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Novel Techniques for the Determination of Cisatracurium Besylate Alone or In the Presence of Its Degradation Product

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ABSTRACT

Four simple, accurate and precise methods have been developed and validated for the determination of cisatracurium besylate in bulk and in its pharmaceutical preparation. The first method was a spectrofluorimetric method based on measuring the native fluorescence intensity (FI) of cisatracurium (CIS) in aqueous micellar medium at 317 nm upon excitation at 235 nm in the range of 0.2- 2.2 µg.mL^{-1}. The other three methods were adopted for the determination of the studied drug in the presence of its alkaline degradation product including a spectrophotometric method namely, first derivative of ratio spectra (DD^{1}) in the range of 8-38 µg.mL^{-1} and two high pressure liquid chromatographic (HPLC) methods, one with diode –array detector (DAD) and the other with fluorescence detector (FLD) in the ranges of 1.0- 40.0 µg.mL^{-1} and 0.25-20.0 µg.mL^{-1}, respectively. Separation was achieved on Supelco Discovery®C_{18} column (150 mm x 4.6 mm, 5 µm) and Agilent Zorbax® SB- CN column (50 mm x 4.6 mm, 1.8 µm) for HPLC - DAD and HPLC- FLD methods, respectively. All the proposed methods were validated and successfully applied for the determination of CIS in bulk and in pharmaceutical preparation with good recovery ranges between 99.92- 100.71. The results obtained by applying the proposed methods were statistically analyzed and compared with those obtained by the manufacturer method, and no significant difference was found.

Keywords: Cisatracurium besylate, Diode-array detector, Fluorescence detector, Spectrofluorimetry and First derivative ratio of ratio spectroscopy.

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INTRODUCTION

Cisatracurium besylate {(1*R*,1'*R*, 2*R*,2'*R*)-2,2- [1,5-pentanediylbis-[oxy(3-oxo-3,1-propanediyl)] -bis[1 -[3,4 –dimethoxyphenyl -methyl] -1,2,3,4 –tetrahydro -6,7- dimethoxy- 2-methylisoquinolinium] dibenzene sulfonate} (Figure1) is a non-depolarizing neuromuscular blocking agent. This active component is presented as white and crystalline powder. CIS is one of ten stereoisomers (*R- cis*, *R*'- *cis*) that compose atracurium, which is approximately threefold more potent than the mixture that constitutes the parent drug[1] but has similar neuromuscular blocking effects in terms of duration and recovery times[2], except for a slower onset and less propensity to release histamine[3]. CIS determination is not yet described in any pharmacopoeias. The literature survey reveals that few analytical methods for the determination of this drug were reported which include HPLC methods for the determination of CIS in human plasma [4-6] and in its pharmaceutical formulation[7]. A spectrofluorimetric method in human serum and pharmaceuticals[8] and an electrochemical study of CIS with carbon paste electrode, along with the determination of traces of it in human urine and human serum by differential pulse voltammetry[9] were also reported. Our study presents four new methods for CIS determination alone and in presence of its alkaline degradation product, namely, micellar spectrofluorometric method, first derivative of ratio spectrophotometric (DD[1]) method, HPLC with diode– array detection (HPLC-DAD), HPLC with fluorometric detection (HPLC-FLD).

