Spectrophotometric, Spectrofluorometric and HPLC Determination of Desloratadine in Dosage Forms and Human Plasma

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Four sensitive, simple and specific methods were developed for the determination of desloratadine (DSL), a new antihistaminic drug in pharmaceutical preparations and biological fluids. Methods I and II are based on coupling DSL with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in borate buffer of pH 7.6 where a yellow colored reaction product was obtained and measured spectrophotometrically at 485 nm (Method I). The same product could be measured spectrofluorometrically at 538 nm after excitation at 480 nm (Method II). Methods III and IV, on the other hand, involved derivatization of DSL with 2,4-dinitrofluorobenzene (DNFB) in borate buffer of pH 9.0 producing a yellow colored product that absorbs maximally at 375 nm (Method III). The same derivative was determined after separation adopting HPLC (Method IV). The separation was performed on a column packed with cyanopropyl bonded stationary phase equilibrated with a mobile phase composed of acetonitrile–water (60 : 40, v/v) at a flow rate of 1.0 ml min^{-1} with UV detection at 375 nm. The calibration curves were linear over the concentration ranges of 0.5—6, 0.02—0.4, 1—10 and 1—30 μg ml^{-1} for Methods I, II, III and IV, respectively. The lower detection limits (LOD) were 0.112, 0.004, 0.172 and 0.290 μg ml^{-1}, respectively, for the four methods. The limits of quantification (LOQ) were 0.340, 0.012, 0.522 and 0.890 μg ml^{-1} for Methods I, II, III and IV, respectively. The proposed methods were applied to the determination of desloratadine in its tablets and the results were in agreement with those obtained using a reference method. Furthermore, the spectrofluorometric method (Method II) was extended to the *in-vitro* determination of the drug in spiked human plasma, with a mean percentage recovery (n=4) of 99.7±3.54. Interference arising from endogenous amino acids has been overcome using solid phase extraction. The proposed methods are highly specific for determination of DSL in the presence of the parent drug loratadine. A proposal for the reaction pathways is postulated.

Key words desloratadine; spectrophotometry; spectrofluorometry; HPLC; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; 2,4-dinitrofluorobenzene

Desloratadine (DSL), 4-(8-chloro-5,6-dihydro-11*H*-benzo-[5,6]cyclohepta [1,2*b*]pyridin-11-ylidene)-1-piperidine, (DSL is the descarboethoxy form of loratadine) (Fig. 1), is a selective peripheral H1 receptor antagonist, devoid of any substantial effect on the central and autonomic nervous systems.[1,2] Desloratadine exhibits qualitatively similar pharmacodynamic activity with a relative oral potency in animals, two to three-fold greater than its parent analogue loratadine, probably due to a higher affinity for histamine H1 human receptors.[3] Nevertheless, the development of drugs with increased potency will continue to challenge the analytical chemist to lower the limit of quantitation (LOQ).

Several analytical methods have been reported for the determination of DSL in biological samples and applied in pharmacokinetic studies. These methods include gas chromatography with nitrogen phosphorous detection,[4] liquid chromatography with fluorescence detection,[5,6] ultraviolet detection,[7] or mass spectrometric detection.[8—11] However, DSL was determined in pharmaceutical preparations using a liquid chromatographic method with UV detection[12] and capillary isotachophoresis.[13]

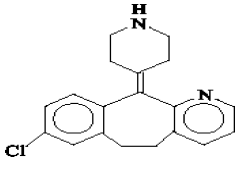

Fig. 1. Structural Formula of Desloratadine (DSL)

As DSL has no functional group that enables absorption in the visible region or the emission of fluorescence, we decided to analyze the drug through derivatization reactions. In this approach, two different labeling agents, namely; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and 2,4-dinitrofluorobenzene (DNFB) (Sanger's reagent), have been used in derivatization reactions based on their reaction with the free secondary amino group in the piperidine ring of DSL. The proposed methods are highly specific for desloratadine in the presence of the parent drug (loratadine). Both reagents are well known to react with primary and secondary amines forming stable condensation colored products.[14] Moreover, the condensation product with NBD-Cl was found to be also a fluorescent derivative. NBD-Cl has been used as a derivatizing agent for many compounds either *per se* or in formulations and biological fluids such as lisinopril,[15] gliclazide,[16] tianeptine,[17] ranitidine and nizatidine,[18] atenolol,[19] fenetrol,[20] trimetazidine,[21] and recently befunolol,[22] some β-blockers[23] and paroxetine.[24] Sanger's reagent (DNFB) on the other hand has been applied as a chromophore reactant in the spectrophotometric determination of norfloxacin,[25] enalapril and lisinopril.[26] DNFB also has been used as a precolumn derivatizing reagent for the HPLC assay of paromomycin,[27] and vertilmicin[28] followed by UV detection.

In the present work, the two colored reaction products obtained with NBD-Cl and Sanger's reagent were measured spectrophotometrically (Methods I, III). Meanwhile, the reaction product with NBD-Cl was measured spectrofluorometrically (Method II). Also, the colored reaction product with DNFB was quantitated by HPLC with UV detection

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(Method IV). The four developed procedures were applied for the determination of DSL in tablets without any interference from the excipients. A low LOQ is a common requirement to support a clinical development program and when this is coupled with a simple procedure for sample preparation and feasible analytical tool, they would be the requirements of an optimized analytical method. Although the previously reported methods that were applied in pharmacokinetic studies of desloratadine possess the required low LOQ, they lack the simple sample preparation and method feasibility that were obtained in our spectrofluorometric method using NBD-Cl as a derivatizing agent where a low LOQ of 0.012 μg/ml was attained which allowed the analytical determination of DSL in spiked and real human plasma.

