DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD TOWARDS DETERMINATION OF RIPASUDIL HYDROCHLORIDE HYDRATE IN PURE AND OPHTHALMIC FORMULATION**

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The goal of the present investigation was to develop and validate the UV-spectrophotometric technique for the quantitative estimation of ripasudil hydrochloride hydrate in pure and ophthalmic formulations. Ripasudil hydrochloride hydrate is a novel drug molecule that has been used to treat glaucoma and ocular hypertension. It was licensed for therapy in Japan for the first time in September 2014. Until now, only an HPLC analytical method for determining it in dosage forms and biological fluids has been disclosed. The objective of this research was to screen a solvent system in which ripasudil hydrochloride hydrate can be completely solubilized, then develop and validate a simple, accurate, precise, and cost-effective UV-spectrophotometric method for estimating ripasudil hydrochloride hydrate in the pure and ophthalmic formulations in accordance with International Conference on Harmonization (ICH) recommendations. The current method is simple, quick, accurate, precise, and cost-effective. Ripasudil hydrochloride hydrate is soluble in HPLC-grade distilled water, so it was used as a solvent to form the solution. The subsequent solution was scanned within the UV range (200–400 nm). The λ_{max} of ripasudil hydrochloride hydrate was found to be 278 nm. Beer's law is valid within the 10 to 50-μg/mL concentration range. The developed analytical approach was extensively validated as per ICH guidelines for linearity, accuracy, precision, robustness, detection limit, and quantitation limit. Linearity was obtained within the 10 to 50 μg/mL range with a correlation coefficient of 0.9981. Limit of detection and limit of quantitation were found to be 0.0785 and 0.2355 μg/mL. The technique presented good recovery and reproducibility; thus, the proposed technique might be employed for regular investigation of ripasudil hydrochloride hydrate in the pure and ophthalmic formulations.

Keywords: *linearity, LOD, LOQ, recovery, intermediate precision, robustness.*

РАЗРАБОТКА И ВАЛИДАЦИЯ УФ-СПЕКТРОФОТОМЕТРИЧЕСКОГО МЕТОДА ОПРЕДЕЛЕНИЯ РИПАСУДИЛА ГИДРОХЛОРИДА ГИДРАТА В ЧИСТОМ ВИДЕ И ОФТАЛЬМОЛОГИЧЕСКИХ ПРЕПАРАТАХ

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Разработан УФ-спектрофотометрический метод количественного определения лекарственного средства для лечения глаукомы и глазной гипертензии рипасудила гидрохлорида гидрата в чистом

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виде и офтальмологических препаратах в соответствии с рекомендациями Международной конференции по гармонизации (ICH). До сих пор для определения рипасудила гидрохлорида гидрата в лекарственных формах и биологических жидкостях применяли только аналитический метод ВЭЖХ. Проведен скрининг системы растворителей, в которой гидрат рипасудила гидрохлорида может быть полностью солюбилизирован. Для приготовления раствора гидрата гидрохлорида рипасудила в качестве растворителя использована дистиллированная вода для ВЖЭХ. В спектре поглощения полученного раствора в УФ-диапазоне 200—400 нм для гидрата рипасудила гидрохлорида зарегистрирован максимум λ_{max} = 278 нм. Закон Бера действует в диапазоне концентраций 10—50 мкг/мл. В соответствии с рекомендациями ICH проведена валидация разработанного метода. Получена линейность в диапазоне 10—50 мкг/мл с коэффициентом корреляции 0.9981. Предел обнаружения и предел количественного определения составляют 0.0785 и 0.2355 мкг/мл. Метод обладает хорошей повторяемостью и воспроизводимостью и может быть использован для регулярного исследования гидрата рипасудила гидрохлорида в чистом виде и офтальмологических препаратах.

Ключевые слова: *линейность, предел обнаружения, предел количественного определения, воспроизводимость, промежуточная точность, надежность.*

Introduction. One of the most common approaches in the pharmaceutical analysis is ultraviolet (UV)-visible spectroscopy. It helps in the determination of how much ultraviolet or visible radiation material the solution absorbs. UV-Visible spectrophotometers are instruments that quantify and qualify the ratio of the intensity of two beams of light in the UV-visible area [1]. UV spectrophotometers are commonly used to acquire specific details on the chromophoric component of compounds. It elucidates the structural properties of various medications that absorb specific wavelengths when exposed to ultraviolet light. The Beer–Lambert law is the fundamental law that regulates quantitative spectrophotometric analysis [2].

