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Spectrophotometric Determination of Levofloxacin in Pharmaceutical Formulations

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A simple, sensitive and accurate spectrophotometric method has been developed for the assay of levofloxacin (LEVO), which is based on the complexation of drug with copper(II) at pH 5.0, using Britton-Robinson (BRT) buffer solution, to produce a green adduct. Developed spectrophotometric method was compared with UV-spectrophotometric method. The absorbances were measured at 581.13 nm and 299.15 nm for proposed method and UV-spectrophotometric method, respectively. The stoichiometric ratio of levofloxacin to Cu(II) ions in the chromophoric complex was also determined to be 1:1 by Job's method. The optimum conditions for Cu(II)-LEVO complex(1:1) were ascertained and a spectrophotometric method was developed for the determination of levofloxacin in the concentration range 8.0-160.0 g mL^{-1}, the detection limit being 2.3 g mL^{-1}. The method was validated for the direct determination of levofloxacin in tablet dosage formulations. The repeatability, reproducibility, precision and accuracy of the method was also investigated. The protonaion constants of the levofloxacin and stability constants of its Cu(II) complexes were also determined by potentiometric titration method in 50 % methanol-water mixtures at 25.00 ± 0.02 ºC under nitrogen atmosphere and ionic strength of 0.10 M sodium chloride. It has been observed that levofloxacin has two protonation constants. It was found that the divalent metal ion Cu(II) was formed CuL, CuL_2, Cu_2L_2 and Cu_2L stable complexes with levofloxacin by potentiometric method.

Key Words: Spectrophotometry, Levofloxacin.

INTRODUCTION

Levofloxacin (LEVO) is a synthetic broad spectrum antibacterial agent for oral and intra venous administration. Chemically, a chiral fluorinated carboxyquinolone, is the pure (-)-(S)-enantiomer of the racemic drug substance ofloxacin[1]. The chemical name is (-)-(S)-9-fluoro-2.3-dihy-dro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxilic acid (Fig. 1).

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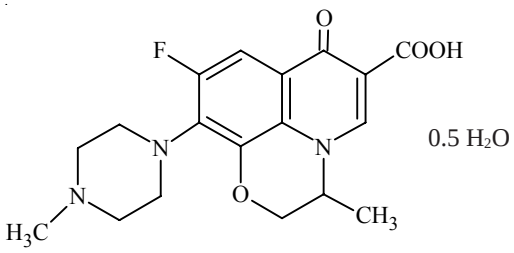

Fig. 1. Chemical structure of levofloxacin

No official (pharmacopoeia) method has been developed for the assay of levofloxacin in its formulations. However, many studies have been reported for the determination of LEVO in pharmaceuticals and biological fluids including synchronization-first-derivative fluorescence spectroscopy[2], colorimetry[3], terbium-sensitized luminescence[4] and capillary electrophoresis[5]. HPLC methods were reported for the determination of LEVO in biological fluids[6-10]. LEVO was determined by voltammetric[11], cyclic voltammetric at glassy carbon electrode[12] and polarographic techniques[13]. Complexation procedures are popular for their sensitivity in the assay of drugs and, therefore, metal complexation spectrophotometry has received considerable attention for the quantitative determination of many pharmaceutical compounds[14-20]. Although complexation reactions are too simple and sensitive, no method for the determination of LEVO has been reported by this procedure. For many years, the use of complexation reactios as an analytical technique was used a lot of areas such as, paints and pigments, textile (mordantion reactions), dye-stuffs and either determination of metals and drugs in a lot of materials (pharmaceuticals, biological samples, natural water samples, alloys and natural tea)[21-28].

The aim of this work was (a) to estabilish a spectrophotometric method with the Cu(II) ion as an analytical reagent without any time-consuming extraction or separation steps prior to drug assay for routine analysis of LEVO from dosage forms; (b) to calculate the stability constant of LEVO-Cu(II) complex by spectrophotometric and potentiometric methods.

