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Research Article

Development of RP-HPLC methods for the analysis of Dexamethasone and Levofloxacin alone and in combinations used in the therapy of Covid-19

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Abstract

In December 2019, the World Health Organization was informed of an outbreak of pneumonia of unknown etiology in Wuhan, Hubei Province, China. On January 7, 2020, a new type of coronavirus was isolated, with the WHO later officially calling it "COVID-19" and the International Committee on Taxonomy of Viruses naming the virus "SARS-CoV-2". On January 30, 2020, the WHO declared the severe acute respiratory syndrome coronavirus 2 outbreak a public health emergency of international concern, making it an unprecedented global public health challenge. From a scientific and literary reference, it was established that the main drugs in the therapy with Covid-19 are Dexamethasone and Levofluoxetine. For this reason, we pay attention to the analysis of these two medicinal products. In the literature, we did not find an analysis of a combination of Dexamethasone and Levofloxacin. Development and validation of a highperformance liquid chromatographic analytical procedure for simultaneously determining Dexamethasone and Levofloxacin in a synthetic mixture is described in this paper. The separation was made with a LiChrosorb RP 18 (250 × 4.6 mm) column, at 25 °C temperature,with isocratic mode andmobile phase,containingt acetonitrile and woter (70-30v/v). Eluent was monitored at 254 nm and the flow rate was 1.0 ml/min. Dexamethasone and Levofluoxetine were effectively separated with retention time (tr) of 4.69 min and 14.51 min,respectively,with in the selected chromatographic conditions.The method was validated for analytical parameters: specificity, linearity, precision, accuracy,andlimits of detection and quantitation. The calibration curves were linear inthe concentration range of 12.5 to 100.0 µg/ml for Dexamethasone and Levofloxacin, and the regression coefficientswere more than 0.999. For Dexamethasone and Levofloxacin the recovery was 100.01% and 100.04%, respectively. This analytical procedure is applicable for the quality control of drug formulations.

Keywords

Dexamethasone, Levofloxacin, RP-HPLC, Validation, Drugs, Quality control, Covid-19

Introduction

In December 2019, the World Health Organization was informed of an outbreak of pneumonia of unknown etiology in Wuhan, Hubei Province, China. On January 7, 2020, a new type of coronavirus was isolated, with the WHO later officially calling it "COVID-19" and the International Committee on Taxonomy of Viruses naming the virus "SARS-CoV-2". (Moore WV 1988) On January 30, 2020, the WHO declared the severe acute respiratory syndrome coronavirus 2 outbreak a public health emergency of international concern, making it an unprecedented global public health challenge. Due to the high infectivity of the virus, its rapid spread and its continuous evolution in the human population, clinical trials have been started all over the world, with the subsequent collection of data on the course of the disease, in search of an effective therapy (Birdsong 1986).

The fastest way to affect COVID-19 is to find that an already approved drug works against it. That is why some researchers have focused on revising old molecules in the hope of finding effective existing drugs and retargeting them, respectively. Others are applying the latest discoveries in synthetic biology, developing sophisticated monoclonal antibody therapies and vaccines (Mellion 1984).

The first group includes antiviral drugs, anti-inflammatory drugs, antimalarial drugs, some antibiotics. These groups also include corticosteroids and fluoroquinolones (Kibble et al. 1987).

Dexamethasone is a synthetic corticosteroid that has an anti-inflammatory and immunosuppressive effect, thanks to which it limits the production and damaging effect of cytokines. It also blocks macrophages, which results in influencing secondary infections.

Fluoroquinolones, as broad-spectrum antimicrobial substances possessing an immunomodulatory effect leading to a weakening of the inflammatory response, are used as adjuncts in the treatment of pneumonia associated with SARS-CoV-2 (Penn 1988).

Despite their financial and physical availability, as well as their ease of administration, corticosteroids and fluoroquinolones are drugs with significant adverse drug reactions, necessitating their use only in specific medical conditions consistent with co morbidities, if any (Limbird 1985).

In this regard, the research and development of reliable analytical methods for the determination of Dexamethasone and Levofloxacin, both alone and in mixtures, in chemical and biological samples is essential to provide fast and reliable information (Strauss et al. 1985; Wagner 1988; De Cock et al. 2001).

