations of standard solution of IOH in triplicates. Calibration curves were plotted and the values of correlation coefficients were found to be close to unity indicating good linearity. Ranges were derived from the corresponding linearity of each method where minimum and maximum values excel over the officially approved values (80 to 120% of the tested concentration of the active substance in the drug product) [10].

2.7.8.3. Accuracy

The accuracy was determined by the recovery of known amounts of IOH. In the accuracy test, three concentrations of IOH were evaluated (10, 20 and 30 µg/mL). Satisfactory results of mean recoveries, and RSD% values were obtained demonstrating good accuracy of the proposed methods.

2.7.8.4. Precision

The precision of the proposed methods was evaluated by measuring the response of three concentrations (18, 26, and 32 µg/mL for DIPH) in three different times of the same day (intra-day) and on three different days (inter-day) and good RSD% values were obtained.

3. RESULTS AND DISCUSSION

The industrial production of iohexol involves a multistep chemical synthesis. The final drug substance must meet the stringent purity standards set by regulatory agencies. Since purity is of ultimate importance, it is important to develop a stability indicating method to quantify iohexol in the presence of its acidic degradate which is also the synthetic precursor [7]. This degradate was obtained upon reflux of iohexol with acid or alkali (Fig. **1**).

The structure of the isolated degradation product was confirmed by mass spectrometry where the peak at *m/z* 556 was found to be corresponding to the acidic degradate. The structure was further confirmed by IR spectrometry which indicated the appearance of a broad band at 3047 cm^{-1} corresponding to the aromatic acidic hydroxyl group instead of the aliphatic alcoholic hydroxyl which appears in the spectrum of the intact iohexol at 3350 cm^{-1}. Moreover, the spectrum of the degradation product shows a distinguished peak at 1705 cm^{-1} corresponding to the acidic carbonyl group while the intact drug shows the carbonyl peak at 1635 cm^{-1} which is the amidic range, (Figs. **2** and **3**).

The zero order absorption spectra of iohexol and its acidic degradate showed that iohexol acidic degradate overlaps with that of intact iohexol and hinders its direct assay (Fig. **4**). The objective of this work was to develop specific, precise and simple stability indicating spectrophotometric methods for the determination of iohexol in its pure and dosage forms in the presence of its acidic degradate.

3.1. Derivative Ratio Method

Derivative ratio spectrophotometric method was designed by Salinas *et al.* [14], which is based on derivatization of the ratio spectra for resolving binary mixtures. The main benefit of the derivative ratio spectrophotometry is that the spectrum of the interferent is totally cancelled, so the selection of the wavelength used for calibration is not critical; thus, easy measurements can be done at the wavelength of highest value either a maximum or a minimum [15, 16].

To optimize the proposed DD^1 method, several concentrations of the acidic degradate 12, 18, and 20 µg/mL were tried as a divisor, while satisfactory result was obtained using 20 µg/mL of the degradate as a divisor. Multiple Δλ and scaling factors were tested, where Δλ = 8 and a scaling factor = 10 were the optimum to facilitate iohexol determination and to minimize the error in reading the signal (Figs. **5** and **6**). The absorption spectra of iohexol in the range of 4-40 µg/mL were divided by the absorption spectrum of 20 µg/mL of the degradate (as a divisor), where the obtained ratio spectra were differentiated with respect to wavelength. DD^1 values showed good linearity at 224.6 nm.

