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Stability indicating methods for the determination of clozapine

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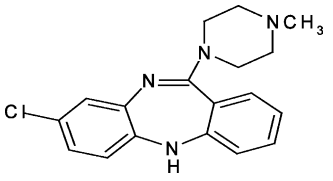
Abstract

Five new selective, precise and accurate methods are described for the determination of clozapine in the presence of its main degradation product. Method A utilizes the second and third derivative spectrophotometry at 315 and 305 nm, respectively. Method B is RSD_1 spectrophotometric method based on the simultaneous use of the first derivative of ratio spectra and measurement at 295 nm. Method C is a pH-induced difference (ΔA) spectrophotometry using UV measurement at 325 nm. Method D is a densitometric one, after separation on silica gel plate using methanol: water as mobile phase, and the spot was scanned at 295 nm. Method E is RP-HPLC using acetonitrile: water (40:60 v/v) as mobile phase at a flow rate of 1 ml/min and UV detection was at 295 nm. Regression analysis showed good correlation in the concentration ranges 3–10, 4–10, 10–25 μg/ml, 200–1000 ng/spot, 5–100 μg/ml with percentage recoveries of 99.4 ± 0.28, 99.8 ± 0.20, 100.05 ± 0.11, 99.41 ± 0.34, 100.11 ± 0.07 and 100.07 ± 0.05% for methods A, B, C, D and E, respectively. These methods are suitable as stability indicating methods for the determination of clozapine in the presence of its main degradation product either in bulk powder or in pharmaceutical formulations.

Keywords: Clozapine; Derivative spectrophotometry; RSD_1 spectrophotometry; ΔA spectrophotometry; TLC-densitometry; RP-HPLC

1. Introduction

Clozapine is 8-chloro-11-(4-methylpiperazine-1-yl)-5H-dibenzo[b, e] [1, 4] diazepine and is used as antipsychotic drug [1]. The structural formula is as follows:



Several methods have been reported for the determination of clozapine including titrimetric [2], electrochemical [3,4], spectrophotometric [5,6] and chromatographic [7–18] methods. None of these reported methods were used for the determination of clozapine in presence of its main acid-induced degradation product.

The main task of this work is to establish simple and accurate stability indicating methods for the determination of clozapine in the presence of its main, acid-induced, degradation product, which can be used for the routine and quality control analysis of clozapine in raw material and pharmaceutical formulations.

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2. Experimental

2.1. Instruments

1. SHIMADZU UV–VISIBLE 1601 PC spectrophotometer.
2. UV lamp with short wavelength 254 nm.
3. TLC plates (20 × 20 cm) coated with silica gel 60 F_{254} (E. MERCK).
4. SHIMADZU—dual wavelength flying spot CS-9301densitometer.
5. SHIMADZU CLASS-LC 10 liquid chromatographic system equipped with SHIMADZU SPD-10 A diiode array uv-detector, ZORBAX® C_{18} (5um) column was used as stationary phase.

2.2. Materials

2.2.1. Pure samples

Clozapine (CL) was kindly supplied by The National Organization for Drug Control and Research (NODCR). The purity of the sample was found to be 99.98% according to the B.P. method (1998).

2.2.2. Dosage forms

Leponex® tablets (Sandoz) each tablet was claimed to contain 25 mg of clozapine; Batch number 023.
Leponex® tablets (Sandoz) each tablet was claimed to contain 100 mg of clozapine; Batch number 332.
Clozapex® tablets (Apex pharma Egypt) each tablet was claimed to contain 25 mg of clozapine Batch number 228.
Clozapex® tablets (Apex pharma Egypt) each tablet was claimed to contain 100 mg of clozapine Batch number 90360.

2.2.3. Reagents

1. Methanol HPLC grade (B.D.H.).
2. Acetonitrile HPLC grade (B.D.H.).
3. Deionized water.
4. 0.1 N hydrochloric acid.
5. 0.1 N sodium hydroxide.
6. 2 N sulfuric acid.

2.2.4. Preparation of the degradation product

Pure CL (− 0.3 g) were heated at reflux with 20 ml 2 N sulfuric acid for 1 h. The solution was allowed to cool and, upon cooling, the amide degradation product separated out. The precipitate was filtered, washed and recrystalized. The obtained powder was identified using IR spectroscopy and thin layer chromatography, then it was used for the preparation of the stock solution of the degradate. The filterate contains *N*-methylpiperazine, which is liquid and miscible with water, so it has been difficult to separate it for further quantitation.

