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Development and Validation of an HPLC Method for the Determination of the macrolide antibiotic Clarithromycin using Evaporative Light Scattering Detector in raw materials and Pharmaceutical Formulations

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Abstract: In this work, ELS-Detector has been used for the development of an HPLC method for the determination of clarithromycin in pharmaceutical formulations (tablets and pediatric suspension). Isocratic reversed phase HPLC approach has been developed using a C-18 column (Waters Spherisorb 5 μm ODS2, 4.6x250 mm) and a mobile phase consisting of acetonitrile / aqueous trifluoroacetic acid as pairing reagent. Experimental parameters (temperature of heated drift tube, flow rate of mobile phase, gas flow rate, mobile phase composition) were optimized. Clarithromycin' s stability was thoroughly examined in different solvent systems. Using the optimized conditions the working range was 5-100 μg/mL (upper limit can be increased considerably), with a detection limit of 4.5 μg/mL ($6x10^{-6}$ M). The method was validated as per ICH guidelines. The retention time was 4.7 min. The method was successfully applied for the content assay of clarithromycin formulations.

Keywords*:* Clarithromycin; Reversed-phase HPLC; Evaporative Light Scattering Detection (ELSD), pharmaceutical formulations.

Introduction

The past twenty years, Evaporative Light Scattering Detection (ELSD) has moved into a detection of choice for a number of HPLC applications, as it offers freedom from some of the limitations of spectroscopic detection. ELSD is based on the universal ability of particles to cause photon scattering when they traverse the path of a polychromatic beam of light, as the chromatographic eluate is nebulized with an inert gas and evaporated into a heated drift tube. ELSD was first demonstrated in 1996 by Ford and Kennard to have a nearly linear mass dependent response irrespective of chemical composition. Sensitivity in ELSD is independent of the analyte's spectral properties and the signal (peak area A) is related to the absolute quantity (m) of the analyte through a logarithmic relationship (logA=blogm + loga). It is compatible with a much wider range of solvents and modifiers and produces a stable baseline even in gradient elution [1-6].

Macrolide antibiotics have been used for the treatment of bacterial infections caused by Gram-positive organismus [7]. Clarithromycin is a relatively new semisynthetic macrolide antibiotic derived from erythromycin A and consists of a 14-membered lactone ring with sugars linked via glycosidic bonds. (Fig. 1) As all macrolides, clarithromycin displays similar antibacterial properties and constitutes an important alternative for patients with penicillin sensitivity and allergy. Moreover, clarithromycin is more stable to the acid environment of the stomach and exhibits better bioavailability and pharmacokinetic behavior.

Several methods have been introduced for the determination of clarithromycin in pharmaceutical samples. These methods are based either on the derivatization of the molecule before the chromatographic column due to its low absorptivity or on the ion-pair formation followed by electrochemical detection with amberometric or coulometric detection [9-15]. There also references using spectrochemical methods [16,17], gas chromatography, microbiological methods, thin layer chromatography (TLC), mass spectrometry [18, 19], capillary electrophoresis [20], HPLC [21,22] and electrophoresis [23].

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Most of these methods are time-consuming because of the analyte's pretreatment and also of lower sensitivity. An evaporative light scattering detection although of lower sensitivity compared to MS detection, is still a detector of choice for novel antibiotics that cannot be detected with a UV detector, due to its much lower price and to its compatibility to HPLC systems as shown in a number of determinations [24-27]. A key feature of ELSD is that operates also in gradient mode and can also be used to set up MS-compatible methods, as the mobile phase constraints are similar [28-29].