Experimental

Materials and Reagents All chemicals were of analytical reagent grade. Desloratadine reference standard was kindly provided by Schering-Plough Corporation, U.S.A. Aerius tablets labeled to contain 5 mg DSL per tablet were purchased from commercial sources at a local pharmacy. Plasma samples were obtained from Mansoura University Hospital, Mansoura, Egypt, and were kept frozen until use after gentle thawing. Sep-Pak® C18 solid phase extraction cartridges, 200 mg (SPE) were purchased from Waters Corporation (Milford, MA, U.S.A.). 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution containing 2 mg/ml was freshly prepared in methanol. 2,4-Dinitrofluorobenzene (DNFB) was also purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution containing 0.25% (v/v) was freshly prepared in methanol. Borate buffer solutions (0.2 M) were prepared by mixing appropriate volumes of 0.2 M boric acid and 0.2 M NaOH and adjusting the pH to 7.6 and 9.0 using a pH meter.[29] Formic acid 85%, acetonitrile and methanol; AR Grade, Aldrich (St. Louis, MO, U.S.A.).

Apparatus A Shimadzu UV–Visible 1601 PC spectrophotometer was used for the spectrophotometric measurements (P/N 206-67001). The recording range was 0—1.0.

The fluorescence spectra and measurements were performed on a Perkin-Elmer UK model LS 45 luminescence spectrometer, equipped with a 150 W Xenon arc lamp, grating excitation and emission monochromators for all measurements and a Perkin-Elmer recorder. Slit widths for both monochromators were set at 10 nm. A 1 cm quartz cell was used.

The HPLC separation was performed with a Perkin-Elmer™ Series 200 chromatograph equipped with a Rheodyne injector valve with a 20 μl loop and a UV/VIS detector. A Total Chrom Workstation was used for data collection and processing (Perkin-Elmer, MA, U.S.A.).

Columns and Mobile Phases: Separation was achieved on a Hibar®, Lichrosorb®, cyanopropyl column (250 mm×4.6 mm ID, 5 μm particle size) from Merck. A Hibar®, Lichrosorb® RP-18 pre-packed column (250 mm×4 mm ID, 5 μm particle size) combined with a guard column from Merck was used for the reported reference method. The columns were operated at ambient temperature. The components of the mobile phase were acetonitrile : water (60 : 40).

Sample Preparation and Procedure A standard solution of DSL containing 200 μg/ml was prepared in methanol and was further diluted with the same solvent as appropriate to obtain the working concentration. The standard solution was kept in the refrigerator and was found to be stable for at least 7 d.

Construction of Calibration Graph for Method I To a set of 10 ml volumetric flasks, aliquot volumes containing the drug in the working concentration range (0.5—6.0 μg/ml) were quantitatively transferred. To each flask 1 ml of borate buffer (pH 7.6) followed by 0.8±0.2 ml of NBD-Cl solution (0.2%) were added and mixed well. The solutions were heated at 60±10 °C for 10 min in a thermostatically controlled water bath. The reaction was quenched by cooling under tap water, and then 0.2 ml of concentrated HCl was added and each flask was made up to volume with methanol. The absorbance was measured at 485 nm against a reagent blank.

The calibration curve for the proposed method was constructed by plotting the absorbance *vs.* the final concentration of the drug (μg/ml). Alternatively, the corresponding regression equation was derived.

Construction of Calibration Graph for Method II The described procedures for the spectrophotometric method were repeated after dilution of the standard solution to obtain the concentration range (0.02—0.4 μg/ml). The fluorescence intensity of the reaction product was measured at 538 nm after excitation at 480 nm. The fluorescence intensity was plotted *vs.* the final concentration of the drug (μg/ml) to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

Construction of Calibration Graph for Method III To a set of 10 ml volmetric flasks, aliquot volumes containing the drug in the working concentration range (1—10 μg/ml) were quantitatively transferred. To each flask 0.2 ml borate buffer (pH 9.0) followed by 2.0±0.5 ml of DNFB solution (0.25%) were added and mixed well. The solutions were allowed to stand for 5 min. Each flask was made up to volume with methanol. The absorbance was measured at 375 nm against a reagent blank. The calibration curve for the proposed method was constructed by plotting the absorbance *vs.* the final concentration of the drug (μg/ml). Alternatively, the corresponding regression equation was derived.

Construction of Calibration Graph for Method IV To a set of 10 ml volumetric flasks, aliquot volumes containing the drug in the working concentration range (1—30 μg/ml) were quantitatively transferred. To each flask 0.2 ml borate buffer (pH 9.0) followed by 2.0±0.5 ml of DNFB solution (0.25%) were added and mixed well. The solutions were allowed to stand for 5 min. Each flask was made up to volume with the mobile phase. Injection into the HPLC was performed at ambient temperature (25 °C). All samples were filtered through 0.45 μm sample filters (RC 25, Sartorius AG, Goettingen, Germany) prior to injection into the HPLC system. Twenty μl aliquots were injected (in triplicate) and the peak area of the derivatized drug was recorded at 375 nm. A calibration curve was constructed by plotting the peak area against the final concentration of the drug. Alternatively, the corresponding regression equation was derived.

Assay Procedure for Tablets Twenty tablets were finely powdered after weighing, and a portion of the tablet powder equivalent to 20.0 mg of DSL was transferred into a small conical flask, and extracted three successive times each with 30 ml of methanol. The extracts were filtered into 100 ml volumetric flask; the conical flask was washed with a few ml of methanol. The wash was added to the same volumetric flask, and then the flask was made up to volume with the same solvent. Aliquots covering the working concentration range cited in Table 1 were transferred into 10 ml volumetric flasks. Proceed as described under "Construction of calibration graph..." adopting any of the four methods. "The nominal content of the tablets was calculated using the corresponding regression equation."