EXPERIMENTAL

LEVO and its dosage forms (Cravit film tablets) were kindly provided by FAKO Pharm. Comp. (Istanbul, Turkey). Copper(II) chloride dihydrate, chemicals and solvents were obtained from E. Merck or Carlo Erba. All chemicals used were of AR grade and were used without further purification. Doubly distilled conductivity (Millipore system) water was used as aqueous medium in potentiometric studies and in order to eliminate undesirable ions in spectrophotometric studies.

The electronic spectra were recorded on a Perkin-Elmer (Lamda 45) spectrophotometer in water. Potentiometric titrations were carried out in a thermostated 80 mL glass vessel. It was equipped with a combined pH electrode (Orion Inlab 412 combined glass electrode), nitrogen inlet and outlet tubes, a magnetic stirrer and titrant inlet. The electrode was modified by exchanging its aqueous NaCl solution consisting of 0.10 M NaCl saturated with AgCl. The e.m.f was measured using an Orion 960 automatic titrator. The pH measurement of proton-ligand and metal-ligand systems of levofloxacin were made with containing carbonate-free NaOH at a known (*ca.* 0.10 M) concentration at 25.00 ± 0.02 °C with ionic strength 0.10 M NaCl. Temperature was maintained constant inside the cell at 25.00 ± 0.02 °C, by the circulating water by a Haake thermostatted bath (precision ± 0.02). The potentiometric cell was calibrated before each experiment to obtain -log[H^+] values (pH) for the titration medium. The ion products ($K_w = [H^+][OH^-]$) were calculated at a constant ionic strength of 0.10 M with NaCl in 50 % aqueous methanol solutions based on measurements of [OH^-] and pH in several series of experiments. The standardization of the combined pH electrode was also checked in the alkaline range by addition of excess NaOH. By assuming the E^0 cell value determined in the acidic range to be reliable and the [OH^-] concentration of a base added in excess, we calculated the reproducible values of pK_w for the examined 50 % aqueous methanol solution. The pK_w value obtained is 14.90 in this medium.

Standard solutions for spectrophotometric determination: A stock solution containing 5 mM of LEVO was prepared in water and diluted as appropriate for further readings. This solution would be stable for 1 week if it was kept in the refrigerator. $CuCl_2 \cdot 2H_2O$ solution 5 mM was prepared in water. Britton-Robinson (BRT) buffer solution prepeared from 0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH. The pH adjusted with 5 M NaOH. Standard solutions were prepared by dilution of the stock solution with EtOH to give solutions containing LEVO in the concentration range of 0.37-0.59 mg mL^{-1} for UV-spectrophotometric method. The calibration plot was constructed by plotting the absorbance against the compound concentration. All validation parameters were also calculated for the comparison study.

Calibration curve: For the calibration graph a series of five standard solutions was prepared by dilution of corresponding stock solution to obtain the concentration range of 8.0-160.0 mg mL^{-1} LEVO in EtOH. Add 1 mL BRT buffer and 1 mL of $CuCl_2 \cdot 2H_2O$ solutions. The solution was mixed well and then heated using a thermostatically controlled water bath at 45 ºC for 10 min, then cooled rapidly.

Distilled methanol was used to complete to the mark. The absorbance was measured at 581.13 nm against a reagent blank (Fig. 2). A calibration curve was calibrated and the regression equation was derived.