2.2.5. Standard solutions

2.2.5.1. Clozapine stock solution (1 mg/ml). An accurately weighed amount of clozapine equivalent to 100 mg was transferred into a 100 ml volumetric flask, 40 ml absolute ethanol was added, shaken for 10 min and completed to volume with methanol.

2.2.5.2. Clozapine working solutions.

(i) For D_2 and D_3, RSD_1 and ΔA spectrophotometric methods (100 μg/ml): An amount equivalent to 10 ml of the previous stock solution was transferred into a 100 volumetric flask and completed to volume with methanol.

(ii) For densitometric method (0.5 mg/ml): an amount equivalent to 50 ml of the previous stock solution was transferred into a 100 volumetric flask and completed to volume with methanol.

(iii) For HPLC method (100 μg/ml): an amount equivalent to 10 ml of the previous stock solution was transferred into a 100 volumetric flask and completed to volume with acetonitrile:water (40:60 v/v).

The degradate was prepared at the same above-mentioned concentrations and solvents for each of the corresponding methods.

2.2.6. Laboratory prepared mixtures

2.2.6.1. For second (D_2) & third derivative (D_3) spectrophotometric method. Accurate aliquots equivalent to (30–100 μg) of CL were transferred from its working solution (100 μg/ml) into a series of 10 ml volumetric flasks, and portions equivalent to 10–80% of the degradate from its working solution (100 μg/ml) were added to the same flasks and the volume was completed to the mark with methanol.

2.2.6.2. For RSD_1 spectrophotometric method. Accurate aliquots equivalent to (40–100 μg) of CL were transferred from its working solution (100 μg/ml) into a series of 10 ml volumetric flasks, and portions equivalent to 10–80% of the degradate from its working solution (100 μg/ml) were added to the same flasks and the volume was completed to the mark with methanol.

2.2.6.3. For ΔA spectrophotometric method. Accurate aliquots equivalent to (100–250 μg/ml) of CL were transferred from its working solution (100 μg/ml) into two sets of 10 ml volumetric flasks, and portions equivalent to 10–60% of the degradate from its working solution (100 μg/ml) were added to the same flasks and the volume was completed to the mark in one set with 0.1 N sodium hydroxide and in the other set with 0.1 N hydrochloric acid.

2.2.6.4. For densitometric method. Accurate aliquots equivalent to (200–1000 μg) of CL were transferred from its working solution (0.5 mg/ml) into a series of 10 ml volumetric flasks, and portions equivalent to 10–100% of the degradate from its working solution (0.5 mg/ml) were added to the same flasks and the volume was completed to the mark with methanol.

2.2.6.5. For HPLC method. Accurate aliquots equivalent to (50–1000 μg) of CL were transferred from its working solution (100 μg/ml) into a series of 10 ml volumetric flasks, and portions equivalent to 10–100% of the degradate from its working solution (100 μg/ml) were added to the same flasks and the volume was completed to the mark with acetonitrile: water (40:60 v/v).

2.3. Procedures

2.3.1. 1-Method A, D_2 and D_3 derivative spectrophotometric method

2.3.1.1. Linearity. Accurate aliquots equivalent to (30–100 μg) of CL were transferred from its working solution into a series of 10 ml volumetric flasks then made up to volume using methanol. The second and third derivative absorption spectra of the uv-spectrum of each solution against methanol as a blank were recorded. The peak heights, using 315 and 305 nm respectively, as maxima and zero crossing line as minima were measured. The calibration curves representing the relationship between the measured peak height and the corresponding concentration were constructed.

2.3.1.2. Assay of prepared mixtures. The D_2 & D_3 spectra of the laboratory-prepared mixtures containing different ratios of CL and its degradate were recorded. The peak heights at315 and 305 nm, respectively were measured. The concentration of CL in the prepared mixtures was calculated from the regression equations.

2.3.2. 2-Method B, RSD_1 spectrophotometric method

2.3.2.1. Linearity. Accurate aliquots equivalent to (40–100 μg) of CL were transferred from its working solution into a series of 10 ml volumetric flasks then made up to volume using methanol.The absorption spectra of the these solutions were divided by the 'the divisor' (3 μg/ml of the degradate) and the ratio spectra thus obtained were smoothed and the first derivatives of the ratio spectra were recorded. The peak amplitude at 295 nm was measured. The calibration curve representing the relationship between the measured amplitude and the corresponding concentration was constructed.

