Nota Técnica

SPECTROPHOTOMETRIC AND SPECTROFLUORIMETRIC DETERMINATION OF SOME DRUGS CONTAINING SECONDARY AMINO GROUP IN BULK DRUG AND DOSAGE FORMS VIA DERIVATIZATION WITH 7-CHLORO-4-NITROBENZOFURAZON

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Sensitive and selective spectrophotometric and spectrofluorimetric methods have been developed for determination of some drugs such as Pramipexole, Nebivolol, Carvedilol, and Eletriptan, which commonly contain secondary amino group. The subject methods were developed via derivatization of the secondary amino groups with 7-Chloro-4-Nitrobenzofurazon in borate buffer where a yellow colored reaction product was obtained and measured spectrophotometrically or spectrofluorimetrically. Concentration ranges were found as 2.0 to 250 μg mL^{-1} and 0.1 to 3.0 μg mL^{-1}, for spectrophotometric and spectrofluorimetric study, respectively. The described methods can be easily applied by the quality control laboratories in routine analyses of these drugs in pharmaceutical preparations.

Keywords: secondary amino groups; 7-chloro-4-nitrobenzofurazan; pharmaceutical preparations.

INTRODUCTION

Pramipexole ((6S)-N^6-propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine, PRM) is a non-ergoline dopamine agonist, initially introduced for the treatment of early and advanced Parkinson's disease[1] and recently approved in US and Europe also for the treatment of idiopathic restless legs syndrome in adults.[2] Nebivolol hydrochloride (NBV) is chemically known as α,α′-[iminobis(methylene)]bis[6-flouro-3,4-dihydro-2H-1-benzopyran-2-methanol]hydro-chloride,[3] it is a highly selective β$_1$-blocker with nitric oxide-mediated vasodilatory actions and beneficial effects on vascular endothelial function. It is a third-generation, cardio-selective beta-blocker used for the treatment of hypertension and chronic heart failure.[4] Carvedilol (CRV), 1-(carbazol-4-yloxy)- 3-((2methoxyphenoxy)ethyl)amino)-2-propanol (Figure 1), is a non-selective beta adrenergic blocking agent.[5] Eletriptan (ETP), [(R)-3-(1-methyl-2-pyrrolidinylmethyl)-5-[2-(phenylsulphonyl)-ethyl]-1H-indole] (Figure 1), which is a new serotonin 5-HT(1B/1D) receptor agonist, has been developed for the treatment of acute migraine attacks.[6] Chemical structures of the subject APIs were given in Figure 1.

There are only a limited number of analytical methods developed for analysis of these drugs in dosage forms. Previously, analysis of Pramipexole in bulk and drug products was carried out by a high performance liquid chromatographic (HPLC) method.[7,8] Five HPLC[9-13] and densitometric[14] methods have been reported for determination of NBV in drug products. Two spectrophotometric methods were reported for the determination of CRV.[15,16] The literature data for ETP indicates only one HPLC method utilized in analysis of Eletriptan in dosage forms.[17]

Although chromatographic methods are sensitive and accurate, their major disadvantages comprise utilization of complex and expensive equipment, usage and disposal of high purity solvents, application of labor-intensive sample preparation procedures as well as longer duration of analyses. In view of that, new spectrophotometric/spectrofluorometric methods were developed via derivatization of the
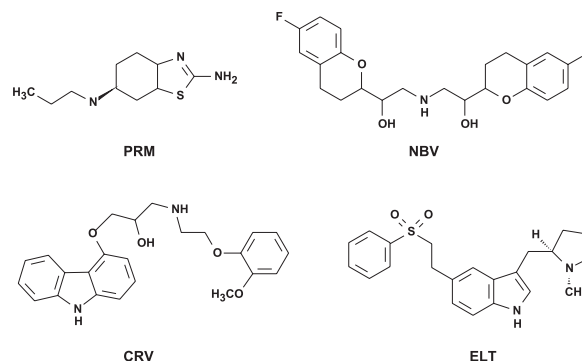

Figure 1. Chemical structures of drugs, (A) Pramipexole, (B) Nebivolol, (C) Carvedilol, (D) Eletriptan

secondary amino group of the subject drugs with 7-Chloro-4-Nitro-benzofurazon (NBD-Cl), which is well known to react with primary and secondary amines forming stable condensation colored products.[18] The condensation products of NBD-Cl have been demonstrated to be suitable for spectrophotometric and spectrofluorimetric assessments therefore, it has been used in analyses of many pharmaceutical formulations or biological fluids.[19-23] The developed methods were also validated successfully for specificity, linearity, accuracy, precision, robustness and they have been applied for quantification of these drugs in tablets, avoiding interferences caused by the excipients. The proposed methods can be easily used in laboratories where modern and expensive apparatus are not available.