Steroids are cyclopentanoperhydrophenanthrenes with lipid properties. Structurally, they are tetracyclic compounds. Steroids differ in the functional groups attached to these rings (Walters et al. 1990; Lurie et al. 1994; Odoardi et al. 2015).

There are many medicinal products with a steroid structure, as well as naturally occurring steroid hormones in the human body, as well as cholesterol (precursor in the biosynthesis of steroid hormones) (Noggle Jr et al. 1990; Sandra et al. 1989). Specific categories of steroids:

- Corticosteroids – hormones important for metabolism and water-electrolyte balance.
- Sex hormones – androgens, estrogens and progestagens (Apffel et al. 1991).

Medicines for the treatment of chronic lung diseases most often contain various bronchodilators, mucolytics, corticosteroids as active ingredients. They are prescribed according to a doctor's prescription, using them in the form of tablets, capsules, inhalers, ampoules for injection. (Saartok et al. 1984; Brown et al. 1991; Kicman et al. 2008) The most common product from the steroid group used in the therapy of severe lung infections, including severe pneumonia of an infectious nature and the newly emerging Covid-19, is the synthetic corticosteroid Dexamethasone, which is the subject of our study, and also has anti-inflammatory, analgesic and antiallergic action and immunosuppressive properties. (Mesmer et al. 1997; Llewellyn et al. 2011; Yadav et al. 2013) Dexamethasone is the first drug to reduce mortality among Covid-19 patients on artificial respiration. According to the WHO, in patients on artificial respiration, the preparation was able to reduce mortality by a third, and in patients needing only oxygen – by a fifth. 24 (Cavrini V et al. 1983) The drug is particularly successful in patients with severe atypical pneumonia. Dexamethasone treatment in patients with milder symptoms has not been as effective (Hintikka et al. 2008; Gosetti et al. 2013; Nola et al. 2015).

Following a large-scale study published in July 2020 in the New England Journal of Medicine, the US National Institutes of Health made the following recommendations for the use of Dexamethasone for the treatment of Covid-19: "Use of Dexamethasone for the treatment of Covid-19 in hospitalized patients , who are mechanically ventilated or require supplemental oxygen" and "not to use Dexamethasone to treat Covid-19 in patients who do not require supplemental oxygen." (Cavrini et al. 1983; Kim et al. 2000).

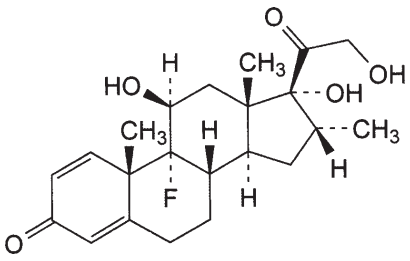

Chart 1. Dexamethasone. 9-Fluoro-11b,17,21-trihydroxy-16a-methylpregna-1,4-diene-3,20-dione.

A simple, rapid, accurate and sensitive method was developed for the quantitative analysis of Dexamethasone Acetate in microemulsions using High Performance Liquid Chromatography (HPLC) with UV detection. Chromatography parameters were Lichrospher 100 RP-18

stainless steel column (250 mm × 4 mm i.d., 5 µm particle size), at 30 ± 2 °C. The isocratic mobile phase was methanol:water (65:35; v/v) at a flow rate of 1.0 mL.min-1. The determination is carried out using a UV-Vis detector, with a wavelength of 239 nm (Cristina et al. 2009).

A rapid differential pulse polarographic method was developed for the determination of Dexamethasone Sodium Phosphate. A well-defined single peak with an Ep value of -1.14 V was obtained in acetate buffer (pH 5.0). The linearity is valid in the range 0.2–1.2 mg/25 mL (r=0.9992) with a minimum detection limit of 7.6×10(-6) M. The precision of the developed method is implied by the relative mean deviation values, the standard deviation, and the coefficient of variation, which are 2.44%, 0.014, and 3.5%, respectively. The method is reproducible and accurate and can be used in the pharmaco-analytical characterization of Dexamethasone Sodium Phosphate. (Jeyaseelan et al. 2002) A chromatographic method for the determination of Dexamethasone Sodium Phosphate by reverse phase HPLC is also described and is quantified by UV absorbance at 254 nm. This method provides a simple, sensitive and reliable technique for determining the amount of the drug molecule in various dosage forms (Kwak et al. 1995).