2.3.2.2. Assay of prepared mixtures. The ratio spectra first derivative curves of the laboratory-prepared mixtures containing different ratios of CL and its degradate were recorded. The peak amplitude at 295 nm was measured, and then the concentration of CL in the prepared mixture was calculated from the regression equation.

2.3.3. Method C, ΔA spectrophotometric method

2.3.3.1. Linearity. Accurate aliquots equivalent to (100–250 μg) of CL were transferred from its working solution (100 μg/ml) into two sets of 10 ml volumetric flasks. The volume was diluted in one set with 0.1 N sodium hydroxide and in the other set with 0.1 N hydrochloric acid. The ΔA spectrum for each concentration was recorded at 325 nm, by placing the alkaline solution in the reference beam and the acid solution in the sample beam. The calibration curve relating the ΔA at 325 nm to CL concentration was constructed.

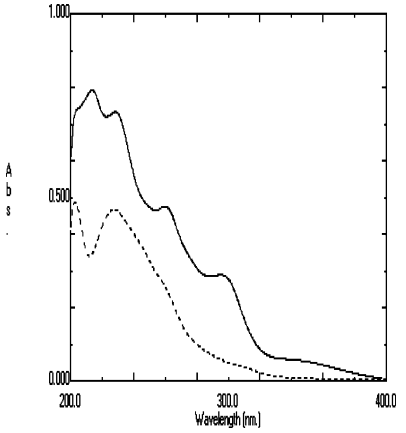

Fig. 1. Zero-order spectra of: -Intact clozapine (10 μg/ml), [_____]. Degradation product (3 μg/ml), [-------].

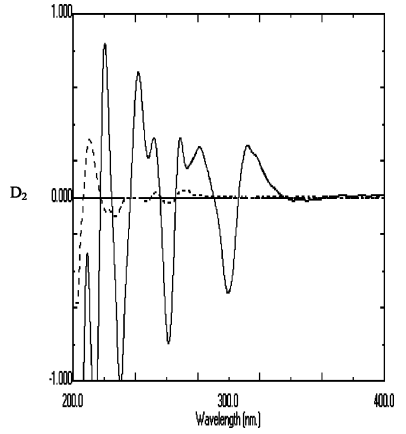

Fig. 2. Second derivative spectra of the methanolic solutions of: -Intact clozapine (10 μg/ml), [_____]. Degradation product (3 μg/ml), [------].

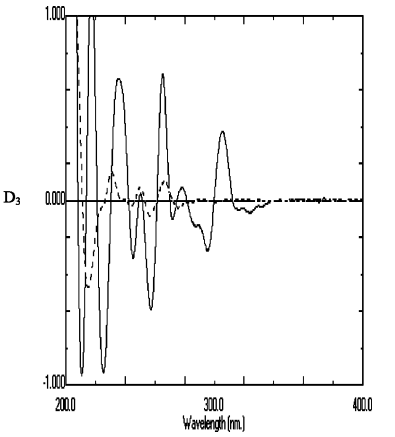

Fig. 3. Third derivative spectra of the methanolic solutions of: -Intact clozapine (10 μg/ml), [_____]. Degradation product (3 μg/ml), [------].

2.3.3.2. Assay of prepared mixtures. The ΔA spectra of the laboratory-prepared mixtures containing different ratios of CL and its degradate were recorded. The peak amplitude at 325 nm was measured, then the concentration of CL in the prepared mixture was calculated from the regression equation.

2.3.4. Method D, densitometric method

2.3.4.1. Linearity. Accurate aliquots equivalent to (200–1000 μg) of CL were transferred from its working solution (0.5 mg/ml) into a series of 10 ml volumetric flasks then the volume was completed with methanol. Ten μl of each solution was applied to a thin layer chromatographic plate (20×20 cm) using 10-μl micro syringe. Spots were spaced 2 cm apart from each other, 1.5 cm from the bottom edge of the plate, the plate was placed in chromatographic tank previously saturated for 1 h with the developing mobile phase

methanol: water (60:40 v/v). The plate was developed by ascending chromatography through a distance of 16 cm, dried at room temperature, the spots were detected under UV lamp, and scanned at 295 nm. (Photomode: reflection and scan mode: zigzag). The calibration curve representing the relationship between the recorded area under the peak and the corresponding concentration was constructed.