Assay Procedures for Spiked Human Plasma A SPE cartridge was preconditioned with 1 ml of methanol followed by 1 ml of 2% formic acid. One milliliter aliquots of spiked human plasma sample were diluted with 2 ml of 2% formic acid solution. The mixture was then applied to the preconditioned SPE cartridge under vacuum. The extraction cartridge was washed sequentially with 1 ml of 2% formic acid followed by 1 ml of 2% formic acid in acetonitrile : methanol (70 : 30, (v/v) %). Elution of the analyte was performed using 1 ml aliquots of 4% ammonium hydroxide in methanol : acetonitrile : water (45 : 45 : 10). The eluate was dried under a nitrogen stream. The residue was then reconstituted with 2 ml of methanol and transferred into a 10 ml volumetric flask. The assay was then carried out as described under Method II. The fluorescence intensity of the resulting solution was measured at 538 nm after excitation at 480 nm. The nominal content of the drug was determined using the corresponding regression equation.

Assay Procedures for Real Human Plasma Aerius® tablets (5 mg/tablet) were orally administered to a healthy fasting volunteer (female, 40 years old). Five milliliters of blood sample were withdrawn after 3 h, 4 ml of citrate solution added, and the mixture centrifuged at 3500 rpm for 15 min to get 3.0 ml of plasma. The procedure described for spiked human plasma was performed. The nominal content of DSL was determined using the corresponding regression equation.

Results and Discussion

The present prescribed four methods are highly specific for the determination of DSL in the presence of the parent drug, loratadine, since the later is deprived from secondary amine which is essential for the reaction with either NBD-Cl or DNFB. However, 3-hydroxy desloratadine is the major active metabolite of DSL which retains the secondary amine group, thus the four methods can not specify DSL in the presence of the metabolite.

Reaction with NBD-Cl (Methods I and II) 4-Chloro-7-

nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), as an electroactive halide reagent, was first introduced as an analytical reagent for the determination of some amines and amino acids.[30] In recent reports, NBD-Cl was further used as a chromogenic reagent for the determination of some primary and secondary amines.[14—24] In the present study, DSL was found to react with NBD-Cl in borate buffer of pH 7.6 producing a yellow color with maximum absorbance at 485 nm; Method I. Figure 2a illustrates the absorption spectrum of the reaction product.

Also, the same reaction product yields a strong fluorescence (Fig. 3) at 538 nm after excitation at 480 nm through the formation of a Meisenheimer complex[31] (Method II).

Study of Experimental Parameters The different experimental parameters affecting the development of the reaction product and its stability were carefully studied and optimized. Such factors were changed individually while others were kept constant. These factors include: pH, volume of the reagent, temperature and heating time. The optimum reaction conditions obtained by measuring the fluorescence intensity of the reaction product were applied to the spectrophotometric procedures.

Methods I and II The influence of pH on the fluorescence intensity of the reaction product was investigated using borate buffer. Maximum fluorescence intensity was obtained at pH 7.6±0.1, after which the fluorescence intensity of the reaction product gradually decreased. Therefore, pH 7.6 was chosen as the optimum pH for such study (Fig. 4).

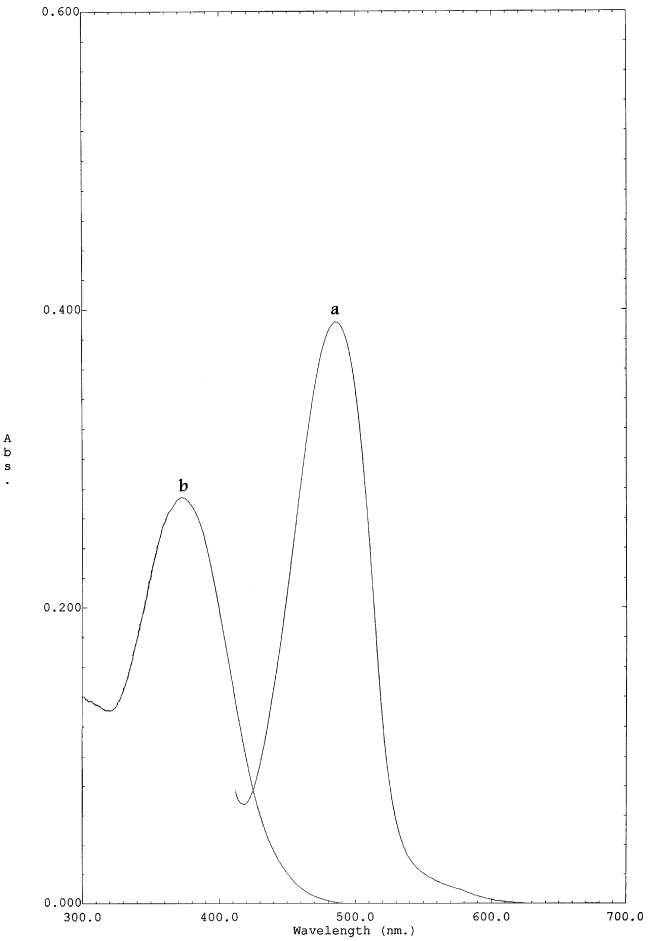

Fig. 2. Absorption Spectra of the Reaction Products of (a) DSL (3 μg/ml) with NBD-Cl at pH 7.6 and (b) DSL (4 μg/ml) with DNFB at pH 9.0

Other buffers having the same pH value such as phosphate and hexamine were attempted and compared with 0.2 M borate buffer. Borate buffer was found to be superior to others as revealed by the high net fluorescence intensity. This different response to different buffers may be attributed to the slow rate of hydrolysis of NBD-Cl to NBD-OH in borate buffer. This result is in agreement with that of Miyano H. *et al.*[32]

The influence of the concentration of NBD-Cl was studied using different volumes of a 0.2% solution of the reagent. It was found that increasing the volume of the reagent produces a proportional increase in the fluorescence intensity of the reaction product up to 0.4 ml. However, no further increase in the fluorescence intensity was observed upon increasing the volume of the reagent up to 1.0 ml, after which further increase produces a gradual decrease in the fluorescence intensity. Therefore, 0.8±0.2 ml of 0.2% of NBD-Cl solution was chosen as the optimal volume of the reagent (Fig. 5).