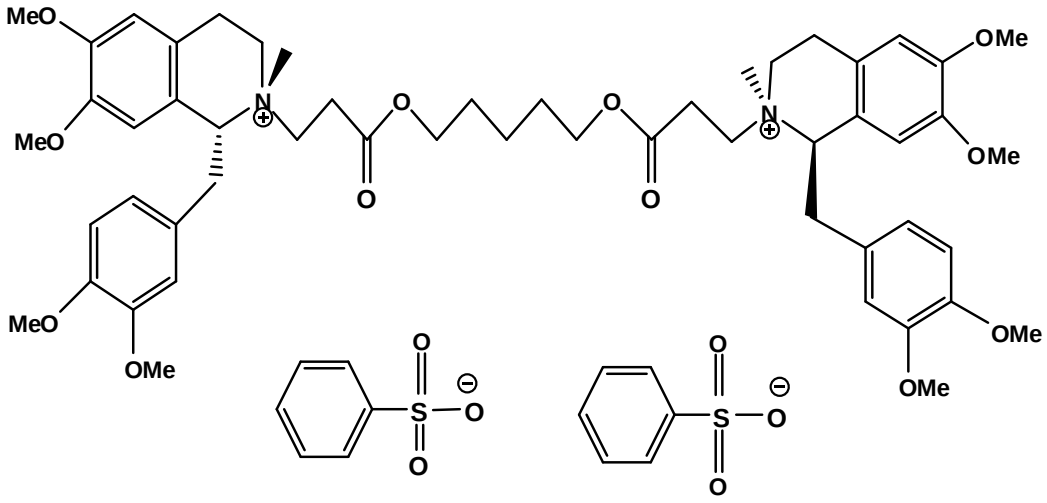

Figure 1: Chemical structure of cisatracurium besylate

MATERIALS AND METHOD

Instrumentation

- HPLC Agilent 1200 SL RRLC series was equipped with binary pump G1312B, autosampler injector FC-ALS G1330B and DAD / SL G1315C (Germany) operated at

235 nm at a flow rate of 1.5 mL.min^{-1}. Separation was performed on a Supelco Discovery® C_{18} column (150 x 4.6mm, 5 µm).

- HPLC Agilent 1200 series was equipped with quaternary pump G1311A, autosampler injector G1329A and FLD G1321A (Germany) operated at 235 nm for excitation and 320 nm for emission at a flow rate of 0.7 mL.min^{-1}. Separation was performed on an Agilent Zorbax® CN column (50 x 4.6mm, 1.8 µm).
- Shimadzu UV-1601 UV/VIS spectrophotometer (Japan) with 1 cm matches quartz cell.
- Shimadzu RF-540 spectrofluorimeter (P/N 204-02900), using quartz cell (1x1x4.5cm) slit width 2.5 nm.
- Mettler Toledo balance (Germany).
- Precisa pH meter (Swiss).
- Bandelin-Sonorex TK sonicator (Germany).

Materials

All chemicals and reagents were of HPLC or analytical grade

- Pure sample of Cisatracurium besylate was kindly supplied and certified from Glaxo Smithkline, Cairo, Egypt. Its purity was assessed according to the manufacturer method and found to be 99.84 % ± 1.13.
- Nimbex® ampoules (B.N. N927) (GlaxoSmithkline, Cairo, Egypt) labeled to contain 2.68mg cisatracurium besylate equivalent to 2 mg cisatracurium.
- Distilled water was produced in-house (Aquatron water still, A4000D, UK).
- Acetonitrile and methanol are of HPLC grade (Scharlau), octane sulphonic acid (SDFCL), ammonium acetate (El nasr), NaOH (0.1*N* in distilled water), HCl (0.1*N* in distilled water), triethylamine (Aldrich), sodium lauryl sulphate (SDS) (Qualikems) (0.1*M* in distilled water), tween 80 (Qualikems), cetyltrimethyl ammonium bromide (CTB) (BDH) and sulphuric acid (0.01*N* in distilled water adjusted to pH 3.6).

Methods

All calculations were performed regarding CIS free base.

Preparation of standard solutions

Stock standard solution of 100.0 µg.mL^{-1} of CIS was prepared in the selected solvent (distilled water adjusted to pH (3.6) with 0.01*N* sulphuric acid).

Working standard solution of 10.0 µg.mL^{-1} of CIS was prepared in the same solvent mentioned above.

Preparation of degradation products

An accurately weighed amount equivalent to 50 mg CIS was dissolved in a 25 mL 0.1*N* NaOH and heated on a water bath at 90 °C for 1 hour for complete degradation, then it was cooled, neutralized using 0.1*N* HCl, filtered and evaporated till dryness, the residue was weighed and dissolved in the selected solvent to obtain a degraded solution of concentration 1.0 mg.mL^{-1}. Then further dilutions in the selected solvent were made to prepare working standard solution of 100.0 µg.mL^{-1}.