2.3.4.2. Assay of prepared mixtures. Ten μl of different samples of the laboratory prepared mixtures were applied to a thin layer chromatographic plate; proceed as mentioned under linearity starting from 'Spots were spaced …'. The area under the peak was recorded and the concentration of CL was calculated from the regression equation.

2.3.5. 5-Method E, RP-HPLC method

2.3.5.1. Linearity. Accurate aliquots equivalent to (50–1000 μg/ml) of clozapine were transferred from its working solution (100 μg/ml) into a series of 10 ml volumetric flasks. Methanol: water (60:40 v/v) was added to volume to give a final concen-

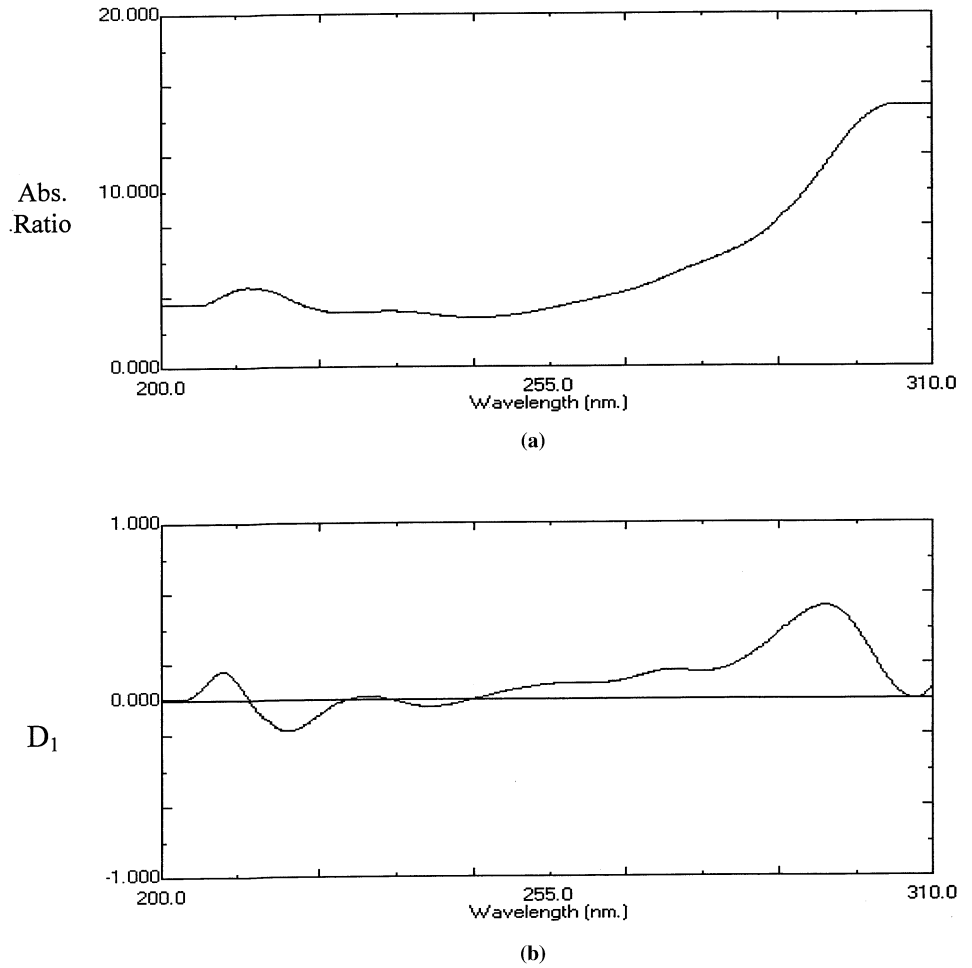

Fig. 4. Ratio-spectra and first derivative curves of the ethanolic solutions of intact clozapine (10 μg/ml) using 3 μg/ml of the degradation product as the divisor [—].