Different temperature settings were investigated with constant heating time. Increasing the temperature was found to produce a proportional increase in the fluorescence intensity of the reaction product up 50 °C, following which it remained constant up to 70 °C. A further increase in temperature resulted in a gradual decrease in the fluorescence intensity. Therefore, heating at a relatively low temperature (60±10 °C) was selected as the most suitable temperature for carrying out the study.

The time of heating is an essential part of the experiment. Different time intervals were tested and it was found that after 10 min, the reaction product reaches the highest fluorescence intensity and then remains constant up to 25 min. It was observed that heating for 10 min is adequate for maximum fluorescence intensity.

Regarding the stability of the produced derivative, it was found to be stable for at least 3 h.

The effect of diluting solvent was tested using different solvents, *viz.* water, methanol, acetone, dimethylsulfoxide and dimethylformamide. Of all the solvents studied, the highest fluorescence intensity was obtained upon using methanol.

The fluorescence intensity or absorbance value of the hydrolysis product of NBD-Cl, namely, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) is quenched by decreasing the pH of the reaction medium to less than 1 by adding 0.2 ml of HCl. Therefore, acidification of the reaction mixture prior to measurement of the fluorescence intensity or absorbance value remarkably decreased the background fluorescence or absorbance due to the formation of NBD-OH without affecting the drug reagent adduct, hence the sensitivity was increased.[33]

Reaction with DNFB (Methods III and IV) DNFB (Sanger's reagent), as an active aryl halide reacts with primary, or secondary amines in aqueous alkaline medium to form a yellow colored product through a nucleophilic substitution reaction.[34] In the present study, DSL was found to react with DNFB in borate buffer of pH 9.0 producing a yellow color with maximum absorbance at 375 nm, Method III (Fig. 2b). Also this colored reaction product was measured by HPLC with UV detection at 375 nm (Method IV). Excess reagent could be easily eliminated through the instrumental background correction (auto zero of blank) in the case of spectrophotometric assay, meanwhile, complete resolution of

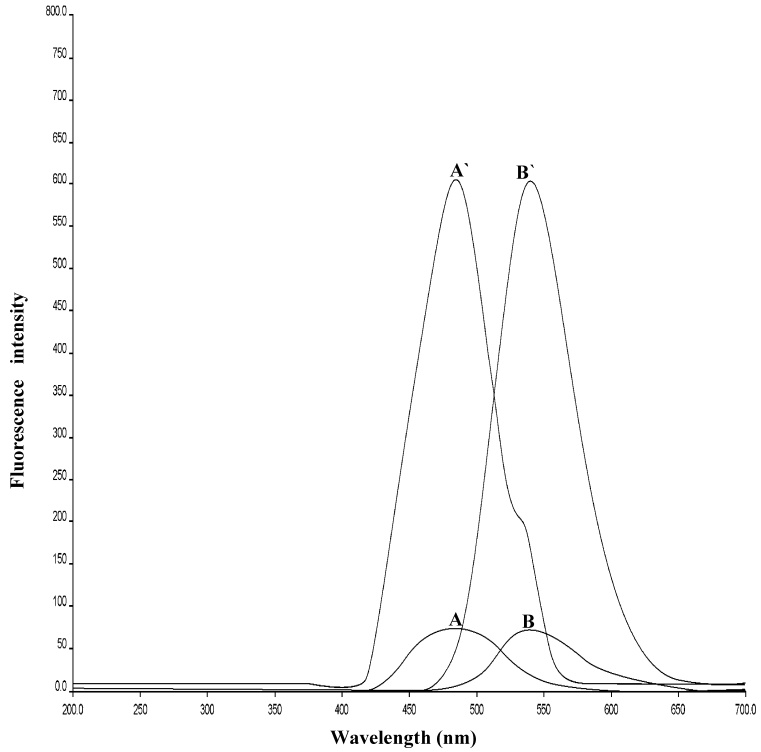

Fig. 3. Fluorescence Spectra of: (A and B) Excitation and Emission Spectra of Blank, 0.02% NBD-Cl at pH 7.6, (A′ and B′) Excitation and Emission Spectra of the Reaction Product of DSL (0.4 μg/ml) with NBD-Cl at pH 7.6

(A, A′) Excitation spectra, (B, B′) emission spectra.