EXPERIMENTAL

Apparatus

Spectrophotometric measurements were carried out using a Shimadzu UV-160 A spectrophotometer and 1-cm glass cells. Fluorescence spectra and measurements were taken on a Shimadzu spectrofluorimeter Model RF-1501 equipped with xenon lamp and 1-cm quartz cells. pH measurements were made with WTW pH 526 digital pH Meter.

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Reagents and solutions

Pramipexole Hydrochloride and Nebivolol Hydrochloride drug substances and its pharmaceutical preparation (Nexivol® containing 5 mg/tablet Nebivolol Hydrochloride) were kindly supplied by Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey). Pexola® containing 1 mg of pramipexole hydrochloride per tablets was obtained from local drugstore. Carvedilol and its pharmaceutical preparation (Kinetra®) containing 25 mg of Carvedilol per tablet were procured from Sanovel (Istanbul, Turkey). Eletriptan Hydrobromide and its pharmaceutical preparation (Relpax film coated tablet®) containing 40 mg of Eletriptan Hydrobromide per tablet were kindly supplied by Pfizer (Istanbul, Turkey). NBD-Cl and other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals and reagents were of analytical-reagent grade. A stock solution of PRM base containing 1 mg mL^{-1} was prepared in water. A stock solution of NBV base containing 10 mg mL^{-1} was prepared in methanol. A stock solution of CRV (1 mg mL^{-1}) was prepared in acetonitrile. ETP stock solution 1.0 mg/mL, calculated as free base was prepared in methanol.

NBD-Cl solution was freshly prepared in methanol at 5 mg mL^{-1} concentration. Buffer solution was prepared as follow: 0.620 g boric acid and 0.750 g potassium chloride were dissolved with 100 mL of water. After adjusting the pH to 8.0 and 8.5 with 0.1 N sodium hydroxide solution, the final solution was diluted to 200 mL with water.

General procedure

Pramipexole

This material was analyzed using both spectrophotometric and spectrofluorimetric methodology. Aliquots of the stock standard solution varied from 0.05-0.3 and 0.015-0.15 mL, for the spectrophotometric and spectrofluorimetric studies, respectively, were transferred into a series of 10.0 mL volumetric flasks and then the volume was adjusted to about 0.3 and 0.15 mL with water, for spectrophotometric and spectrofluorimetric study, respectively.

Nebivolol

Aliquot of the associated stock standard solution ranged between 0.005 and 0.060 mL were transferred into a series of 10.0 mL volumetric flasks and then the volume was adjusted to about 0.060 mL with methanol.

Carvedilol

Aliquots of its stock standard solution between 0.035 and 0.300 mL were individually transferred into glass stoppered tubes and their volumes were brought to 0.300 mL with acetonitrile.

Eletriptan

Aliquots of its stock standard solution (0.15-1.25 mL) were individually transferred into glass stoppered tubes and their final volumes were brought to 1.25 mL with methanol. Following addition of 0.2 mL of buffer solution to each drug solutions, 0.7, 0.8, 0.3 and 0.5 mL of NBD-Cl solutions were added to PMP, NBV, CRV and ELT, respectively and the mixtures treated in water bath with thermostat at 70 °C for 30 min for NBV and 80 °C for 7, 20 and 30 min for PMP, CRV and ELT, respectively. The mixtures were then cooled within a ice bath and acidified with 0.2 mL of 0.1 M HCl solution. The derivatives of individual drug substances formed by coupling with NBD-Cl, were extracted using 5 mL aliquots of chloroform (for PRM and CRV), dichloromethane (for NBV) and ethyl acetate (for ELT) by mixing vigorously with a vortex mixer. The phases were separated by centrifugation. For the spectrophotometric studies, absorbance values of the derivatives extracted to the organic layers

were measured at their respective maximum wavelengths against a blank solution prepared similarly.