LABORATORY-PREPARED MIXTURES

Spectrophotometric method (DD^{1})

Aliquots equivalent to 270.0 - 90.0 µg of CIS from its stock standard solution **(**100.0 µg.mL^{-1}**)** were transferred into series of 10 mL volumetric flasks. Aliquots of degraded solution (100.0 µg.mL^{-1}) equivalent to 10-70% of the intact solution were added to the same flasks.

HPLC-DAD method

Aliquots equivalent to 360.0 - 40.0 µg of CIS from its stock standard solution **(**100.0 µg.mL^{-1}**)** were transferred into series of 10 mL volumetric flasks. Aliquots of degraded solution (100.0 µg.mL^{-1}) equivalent to 10-90% of the intact solution were added to the same flasks.

HPLC-FLD method

Aliquots equivalent to 180.0 - 20.0 µg of CIS from its stock standard solution **(**100.0 µg.mL^{-1}**)** were transferred into series of 10 mL volumetric flasks. Aliquots of degraded solution (100.0 µg.mL^{-1}) equivalent to 10-90% of the intact solution were added to the same flasks.

Procedure

Calibration Curves

Micellar enhanced spectrofluorimetric method

Different aliquots of working standard solution (10.0 µg.mL^{-1}) equivalent to 2.0- 22.0 µg of CIS were transferred into a series of 10 mL volumetric flasks. To each flask 1 mL of 0.1*M* SDS was added and the volume was completed by the selected solvent at ambient temperature. The FI of each dilution was recorded at 235 nm for excitation and 317 nm for emission. The calibration curve was obtained representing the relationship between the FI and the corresponding concentration, then the linear regression equation was computed.

First derivative of ratio spectra method

Different aliquots of stock standard solution (100.0 µg.mL^{-1}) equivalent to 80.0- 380.0 µg of CIS were transferred into a series of 10 mL volumetric flasks and completed to the mark with the selected solvent. The spectrum of each concentration was divided by the spectrum of the alkaline

degradation product (10.0 µg.mL^{-1}). The first derivative of each ratio spectra was recorded with $\Delta\lambda$ = 2 nm and scaling factor = 10 and the first derivative of ratio spectra (DD^1) value was measured at 294.4 nm.The calibration curve was constructed and the corresponding regression equation was then computed.

HPLC-DAD method

Different aliquots of stock standard solution (100.0 µg.mL^{-1}) equivalent to 10.0- 400.0 µg of CIS were transferred into a series of 10 mL volumetric flasks and the volume was completed by the solvent. Twenty microliters of each concentration was injected into Agilent HPLC- DAD using Supelco Discovery® C_{18} column (150 mm x 4.6 mm, 5 µm) at a flow rate of 1.5 mL.min^{-1}, The DAD was operated at 235 nm. The mobile phase consisted of water: acetonitrile: triethylamine (66.5: 33.5: 0.25, v/v/v) adjusted to pH (3.0) with *o*- phosphoric acid .The peak areas of all concentrations were detected and plotted against the corresponding concentration and the linear regression equation was computed.

HPLC-FLD method

Different aliquots of stock standard solution (100.0 µg.mL^{-1}) equivalent to 25.0- 2000.0 µg of CIS were transferred into a series of 100 ml volumetric flasks and the volume was completed by the solvent. Five microliters of each concentration was injected into Agilent HPLC- FLD using Agilent Zorbax® CN column (50 mm x 4.6 mm, 1.8 µm) at a flow rate of 0.7 mL.min^{-1}. The FLD was operated at an excitation wavelength of 235 nm and emission wavelength of 320 nm. The mobile phase consisted of buffer mixture (0.6 % of both octane sulphonic acid and ammonium acetate): methanol: acetonitile: triethylamine (43: 43: 14: 1.5 v/v/v/v) adjusted to pH (4.5) with *o*-phosphoric .The peak areas of all concentrations were detected and plotted against the corresponding concentration and the linear regression equation was computed.