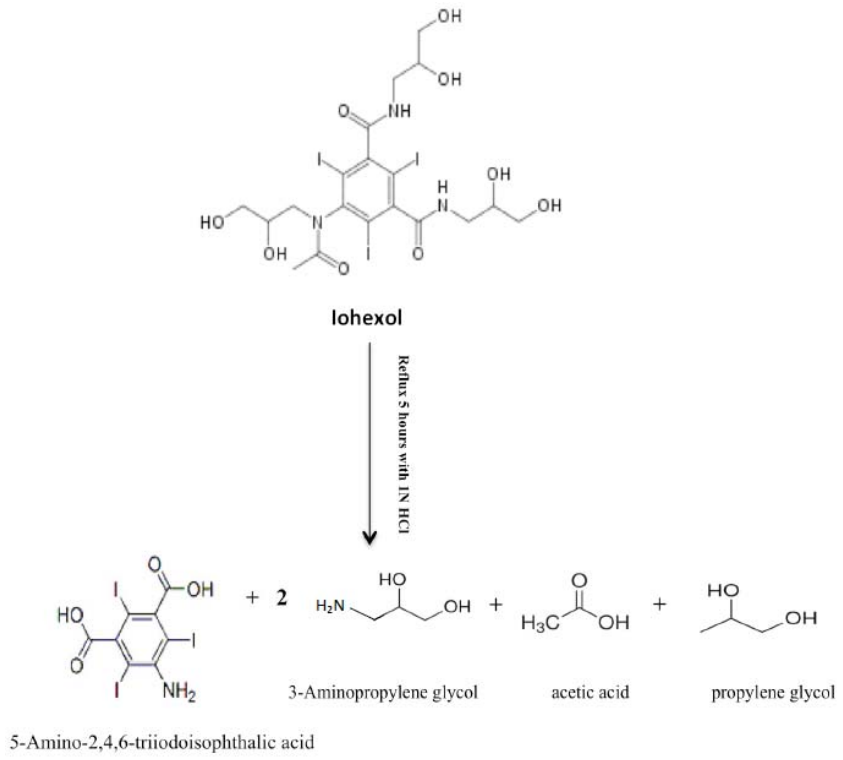

Fig. (1). The degradation mechanism of iohexol [1].

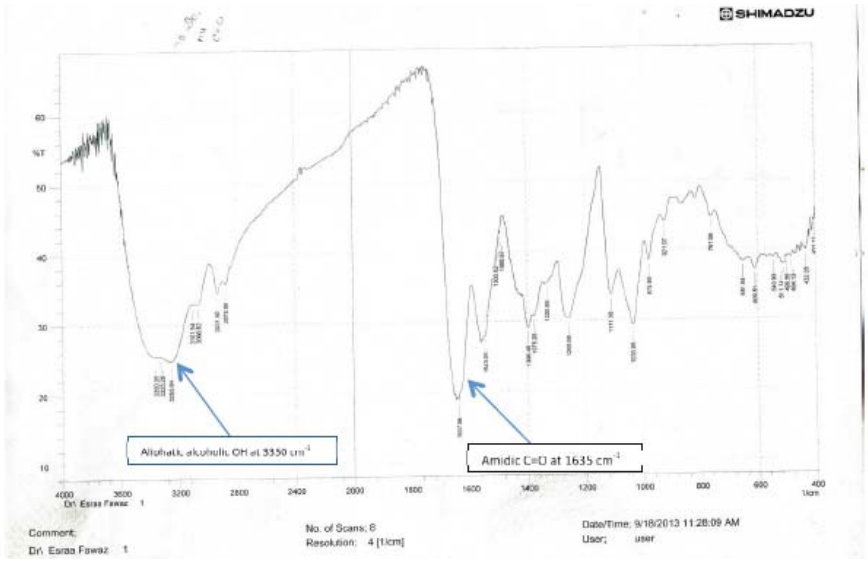

Fig. (2). IR spectrum of intact Iohexol.

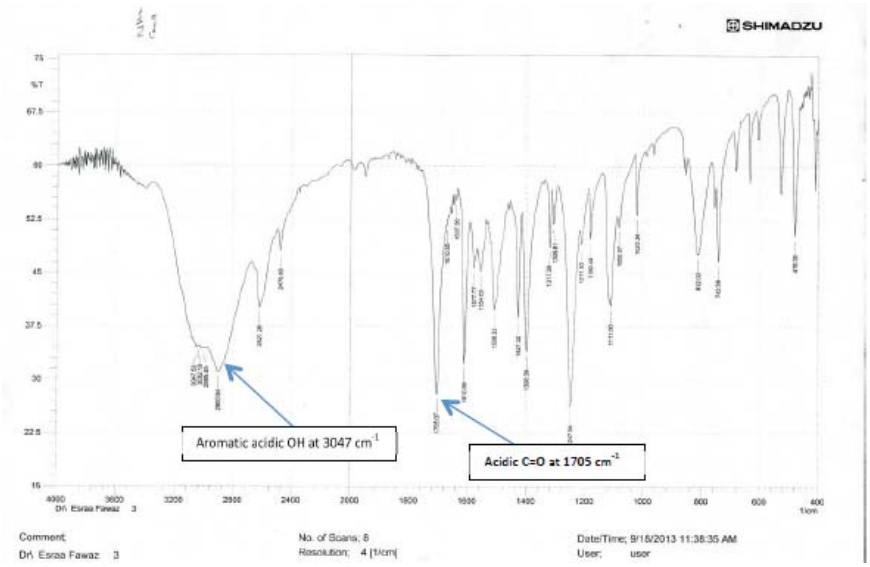

Fig. (3). IR spectrum of Iohexol degradation product.