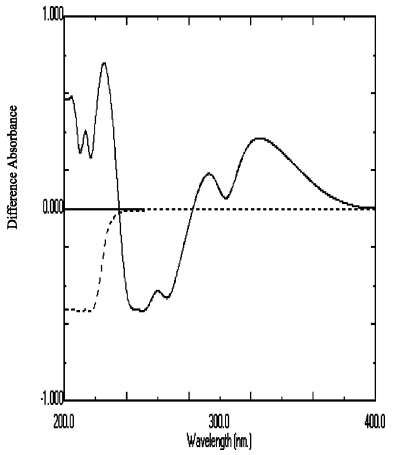

Fig. 5. Difference spectra of: -Intact clozapine (15 μg/ml), [_____]. Degradation product (5 μg/ml), [------].

tration range from 1 to 50 μg/ml. Twenty μl of the solution from each of the above was injected and the chromatograms were recorded maintaining the flow rate at 1 ml/min and monitoring the effluent at 230 nm. Peak area values were then plotted as a function of clozapine concentration to obtain the calibration curve.

2.3.5.2. Assay of prepared mixtures. The specified HPLC method was followed for the analysis of laboratory prepared mixtures containing different ratios of clozapine and its degradate. The peak area values for clozapine were recorded then the concentration of clozapine in the prepared mixtures was calculated from the regression equation.

2.3.6. Assay of pharmaceutical formulation

The contents of ten tablets of each of the pharmaceutical formulations were thoroughly powdered and mixed, an amount of the powder equivalent to 100 mg of CL was weighed accurately in 250 ml beaker, 70 ml of methanol was added, stirred magnetically for about 30 min then filtered through a filter paper into a 100 ml volumetric flask, the beaker and the funnel were washed and the volume was completed with methanol. The solutions were diluted to the same concentrations of the working standard solutions and treated according to linearity for each method.

3. Results and discussion

In this work, clozapine was determined in the presence of its main acid-induced degradation product. The degradation product was prepared via acid hydrolysis of clozapine.

The investigated drug and its degradation product were stable under the specified conditions for each of the prposed methods.

3.1. Method A, second & third derivative spectrophotometric method

Zero order absorption spectra of clozapine and its degradate in methanol show severe overlapping which interferes with the direct determination of pure clozapine (Fig. 1).

As shown in Figs. 2 and 3, it is clear that the overlapping observed in the zero order absorption spectra was eliminated and sharply defined, well separated peak at 315 and 305 nm for the intact molecule which lies at the zero crossing of its degradate was obtained and used for the D_2 and D_3 spectrophotometric determination of intact clozapine in presence of its degradate.

By applying the D_2 and D_3 spectrophotometric method, a linear correlation was obtained between the peak height and the concentration over the range 3–10 μg/ml for pure clozapine and the following regression equations were obtained:

$$H_1 = 0.06C_1 + 0.04, \quad r_1 = 0.9997$$

$$H_2 = 0.042C_2 + 0.013, \quad r_2 = 0.9997$$

where 'H_1' and 'H_2' stand for the peak heights in millimeter at 315 and 305 nm respectively, 'C_1' and 'C_2' for the concentrations in μg/ml and 'r_1' and 'r_2' for the correlation coefficients.

3.2. Method B, RSD_1 spectrophotometric method

Fig. 1 shows the absorption spectra of clozapine and its degradate which overlap seriously. Fig. 4 shows the ratio spectrum of clozapine (spectrum divided by the spectrum of 3 μg/ml of the degradate) and its first derivative curve. The peak at 295 nm for intact clozapine can be adopted for the determination of clozapine in presence of its degradate and in pharmaceutical formulations.

Calibration curve was obtained by plotting the peak amplitude at 295 nm of the first derivatives of the ratio spectra of clozapine versus concentration, which shows linear relationship in the range of 4–10 μg/ml. From which the following regression equation was calculated:

$$A = 0.045C - 0.032, \quad r = 0.9998$$

where 'A' stands for the peak amplitude at 295 nm, 'C' for the concentration in μg/ml and 'r' is the correlation coefficient.