Procedure for pharmaceutical preparation (Nimbex® ampoule)

Five milliliters of Nimbex® ampoule containing (10,000 µg CIS) was diluted with the selected solvent in a 100 mL volumetric flask to get a final concentration of (100.0 µg.mL^{-1}). Subsequent dilutions were used for the determination of CIS by HPLC-DAD, HPLC- FLD, micellar spectrofluorimetry and DD^1 methods. The same procedure was repeated applying the standard addition technique.

RESULTS AND DISCUSSION

Micellar spectrofluorimetic method

The present work depends on measuring native FI of CIS in aqueous micellar-medium at 317 nm

upon excitation at 235 nm (Figure 2).The influence of micellar medium on fluorescence emission was studied. The fluorimetric properties of CIS were studied in different micellar media by diluting 1 mL of working standard solution of CIS (10 µg.mL^{-1}) with 1 mL of each of 0.1 *M* of surfactants in 10 mL volumetric flasks and completed the volume with the selected solvent. The enhancement ability sequence was: SDS > CTB > Tween 80 .The anionic surfactant SDS showed strong sensitizing effect on fluorescence. The effect of different solvents on the FI was studied. The highest FI was in order: distilled water adjusted to pH (3.6) with 0.01 *N* sulphuric acid (selected solvent) > isopropanol > water > acetonitrile > methanol. This permits the development of a very sensitive method of assay for CIS in pure and pharmaceutical dosage form. The parameters of regression equation and correlation coefficient were given in table 1.

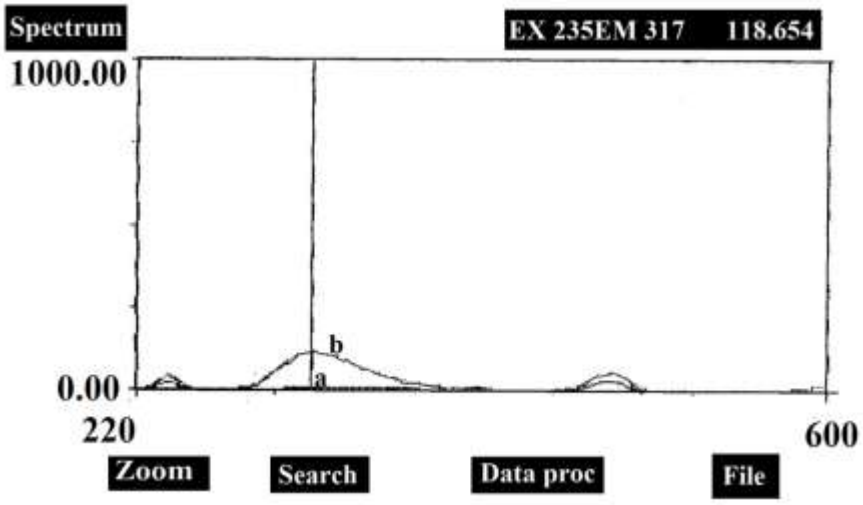

Figure 2: Fluorescence emission spectrum of: (a) blank and (b) 1 µg.mL^{-1} cisatracurium standard in aqueous micellar solution.

Table 1 Results obtained by the four proposed methods for the determination of cisatracurium in bulk and its pharmaceutical formulation.

Parameters	Micellar-spectrofluorimetric method	DD[1] method	HPLC-DAD method	HPLC-FLD method
Calibration range	0.2- 2.2 µg.mL^{-1}	8- 38 µg.mL^{-1}	1- 40 µg.mL^{-1}	0.25- 20 µg.mL^{-1}
Limit of detection (LOD)	0.016	0.277	0.038	0.007
Limit of quantitation (LOQ)	0.047	0.839	0.116	0.02
Slope (b)	96.827	0.904	33.12	71.504
Standard deviation of the slope (Sb)	0.542	0.008	0.126	0.328
Confidence limit of the	96.827±1.505	0.904±0.022	33.12±0.325	71.504±0.84

slope				4
Intercept (a)	18.949	-0.096	-0.011	- 6.132
Standard deviation of the intercept (Sa)	0.749	0.196	2.632	3.591
Confidence limit of the intercept	18.949±2.079	-0.096±0.543	-0.011±6.765	-6.132±9.23
Regression coefficient (r^2)	0.9999	0.9997	0.9999	0.9999
Standard error of Estimation	0.907	0.195	4.623	6.086
Intra-day (% RSD) *	0.16- 1.16	0.16- 0.64	0.05- 0.54	0.06- 0.3
Inter-day (% RSD) *	0.2- 0.87	0.76- 1.56	0.07- 0.73	0.12- 0.38