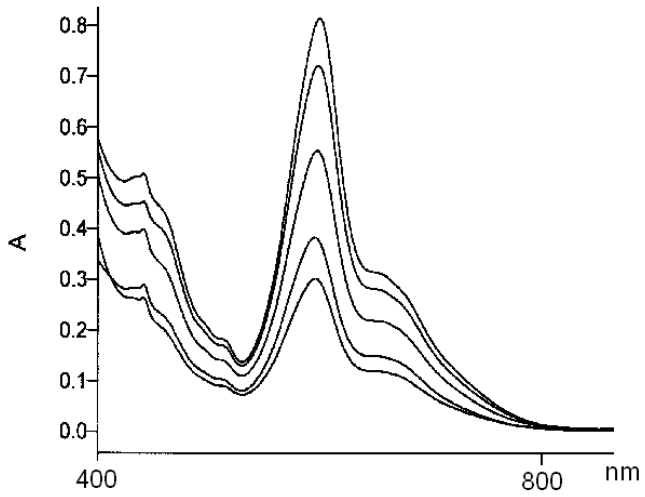

Fig. 2. Absorption spectra of LEVO-Cu(II) complex

Procedure for tablets: A quantity of the powder equivalent to 40 mg LEVO from which 10 tablets were weighed and pulverized was transferred into a small conical flask. Extraction was performed 4 times with 20 mL of water. The solution was filtered into a 100 mL volumetric flask and then completed to the mark with water. Aliquot volumes were transferred into a 10 mL volumetric flask and the procedure for calibration curve was applied as described. The nominal content of the tablet was determined either from the regression equation or using the calibration graph.

Job's method: Job's method[29] was used to determinate, the stoichiometric ratios for the reactions between the LEVO and the copper(II) ion in ethanol. The solution were prepared by mixing solutions of both components with equal molar concentrations (1.0×10^{-4} M) in ratios varying from 1:0 to 9:1.

The stability constant of LEVO-Cu(II) complex was found from the equation as follows:

$$(A–A_0)/A_{ML}–A = K_s[M]$$

where A is the absorbance of the solution at chosen wavelength after addition of given amount of cation at a concentration [M], A_0 is the absorbance of the free ligand at given wavelength, before the addition of the cation, A_{ML} is the absorbance at given wavelength in the presence of an excess of cation such that the ligand is fully complexed[30].

Standard solutions for potentiometric titrations: Stock solution of LEVO was prepared in the purified methanol[31]. Stock solutions of 0.03 M $CuCl_2$ were standardized using an appropriate indicator by EDTA titrations[32]. Sodium hydroxide solutions were prepared as 50 % methanol-water mixture and its concentration and absence of carbonate ions were frequently checked

by means of Gran plots[33] using potassium hydrogen phthalate (Merck) as the acid. 0.10 M acid solutions prepared from Merck p.a. hydrochloric acid were titrated against standardized 0.10 M sodium hydroxide solution[34]. The ionic strength of each solution was adjusted to 0.10 M by the addition of NaCl as supporting electrolyte.

Potentiometric titrations were carried out at constant temperature in an inert atmosphere of nitrogen with CO_2-free standardized 0.10 M NaOH in a 50 mL solution containing 0.10 M NaCl: (i) 2.00×10^{-3} M HCl (for cell calibration); (ii) 3.00×10^{-3} M HCl + 1.50×10^{-3} M levofloxacin (for the protonation constants of levofloxacin); (iii) 3.00×10^{-3} M HCl + 1.50×10^{-3} M levofloxacin + 1.50×10^{-3} M $CuCl_2$ (for the stability constant of the 2:2 M:L complex); (iv) 6.00×10^{-3} M HCl + 3.00×10^{-3} M levofloxacin + 1.50×10^{-3} M $CuCl_2$ (for the stability constant of the 1:2 M:L complex).

Data processing: The protonation and stability constants of levofloxacin were evaluated by iterative non-linear least squares fit of potentiometric equilibrium curves through mass balance equations for all the components expressed in term of known and unknown equilibrium constants using a computer program BEST[35]. All the models converged at $r < 0.03$ pH units of the observed pH values, which is considered to be an acceptable fit. The equilibrium constants reported in this paper were obtained as averaged values of three titrations. Selection of the equilibrium models was based on critical evaluation of the least squares fitting results, namely analysis of the statistical parameters.

RESULTS AND DISCUSSION

Copper(II) is a labelling reagent for primary and secondary amine or several acide groups. Several pharmaceutical compounds have been determined through this approach, such as, atenolol, acebutolol, propranolol and furosemide[14,15].