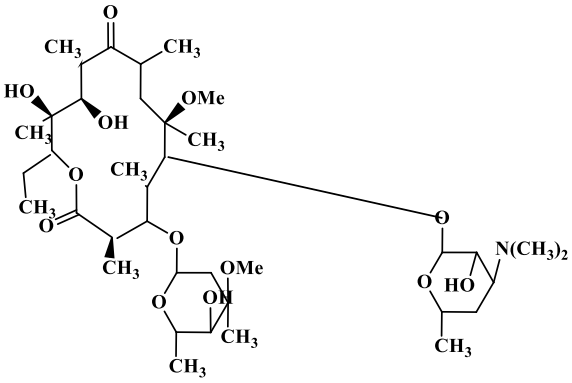

Figure 1. Structure of clarithromycin

The aim of the present work was to propose a rapid, simple and sensitive method for the determination of clarithromycin in raw materials and pharmaceutical formulations and to investigate the ability of ELSD to perform quantitation with no need of derivatization of non-absorbing analytes. The sample pretreatment was simple and fast, the analyte was well separated from all its excipients and the chromatographic characteristics of the method meet all the European Pharmacopeia requirements. The developed method was also applied in order to test the solubility and stability of the analyte in different solvent and solvent systems and conclusions for the preparation and handling of the analyte solutions were made. To the best of our knowledge the use of ELSD in the determination of pharmaceutical formulations of clarithromycin is not widely explored.

Experimental

Instrumentation

The chromatograph used consisted of a Shimadzu solvent delivery module LC-10AD (controller, pump, injector equipped with a 20 μL sample loop) and a SEDERE - ELS Detector (SEDEX MODEL 75). Nitrogen gas was used for the nebulization and evaporation of mobile phase. The above system was controlled by a Shimadzu CLASS-VP Chromatography Data System, running on a personal computer. A Waters C18 column (3.9 × 150 mm), packed with silica, 4 μm particle size was used. A filtration system (Millipore, Bedford, M.A USA) with type HVLP Millipore filters (diameter 47 mm, pore size 0.45 μm) was used for degassing the mobile phase under vacuum, while helium was purged through mobile phase. Water of HPLC grade was produced by Milli-Q® Plus Reagent Grade Water Purification System. For pH measurements, a pH Meter 3310 Jenway was used. A sonicator bath (Metason 60, Struers) was used for assisting mixing and dissolution.

Reagents and standards

All chemicals were of analytical grade except otherwise stated. Acetonitrile (ACN), 99.9% was of HPLC grade (LabScan), Formic acid (HCOOH) 98% was obtained from Merck, Trifluoroacetic acid (TFA) was from Fluka Chemica, Acetic Acid ACS-ISO was from Panreac. Methylene chloride 99.9 % HPLC grade (Labscan), propanol 99.5 % was from Merck, diethylether 99.5%, hexane 95% and ethyl acetate were all from Labscan.

Water of HPLC grade was used throughout the experimental section where water is mentioned. The analysed pharmaceutical formulations were obtained from commercial sources. Clarithromycin reference standard (98% purity) was purchased from Sigma Aldrich.

Stock solution of pure clarithromycin was prepared by dissolving 0.050 g of clarithromycin in acetonitrile (ACN), the dissolution being assisted by sonication. The concentration of the stock solution was 500 mg/L and was stored in the refrigerator. Working standard solutions were prepared daily from the stock solution by appropriate dilution with ACN in the range of 5-100 mg/L. The optimized mobile phase consisted of ACN/formic acid (HCOOH)/water (H_2O)/trifluoroacetic acid (TFA) (70/15/14.9/0.1 (% v/v)) and was prepared by manual mixing just before use.

Pharmaceutical formulations preparation

The formulations examined were tablets 250/500 mg and pediatric suspension 250 mg/5 ml.
Four tablets of clarithromycin were weighed and powdered. A proper amount of this powder, containing 0.0625 g of clarithromycin, was taken and dissolved in 100 mL (methanol) MeOH slightly acidified with TFA and then diluted to final volume of 250mL with acetonitrile by assisting with mechanical shaking for at least 40 min. Any insoluble matter was allowed to settle. The use of methanol in pharmaceutical formulations gave better solubility results and the TFA presence assisted the tablets powder dissolution. The supernatant liquid was filtered and by proper dilution in ACN working solutions were prepared. For the 125 mg/5 mL suspension, a weighed portion of the granules equivalent to 0.0625 g of clarithromycin, was transferred to a 250-mL volumetric flask and the preparation of working solutions was the same as described for tablets without the presence of TFA.