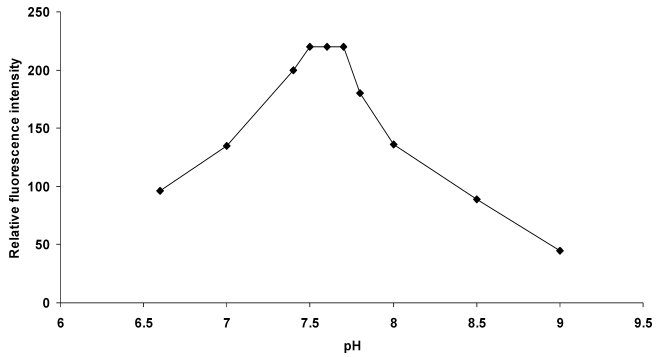

Fig. 4. Effect of pH on the Fluorescence Intensity of the Reaction Product of DSL (0.16 μg/ml) with NBD-Cl at 538 nm

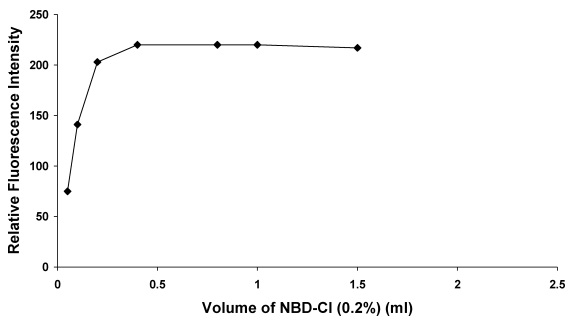

Fig. 5. Effect of Volume of NBD-Cl (0.2%) on the Fluorescence Intensity of the Reaction Product with DSL (0.16 μg/ml) at pH 7.6

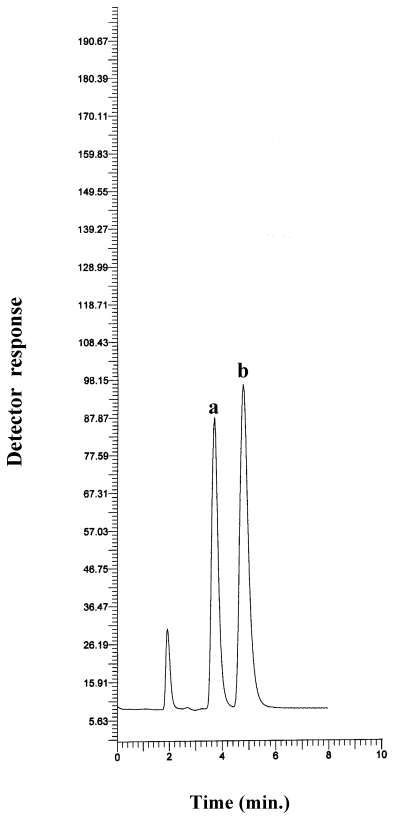

Fig. 6. Typical Chromatogram of (a) Blank Reagent; (b) Derivatized Drug (25 μg/ml)

Chromatographic system; column: cyanopropyl (5 μm) 250×4.6 mm. Mobile phase, acetonitrile–water (60 : 40) (v/v). Flow rate = 1 ml/min, UV detection at 375 nm; column temperature: ambient.

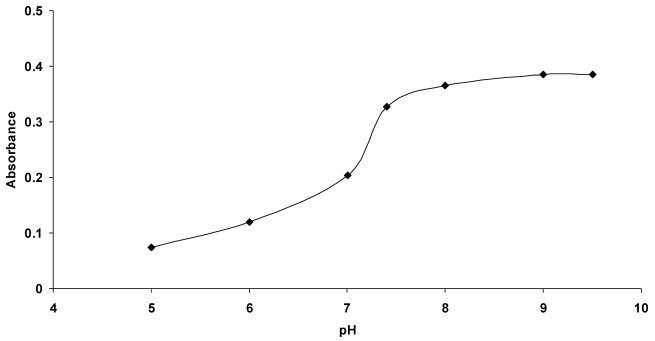

Fig. 7. Effect of pH on the Absorbance of the Reaction Product of DSL (5 μg/ml) with 2,4-Dinitrofluorobenzene

the peaks corresponding to the reagent and the reaction product was achieved in the HPLC method (Fig. 6).

The different experimental parameters affecting the color development and its stability were carefully studied and optimized. These parameters include pH, volume of the reagent, and time. The optimum conditions have been used for the spectrophotometric method and the precolumn derivatization for the HPLC method.

According to the literature, the substitution reaction of DNFB with amines was carried out in alkaline medium. Therefore, the reaction was investigated using borate buffer over the pH range from 5 to 9.5. It was found that increasing the pH resulted in a corresponding increase in the absorbance of the reaction product up to pH 9.0 after which it remained constant. Therefore, pH 9.0 was chosen as the optimum pH throughout this study (Fig. 7).

The effect of buffer concentration was investigated using increasing volumes of 0.2 molar solution of borate buffer (pH 9.0). It was observed that increasing the volume of the buffer produces a corresponding increase in the absorbance value of the reaction product up to 0.004 M, and it remained constant up to 0.016 M (final concentration). Therefore, 0.2 ml of borate buffer (pH 9.0), *i.e.*, 0.004 M, was chosen as the optimum buffer concentration.

The influence of the concentration of DNFB was studied using different volumes of 0.25% solution of the reagent. It was found that the reaction of DNFB with DSL started upon using 0.1 ml of the reagent in the presence of borate buffer (pH 9.0). Increasing the volume of the reagent produces a proportional increase in the absorbance of the reaction product up to 1.5 ml, after which it remains constant up to 2.5 ml. A further increase produces a gradual decrease in the absorbance value. Therefore, 2.0±0.5 ml of 0.25% DNFB was chosen as the optimal volume of the reagent.

The effect of temperature on the development of the color of the reaction product was studied using different temperature settings with a constant heating time. A gradual increase in the heating temperature up to boiling did not produce a significant increase in the absorbance of the reaction product. Therefore, the reaction was carried out at room temperature after gentle shaking for 5 min, which is adequate for complete color development.

The effect of diluting solvent was investigated using different solvents *viz.* water, methanol, ethanol, isopropanol, acetone, dimethylsulfoxide, and dimethylformamide. Of all the solvents studied, the highest absorbance was obtained using methanol.