* The interday and the intraday (n = 3), average of three concentrations (0.5, 1, 1.5 µg.mL^{-1}) for micellar spectrofluorimetry, (10, 15, 25 µg.mL^{-1}) for DD^1, (5, 15, 30 µg.mL^{-1}) for HPLC-DAD and (5, 10, 15 µg.mL^{-1}) for HPLC-FLD

First-derivative of ratio spectra (DD^1) method.

The zero-order spectra (D^0) of the drug and its degradation products showed a severe overlap; as shown in (Figure 3) which interferes with the analysis of the drug. By the application of the first-derivative of ratio spectra (DD^1) CIS can be quantitatively determined at 294.4 nm without any interference from its degradation products as shown in (Figure 4). Careful choice of the divisor and the working wavelength were of great importance so different concentrations of degradation product were tried as a divisor (10, 15, 20, 25 and 30 µg.mL^{-1}), the best one was 10 µg.mL^{-1} as it produced minimum noise and gave better results in accordance with selectivity. The parameters of regression equation and correlation coefficient were given in table 1.

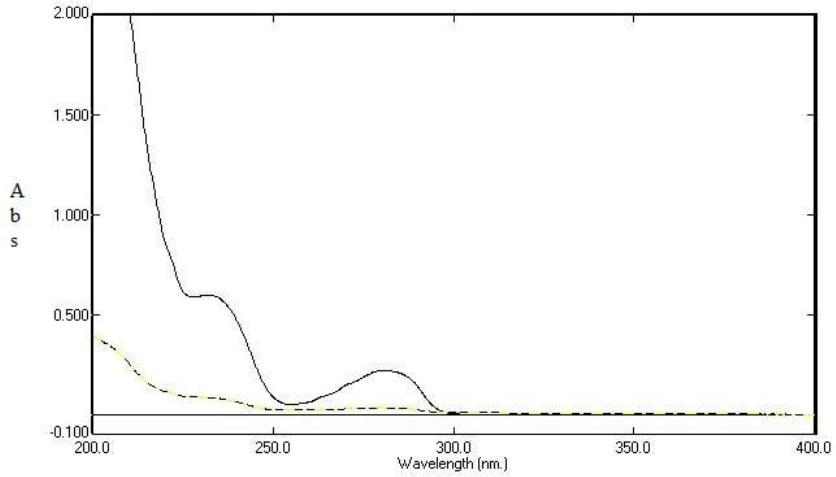

Figure 3: Zero –order absorption spectra of cisatracurium standard solution (—— 20µg.mL^{-1}) and its alkaline degradation product (- - - - - - 20 µg.mL^{-1})

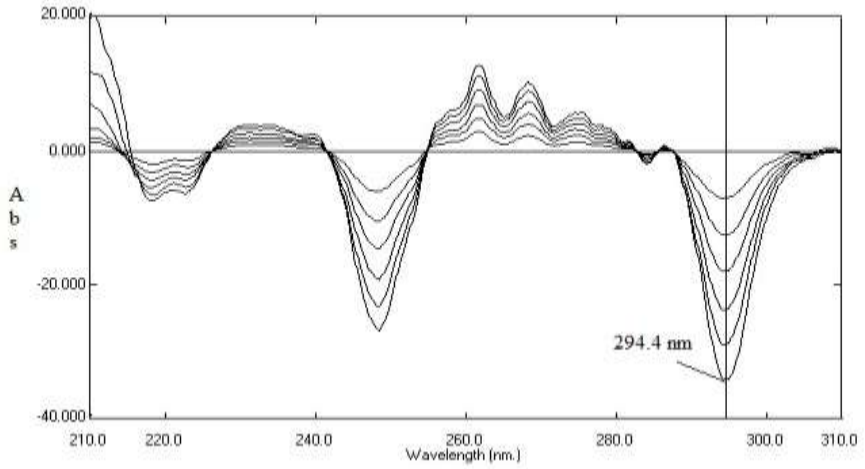

Figure 4: First derivative of ratio spectra of cisatracurium (8, 14, 20, 26, 32 and 38 µg.ml^{-1}) using the spectrum of 10 µg.mL^{-1} degradation product as a divisor.