Analytical Procedure

The chromatographic elution was performed at room temperature (25 ºC) in isocratic mode at 1.0 mL/min flow rate. Gas flow was set at 3.4 mL/min. Sample working solutions were filtered through a 0.45 μm membrane filter before usage. Flow path was rinsed for about 15 min, until baseline noise became stable.

Results and Discussion

Choice of stationary, optimization of mobile phase and ELSD parameters

A non-polar C-18 analytical chromatographic column was chosen as the stationary phase for the separation / determination of a molecule, such as clarithromycin in the form of ion-pair. The 4 μm particle size provides well separated narrow peaks.

An intial study of the influence of mobile composition to ELSD response took place through Flow Injection Analysis Systems which showed Enhanced ELSD response with the increase of the mobile phase volatility and ionic species presence forming ion pair with the analyte and therefore increase of the mass of the detected particle.

For the mobile phase optimisation, a solvent system suitable for the proper extraction of the analyte and also of good response to the ELS detector should be chosen. Therefore, a number of elution systems of solvents were examined. ACN, as the solvent of choice for clarithromycin dissolution and of good ELSD response was also a component of mobile phase solvent mixture. Clarithromycin has poor solubility in usual organic solvents and that was a further reason for the presence of ACN in mobile phase[30]. Binary mixtures of ACN/ H_2O at any ratio resulted in insufficient elution of clarithromycin due to its chemical affinity to the stationary phase. The need for acidification of the extraction solvent has been also demonstrated in literature [31], as the solubility of the analyte seems to be better in lower pH. For this reason, the ion-pair approach using TFA was chosen in order to provide lipophilicity to the analyte. TFA has sufficient volatility and it is suitable for detectors such as ELSD. However, the elution of clarithromycin was still insufficient, probably because of the low concentration of TFA, which was unable for both acidification and ion-pairing. So, formic acid and acetic acid were added to the mobile phase as an ion pair reagent. Formic acid provided a rather stable baseline and a better elution of the analyte compared to acetic acid. Different ratios of the solvents ACN, water, TFA and formic acid showed that the presence of ACN in the range of 65-70 % v/v and an aqueous phase of 30% v/v provide better elution of the analyte and symmetry and baseline noise. The mobile phases examined and their characteristics as far as the elution of the analyte is concerned are depicted in Table 1. The optimized mobile phase consisted of ACN/ HCOOH) / water/ TFA (70/15/14.9/0.1 (% v/v)) and was prepared by manual mixing just before use.

Applying the optimized mobile phase with univariate optimization the evaporation temperature was selected to be 50º C. The evaporation temperatures examined were 40, 45, 50, 55 º C and the obtained peak area of clarithromycin solution of 75 mg/L was examined. Increased peak area was gained at 50º C.

At a second step, the influence of the pressure of carrier gas was optimized through changes of the pressure of carrier gas at 2.5, 3.0 and 3.5 bar with better results at pressure 3.0 bar giving better peak area and symmetry factor.

Table 1. Tested mobile phases and their influence into the elution of the analyte.

Tested mobile phases	***Composition of mobile phases (%v/v)***	***Comments***
ACN	100	Not eluted
MeOH	100	Not eluted
MeOH/ ACN	50/50	Not eluted
MeOH/H_2O	50/50	Not eluted
ACN/ H_2O	80/20	Deficient elution of the analyte, high symmetry factor
ACN/ H_2O	70/30	Deficient elution of the analyte, high symmetry factor
ACN/ H_2O	50/50	Deficient elution of the analyte, high symmetry factor
ACN/ H_2O (pH adjusted to 3,5 with TFA)	70/30	Slow elution
ACN/ H_2O (pH adjusted to 4,0 με TFA)	60/40	Slow elution
ACN/ $HCOOH_{(aq)}$ (c=0,05 mM) (pH adjusted to 4,0 with NH_3)	60/40	Better elution, Chromatographic peak symmetry improvement
ACN/$CH_3COONH_{4(aq)}$ (5%w/v)	70/30	Increased background signal (low volatility of CH_3COONH_4 to ELSD)
ACN/$CH_3COONH_{4(aq)}$ (20 mM)	50/50	Increased background signal
ACN/$CH_3COONH_{4(aq)}$ (20 mM)	80/20	Increased background signal