The reaction product was found to be stable for at least 60 min at room temperature.

HPLC Method Development A simple and reliable HPLC method was developed for the determination of the formed condensation product with DNFB. The separation and resolution of the peaks of the blank reagent and the derivatised drug could be achieved using a mixture of acetonitrile–water (60 : 40, v/v) as the mobile phase with UV detection at 375 nm.

The effect of pH on the retention of the derivatized drug and blank reagent was investigated by replacing the aqueous portion of the mobile phase with 0.02 M sodium phosphate buffer over the pH range 3.0 to 7.0. The optimum separation was accomplished upon using aqueous solution. Also, the acetonitrile ratio was studied over the range from 20 to 80% (v/v in water). Optimum resolution was obtained over the ratio 55 to 65% after which overlapping of the two peaks (blank and derivatized drug) was observed. At lower acetonitrile ratios, longer retention times were obtained.

Acetonitrile at the same percentage (60%) was replaced with methanol. The separation was possible, although with an asymmetric peak, thus acetonitrile was preferred.

Characterization of the Chromatographic Peak: According to the conditions described, the retention times were about 3.7 and 4.8 min for DNFB and the derivatized drug, respectively.

A typical chromatogram of DSL after derivatization with DNFB is shown in Fig. 6. Separation was possible in a short retention time, less than 5 min, with a resolution factor of about 2.75.

Analytical Performance. Validation of the Proposed Methods The validity of the methods was tested regarding linearity, specificity, accuracy, repeatability and precision according to ICH Q2B recommendations.[35)]

Linearity By using the above procedures, linear regression equations were obtained. The regression plots showed that there was a linear dependence of the analytical response in the four methods to the concentration of the drug over the ranges cited in Table 1. Linear regression analysis of the data gave the following equations. For Method I, $A=-0.007+0.135C$ with a correlation coefficient, $r=0.9998$, for method II, $F=1.111+1333C$ having a correlation coefficient, r of 0.9995. For method III, $A=0.0022+0.079C$ with $r=0.9998$ and for method IV, $P=1.78\times10^4+1.61\times10^5C$ with $r=0.9998$. Where A is the absorbance, F is fluorescence intensity, P is the peak area, C is the concentration of the drug (μg/ml), and r is the correlation coefficient.

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2B.[35)] The results are shown in Table 1. The limits of detection (LOD) were determined by establishing the minimum level at which the analyte can be reliably detected, and the results are also summarized in Table 1.

LOQ and LOD were calculated according to the following equation[35)]

$$\mathrm{LOQ}=10\sigma/S$$

$$\mathrm{LOD}=3.3\sigma/S$$

Where σ is the standard deviation of the intercept of the re-

Table 1. Performance Data of the Proposed Method[a)]

Parameter	Method I	Method II	Method III	Method IV
Concentration range (μg/ml)	0.5—6.0	0.02—0.4	1—10	1—30
Limit of detection (μg/ml)	0.112	0.004	0.172	0.290
Limit of quantification (μg/ml)	0.340	0.012	0.522	0.890
Correlation coefficient (r)	0.9998	0.9995	0.9998	0.9998
Slope	0.135	1333.14	0.079	1.614×10^{4}
Intercept	−0.007	1.11	0.002	1.078×10^{4}
$S_{y/x}$	4.24×10^{-3}	0.409	4.65×10^{-3}	3.276×10^{4}
S_a	4.56×10^{-3}	1.61	4.12×10^{-3}	1.432×10^{4}
S_b	8.44×10^{-4}	1.29	5.96×10^{-4}	9.899×10^{2}
% Error	0.39	0.45	0.48	0.438

a) $S_{y/x}$=standard deviation of the residuals. S_a=standard deviation of the intercept of regression line. S_b=standard deviation of the slope of regression line. % Error=RSD%/$\sqrt{n}$.

Table 2. Application of the Proposed and Reference Methods to the Determination of DSL in Pure Form[a)]

Parameters	Method I	Method II	Method III	Method IV	Reference method[37)]
No. of experiments	7	6	7	8	3
Mean found, %	99.76	99.88	99.77	100.84	99.83
±S.D.	1.03	1.11	1.28	1.25	0.81
RSD, %	1.03	1.11	1.28	1.24	0.66
Variance	1.06	1.23	1.64	1.56	
Student's t-value	0.104 (2.31)	0.07 (2.37)	0.08 (2.31)	1.29 (2.26)	
Variance ratio F-test	1.62 (5.14)	1.87 (5.79)	2.50 (5.14)	2.38 (4.74)	

a) Figures between parentheses are the tabulated t and F values respectively, at $p=0.05$.[36)]

gression line. S: is the slope of the calibration curve.

The proposed methods were evaluated for the accuracy as percent relative error (% Er) and the precision as percent relative standard deviation (% RSD) (Tables 1, 2).

Accuracy To test the validity of the proposed methods they were applied to the determination of authentic sample of DSL over the concentration range cited in Table 2. The results obtained were in good agreement with those obtained using the reference method. Student's t-test and the variance ratio F-test[36)] revealed no significant differences between the performance of the two methods regarding the accuracy and precision, respectively (Table 2). An HPLC reference method was adopted using acetonitrile–phosphate buffer of pH 2.6 (1 : 1) as a mobile phase with UV detection at 262 nm.[37)]

The validity of the methods was proved by statistical evaluation of the regression lines, using the standard deviation of the residuals ($S_{y/x}$), the standard deviation of the intercept (S_a) and standard deviation of the slope (S_b). The results are abridged in Table 1. The small values of the figures indicate low scattering of the calibration points around the calibration line and high precision.