Rho-associated coiled-coil-containing protein kinase (ROCK) inhibitor, ripasudil hydrochloride hydrate decreases intraocular pressure (IOP) by improving normal aqueous outflow via the trabecular meshwork and Schlemm's canal [3]. The International Union of Pure and Applied Chemistry name ripasudil hydrochloride hydrate is 4-fluoro-5-[[(2S)-hexahydro-2-methyl-1H-1,4-diazepin-1-yl]sulfonyl]isoquinoline hydrochloride, K-115 hydrochloride, with its molecular formula being $C_{15}H_{18}FN_3O_2S$. The average molecular weight of ripasudil hydrochloride hydrate is 395.87 Da. Ripasudil is an "out-flow" drug that reduces IOP by stimulating the movement of aqueous humor from the ciliary body away from the eye [4]. The *S* configuration at the 2-position on the 1,4-diazepane ring of ripasudil is what gives the drug its characteristic effect. The absorption of the drug via the cornea is well established with wide distribution into most tissues, and high levels of the drug are observed in the liver, kidneys, and in urine. It has plasma protein binding of 55.4–59.8%. Its major inactive metabolite is hydroxylation-derivative M1 in the human plasma formed by aldehyde oxidase. Ripasudil is metabolized to M2 by CYP3A5 and 3A4, and M4 by CYP3A5, 3A4, and 2C8 [5]. It is eliminated primarily by the kidneys with an elimination half-life ($t_{1/2}$) of 0.455 h. The partition coefficient of the drug is 0.88 with a 60% absolute bioavailability [6].

The analysis technique illustrates how the analytical procedure has to be carried out. It comprises a detailed description of the steps required to complete each analytical test. This includes but is still not be restricted to, the preparation of the sample, the reference standard, and the reagents, the usage of the apparatus, the development of the calibration curve, the calculation formulae, etc. [7].

Ripasudil hydrochloride hydrate is not yet listed in any pharmacopeia, and only an HPLC analytical method for determining it in ophthalmic formulations and biological fluids has been disclosed [8]. Because of the simplicity, specificity, and low cost, UV spectroscopic analysis remains one of the commonly employed methods for determining drug concentrations. A novel UV spectrophotometric method for identifying ripasudil hydrochloride hydrate in bulk and pharmaceutical compositions is presented in this work. The goal of this research was to develop and validate a UV-spectrophotometric technique for estimating ripasudil hydrochloride hydrate in bulk and pharmaceutical formulations in accordance with International Conference on Harmonization (ICH) recommendations [7].

Experimental. Ripasudil hydrochloride hydrate was purchased from Sigma Aldrich, Delhi, India and was used as the reference standard. All chemicals and reagents were of analytical or HPLC grade. For the measurement of absorbance, a Shimadzu UV-Visible spectrophotometer (UV mini-1700, Shimadzu Corporation, Kyoto, Japan) was utilized with matched quartz cells.

Various solvents were used to investigate the solubility of ripasudil hydrochloride hydrate. The solvents include HPLC-grade distilled water, methanol, ethanol, and acetonitrile. A saturated solution was generated

by dissolving excess amounts of the drug in 10 mL of each solvent. At room temperature (25±1°C), a magnetic stirrer was used to stir the saturated drug solution for 24 h at 100 rpm. The sample was then centrifuged at 10,000 rpm for 10 min. A 0.22 μm syringe filter was used to recover clear supernatant, which was then scanned with a UV spectrophotometer. The results were observed and noted.

One hundred milligrams of ripasudil hydrochloride hydrate was accurately weighed and placed into a 100-mL volumetric flask, diluted, and then made up to the mark with HPLC-grade distilled water to prepare a standard stock solution (SS-I) of 1000 μg/mL.

In a volumetric flask of 100 mL capacity, 10 mL of the SS-I was diluted with HPLC-grade distilled water up to 100 mL to obtain a concentration of 100 μg/mL, which was utilized as the working stock solution (WSS). The UV spectroscopic scanning (200–700 nm) was conducted with a WSS to determine the λ_{max} of ripasudil hydrochloride hydrate against HPLC-grade distilled water as blank.

Validation of the developed analytical technique was implemented as per the ICH guidelines Q2 (R1) (ICH, 2005) [7]. The proposed analytical technique was validated for distinct parameters such as linearity and range, precision, accuracy, specificity, robustness, limit of detection (LOD), limit of quantitation (LOQ), and assay.

Working stock solutions of 100 μg/mL were diluted further using HPLC-grade distilled water to yield dilutions of 10, 20, 30, 40, and 50 μg/mL. To identify the wavelength of maximum absorbance of the drug, the solutions were scanned in a UV-Visible spectrophotometer within the range 200–700 nm. The regressed equation was developed after plotting absorbance vs concentration to obtain the calibration curve [9].

The technique variability is measured by the system precision. Three repeat analyses of the identical working solution were used to determine it. The results of intraday (repeatability) and interday (intermediate precision) variation revealed the method's precision. The intraday precision of the devised UV technique was estimated by preparing samples from the very same batch in nine determinations on the same day, i.e., at zero hours, at the fourth hour, and at the eighth hour, each with three concentrations (5, 10, and 20 μg/mL) and three replicates (n = 3). The method precision was assessed using the % RSD of the results. The interday precision was established by analyzing samples in triplicate (n = 3) for three days in a row [9].