ACN/$NH_{3\,(aq)}$(1%v/v)	70/30	Deficient and slow elution
H_2O/$CH_3COONH_{4(aq)}$ (20 mM)	50/50	Deficient elution, high symmetry factor
ACN/$CH_3COONH_{4\,(aq)}$ (20 mM)$CH_3COOH_{(aq)}$ (17,5 mM)	70/20/10	Deficient elution, high symmetry factor
MeOH/ACN/ $HCOOH_{(aq)}$ (c=0,05 mM) (pH adjusted to 5,5 με NH_3)	30/30/40	Deficient elution, high symmetry factor
ACN/$TFA_{(aq)}$(32,39 mM)	80/20	Increased background signal
ACN/$TFA_{(aq)}$(64,77 mM)	80/20	Increased baseline noise, faster elution
ACN/ $TFA_{(aq)}$(43,17 mM)	70/30	Better sensitivity (lower background signal)
ACN/ $TFA_{(aq)}$(129,2 mM)	90/10	Increased background signal
ACN/$TCA_{(aq)}$ (7,44 mM) /$TFA_{(aq)}$(129,2 mM)	80/10/10	Unstable baseline
ACN/$TCA_{(aq)}$ (12,32 mM) /$TFA_{aq)}$(82,88 mM)	75/12,5/12,5	Unstable baseline
ACN /HCOOH / $TFA_{(aq)}$(43,16 mM)	60/10/30	Good elution and symmetry
ACN/H_2O/HCOOH / $TFA_{(aq)}$(51,8 mM)	55/20/25	Good elution and symmetry
ACN/CH_3COOH / $TFA_{(aq)}$ (51,8 mM)	55/20/25	Good elution, high symmetry factor
ACN/HCOOH / $TFA_{(aq)}$(43,16mM)	55/15/30	Good elution and symmetry
ACN/H_2O/HCOOH/CH_3COOH/ $TFA_{(aq)}$ (32,3 mM)	45/10/5/40	Slow elution
ACN /HCOOH/ $TFA_{(aq)}$(43,16 mM)	70/10/30	Fast elution, good symmetry
ACN/HCOOH/ $TFA_{(aq)}$(86,13 mM)	**70/15/15**	**Better chromatographic characteristics / mobile phase of choice**

ACN: acetonitrile, MeOH: methanol, TFA: trifluoroacetic acid, TCA: trichloroacetic acid

Chromatographic characteristics

Using the selected column and mobile phase the obtained retention time (t_R) of clarithromycin was 4,5 min, which was used for the identification of clarithromycin in pharmaceutical formulation. Resolution factor (Rs) of clarithromycin to the other formulation substances was sufficient. The substances studied, were vanillin, sucrose, sorbic acid and citric acid. In every case resolution factor was more than 4.5 (eq 1). The symmetry factor (A_f) of clarithromycin chromatographic peak ranged from 1.25 to 1.65 (eq.2). Representative chromatogram of clarithromycin in mobile phase is shown in Fig. 2.

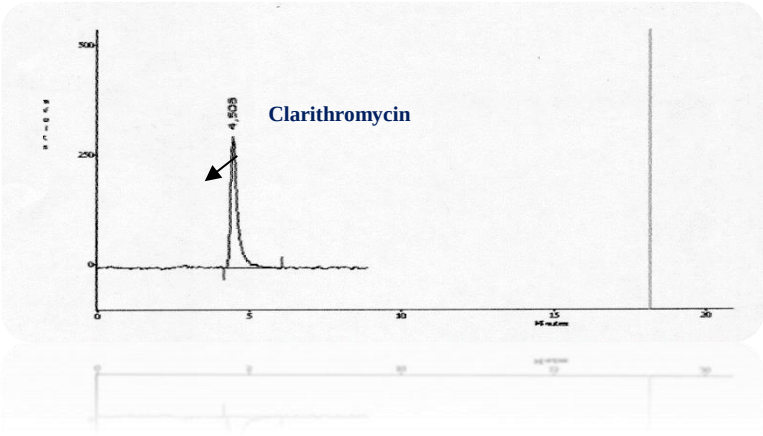

Figure 2. Typical chromatogram of standard solution of clarithromycin (50μg/mL) in ACN. Retention time is 4,508 min.