Currently, the therapy of the newly emerging Covid-19 is related to the application and combination of medicinal products characterized by basic anti-inflammatory, immunomodulatory and enzyme-inhibiting mechanisms, influencing the possibility of the formation of severe pulmonary complications. Colchicine, Bromhexine, Levofloxacin and others stand out as such.

Fluoroquinolones are broad-spectrum synthetic antimicrobial agents that are chemical derivatives of quinolone. Interestingly, fluoroquinolones can exhibit antiviral activity against vaccinia virus, papovavirus, CMV, VZV, HSV-1, HSV-2, HCV, and HIV. An in silico study shows that fluoroquinolones can inhibit the replication of SARS-CoV-2 by exhibiting a stronger capacity to bind to its core protease. Fluoroquinolones exhibit multiple immunomodulatory properties leading to attenuation of the inflammatory response by inhibiting proinflammatory cytokines.

The respiratory fluoroquinolones, Levofloxacin and Moxifloxacin, are first-line therapeutic agents for the treatment of severe pneumonia. They are characterized by favorable pharmacokinetic properties; higher lung concentrations and an excellent safety profile comparable to other antibiotics used to treat respiratory infections, such as macrolides and b- lactams. Based on their potential antiviral activity and immunomodulatory properties, favorable pharmacokinetics and safety profile, respiratory fluoroquinolones are adjunctive agents in the treatment of SARS-CoV-2-associated pneumonia (Karampela et al. 2020). Levofloxacin is not described in the European Pharmacopoeia. A sensitive, isocratic stability, RP-UPLC method for the quantitative analysis and purity investigation of Levofloxacin was developed and validated. The accuracy of the analysis is 99.77% and 101.55% (Batuk et al. 2013).

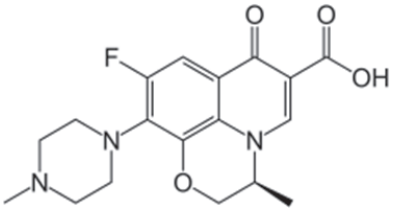

Chart 2. Levofloxacin. (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid.

An efficient approach for the determination of Levofloxacin using high performance liquid chromatography and UV spectrophotometry is described. A Phenomenex C18 reversed- phase column (150×4.6 mm id, 4 µm particle size) was used, eluting isocratically with a mixture of water:acetonitrile:phosphoric acid 0.025 M, pH adjusted to 3.0 with triethylamine (6.00 :20, v /v/v) as the mobile phase at room temperature and at a flow rate of 1.0 mL/min. The UV Detector was set at 294 nm and the UV-vis spectrophotometer at 292 nm (Hurtado et al. 2007).

Materials and methods

HPLC AnalysisThe HPLC analysis was performed by isocratic elution with a flow rate 1.0 ml/min. For the analysis, a HPLC system (SHIMADZU Corporation, LC-20 AD quaternary pump) was used with a UltiMate Dionex 3000 SD, Chromeleon 7.2 SR3 Systems, Thermo Fisher Scientific Inc. Separation was carried out at 25 °C, using LiChrosorb RP-18 (250 × 4.6 mm) column, packed with octadecylsilyl silica gel 5-µm. The mobile phase, acetonitrile -water (70/30 v/v) was sonicated for 20 min and then filtered through a 0.45-µm filter paper. The analysis was performed at 254 nm with 20 µl aliquots of the solution were injected. The chromatogram is shown in Fig. 1.

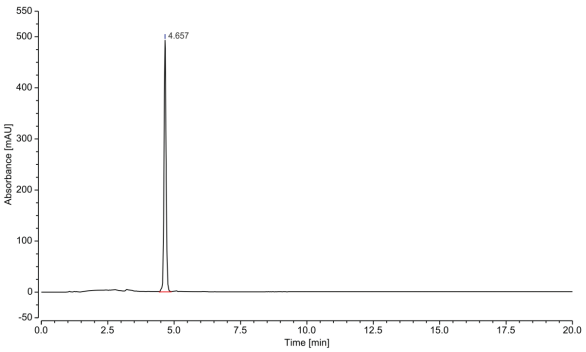

Figure 1. Retention time of Dexametahasone.