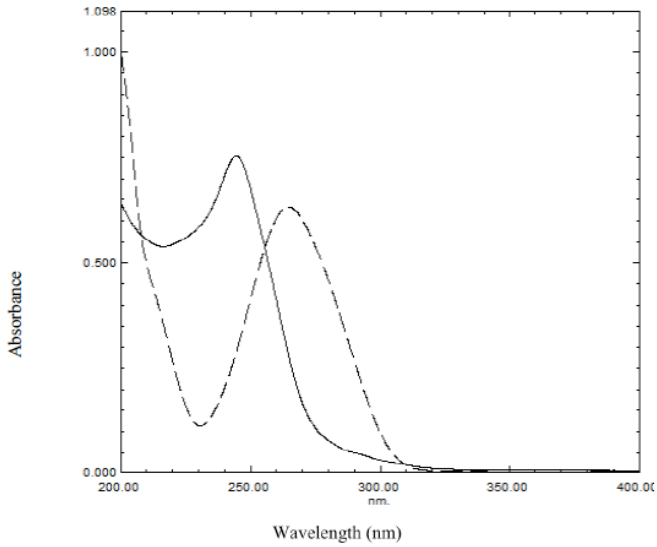

Fig. (4). Zero order absorption spectra of Iohexol 20 µg/mL(——) and the degradate 20 µg/mL (-----).

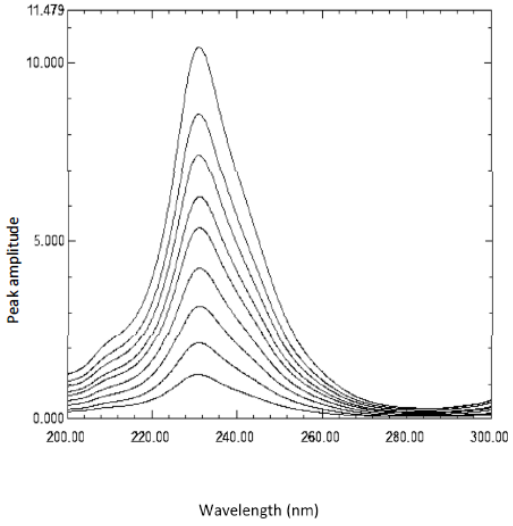

Fig. (5). Ratio spectra of Iohexol 4, 8, 12, 16, 20, 24, 28, 32, and 40 µg/mL using the spectrum of 20 µg/mL of its acidic degradate as a divisor.

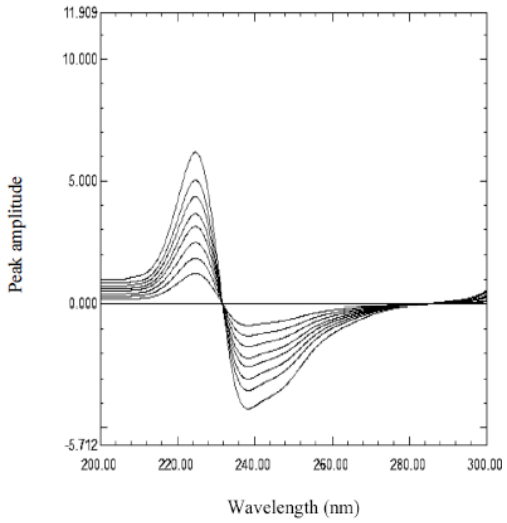

Fig. (6). First derivative of ratio spectra of Iohexol (4, 8, 12, 16, 20, 28, 32, and 40) using the spectrum of degradate (20 µg/mL) as a divisor.

The linearity of the peak amplitudes of the DD^1 curves at 224.6 nm was investigated and a good linear relationship was obtained in the range of 4-40 µg/mL for the iohexol, and the regression parameters were calculated (Table **1**). The method was checked by analysis of laboratory prepared mixtures of iohexol and its acidic degradate in different percentages as presented in Table **2**. Iohexol could be accurately measured in the presence of up to 90% of its acidic degradate with mean percentage recovery of 100.12± 1.036%.

3.2. Ratio Difference Method

Ratio difference method was developed recently as a new, simple and selective method for the determination of components with overlapping spectra, having the advantages of minimal data processing and wide range of applications [17].

Ratio difference spectrophotometric method was applied to solve the problem of the overlapped absorption spectra of iohexol and its degradate as upon dividing the spectrum of iohexol by a divisor of a certain concentration of the degra-

Table 1. Assay validation sheet of the proposed methods for the determination of pure Iohexol.