HPLC methods

Method development

HPLC-DAD:

Various reversed phase chromatographic columns and a series of aqueous mobile phases containing different organic modifiers at different ratios were tested to adjust the optimum conditions. A satisfactory mobile phase was obtained consisting of water: acetonitrile: triethylamine (66.5: 33.5: 0.25, v/v/v) adjusted to pH (3) with o-phosphoric at a flow rate of 1.5 ml.min^{-1}. The ion pairing reagent, triethylamine was used in order to reduce tailing and improve resolution. The pH of the mobile phase was found to be critical in achieving the separation between the drug and its degradation. PH (3 ±0.2) showed the best resolution.Quantitation was achieved with DAD detection at 235 nm. Retention times were 4.352 and 2.294 min for CIS and its alkaline degradation product, respectively (Figure 5). The parameters of regression equation and correlation coefficient were shown in table 1.

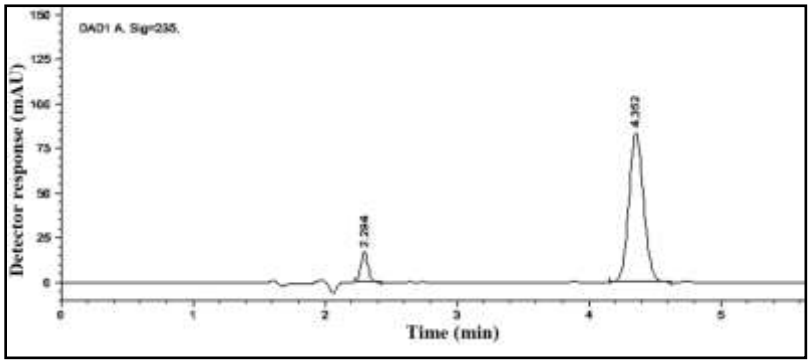

Figure 5: HPLC-DAD chromatogram of 20 µg.mL^{-1} of both cisatracurium and its alkaline degradation product at retention times (4.352 and 2.294 min), respectively.

HPLC-FLD:

Various reversed phase chromatographic columns and a series of aqueous mobile phases containing different organic modifiers at different ratios were tested to adjust the optimum conditions. A satisfactory mobile phase was obtained consisting of buffer mixture (0.6 % of both octane sulphonic acid and ammonium acetate): methanol: acetonitrile: triethylamine (43: 43: 14: 1.5, v/v/v/v) adjusted to pH (4.5) with o-phosphoric at a flow rate 0.7 mL.min^{-1}. The ion pairing reagent, octane sulphonic acid and triethylamine were used in order to reduce tailing and improve resolution. The pH of the mobile phase was found to be critical in achieving the separation between the drug and its degradation product. PH (4.5 ±0.2) showed the best resolution. Quantitation was achieved with FLD at 235 nm for excitation and 320 nm for emission. Retention times were 3.054 and 1.433 min for CIS and its alkaline degradation product, respectively (Figure 6). The parameters of regression equation and correlation coefficient were shown in table 1.