For the spectrofluorimetric studies, upon completion of the extraction process, 1 mL aliquots of the organic phase were transferred to 10 mL volumetric flasks and their volumes were diluted to 10 mL with chloroform (for PRM) or dichloromethane (for NBV) and mixed well. The fluorescence intensities of these derivatives were measured at 529 and 530 nm (for PRM and NBV) while exciting at 470 and 473 nm (for PRM and NBV) measured against blank solutions prepared similarly. The fluorescence intensity of the reference standard, sodium fluoressein, solution was also measured at the same wavelength combination. The relative fluorescence intensity (I_F) was then calculated by the following equation: $I_F = x/y \times 100$. x and y represent the fluorescence intensities of the sample and reference standard solutions, respectively ($n=5$).

Assay procedure for tablets

Pramipexole

Tablet powder equivalent to 50 mg of PRM base was accurately weighed and transferred into a 50 mL calibrated volumetric flask. About 25 mL of water was added and then extraction was performed by shaking mechanically for 20 min and sonicating for 20 more min. Upon completion of the extraction step, the volume of the mixture was brought to 50 mL with water and mixed well by vortexing. Then, final solution was filtered through a 0.45 um nylon filter.

Nebivolol

Tablet powder equivalent to 50 mg of NBV base was accurately weighed and transferred into a 50 mL calibrated flask. About 25 mL of methanol was added and then extraction was performed mechanically for 20 min and sonicated for 20 more min. The volume was brought to 50 mL with methanol and final solution was filtered.

Carvedilol

Ten tablets were individually weighed to get the average weight of the tablets. A sample of the powdered tablets, claimed to contain 100 mg of CRV was transferred to 100 mL volumetric flask. About 75.0 mL of acetonitrile was added and then extraction was performed mechanically for 20 min and sonicated for 20 more min. The volume was brought to 100 mL with acetonitrile and its contents were centrifuged for 10 min at 3000×g.

Eletriptan

Ten film-coated tablets were transferred to 500 mL volumetric flask. About 400 mL of methanol was added and then extraction was performed mechanically for 20 min and sonicated for 20 more min. The volume was brought to 500 mL with methanol and final solution was filtered. Then 1 mL of this solution was further diluted to 100 mL using methanol.

Aliquots of the filtrates were further diluted with solvents stated above then proceeded with the solution preparation scheme as described under of the general procedure section. The nominal contents of the tablets were calculated using either the calibration graph or the corresponding regression equation.

RESULTS AND DISCUSSION

NBD-Cl was selected as a derivatizing reagent because it forms fluorescent/chromogenic derivatives with primary or secondary amines requiring relatively mild reaction conditions.[19-23] PRM, NBV, CRV and ELT contain secondary amino group which is known to react with NBD-Cl in alkaline medium (Figure 2). The absorbance of

the generated derivatives were then measured at their respective λ_{max} which were outlined in Table 1. Figure 3 shows absorption spectra of drug-NBD derivatives against the blank prepared similarly.
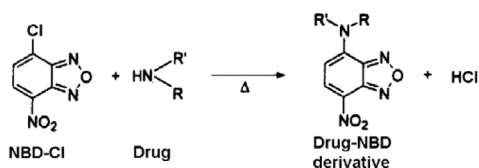
Figure 2. *Proposed reaction pathway between drug containing secondary amine and NBD-Cl*

Under the developed experimental conditions, the maximum fluorescence intensities for PRM and NBV were determined at 529 and 530 nm while excitation took place at 470 and 473 nm, respectively. Figure 4 shows the excitation and emission spectra for PRM-NBD and NBV-NBD derivatives. When NBD derivatives of CRV and ELT were measured against a blank prepared using a spectrofluorimetry, no substantial differences have been determined. Therefore, NBD derivatives of CRV and ELT were studied utilizing spectrophotometric methods.

Study of experimental parameters

The different experimental parameters affecting the development of the reaction product were carefully studied and optimized. The variability/optimization of the main reaction parameters such as pH, volume of the reagent, temperature, and heating time were studied individually.

Effect of pH and HCl concentration
The pH study was completed within the range of 7-10 using borate buffers. The maximum absorbance was obtained at pH 8.5 for PRM and NBV and at pH 8 for ELT and CRV. The test results for pH studies are summarized in Figure 5.