Accurately weighed 0.01 g Dexamethasone and 0.01 g Levofloxacin working standards are transferred to a volumetric flask (25 ml). Dissolve with acetonitrile until a good solution is obtained.

Several mobile phase combinations were trialed for optimization of the RP-HPLC conditions.Taking into

account the protolysis constants (Ka) and solubility of both the compounds, several important parameters, such as, percentage and type of organic modifier, pH of the mobile phase, and concentration of the acid,were studied. Resolution wasthe most important criterion for the method and it wasimperative to achieve adequate separation. The trials showed that a mobile phase, consisting of acetonitril/water , in the proportion of 70/30v/v,with reverse phase LiChrosorb RP-18 (250 × 4.6 mm) column,yielded symmetric and sharp peaks. Greatly improved detector responses for both drugs were obtained at the optimum wavelength of 254 nm. As shown in Fig. 1, the retention times were 4.69 min for Dexamethasone and 14.51 min for Levofloxacin (Figs 2, 3).

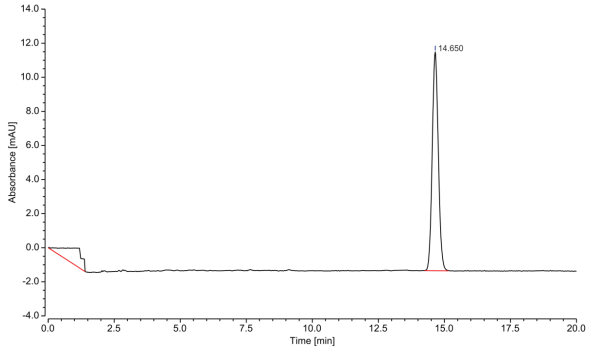

Figure 2. Retention time of Levofloxacin.

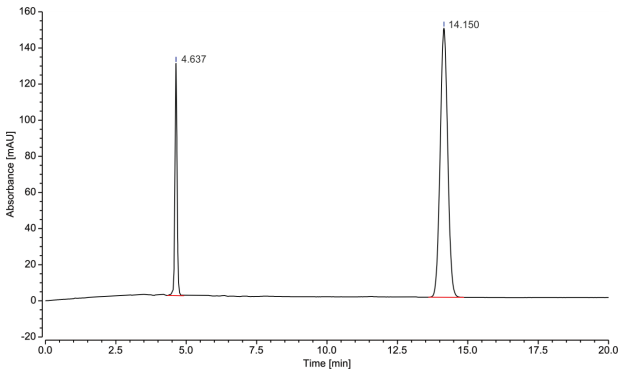

Figure 3. The chromatogram is shown Dexamethasone and Levofloxacin combination.

The method was simple and hadashorter run time (20 min) in comparison with that reported by Patel (2011) and Bind et al. (2015). The proposed method was validated in terms of specificity, linearity, accuracy, precision, and limits of detection and quantitation,according to guidelines of the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH guidelines 2005) Method ValidationThe selectivity of the current method demonstrated adequate separation of the two active ingredients (Dexamethasone and Levofloxacin). The matrix components, e.g., excipients, did not interfere with the two analytes. For examining linearity, standard solutions containing Dexamethasone (12.50–100.0µg/ml) and Levofloxacin (12.50–100.0µg/ml) were prepared in the solvent. Each concentration level was triplicated in chromatography, using 20 µl injections for each standard solution.In addition, studies were prepared with seven different concentrations of mixtures for both drugs. The responses, measured as peak areas, are shown in Table 1. The calibration curves showed linearity in the selected concentration range for both drugs. The linear regression equations for Dexamethasone and Levofloxacin were as follows: y = 11827.3×–31.6; and y = 14929.5×–10454.4, respectively. The regression coefficients (r) were greater than 0.999, which indicated a high degree of linearity for both drugs (Figs 4, 5).

Table 1. The linear data for Dexamethasone and Levofloxacin.