LEVO has carboxilic acid group, that was found to react with Cu(II) with the formation of the mononuclear complex resulting in green adduct. Under the described experimental conditions, the green adduct has a characteristic absorption spectrum with maximum absorption at 581.13 nm as shown in Fig. 2.

The different experimental parameters affecting the produced colour were extensively studied in order to determine the optimal conditions for the determination of the drug.

Effect of pH: Firsly, the influence of pH on the absorption was studied. The maximum absorption occured at aproximately pH 5.0 using BRT buffer (Fig. 3). Other buffers having the same pH value such as carbonate or phosphate buffers were studied and compared with BRT buffer which proved to be superior over carbonate and phosphate buffers because the absorbance readings were higher. These results are in agreement with that of Miyano *et al.*[36].

Effect of temperature: The effect of temperature on the produced adduct was studied and found that heating at 40 ºC for 10 min was better than heating at a higher temperature for a shorter period (Fig. 4).

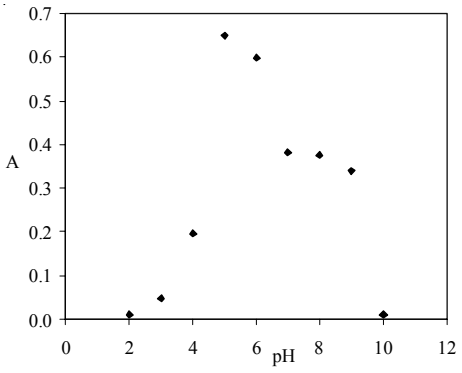

Fig. 3. Effect of pH on the development of the complex of LEVO with Cu(II)

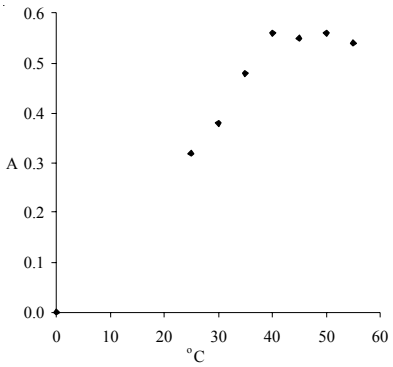

Fig. 4. Effect of temperature on the development of the complex of LEVO with Cu(II)

Effect of reagent concentration: The most important factor affecting the formed green adduct was the volume of Cu^{2+}. Fig. 5 is shown that 1 mL of 5 mM Cu^{2+} solution give maximum sensitivity. Increasing the volume of Cu^{2+} leads to decrease in the absorbance, this may be due to the high background absorbance of the reagent. The absorption of the hydrolysis product of Cu^{2+}, namely $Cu(OH)_2$, completely disappeared at pH less than 4. Therefore, acidification of the reaction solution prior to the measurement remarkably decreased the background absorbancy without affecting the drug-metal adduct, hence, the sensitivity of the procedure was increased.

Stoichiometric relationship: The complex formation was studied at the same concentration of LEVO (1×10^{-4} mol L^{-1}) and in a metal ion [Cu(II)] concentration (6×10^{-3} mol L^{-1}). A low and unchanged concentration of the electroneutral organic ligand allowed us to exclude from consideration the theoretically possible formation of biligand complex (according to the equilibrium or $2L + 2M = M_2L_2$), whose detection, in present experimental conditions, requires the complex formation constant to be higher than 10^7 mol L^{-1}.

The stoichiometry ratio of the metal-ligand complexes of compound LEVO was obtained from the continuous variation method[37] and potentiometric studies. Job's plot for LEVO-Cu(II)) complex is presented on Fig. 6. It was found from the Job's plots for LEVO-Cu(II)) complex that $X_{max} = 1/2$ for this complex, hence, the stoichiometry of the metal-ligand complex of LEVO was found to be 1:1 at used concentration of the ligand (1×10^{-4} mol L^{-1}). The log β_{ML} was found to be 7.59 ± 0.02.