NBD-Cl is hydrolyzed in alkaline medium by formation of NBD-OH having a maximum absorbance at 460 nm. In order to decrease high background effect, it was necessary to acidify the reaction mixture. The required amount of HCl for acidification was determined as 0.2 mL of 0.1 M HCl.

Effect of temperature and time
In order to determine the optimum temperature and time required for the completion of the reaction, the derivatization reaction was carried between the range of 60 to 80 °C. Complete color development was attained after 7, 20 and 30 min for PMP, CRV and ELT, respectively in water bath at 80 °C while heating at 70 °C for 30 min was sufficient to fulfill the optimum reaction requirement for NBV(Figure 6).
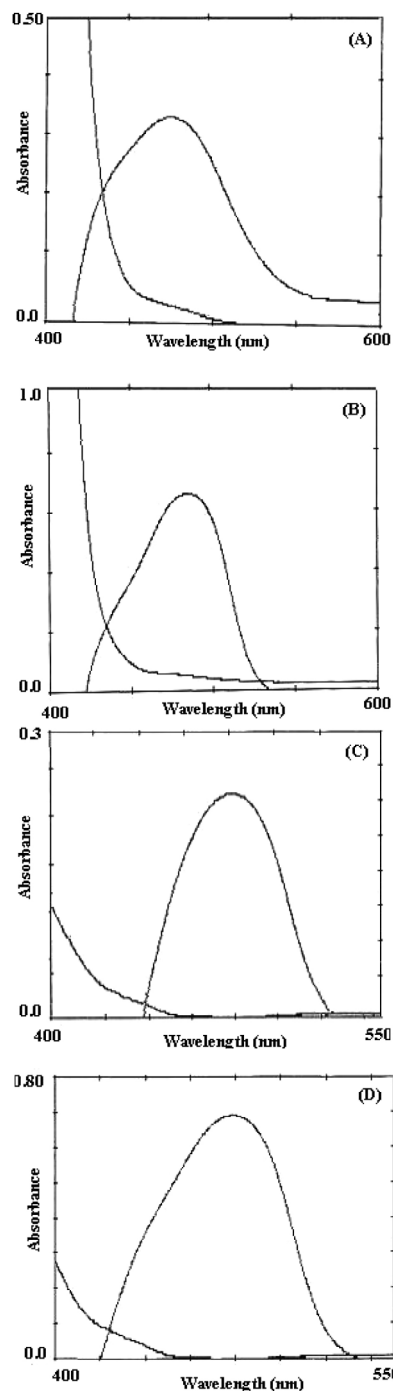

Figure 3. *Absorption spectra of drug-NBD derivative against reagent blank (A) Pramipexole, (B) Nebivolol, (C) Carvedilol, (D) Eletriptan*

Table 1. Optimization of variables affecting the reaction of drugs with NBD-Cl

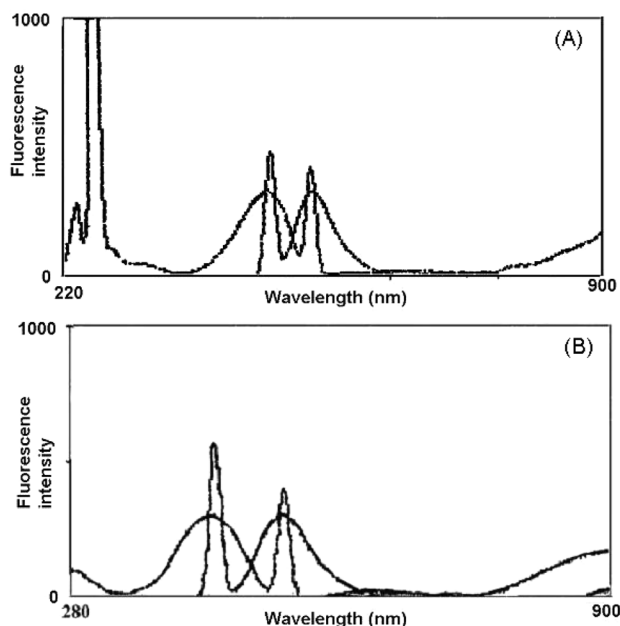| Variable	Optimum condition			
	For PRM-NBD	For NBV-NBD	For CRV-NBD	For ELT-NBD
pH	8.5	8.5	8.0	8.0
HCl (M), mL	0.1 M, 0.2 mL	0.1 M, 0.2 mL	0.1 M, 0.2 mL	0.1 M, 0.2 mL
Temperature (°C)	80	70	80	80
Time (min)	7	30	20	30
NBD-Cl (%, w/v) and mL	0.5 (%, w/v) and 0.7 mL	0.5 (%, w/v) and 0.8 mL	0.5 (%, w/v) and 0.3 mL	0.5 (%, w/v) and 0.5 mL
Solvent	chloroform	dichloromethane	chloroform	ethyl acetate