The equations used for the calculation of resolution factor and symmetry factor are [32]:

$$\text{Resolution } R_s = 1{,}18\,\frac{t_{R,B} - t_{R,A}}{(wh_A + wh_B)} \quad \text{(eq. 1)}$$

$$\text{Symmetry factor } A_{s,f} = \frac{w_{0,05}}{2d} \quad \text{(eq.2)}$$

Where t_R retention times or distances along the baseline from the point of injection to the

perpendiculars dropped from the maxima of 2 adjacent peaks, wh_A and wh_B: peak widths at half-height., $w_{0,05}$: width of the peak at one-twentieth of the peak height, d: distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

A resolution of greater than 1.5 corresponds to baseline separation and a value of symmetry factor of 1.0 signifies complete (ideal) symmetry

Solubility of clarithromycin solutions

Taking into account the poor solubility of clarithromycin in the most organic solvents, a study of the solubility of clarithromycin in different solvents was performed. The solvents used were methylene chloride, propanol, ether, hexane, acetonitrile, ethyl acetate, methanol and aqueous formic acid. Saturated solutions of clarithromycin were prepared at 25ºC and then in proper dilutions injected to chromatographic system. For the concentration calculation, a calibration curve at the optimum chromatographic conditions described in 3.1 was used. From the obtained results clarithromycin showed an excellent solubility in acidic environment, but these solutions were rather unstable. Methylene chloride, on the other hand, was unsuitable due to the formation of emulsions in the aqueous environment, despite the good solubility of the analyte. In propanol and ether, the calculated solubility in our experimental and chromatographic conditions was 10.2 and 7.8 g/L respectively, while in methanol there was a slightly higher solubility. ACN was chosen due to the sufficient solubility of clarithromycin (12.7 g/L) and its good behavior with ELSD detectors. A slight amount of methanol acidified with TFA was used for clarithromycin pharmaceutical tablets dissolution before the addition of ACN since it gave better chromatographic results.

Stability of clarithromycin solutions

Following the solubility experiments then the stability of clarithromycin solutions in different solvent systems was examined measuring the peak area of the analyte from 0-150 min. Clarithromycin solutions in ACN seem to be stable in the experimental conditions at room temperature. However, clarithromycin solutions in mobile phase or in other acid environment are rather unstable. The precense of TFA and formic acid in working solution causes a dramatic decrease of the peak area of clarithromycin of about 60 % in an hour. (Fig. 3). Additionally, the storage of the stock solution in the refrigerator at -20º C in ACN seemed to have no influence in the stability of the working solutions.

For that reason the working solutions used in our experiments were prepared daily in acetonitrile and not in mobile phase, just before their injection to the chromatographic system.The ACN solutions of clarithromycin were stable for at least 5 hours in room temperature.