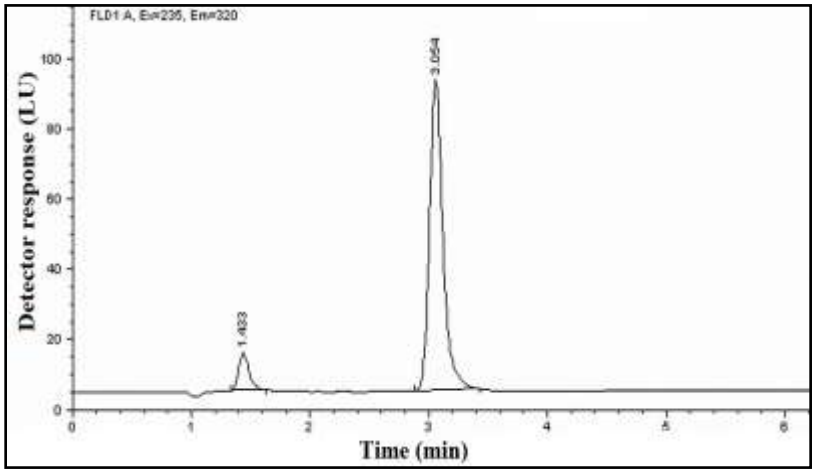

Figure 6 : HPLC-FLD chromatogram of 10 µg.mL^{-1} of both cisatracurium and its alkaline degradation product at retention times (3.054 and 1.433 min), respectively.

System suitability tests

System suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. System suitability tests were used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters of these tests are column efficiency (number of theoretical plates) (N), height equivalent to theoretical plate (HETP), peak resolution (R), peak tailing (T), separation factor (relative retention) (α), and capacity factor (K)[10]. The results of these tests for both HPLC methods are listed in table 2.

Table 2: System suitability tests for the determination of cisatracurium in the presence of its alkaline degradation product by the two HPLC proposed methods.

Parameters	HPLC-DAD method	HPLC -FLD method	Reference value
Resolution (R)	13.6	9.17	R > 1.5
Tailing factor (T)	1.101	1.341	≤ 2 (T=1 for a typical symmetric peak)
Capacity factor (K)	3.4	1.13	1 – 10 acceptable
Relative retention (α)	2.63	2.14	> 1
No. of theoretical plates (N) CIS	7298	3893	≥ 2000

METHOD VALIDATION

Linearity and Range

Linearity range for CIS estimation was found to be 0.2 – 2.2 μg.mL^{-1} for the micellar-enhanced spectrofluorimetic method, 8 -38 μg.mL^{-1} for the first-derivative of ratio spectra (DD^1) method, 1.0 - 40.0 μg.mL^{-1} for the HPLC-DAD method and 0.25 – 20.0 μg.mL^{-1} for HPLC-FLD method.

Precision

Precision of the method was made by analysis of three independent drug preparations. The determination of RSD% value obtained from three assay values in order to calculate intraday variations. These studies were repeated for three different days for the determination of inter-day variation. The RSD% values for intraday and interday variation study were found to be satisfactory (not more than 2) which confirm a suitable precision of the proposed methods as shown in table 1.

Accuracy

The four methods were tested for accuracy, calculated by % recovery ± S.D. of different concentrations of CIS as shown in table 3. The accuracy was assessed also by standard addition technique applied for Nimbex® ampoules, all carried out in triplicates. The results obtained were reproducible with low S.D. as shown in table 4.

Table 3: Determination of cisatracurium in bulk powder by using the four proposed methods

	Conc. taken (μg.mL^{-1})	Conc. found (μg.mL^{-1})	Recovery %	Average recovery%(mean±S.D)
Micellar spectrofluorimetric method	0.4	0.396	99.00	100.72 ±1.00
	0.7	0.701	100.14	
	1.1	1.119	101. 73	
	1.3	1.317	101.31	
	1.6	1.614	100.88	
	1.9	1.924	101.26	

DD[1] method	10	10.046	100.46	100.34±0.96
	15	15.040	100.27	
	18	18.099	100.55	
	25	25.101	100.40	
	30	30.505	101.68	
	35	34.535	98.67	
HPLC-DAD method	2	2.007	100.34	99.92±1.04
	6	5.898	98.30	
	12	12.003	100.03	
	25	25.283	101.13	
	35	34.925	99.79	
HPLC-FLD method	1	1.012	101.23	100.18±0.90
	3	3.012	100.41	
	6	5.96	99.33	
	9	8.900	98.88	
	13	13.110	100.84	
	15	15.058	100.38	