Parameter	Derivative Ratio Method	Ratio Difference Method	Mean Centering Method
Range	4-40 (µg/mL)	4-40 (µg/mL)	4-40 (µg/mL)
Linearity			
Slope	0.1529	0.0135	0.0516
Intercept	0.0529	0.0513	0.0333
Correlation coefficient (r)	0.9998	0.9997	0.9998
Specificity	100.12 ± 1.036	100.58 ± 1.031	100.59± 0.905
Accuracy (mean±RSD)	99.84 ± 1.046	100.38 ± 0.826	99.93 ± 0.882
Precision (RSD%)			
Repeatability[a]	0.731	1.066	1.248
Intermediate precision[b]	0.824	0.974	0.502

[a] The intraday and [b] the inter-day RSD values of samples of concentration of 18, 26 and 32 µg/mL of iohexol.

Table 2. Determination of Iohexol in laboratory-prepared mixtures by the proposed methods.

% Degradation Product	Concentration (µg/mL)	Derivative Ratio Method	Ratio Difference Method	Mean Centering Method
10	4	100.72	98.75	99.18
20	8	101.18	101.38	101.43
30	12	100.68	101.83	99.92
40	16	100.42	100.88	99.89
50	20	98.05	100.70	101.72
60	24	99.19	101.42	99.94
70	28	101.22	99.18	101.65
80	32	100.13	100.16	101.00
90	36	99.50	100.92	100.60
mean		100.12	100.58	100.59
RSD%		1.036	1.031	0.905

date, a ratio spectrum results, and a linear relationship between the difference in amplitudes at any two wavelengths and the corresponding concentration of iohexol results, while the ratio spectrum of the degradate become a straight line of constant amplitude parallel to the *x*-axis and the difference in amplitudes at any two wavelengths will be zero [18, 19].

The method contains two critical steps; the first step is the selection of the divisor concentration; the selected divisor should provide minimal noise and maximum sensitivity [20, 21]. Different concentrations of the degradate were tested at 6, 10, 20, and 24 µg/mL and the divisor concentration of 20 µg/mL was found to be the optimum when used for the prediction of iohexol concentration in pure form and in laboratory prepared mixtures (Figs. **5** and **7**). The second step is the selection of the wavelength values at which the measurements are taken. Any two wavelengths can be chosen, if they show different amplitudes in the ratio spectrum and a good linearity is present at each wavelength individually. The wavelength pairs 227-240, 227-255, and 227-265 nm could not be used as 227 nm showed poor linearity. 255-250 and 255-265 nm were also tried and 255-265 nm showed the best results.

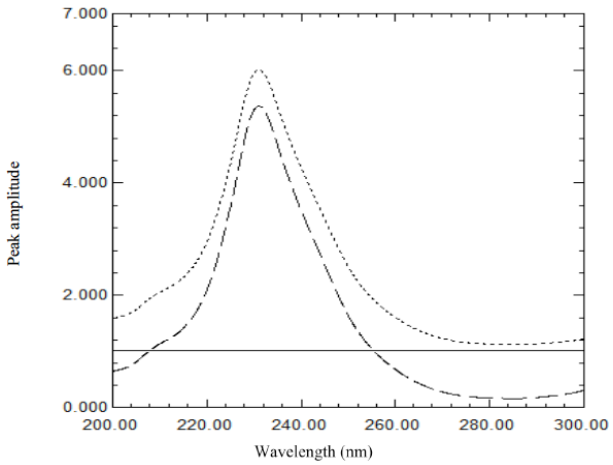

Fig. (7). Ratio spectra of Iohexol 20 µg/mL (- - -), degradate 20 µg/mL (————), and a laboratory prepared mixture containing 20 µg/mL of both (…….) using a divisor of 20 µg/mL degradate.

Linear correlation was obtained from the differences in amplitude at 255-265 nm for iohexol in the range of 4-40 µg/mL, and the regression equation was computed (Table **1**). The method was checked by the analysis of laboratory prepared mixtures of iohexol and its acidic degradate in different ratios as presented in Table **2**. Iohexol could be determined in the presence of up to 90% of its acidic degradate, with mean percentage recovery of 100.58% ± 1.03.

3.3. Mean Centering Method

For further improvement of selectivity, mean centering method was applied. It is a recently developed simple method that depends on the manipulation of the ratio spectra by the MATLAB® software to cancel the effect of one of the mixtures to determine the other one. It eliminates the derivative step and therefore, the signal-to-noise ratio is enhanced [22, 23]. The absorption spectra of iohexol (4-40 µg/mL) were divided by the spectrum of the acidic degradate 20 µg/mL, then the ratio spectra were mean centered (Fig. **8**). Calibration curve was constructed between the concentration of iohexol and the amplitude of the mean centered ratio spectra at 234 nm. The method was checked by the analysis of laboratory prepared mixtures of iohexol and its acidic degradate in different ratios as presented in Table **2**.