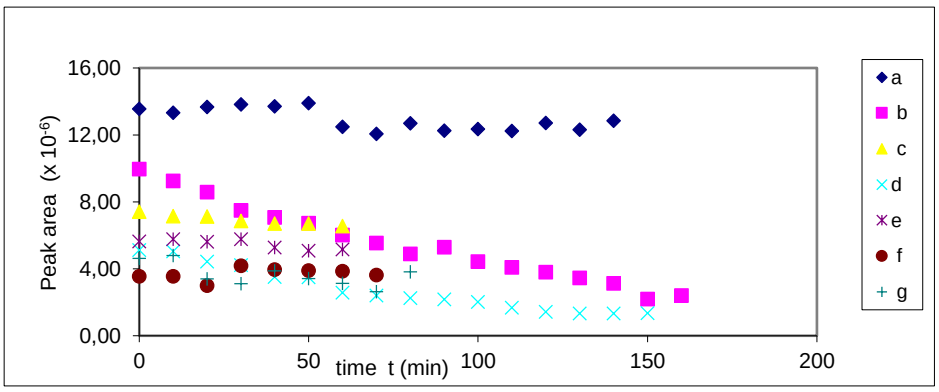

Figure 3. Stability of standard clarithromycin solution of 75mg/L in the following solvent systems (%v/v): **a.** ACN:100, **b**. ACN/TFA: 99.9/0.1, **c.** ACN/H2O/TFA:70/29.9/0.1 **d.** Mobile phase **e.** ACN/H2O/HCOOH: 70/25/5 **f.** ACN/H2O/HCOOH: 70/20/10 g. ACN/H2O/TFA: 65/34.9/0.1

Method validation

The method was validated for the following parameters selectivity, range and linearity, accuracy and precision, robustness, limit of detection and quantitation [33,34].

Selectivity and Placebo Interference

Specificity of the method is important to check interference of excipients and dissolution medium on the response of the drug substance. A composite solution of placebo was prepared from all excipients of tablets without the active ingredient in the same solvent system. This solution was analyzed using the same chromatographic conditions. Placebo interference was also ensured by spiking the reference solution with appropriate levels of excipients and evaluating for any interference or additional peak other than the usual chromatographic peak of the analyte.

Clarithromycin standard solutions of 50 μg/mL were spiked with 50,100 and 200 μg/mL of the common excipients (sucrose, citric acid, sorbic acid and vanillin) and at each concentration level there was no interference caused from the excipients. Sucrose was eluted at 2,63 min, citric acid at 2,24 min, while sorbic acid and vanillin were not detected at the

chromatographic conditions of the method as depicted in Figure 4. Resolution values were found over 4.5(Rf>4,5). An indicative chromatogram of pharmaceutical formulation is depicted in Figure 5.

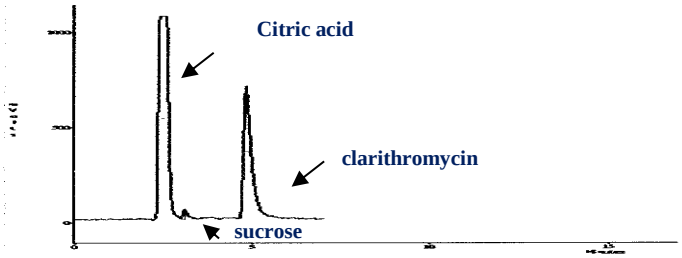

Figure 4. Chromatogram of standard solution of clarithromycin (50μg/ mL, t_R=4,89min), sucrose (100μg/mL, t_R=2,63min), citric acid (100μg/mL, t_R=2,24min), sorbic acid (100μg/mL) and vanillin (100μg/mL) (no peaks detected).

Linearity

Calibration graphs were constructed using the external standard approach following linear regression analysis. Under the experimental conditions described above, linear calibration curves were obtained for clarithromycin standard solutions throughout the range of concentrations studied (5-100 mg/L) as shown in Table 2.

Table 2. Calibration curve characteristics of clarithromycin standard solutions.

Form	Range (mg/L)	Intercept	Slope	Standard deviation of intercept $S_{y/x}$	Correlation coefficient	Replicates at each concentration level (N)
Standard	5-100	4.523	1.201	0.071	0.996	4