Table 4: Application of standard addition technique by the four proposed methods

Method	**CIS dosage form**	**Found% [a]**	**Conc. added**	**Conc. found [b]**	**Recovery %**	**Average recovery% (mean±S.D)**
Spectrofluorimetric method	N927	99.86±1.09	0.2	0.199	99.50	
			0.4	0.398	99.50	
			0.6	0.600	100.00	99.92±0.54
			1	1.008	100.80	
			1.4	1.397	99.79	
DD[1] method	N927	99.61±1.25	8	8.146	101.83	101.02±0.64
			10	10.073	100.73	
			15	15.081	100.54	
			20	20.314	101.57	
			25	25.102	100.41	
HPLC-DAD method	N927	99.15±0.51	2	2.030	101.51	101.16±0.64
			5	5.040	100.80	
			10	10.190	101.90	
			15	15.197	101.32	
			25	25.065	100.26	
HPLC-FLD method	N927	100.27±0.35	3	2.977	99.23	100.08±1.08
			6	5.980	99.66	
			9	9.151	101.67	
			11	10.975	99.77	

LOD and LOQ

The LOD and LOQ of the proposed methods were determined using calibration standards. LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S respectively where S is the slope of the calibration curve and σ is the standard deviation of the response. LOD and LOQ values of the four proposed methods were assessed and given in table 1. These low values indicated the good

sensitivity of the proposed methods.

Selectivity

The selectivity of the method was checked by analyzing CIS in laboratory prepared mixture with its alkaline degradation product. The mean percentage recovery ± S.D. of intact in the laboratory mixtures were calculated for the three stability indicating methods. They were successfully applied for the determination of CIS in the presence of its alkaline degradation product up to 70% in DD[1] method and 90% for the two HPLC methods as shown in table 5.

Table 5: Determination of cisatracurium in laboratory-prepared mixtures with its degradation by the proposed methods

Method	Sample no	%degradation	St. conc.	Deg. Conc.	Recovery %	Average recovery% (mean ± S.D)
DD[1]	1	10	27	3	101.28	99.91 ±0.85
	2	30	21	9	98.99	
	3	40	18	12	100.55	
	4	50	15	15	99.70	
	5	60	12	18	99.58	
	6	70	9	21	99.36	
HPLC-DAD	1	10	36	4	98.15	99.36±1.27
	2	30	28	12	100.23	
	3	40	24	16	98.84	
	4	50	20	20	98.30	
	5	60	15	25	101.46	
	6	90	4	36	99.17	
HPLC-FLD	1	10	18	2	101.40	101.08±0.61
	2	30	14	6	100.87	
	3	50	10	10	100.23	
	4	70	6	14	101.86	
	5	90	2	18	101.02	

Table 6: Statistical comparison between the results obtained by the four proposed methods for the analysis of Cisatracurium and the manufacturer method

Statistical terms	Micellar spectrofluorometric method	DD[1] method	HPLC-DAD method	HPLC-FLD method	Manufacturer Method**
Mean	100.72	100.34	99.92	100.18	99.84
S.D.	1.00	0.96	1.04	0.90	1.13
S.E.	0.41	0.39	0.47	0.37	0.51
Variance	1.00	0.92	1.08	0.81	1.28
N	6	6	5	6	5
t-test	1.307 (2.262)*	0.795 (2.262)*	0.116 (2.306)*	0.556 (2.262)*	
F-ratio	1.28 (5.19)*	1.391 (5.19)*	1.185 (6.39)*	1.580 (5.19)*	

** Figures in parenthesis are the theoretical t and F values at confidence limit 95%.*

*** HPLC method according to manufacturer file.*

Statistical analysis of the results

A statistical analysis of the results obtained by the proposed methods and the manufacturer method[11] was carried out showing no significant difference among the methods with respect to t-test and F-ratio[12] as shown in table 6

CONCLUSION

The proposed four methods were simple, rapid, accurate and precise and could be used for the analysis of CIS in pure form and in pharmaceutical dosage form (either alone or in the presence of its degradation product). The sample recovery for all four methods was in good agreement with their respective label claims which suggested no interference of formulation additives in estimation.

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