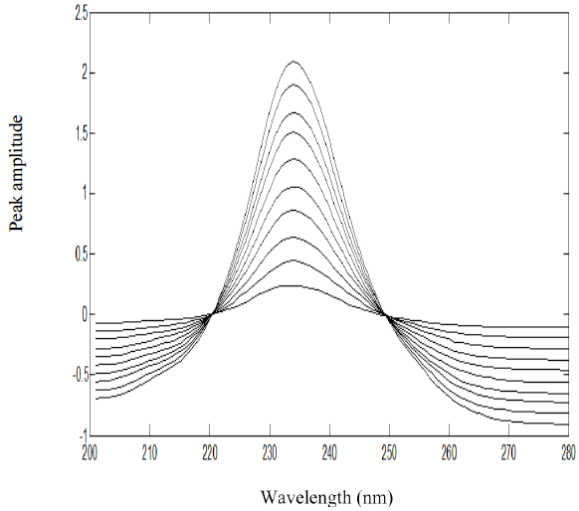

Fig. (8). Mean centered ratio spectra of Iohexol (4-40 µg/mL) using the spectrum of degradate (20 µg/mL) as a divisor.

The usefulness of the proposed methods was successfully applied to the analysis of iohexol in its pharmaceutical formulation and was studied in the presence of additives by assaying Omnipaque® vial. The validity of the methods was assessed by applying the standard addition technique (Table **3**).

One-way ANOVA was performed for comparing the three proposed methods and non-significant differences were obtained (Table **4**). Moreover, results obtained by the proposed procedures for the determination of pure samples of the drug were statistically compared to those obtained by the official US pharmacopeial method and no significant difference was observed (Table **5**). The official method depends on using standard 0.1N $AgNO_3$ as a titrant and the end point was detected potentiometrically. Method validation was performed according to the USP guidelines for all the proposed methods [10]. Table **1** shows results of accuracy, repeatability and intermediate precision of the methods.

The three proposed methods depend on calculating the ratio spectra which are then manipulated mathematically by different ways to get the concentration of the component of interest. Unlike the derivative ratio method, the ratio difference method allows the measurement at any two wavelengths without the need of a derivatization step. Ratio difference method also has an advantage over mean centering method as it does not require special software such as MATLAB® for further manipulation of the ratio spectra. Being a simple one-step method may show the superiority of ratio difference method over the other two methods.

Table 3. Quantitative determination of iohexol in Omnipaque® vial by the proposed methods and the results of application of standard addition technique

Omnipaque® Vial	Derivative Ratio Method	Ratio Difference Method	Mean Centering Method
	Recovery ± SD [a]		
Batch no. 12062934	101.37 ± 0.664	101.03 ± 0.673	993.36 ± 0.881
Recovery% of standard added	99.73± 0.485	99.68 ± 0.641	100.60 ± 0.856

[a]Average of three determinations.

Table 4. Results of ANOVA (one way) for comparison of the proposed methods for the determination of iohexol.

Source of Variation	SS	df	MS	F	P-value	F-crit
Between Groups	2.84	2.00	1.42	2.18	0.05	3.35
Within Groups	17.56	27.00	0.65			
Total	20.40	29.00				

Table 5. Statistical analysis of the results obtained by the proposed methods and the official method for the determination of iohexol in pure powder form.

Item	Derivative Ratio Method	Ratio Difference Method	Mean Centering Method	Official Method [a]
Mean	99.84	100.38	99.93	100.28
SD	1.04	0.83	0.88	0.71
Variance	1.08	0.69	0.78	0.51
n	10	10	10	5
Student's t-test [b]	1.81 (2.13)	0.229 (2.13)	0.654 (2.13)	
F-value [b]	1.15 (6.79)	1.353 (6.79)	1.53 (6.79)	

[a] Precipitimetric titration using standard 0.1N $AgNO_3$ and the end point was detected potentiometrically.
[b] Figures between parentheses represent the corresponding tabulated values of t and F at $P = 0.05$.

CONCLUSION

The proposed spectrophotometric methods have the advantages of being simpler and can analyze iohexol in its pure and dosage forms in the presence of up to 90% of its acidic degradate with no lengthy calculations or complex procedures. Unlike the chromatographic methods, these methods do not need expensive solvents, sample pre-treatment, buffers, or sophisticated techniques and instruments. The methods are suitable and valid for application in quality control laboratories. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration graphs and the validity of Beer's law. Ratio difference method showed an advantage over the two other proposed methods. From the results obtained, we concluded that the suggested methods showed high sensitivity, accuracy, reproducibility and specificity can be used as stability indicating methods in quality control laboratories.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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