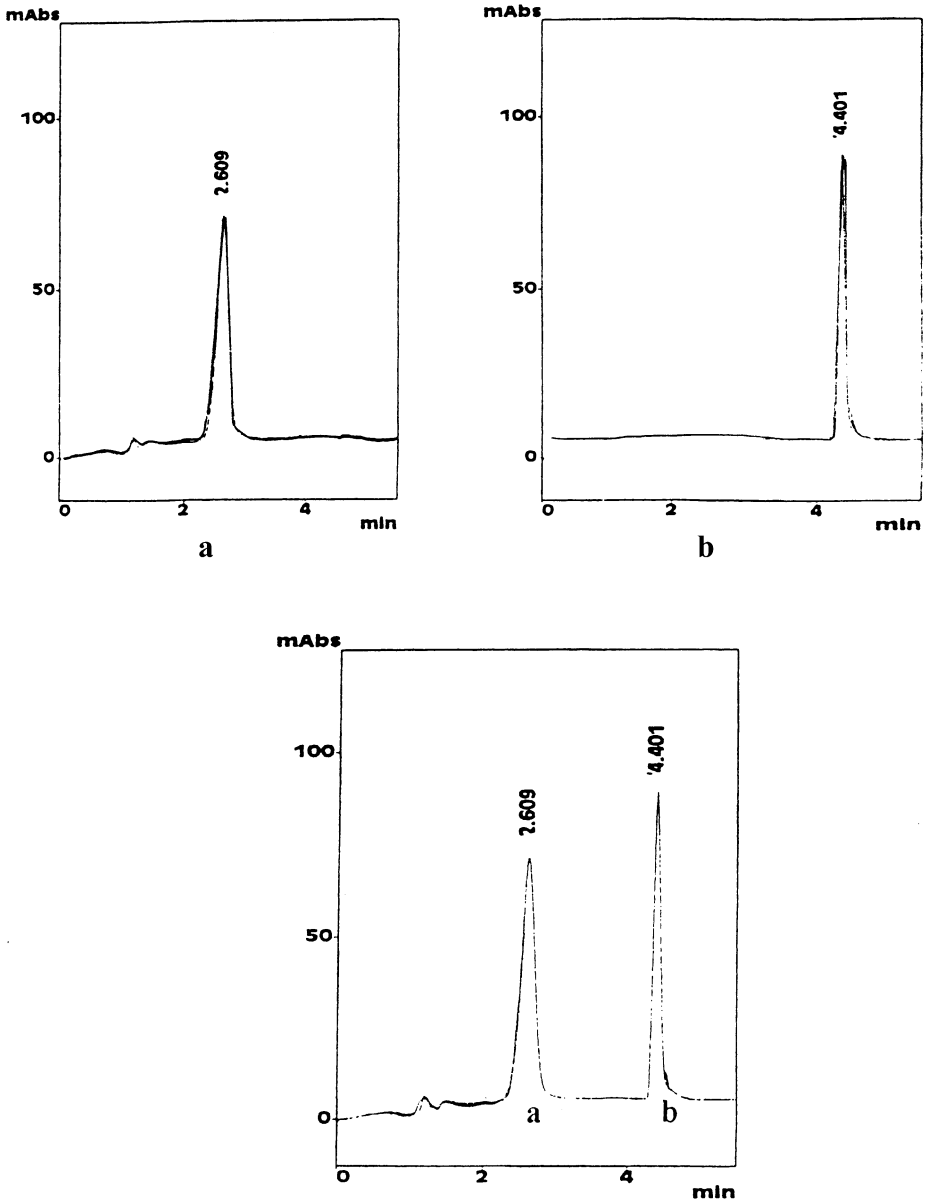

Fig. 6. HPLC chromatograms of (a) degradation product, 10 μg/ml (b) pure clozapine, 20 μg/ml.

Table 1
Comparison between the proposed methods and the reported one for the determination of clozapine in the presence of its degradate

Sample number[a]	Percent of degradate	D_2 method	D_3 method	RSD_1 method	ΔA method	Densitometric method	RP-HPLC method	B.P. (1998) method
		Found (%)						
1	10	99.23	99.73	100.12	99.56	99.98	99.98	100.20
2	20	99.21	100.25	100.21	99.62	100.02	99.96	106.96
3	30	99.99	100.47	100.14	99.75	100.05	100.02	113.20
4	40	99.21	100.91	100.17	99.88	100.11	100.05	116.56
5	50	98.92	99.78	100.05	99.98	100.12	100.01	125.12
6	60	98.89	99.36	100.12	99.47	100.13	99.98	150.35
7	70	99.52	93.65[b]	100.33	92.32[b]	100.06	99.97	176.58
8	80	92.64[b]	92.85[b]	96.45[b]	92.52[b]	100.15	100.02	188.56
Mean ± S.D.		99.28 ± 0.37	100.1 ± 0.6	100.16 ± 0.088	99.71 ± 0.19	100.08 ± 0.05	100.003 ± 0.02	

[a] Average of four experiments.
[b] Percentage rejected.