Precision (a) Repeatability: The repeatability was tested by applying the proposed methods for the determination of two concentrations of DSL in pure form on three successive occasions. The results are presented in Table 3.

(b) Intermediate Precision: Intermediate precision was tested by repeated analysis of DSL in pure form applying the four methods, using the concentrations shown in Table 3 for a period of 3 successive days. The results are summarized in Table 3.

Robustness of the Method The robustness of the method adopted is demonstrated by the constancy of the fluorescence intensity or absorbance with the deliberated minor changes in the experimental parameters such as pH 7.5±0.1, and change in the volume of NBD-Cl (0.2%), 0.8±0.2 ml and the change in the heating temperature (60±10 °C) for Methods I and II respectively. Meanwhile, for Methods III

Table 3. Validation of the Proposed Method for the Determination of DSL Raw Material[a)]

Concentration added (μg/ml)	% Recovery	% RSD	% Error
Method I			
Intra-day			
2.0	99.74±0.79	0.79	0.46
4.0	100.57±0.96	0.96	0.55
Inter-day			
2.0	99.49±1.16	1.16	0.67
4.0	100.58±1.01	1.01	0.58
Method II			
Intra-day			
0.10	100.24±0.68	0.68	0.39
0.20	100.09±0.28	0.28	0.16
Inter-day			
0.10	99.50±1.14	1.14	0.66
0.20	100.12±0.78	0.78	0.45
Method III			
Intra-day			
4.0	100.31±0.62	0.62	0.36
8.0	100.00±0.68	0.68	0.39
Inter-day			
4.0	99.69±0.82	0.82	0.47
8.0	100.36±1.18	1.18	0.68
Method IV			
Intra-day			
10.0	99.77±0.48	0.48	0.28
20.0	99.70±1.14	1.14	0.66
Inter-day			
10.0	99.78±1.44	1.44	0.83
20.0	99.32±1.32	1.32	0.76

a) Each result is the average of three separate determinations.

and IV these changes include pH 9.3±0.3, and volume of DNFB (0.25%) 2±0.5 ml. These minor changes that may take place during the experimental operation did not affect the fluorescence intensity or absorbance or peak area of the reaction products.

Pharmaceutical Applications The proposed methods were then applied to the determination of DSL in its tablets. The methods were tested for linearity, specificity, accuracy, repeatability, and precision according to ICH Q2B recommendations.

Specificity The specificity of the methods was investigated by observing any interference encountered from the common tablet excipients, such as talc, lactose, starch, avisil, gelatin, and magnesium stearate. These excipients did not interfere with the proposed methods.

Accuracy The results of the proposed methods were statistically compared with those obtained using the reference method. Statistical analysis of the results, using Student's *t*-test and the variance ratio *F*-test revealed no significant difference between the performance of the proposed and reference methods regarding the accuracy and precision, respectively (Table 4).

Biological Applications The high sensitivity of the proposed spectrofluorometric method (method II) allowed the determination of DSL in biological fluids. Hence, the method was further applied to the *in-vitro* and *in vivo* determination of DSL in human plasma.

Following oral ingestion of a single dose of 5 mg, DSL gives a mean peak plasma concentration of 0.06 μg/ml after 3 h. This value lies within the working concentration range of the spectrofluorometric method. Interference arising from the endogenous amino acids has been overcome and removed by efficient separation using solid phase extraction.

Effect of Co-administered Drugs Interference likely to be introduced from co-administered drugs, such as cimetidine, fluoxetine, and fexofenadine, was studied. These drugs interfered since they contain secondary amino groups and thus, they should be eliminated before carrying out the assay in biological fluids. On the other hand, other co-administered drugs such as ketoconazole, erythromycin and azithromycin did not interfere with the assay since they do not contain primary or secondary amines.

Precision The within-day precision was evaluated through replicate analysis of plasma samples spiked with different concentrations of the drug (0.02—0.2 μg/ml). The mean percentage recoveries based on the average of four separate determinations were 99.7±3.54. The results are abridged in Table 5.

The inter-day precision was also evaluated through replicate analysis of plasma samples spiked with 0.1 μg/ml of drug on four successive days. The percentage recoveries based on the average of four separate determinations were 97.7±3.2. The accuracy and precision of the results shown in Table 5 are satisfactory. On the other hand, the % recoveries of DSL in real human plasma were 95.28±7.33 (Table 5).

Mechanism of the Reaction The stoichiometry of the two reactions was studied adopting the limiting logarithmic method.[38] The two straight lines were obtained using increasing concentrations of the reagent while keeping the concentration of the drug constant and using increasing concentrations of the drug while keeping the concentration of the

Table 4. Application of the Proposed and Reference Methods to the Determination of DSL in Aerius Tablets[a]

Method	Conc. taken (μg/ml)	Conc. found (μg/ml)	% Recovery	Reference method[37]
Method I	2.0	1.985	99.25	99.02
	3.0	2.947	98.23	98.28
	4.0	3.978	99.45	98.64
	5.0	5.037	100.74	
	6.0	6.007	100.12	
Mean found (%)			99.56	98.65
±S.D.			0.95	0.37
t-value			1.55 (2.45)	
F-value			6.59 (6.94)	
Method II	0.04	0.0397	99.25	
	0.08	0.0787	98.36	
	0.10	0.0994	99.40	
	0.20	0.2007	100.35	
	0.40	0.4008	100.20	
Mean found (%)			99.51	
±S.D.			0.80	
t-value			1.71 (2.45)	
F-value			4.67 (6.94)	
Method III	2.0	1.979	98.95	
	4.0	4.026	100.65	
	5.0	4.968	99.36	
	6.0	5.950	99.17	
	8.0	7.937	99.21	
Mean found (%)			99.47	
±S.D.			0.68	
t-value			1.89 (2.45)	
F-value			3.38 (6.94)	
Method IV	2.0	1.997	99.85	
	4.0	4.025	100.63	
	8.0	7.938	99.23	
	10.0	9.985	99.85	
	20.0	19.686	98.43	
Mean found (%)			99.60	
±S.D.			0.82	
t-value			0.36 (2.45)	
F-value			4.91 (6.94)	

a) Aerius tablets labeled to contain 5 mg DSL/tablet, product of Schering-Plough N. V. Labo-Belgium. Batch #5 STBA 52 B01. The figures between parentheses are the tabulated *t* and *F* values respectively at $p=0.05$.[36]