Figure 4. *Excitation and emission spectra of the reaction product of drugs with NBD-Cl (A) Pramipexole, (B) Nebivolol*
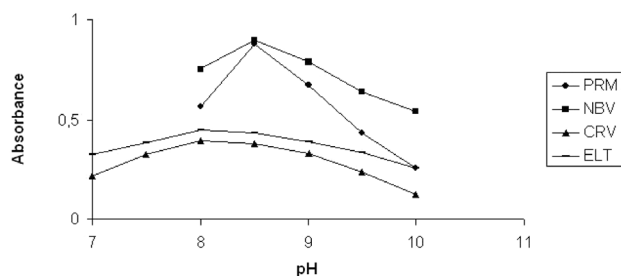

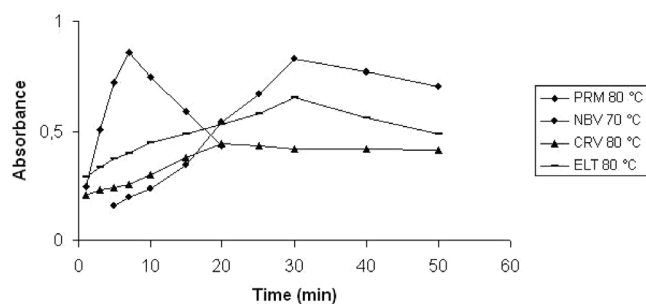**Figure 5.** *Effect of pH on the development of the reaction product of drugs with NBD-Cl*

Figure 6. *The effect of time of heating on the reaction between drugs with NBD-Cl at optimum temperature*

Effect of NBD-Cl concentration

The concentration range of NBD-Cl was also investigated. Additions of 0.7, 0.8, 0.3 and 0.5 mL of 0.5% NBD-Cl solution were found to be sufficient for obtaining maximum and reproducible color intensity for PMP, NBV, CRV and ELT, respectively. Refer to Figure 7 for the summarized related data points.

Effect of organic solvents and stability of the derivative

Different organic solvents were tested to determine the most appropriate solvent: methanol, ethyl acetate, dichloromethane, chloroform and acetonitrile. Chloroform (for PRM and CRV), dichloromethane (for NBV) and ethyl acetate (for ELT) were found to
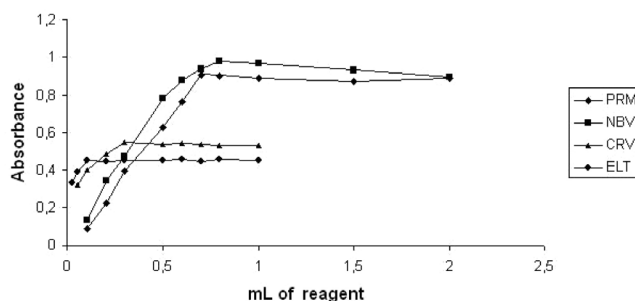

Figure 7. *Effect of volume of NBD-Cl (0.5%, w/v) on the development of the reaction product of drug with NBD-Cl*

be the suitable solvents providing the highest absorbance values. The effect of time on the stability of the drug-NBD derivative in organic solvents was studied at different time intervals. The color remains stable at least for 12 h.

A summary for the optimization of the variables affecting the reaction of these drugs with NBD-Cl are given in Table 1.

Stoichiometry of the reaction

The molar ratio of NBD-Cl and the subject API in the reaction mixture was studied according to Job's method of continuous variation.[24] Utilizing equimolar solution of drugs and NBD-Cl, the reaction stoichiometry was found to be a good approximation 1:1 ratio (drug/reagent).