Table 2
Comparison between the proposed methods and the reported one for the determination of clozapine in pure form and in its pharmaceutical formulation

Preparation	D_2 & D_3 method (Found (%))	Standard addition (Recovery % ± S.D.)	RSD_1 method (Found (%))	Standard addition (Recovery % ± S.D.)	ΔA method (Found (%))	Standard addition (Recovery % ± S.D.)	Densitometric method (Found (%))	Standard addition (Recovery % ± S.D.)	RP-HPLC method (Found (%))	Standard addition (Recovery % ± S.D.)	B.P. method (Found (%))
Pure sample	99.4 ± 0.28 & 99.8 ± 0.2		100.05 ± 0.11		99.41 ± 0.34		100.11 ± 0.07		100.07 ± 0.05		99.36 ± 0.32
Leponex tablets 25 Batch number 023		100.86 ± 0.15 & 100.29 ± 0.36		100.12 ± 0.11		99.77 ± 0.08		99.69 ± 0.17		99.53 ± 0.28	98.81 ± 0.35
Leponex tablets 100 Batch number 332		99.98 ± 0.1 & 99.6 ± 0.52		99.68 ± 0.17		99.79 ± 0.3		99.93 ± 0.015		99.01 ± 0.53	99.94 ± 0.62
Clozapex tablets 25 Batch number 228		99.02 ± 0.17 & 99.55 ± 0.33		99.99 ± 0.09		100.12 ± 0.13		99.84 ± 0.18		100.11 ± 0.11	98.98 ± 0.28
Clozapex tablets 100 Batch number 90360		99.54 ± 0.15 & 99.08 ± 0.09		99.71 ± 0.088		100.006 ± 0.037		99.48 ± 0.07		99.52 ± 0.05	99.54 ± 0.34

Table 3
Assay validation of the proposed methods for the determination of diloxanide furoate

Parameter	D_2 method	D_3 method	RSD_1 method	ΔA method	Densitometric method	RP-HPLC method
Range	3–10 (μg/ml)	3–10 (μg/ml)	4–10 (μg/ml)	10–25 (μg/ml)	200–100 (ng per spot)	5–100 (μg/ml)
LOD	1.21 (μg/ml)	1.35 (μg/ml)	1.59 (μg/ml)	3.85 (μg/ml)	35.89 (ng per spot)	1.65 (μg/ml)
LOQ	2.85 (μg/ml)	2.94 (μg/ml)	3.82 (μg/ml)	9.74 (μg/ml)	198.32 (ng per spot)	4.97 (μg/ml)
Correlation coefficient (r)	0.9997	0.9997	0.9998	0.9999	0.9999	0.9996
RSD[a]	1.134	1.123	1.252	1.553	1.274	1.227
RSD[b]	1.421	1.154	1.221	1.315	1.324	1.312

[a] The intraday ($n = 4$) variations for the proposed methods.
[b] The interday ($n = 9$) variations for the proposed methods.

3.3. Method C, ΔA spectrophotometric method

The ΔA spectra between 0.1 N sodium hydroxide and 0.1 N hydrochloric acid for intact clozapine and its degradate, were recorded and from these spectral characteristics it is clear that the ΔA peak at 325 nm for the intact clozapine between 0.1 N hydrochloric acid and 0.1 N sodium hydroxide could be considered as the λ_{max} most suitable for adopting the ΔA technique for the selective determination of intact clozapine in presence of its degradate as at this maxima, ΔA for the latter reads zero (Fig. 5).

A calibration curve was constructed relating the ΔA values at 325 nm to drug concentration showing perfect linearity in the range of 10–25 μg/ml from which the following regression equation was calculated:

$$A = 0.007C - 0.05, \quad r = 0.9999$$

where 'A' stands for the peak amplitude at 325 nm, 'C' for the drug concentration in μg/ml and 'r' is the correlation coefficient.