Table 5. Application of Method II for Spectrofluorimetric Determination of DSL in Spiked and Real Human Plasma[a]

Sample	Amount added (μg/ml)	Amount found (μg/ml)	% Recovery
Spiked plasma			
Intra-day precision	0.02	0.0203	101.50
	0.04	0.0413	103.25
	0.10	0.0951	95.10
	0.20	0.1979	98.95
Mean			99.70
±S.D.			3.54
Inter-day precision	0.10	0.0951	95.10
	0.10	0.0970	97.00
	0.10	0.1024	102.40
	0.10	0.0963	96.30
Mean			97.70
±S.D.			3.23
Real plasma	0.06	0.0532	88.67
	0.06	0.0564	94.0
	0.06	0.0619	103.17
Mean			95.28
±S.D.			7.33

a) Each result is the average of three separate determinations.

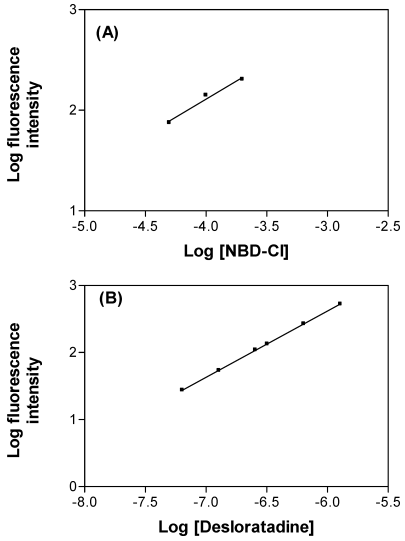

Fig. 8. Limiting Logarithmic Plots for the Molar Reactivity of Desloratadine with NBD-Cl: (A) log *F vs.* log[NBD-Cl] with [DSL] Kept Constant; (B) log *F vs.* log[DSL] with [NBD-Cl] Kept Constant

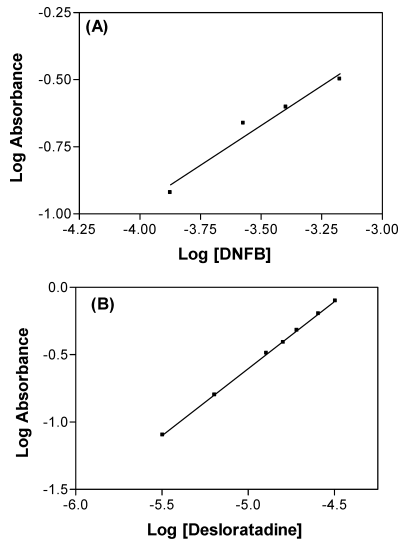

Fig. 9. Limiting Logarithmic Plots for the Molar Reactivity of Desloratadine with DNFB: (A) log *A vs.* log[DNFB] with [DSL] Kept Constant; (B) log *A vs.* log[DSL] with [DNFB] Kept Constant

reagent constant. Plots of log fluorescence *versus* log[NBD-Cl] and log[DSL] gave two straight lines, the slopes of which were 0.72/0.98 for NBD-Cl and DSL, respectively (Fig. 8). Hence, it is concluded that the reaction proceeds in the ratio of 1 : 1, confirming that one molecule of the drug condenses with one molecule of NBD-Cl.

Also, plots of log absorbance *versus* log[DNFB] and log[DSL] gave straight lines, the values of their slopes were 0.60 for DNFB and 0.99 for DSL (Fig. 9). Hence, it is concluded that the reaction proceeds in the ratio of 1 : 1, confirming that one molecule of the drug reacts with one molecule of DNFB. DSL in alkaline medium reacts with NBD-Cl or DNFB to give the following final reaction products.

Based on the observed molar ratios, proposed reaction pathways are given in Charts 1 and 2, respectively.

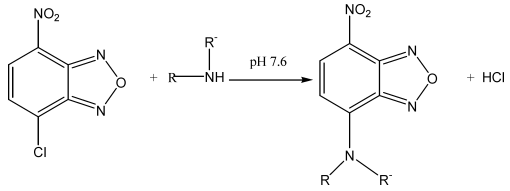

Chart 1. Proposed Reaction Pathway between NBD-Cl and DSL

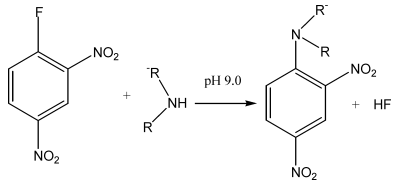

Chart 2. Proposed Reaction Pathway between DNFB and DSL

Conclusion

Four sensitive, simple and time saving methods were developed for the determination of DSL either *per se* or in pharmaceutical preparations. By virtue of the high sensitivity of the proposed fluorometric method, it could be applied to the determination of DSL in spiked and real human plasma with good accuracy and precision. Meanwhile, the proposed methods are highly specific for the determination of DSL in the presence of the parent drug (loratadine).

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