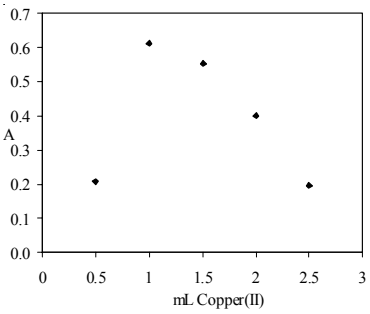

Fig. 5. Effect of volume of Cu(II) on the development of the complex of LEVO with Cu(II)

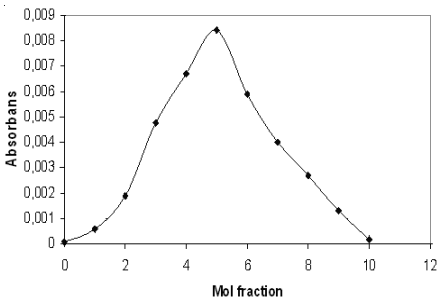

Fig. 6. Continuous variation plot for the stoichiometry of the reaction of LEVO and Cu(II)

Validation of the methods: The possibility to use complex formation between Cu(II) ions and LEVO for quantitative determination of LEVO in pharmaceutical formulations was tested for specificity, linearity, LOD/LOQ values, repeatability, accuracy (recovery), stability and ruggedness. The specificity of the method was checked by observing if there was any interference of the tablet excipient in the LEVO-Cu(II) complex formation. Spectrophotometric measurements showed that placebo sample did not have any absorption under described experimental conditions. However, as a non-separative method it is not specific in relation to degradation products/ LEVO related compounds and impurities, hence it cannot be used as a stability-indicating method. Beer's law was verified in the entire investigated acidity range.

A linear relationship between the absorbance and the concentration of LEVO was obtained over the concentration range 8.0-160.0 μg mL^{-1}. For example, in water solution the regression equation was y = 0.003701x-0.219549 with correlation coefficient (r) of 0.9994, indicating good linearity. The limit of detection (LOD) was 2.3 μg mL^{-1} of LEVO defined as the concentration that gives rise to a signal that is three times the noise of the method. Limit of quantitation (LOQ) was 6.1 μg mL^{-1}, accepted to be ten times the noise signal. The precision of the proposed spectrophotometric method was accessed by analyzing laboratory mixtures (LEVO and excipient) and LEVO tablets containing known quantity of drug. The results are presented in Table-1, as the mean value of ten determinations. It is evident that developed method is of satisfactory repeatability, since the relative standard deviations (RSD) were 0.83 and 0.66 % for laboratory mixture and LEVO tablets, respectively.

The results of the recovery of LEVO from laboratory mixtures are also presented in Table-1. The recovery values varied from 97.75 to 100.12 % indicating that the developed method is quite efficient. Dissolution test results

TABLE-1
REGRESSION DATA OF THE CALIBRATION LINES FOR QUANTITATIVE DETERMINATION OF LEVO BY COMPLEXATION AND SPECTROPHOTOMETRIC METHOD

	Complexation method	Spectrophotometric method
Absorbance (nm)	581.13	299.15
Linearity range (μg mL^{-1})	8.0-160.0	0.37-0.59
Slope	0.003701	0.93
Intercept	0.219549	0.16
Correlation coefficient (r)	0.9994	0.9985
SE of slope	0.1210	2.54×10^{2}
SE of intercept	1.30×10^{-2}	7.82×10^{-3}
LOD (μg mL^{-1})	2.3	0.062
LOQ (μg mL^{-1})	6.1	0.113
Repeatability of absorbance (RSD %)	1.13×10^{-4}	0.87
Repeatability of peak wavelength (RSD %)	1.12	1.32
Reproducibility of absorbance (RSD %)	1.85×10^{-2}	1.09
Reproducibility of peak wavelength (RSD %)	1.71×10^{-3}	0.73

were within permitted and declared limits (minimun 80 %) for LEVO-Cu(II) system. The stability of the complex was confirmed by measuring the absorbance eight times within 4 h with the RSD = 2.55 %. The ruggedness of the method was studied by measuring the absorbance for different time and analytical wavelengths were found as 580-619.8 nm. It was concluded that the sensitivity of the method increased with the decrease of long time and the decrease in wavelength.