Validation of the methods

A linear correlation was established between the absorbance and concentration values in the ranges as outlined in Table 2. The correlation coefficients, intercepts and slopes for the calibration data for the four cited APIs are calculated using the least squares method. These values are illustrated in Table 2. Under the optimum reaction conditions relative fluorescence intensity was found to be linearly correlated with the API concentration over the range of 0.3-3.0 μg mL^{-1} for PRM and 0.10–1.2 μg mL^{-1} for NBV. Linear regression analysis of the concentration-fluorescence intensity data gave the following equation: I_f=22.181 C +8.0837 for PRM and I_f=722.1 C +152.69 for NBV where C is the concentration in μg mL^{-1} and I_f is the fluorescence intensities (n=5). The correlation coefficient was 0.9983 and 0.9999 for PRM and NBV methods showing excellent linearity. The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ = κSDa/b, where κ= 3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The parameters for the analytical performance of the proposed methods are summarized in Table 2.

To check accuracy of the proposed methods, the standard addition technique was applied. Aliquots of the sample solution was spiked with three different concentrations of the standard solution. The percent recoveries of the added standards were calculated using the Equation 1.

$$\text{Recovery \%} = [(C_t\text{-}C_u) / C_a] \text{ x } 100 \qquad (1)$$

where C_t is the total concentration of the analyte found; C_u is the concentration of the analyte present in the formulation; and C_a is the concentration of the pure analyte added to the formulation. The results of analysis of the commercial dosage forms and the recovery study are shown in Table 3. The average percent recoveries ranged from 98.20 to 101.27% indicating good accuracy of the methods.

The specificity of the methods was studied by evaluating the interference liabilities from the common tablet excipients, such as talc, lactose, starch, mannitol and magnesium stearate. No interference

Table 2. Results of analytical parameters of the proposed methods

Parameters	PRM		NBV		CRV	ELT
	Spectrophotometric method	Spectrofluorimetric method	Spectrophotometric method	Spectrofluorimetric method	Spectrophotometric method	Spectrophotometric method
Wavelength (nm)	λmax:474	λex : 470, λem :529	λmax: 484	λex : 473, λem :530	λmax: 480	λmax: 480
Concentration range [a] (μg mL^{-1})	10-60	0.3-3.0	2.0-40.0	0.1-1.2	7.0-60.0	30-250
Regression equation[b]						
Intercept ± SD	- 0,0969 ± 0.063	8.0837 ± 0.55	0.0523 ± 0.0007	152.69 ± 2.19	0.0138 ± 0.00027	-0.1578 ± 0.002383
Slope ± SD	0,0906 ± 0.096	22.181 ± 0.89	0.0191 ± 0.0002	722.1 ± 5.6	0.0149 ± 0.00110	0.1009 ± 0.004222
Correlation coefficient (r^2)	0.9997	0.9983	0.9994	0.9999	0.9997	0.9995
Precision						
Intra-day [c], RSD %	0.55	0.85	0.91	0.68	0.98	0.98
Inter-day[d], RSD %	0.89	1.14	1.02	0.95	1.45	1.43
LOD (μg ml^{-1})	2.08	0.074	0.110	0.010	0.055	0.070
LOQ (μg ml^{-1})	6.95	0.247	0.367	0.0334	0.185	0.236

[a] Average of five determinations. [b] $A = a + bC$ (where C is the concentration of drug in μg mL^{-1}). [c] Results of 5 different days. [d] n=5 correspond to replicate analysis for each level

Table 3. Results of recovery studies by standard addition method

	Methods	Amount taken (μg mL^{-1}) [a]	Amount added (μg mL^{-1})	Total amount found [b] (μg mL^{-1}) (Mean± S.D.[c])	Recovery (%)	RSD (%)
PRM	Spectrophotometric Method	30.0	10.0	40.120±0.32	101.20	0.80
			20.0	49.980±0.55	99.90	1.10
			30.0	60.140±0.68	100.47	1.13
	Spectrofluorimetric Method	1.0	0.3	1.301±0.012	100.33	0.92
			1.0	2.013±0.025	101.34	1.24
			2.0	2.986±0.038	99.30	1.27
NBV	Spectrophotometric Method	5.0	2.0	6.99±0.050	99.50	0.72
			15.0	20.190±0.210	101.27	1.04
			35.0	40.150±0.265	100.43	0.66
	Spectrofluorimetric Method	0.70	0.10	0.799±0,004123	99.99	0.52
			0.30	1.00±0.005765	100.14	0.58
			0.50	1.20±0.007987	100.22	0.67
CRV	Spectrophotometric Method	5.0	2.0	7.01±0.054	100.50	0.77
			25.0	29.65±0.34	98.60	1.15
			55.0	59.01±0.67	98.20	1.14
ELT	Spectrophotometric Method	50	30	79.71±0.502	99.03	0.63
			100	149.79±0.839	99.79	0.56
			200	249.61±1.068	99.81	0.43