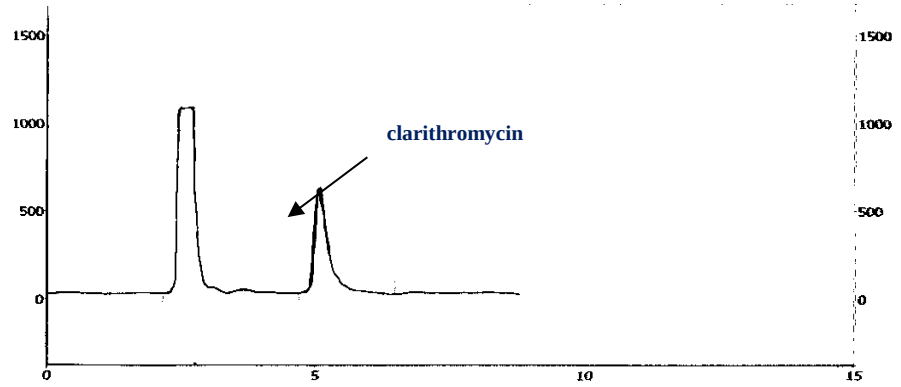

Figure 5. Typical chromatogram from recovery experiment of pharmaceutical formulation granules (125 mg/5mL). Nominal concentration of clarithromycin is 75μg / mL and retention time 5,06 min.

Robustness

The results showed that minor changes of about 2 % of the given values in chromatographic conditions (flow rate, percentage of ACN in the mobile phase, evaporation temperature of ±5° C) do not influence the analyte peak area determination. The value of % RSD for replicates (N = 3) at each concentration (i.e., 25 and 75 mg/L) and the precision calculated at the slightly altered experimental conditions (%RSD less than 1.5%) indicate that the method is robust and suitable for routine analysis assay and dissolution of clarithromycin.

Precision

The intra –day precision of the method was evaluated by replicate measurements (N=4) of clarithromycin at each concentration level. The % RSD calculated was less than 1.2 %. The between day precision was performed on four different days at three concentration levels (20, 50, 100 mg/L) and the % RSD at each concentration level was calculated. At each concentration level, the between day % RSD was less than 1.4%.

Accuracy

Accuracy of the method was studied by preparing the pharmaceutical formulation according to the formulation preparation procedure. A known quantity of clarithromycin solution was added at the same proportion as the nominal concentration in the formulation Results have shown that the recovery of clarithromycin was within 95-98 %, and the RSD is lower than 1.2%. (Table 3)

Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ of the proposed method were dermined on the basis of 3.3 times and 10 times, respectively, the standard deviation of the least dilute standard or more accurately the standard error of the regression ($s_{y/x}$). For both calculations the results were the same. The calculated LOD is 1.6 mg/L and the LOQ 4.7 mg/L.

Recovery of clarithromycin from pharmaceutical formulations

In order to evaluate the efficiency of the extraction of the analyte from commercial formulations the experimental concentrations of the formulations working solutions were calculated through the calibration curve obtained and compared with their nominal values. The nominal value percentage at each concentration level was found at the range of 94-98.5 % as shown in Table 4.

Table 3 Accuracy- recovery study of clarithromycin by standard addition method.

Spiked concentration (mg/L) (N=3)	*% RSD*	*% recovery*
20	1.12	96.47
50	0.75	95.34
100	0.62	98.02

Table 4 Chromatographic determination of clarithromycin formulation.

Nominal Concentration of formulation solutions (mg/L) (N=3)	*Mean calculated concentration through calibration curve (mg/L)*	*% recovery*
tablets (250 mg)		
50	47.7±1.4	95.4
75	72.0±1.2	96.0
100	98.2±0.9	98.2
tablets (500 mg)		
50	47.9±1.6	95.8
75	74.1±1.1	98.8
100	97.9±0.7	97.9
Suspension 125 mg/5 mL		
50	47.8±1.5	95.6
75	73.1±1.2	97.5
100	97.4±1.2	97.4

Conclusion

A novel reliable, fast (6 min), chromatographic isocratic reversed phase HPLC –ELSD method for the determination of clarithromycin was developed, optimized and validated. The developed method can be used for determination of clarithromycin in pharmaceutical formulations with simple preparation steps. ELSD was found as a good alternative and suitable detector for the determination of clarithromycin and also can be used for routine analysis of pharmaceutical formulations. The method offers a fast and easily applied alternative for the determination of clarithromycin in formulations instead of the HPLC-UV proposed by pharmacopoeia and expands the field of the ELSD applications.

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