LEVO pharmaceutical dosage forms were also determined with the UV-spectrophotometric method, which is proposed for the comparison with complexation techniques. All validation parameters were found for the UV-spectrophotometric method (Table-2). The results obtained for the pharmaceutical dosage forms were also listed in Table-2. Recovery experiments were also realized for the UV-spectrophotometric method. Table-2 compares the results of the analysis of LEVO between the complexation and UV-spectrophotometric methods.

All methods showed similar accuracy and precision. According to the student's t- and F test, the calculated t and F values did not exceed the theoretical value for a significance level of 0.05 statistical analysis of the results showed no significant difference between the performance of the compare UV-spectrophotometric. Complexation assays are very rapid, used without any filtration steps and have better accuracy, precision, linearity range and determination limits than UV-spectrophotometric assay.

TABLE-2
COMPARISON THE RESULTS OF THE ANALYSIS OF LEVO BETWEEN THE COMPLEXATION AND UV-SPECTROPHOTOMETRIC METHODS

Sample (n = 10)	LEVO Tablets		Laboratory mixture	
	CM	SM	CM	SM
Labeled claim	500.00	500.00	500.00	500.00
Amount found[a]	497.06	494.44	500.05	500.27
RSD (%)	0.66	0.413	0.83	0.68
Bias (%)	0.732	1.391	-0.05	-0.27
T_{value}	$t_{calcd.}$: 0.06	$t_{theoretical}$: 2.31	$t_{calcd.}$: 0.33	$t_{theoretical}$: 2.31
F_{value}	$F_{calcd.}$: 0.37	$F_{theoretical}$: 2.60	$F_{calcd.}$: 0.71	$F_{theoretical}$: 2.60
Added (mg)	20.00	20.00	20.00	20.00
Found (mg)[b]	19.38	20.08	20.09	20.01
Recovery (%)	100.12	100.08	97.75	100.51
RSD (%) of recovery	1.92	0.51	1.95	0.276
Bias (%)	0.25	-0.39	0.25	-0.1
Dissolution (%)	83.94	-	84.54	-

CM = Complexation method; SM = Spectrophotometric method
[a]LEVO + excipient; [b]n = 10.

Potentiometric studies

Protonation constants of LEVO: The methanol-water 50:50 % (v/v) was the chosen solvent for present study. In such a medium, the studied LEVO and its Cu(II) complexes are soluble giving stable solutions. The stoichiometric protonation constants of the investigated LEVO were determined in 50 % methanol-water mixture at 25.00 ± 0.02 ºC and these constants, log K_1 and log K_2 are 7.92 ± 0.03 and 7.00 ± 0.02, respectively (Fig. 7). When it was investigated literature value of protonation constants of LEVO, it was seen that the present protonation constants are similar to literature