[a] Pexola® tablet (1 mg) (for PRM), Nexivol® tablet (5 mg) (for NBV), Kinetra® tablet (25 mg) (for CRV), Relpax film coated tablet (for ELT). [b] Five independent analyses. [c] Standard deviation

was observed from these excipients.

The inter- and intra-day precision were examined by analysis of drugs (*n*=5) for 7 consecutive days. The RSD values for intra-day precision and %, and inter-day precision and %, for spectrophotometric and spectrofluorimetric methods indicated good precision. The obtained results are summarized in Table 2.

Robustness was assessed by testing the susceptibility of measurements to deliberate variation of the analytical conditions. It was verified that slight variations in concentration of NBD-Cl (0.45–0.55%, w/v), temperature (optimum ± 2 °C), and time (optimum ± 0.25 min)

did not have an adverse impact on the subject derivatization procedure. All precision studies yielded RSD values not more than 2%. The most critical factor affecting the test results was determined to be the pH which was adjusted to be in the range of 8.5 ± 0.2 in order to generate the maximum response or 8.0 ± 0.2.

Applications of the method

The proposed methods were successfully applied to the analyses of marketed products (Pexola® tablet, Nexivol® tablet, Kinetra®

Table 4. Statistical evaluations of the results obtained by proposed and reference (6,1) methods for the assay of drugs in pharmaceutical preparations (*n*=5)

Statistical value	PRM			NBV			CRV		ELT	
	Spectro-photometric method[a]	Spectro-fluorimetric method[a]	Ref. Method[7]	Spectro-photometric method[a]	Spectro-fluorimetric method[a]	Ref. Method[11]	Spectro-photometric Method[a]	Ref. Method[16]	Spectro-photometric method[a]	Ref. Method[17]
Mean[b]	1.004	1.002	1.014	5.07	5.01	5.03	24.94	25.31	40.04	40.08
Recovery (%)	100.40	100.20	101.4	101.40	100.28	100.52	99.75	101.25	101.03	100.21
RSD (%)	1.34	1.64	1.49	1.32	1.22	1.11	0.25	1.48	0.92	1.09
t-test of significance[c]	1.13	1.10		1.12	0.33		0.29		1.28	
F-test of significance[c]	1.28	0.85		0.703	0.84		2.37		1.39	

[a] Pexola® tablet (1 mg) (for PRM), Nexivol® tablet (5 mg) (for NBV), Kinetra® tablet (25 mg) (for CRV), Relpax® film coated tablet (40 mg) (for ELT). [b] 5 independent analyses. [c] $p = 0.05$, $t = 2.23$, $F = 5.05$

tablet and Relpax® film coated tablet) and the results obtained are summarized in Table 4. Statistical analysis of the results obtained by the proposed method and reference HPLC methods[6,11,17] for PRM, NBV and ELT, respectively and UV spectrophotometric method[16] for CRV were performed using the Student's *t*-test and the variance ratio *F*-test. The calculated values were comparable with that of the theoretical test results indicating no significance difference between the compared methods regarding accuracy and precision.

CONCLUSION

New simple, sensitive and selective spectrophotometric and spectrofluorimetric methods, which comprise NBD-Cl as derivatization reagent, have been developed and validated for determination of PRM, NBV, CRV and ELT. The proposed methods were successfully applied for the subject drug substances and their respective drug products,and proven to be selective, highly sensitive, accurate, and precise. Therefore, the proposed methods are suitable for routine assay determination of PRM, NBV, CRV and ELT .

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br in PDF format, with free access.

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Sensitive and selective spectrophotometric and spectrofluorimetric methods have been developed for determination of some drugs such as Pramipexole, Nebivolol, Carvedilol, and Eletriptan, which commonly contain secondary amino group using 7-Chloro-4-nitrobenzofurazon.

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