Linearity level	Dexamethasone		Levofloxacin	
	Concentration (µg/ml)	Average area (µV/ml)	Concentration (µg/ml)	Average area (µV/ml)
1	12,5	154 126	12,5	184 122
2	25	223 653	25	323 853
3	50	567 784	50	499 784
4	75	897 490	75	797 590
5	100	1 034 574	100	934 274
6	125	1 599 204	125	1 399 204
7	150	1 803 719	150	1 503 719
N = 7				
r		0.999		0.999

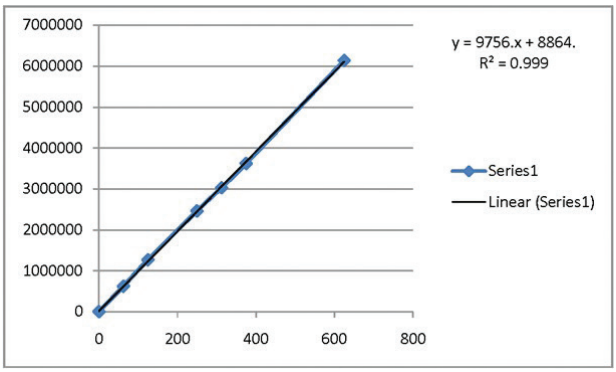

Figure 4. Calibration curve for Dexamethasone average area of peks (µV/sec) against concentration (µg/ml).

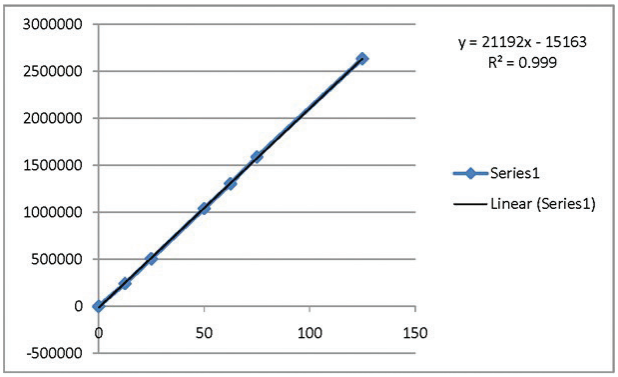

Figure 5. Calibration curve for Levofloxacin average area of peks (µV/sec) against concentration (µg/ml).

Table 2. Results of the analysis for the proposed method.

Parameters	Dexamethasone			Levofloxacin		
	Taken (µg/ml)	Found (µg/ml)	Recovery %	Taken (µg/ml)	Found (µg/ml)	Recovery %
	12,5	12,1	99,2	12,5	12,2	102,56
	25	25,2	100,20	25	25.1	99.60
	50	50,2	100,40	50	50.1	100,10
	75	75,05	100,23	75	74,9	99,70
	100	99,9	99,80	100	99.9	99,94
Mean			100,01			100.20
±SD			0,59			1,21
±Relative SD			0,59			1,19
±SE			0,26			0,55

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the method based on signal to noise ratio (3:1 for LOD and 10:1 for LOQ) was adopted(ICH guidelines (2005)). The limit of detection for Dexamethasone was 0.125 µg/ml and for OFL 0.250 µg/ml, while the limit of quantitation for Dexamethasone was 0.250 µg/ml and for OFL, 0.300 µg/ml.Intra-daily precision was evaluated by calculating the standard deviation (SD) of six replicate determinations using the standard solutions. The SD values revealed the high precision of the method (values were in the range of 0.77 to 0.98 for both drugs). For inter-daily reproducibility, a series was run, in which the standard drug solutions were analyzed over five consecutive days. The SD values were in the range of 0.98 to 1.90. The accuracy of the method was specified by preparing samples of 25%, 50%, 100%, 150%, and 200% of the target concentration. Each concentration level was injected three times. The results showed perfect recoveries (Table 2).

Conclusion

The high performance liquid chromatographic method described in this paper was developed for quantitative control and determination of the contents of Dexamethasone and Levofloxacin. It can be applied in clinical pharmacy, toxicology, and for routine analysis oftablet formulations. The method has high degree of accuracy and precision (less than to 2% Relative SD) and requiresa markedly short performance time (7 min). The parameters for validation of the method (linearity, accuracy, and precision) meet all requirements of the International Council for Harmonization (ICH). One advantage of this method is that it does not use buffers, which are onerous to prepare. The procedure is also simple and easy for implementation.

Acknowledgments

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