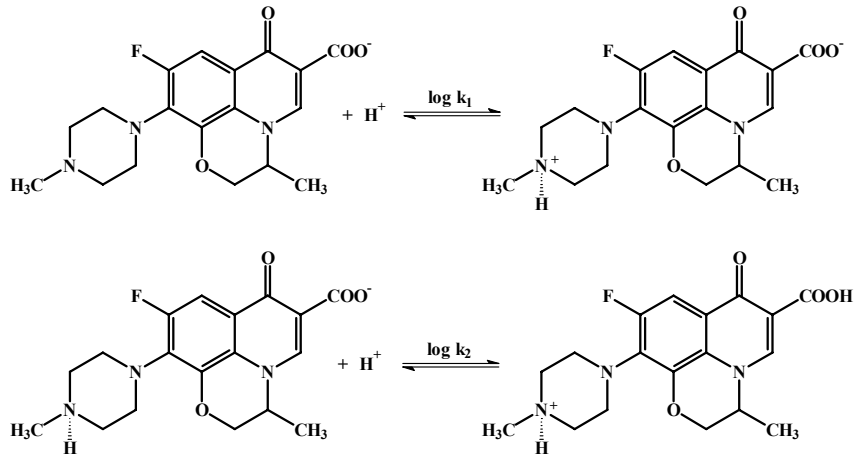

Fig. 7. log K_1 and log K_2 equilibrium reactions of LEVO

value. For example, Ross *et al.*[38] found that the value of protonation constant of LEVO in aqua media as 6.05 and 14.27. However in literature protonation constants of LEVO could not be founded in methanol-water mixture. Therefore, the protonation constant calculated in this study may significantly contribute to the literature.

As the titration curve of LEVO in Fig. 8, which is drawn on LEVO, it can be seen that there is one end-point at a = 2 due to overlap equilibrium. According to the results obtained from this titration curve it can be concluded that the LEVO studied here have two protonation constants. This is also illustrated in the species distribution of the LEVO ligand in Fig. 9. At pH < 6, the LEVO exists in the fully protonated form H_2L. As the pH is increased, the LEVO loses its proton to become HL, which is the predominant species in pH range of 6-9. Under more alkaline conditions the protonated LEVO is transformed to the free ligand L (pH > 9).

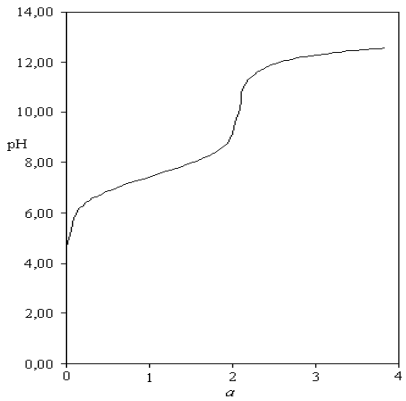

Fig. 8. Potentiometric titration curves for LEVO(L) as a function of added NaOH (a = Moles of base added per mole of ligand)

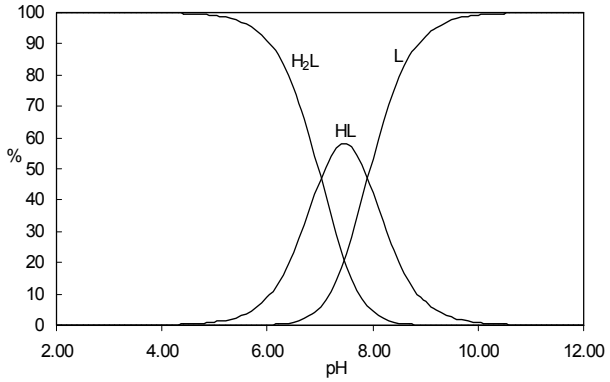

Fig. 9. Species distribution diagram for the systems LEVO (L) as a function of pH. μ = 0.10 mol L^{-1} NaCl, t = 25.00 ± 0.02 ºC, TL = 1.50 × 10^{-3} mol L^{-1}, % = Percentage concentration of species

Stability constants of LEVO-Cu(II)complexes: The potentiometric titration curves of the LEVO with equivalents of LEVO to metal ion for Cu(II) are shown in Fig. 10. The metal ion Cu(II) depresses the titration curve of the free ligand by the release of protons according to the abilities of the metal ions to bind to the LEVO. As the titration curves of the complexes formed by copper with the LEVO are examined, two inflection points can be observed approximate at a = 2 and a = 4 for the LEVO. This inflection points can be explained by the formation of complexes of CuL and CuL_2. The stoichiometric stability constants of Cu(II) complexes of LEVO were determined in 50 % methanol-water mixture at 25.00 ± 0.02 ºC and these constants are tabulated in Table-3. From Table-3, it was shown that the stability constants of LEVO-Cu(II) complex was 7.19 ± 0.02. This value was in agreement with the value which was determined by spectrophotometric method.