3.4. Method D, densitometric method

This method was applied for the determination of clozapine. Complete separation of clozapine was obtained using methanol: water (60:40 v/v) as developing mobile phase. Quantitatively the chromatogram was scanned densitometrically at 295 nm. By applying this technique a linear correlation was obtained between the area under the peak and the concentration of clozapine in the range of 200–1000 ng per spot. The following regression equation was calculated for clozapine:

$$A = 0.014C - 0.063, \quad r = 0.9999$$

where 'A' is the area under the peak, 'C' is the corresponding concentration in μg/ml and 'r' is the correlation coefficient.

3.5. Method E, RP-HPLC method

A simple and stability indicating isocratic HPLC method was adopted for the analysis of clozapine in presence of its degradate and in pharmaceutical formulations. The best peak shape was obtained with acetonitrile: water (40:60 v/v) with retention time of 4.4 min. The final dilution of samples has been done using acetonitrile: water (40:60 v/v) to avoid frontal peak tailing.

A typical chromatogram of pure clozapine is shown in Fig. 6. The chromatograms as shown in Fig. 6 showed no peak interferences between the drug and its degradate.

The calibration curve for clozapine was constructed by plotting concentration versus peak area showed good linearity in the range of 5–100 μg/ml. The regression equation was calculated and found to be:

Table 4
Statistical analysis of the results obtained by the five proposed methods and B.P. (1998) method for the determination of clozapine

	B.P. (1998) method	D_2 spectrophotometric method	D_3 spectrophotometric method	RSD_1 spectro-photometric method	ΔA spectrophotometrie method	Densitometric method	HPLC method
Concentration range	300 mg	3–10 (μg/ml)	3–10 (μg/ml)	4–10 (μg/ml)	10–25 (μg/ml)	200–1000 (ng/spot)	5–100 (μg/ml)
Mean (%)	99.44	99.4	99.8	100.05	99.41	100.11	100.07
S.D.	0.86	0.28	0.2	0.11	0.34	0.07	0.05
N	8	8	8	8	8	8	8
Variance	0.740	0.112	0.098	0.015	0.132	0.009	0.008
Student's *t*-test		0.215 (1.761)	0.325 (1.761)	0.698 (1.761)	0.604 (1.761)	0.563 (1.761)	0.747 (1.761)
F-test		1.09(3.575)	1.05 (3.575)	1.01 (3.575)	1.03 (3.575)	1.21 (3.575)	1.08 (3.575)

*, Values in parenthesis are the theoretical values of t and F (at $P = 0.05$).

$A = 0.013C + 0.023, \quad r = 0.9996$

where 'A' is the peak area, 'C' is the corresponding concentration and 'r' is the correlation coefficient.

To assess the stability indicating selectivity of the proposed methods for the analysis of clozapine without interference from its degradation product, separate aliquots of the degradation product of clozapine were mixed with the intact drug in different ratios and analyzed by the proposed methods. The results obtained are shown in Table 1. It is clear that the accuracy of the proposed methods are not affected by the presence of up to 70, 60, 70, 60, 90 and 90% of the degradation product in the D_2, D_3, RSD_1 and ΔA spectrophotometric, TLC- densitometric and RP-HPLC methods, respectively. The proposed methods were applied successfully for the analysis of clozapine in its dosage form and its validity was further assessed by applying the standard addition technique. Results obtained are presented in Table 2.

Table 3 shows the full validation parameters for the proposed methods.

The results obtained by applying the proposed methods were statistically compared with those obtained by applying the reference method. Table 4 shows that the values of calculated t and F are less than the tabulated ones indicating that there is no significant difference between the methods. Thus, the proposed methods could be applied for the routine and quality control analysis of clozapine in raw material and pharmaceutical formulations.

4. Conclusion

The suggested methods are simple, accurate, selective and sensitive with no significant difference in the precision. However, the spectrophotometeric methods of analysis including D_2, D_3, RSD_1 and ΔA methods are more preferred due to their low cost, simplicity and availability of reagents. Application of the proposed methods to the analysis of clozapine in laboratory prepared mixtures and pharmaceutical formulations shows that neither the degradation product nor the excipients interfere with the determination, indicating that the proposed methods could be applied as stability indicating methods for the determination of clozapine either in bulk powder or in pharmaceutical formulations. Statistical analysis of the results obtained by the five proposed methods and by the non-aqueous titration method of B.P. (1998), revealed no significant difference within a probability of 95%. However the proposed methods are far more sensitive than the B.P. method. Moreover, the suggested methods are more selective, since the B.P. method does not differentiate between the intact drug and its degradation product.

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