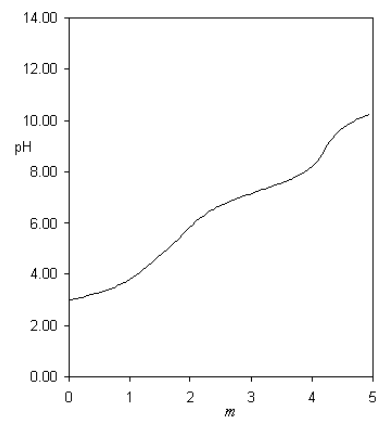

Fig. 10. Potentiometric titration curves for the LEVO and 1:2 stoichiometries of Cu(II) to the LEVO as a function of added NaOH. (m = Moles of base added per mole of metal ion present)

TABLE-3
STABILITY CONSTANTS OF LEVO-Cu(II) COMPLEXES IN 50 % METHANOL-WATER MIXTURE (μ = 0.10 M NaCl, t = 25.00 ± 0.02 ºC)

$L + Cu^{2+}$	⇌	CuL	$\log \beta_{ML} = 7.19 \pm 0.02$
$2L + Cu^{2+}$	⇌	CuL_2	$\log \beta_{ML_2} = 10.65 \pm 0.02$
$2L + 2Cu^{2+}$	⇌	Cu_2L_2	$\log \beta_{M_2L_2} = 15.10 \pm 0.04$
$L + 2Cu^{2+}$	⇌	Cu_2L	$\log \beta_{M_2L} = 4.33 \pm 0.03$

In order to investigate change with pH in the concentration of the complexes, which LEVO formed with Cu(II), the stability constant values are evaluated, using SPE computer program[33] and the species distribution curves are drawn. In Fig. 11, if the distribution diagram for LEVO-Cu(II) system is examined

it has been observed that the complex form with CuL is dominant in between pH = 4-10. This species forms *ca.* 50 % at pH = 5.0. CuL_2 complex forms between pH 6-10. This CuL_2 complex forms 30 % at pH = 7.

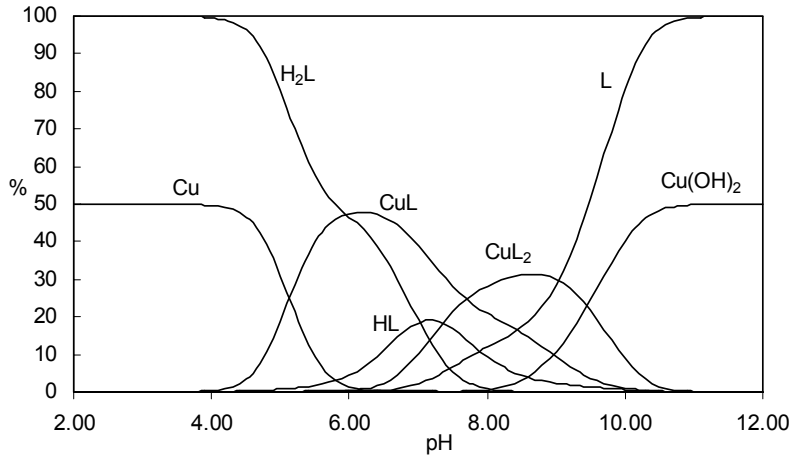

Fig. 11. Species distribution diagram for the systems Cu-LEVO (L) in 1:2 molar ratio as a function of pH. m = 0.10 mol L^{-1} NaCl, t = 25.00 ± 0.02 ºC, TL = 3.0×10^{-3} mol L^{-1}, TCu = 1.50×10^{-3} mol L^{-1}, % = Percentage concentration of species

Conclusion

This method is applied for the routine quality control analysis of LEVO in pharmaceutical formulations. The proposed method does not require any laborious clean up procedure prior to analysis and therefore, it can be frequently used in the laboratories of research, hospitals and pharmaceutical industries. It has extremely high sensitivity, selectivity and low limit of detection.

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