Recovery experiments were used to understand the method's accuracy at five levels (80, 90, 100, 110, and 120%) using the typical addition method. The mean percentage recovery was determined by noting down the absorbance of all five levels [10].

The robustness of an analytical method is a measure of its ability to stay unaffected by minor but determined modifications in method parameters during routine use. It was characterized by two experts performing the analysis at two distinct temperatures, 200 and 300°C. The absorbance was taken six times and the assay was computed [11, 12]. The percentage RSD has been used to express the result.

The limit of detection (LOD) of an individual analytical procedure is the smallest amount of analyte in a sample that can be identified but not generally quantified as an exact figure. The results from the linearity investigations were used to calculate the LOD. The slope of the linearity plot was determined. The standard deviation of the responses was calculated for each of the ten replicate measurements of the same concentration (20 μg/mL). The LOD may be expressed as LOD = $3.3\sigma/S$, where σ is relative standard deviation of the response, S is the slope of the calibration curve (of the analyte) [13, 14]. The lowest amount of analyte in a sample that can be quantitatively measured with sufficient precision and accuracy is the quantitation limit of an analytical process [13, 15]. The LOQ may be expressed as LOQ = $10\sigma/S$, where σ is relative standard deviation of the response, S is the slope of the calibration curve (of the analyte).

In order to examine commercial formulations, in a volumetric flask of 100-mL capacity, 5 mL of ripasudil hydrochloride hydrate eye drop solution was placed, and the volume was filled up to the mark with HPLC-grade distilled water to achieve a concentration of 100 μg/mL. Then, 2 mL was taken and transferred to a 10-mL volumetric flask, which was then made up to the mark with HPLC-grade distilled water to give a concentration of 20 μg/mL. A UV spectrophotometer was used to scan it within the UV range 200–400 nm. At specified absorption maxima, the spectra were recorded. The linear regression equation was used to calculate the drug concentrations.

Result and discussion. Ripasudil hydrochloride hydrate was found to be freely soluble in HPLC-grade distilled water, slightly soluble in ethanol and methanol, and almost insoluble in organic solvent, i.e., acetonitrile. As a result, HPLC-grade water was chosen, and only that was used for further dilution. The standard stock solution was successfully prepared to get the solution with a concentration of 1000 μg/mL. Furthermore, the WSS was successfully prepared by diluting the standard stock solution to obtain a 100-μg/mL concentration. The WSS was then scanned within the range 200–400 nm in a UV-Visible spectrophotometer

to get the absorption maxima of the drug. The unique absorption maxima of ripasudil hydrochloride hydrate of 278 nm were obtained. The scan is illustrated in Fig. 1. The proposed method was validated as per ICH Q2 (R1) guidelines.

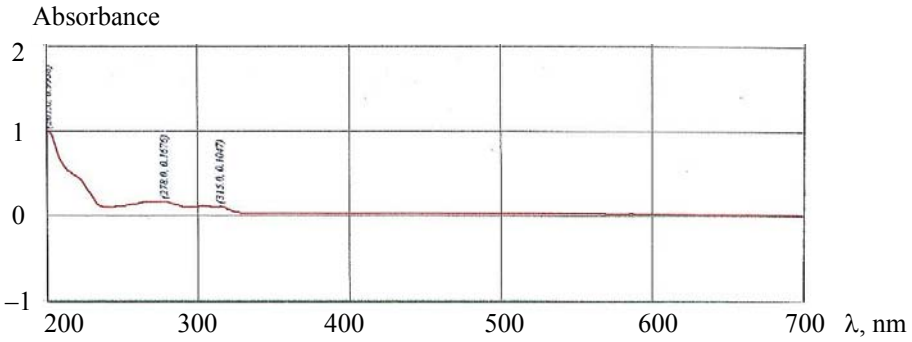

Fig. 1. UV scan of ripasudil hydrochloride hydrate in HPLC-grade distilled water within the range 200–700 nm.

Six points were used to create the analytical curves, which covered the concentration range 10–50 μg/mL. Over this concentration range, Beer's law was followed. The regressed equation obtained was $y = 0.0139x + 0.0034$, where y and x are ripasudil hydrochloride hydrate absorbance and concentration in μg/mL, respectively. The high value of the correlation coefficient ($r^2 = 0.9981$) and small y-intercept value proved the linearity. Table 1 presents the data for linearity with absorbance and %RSD. Figure 2 highlights the calibration curve for the ripasudil hydrochloride hydrate along with the regressed equation and the value of the regression coefficient. Figure 3 highlights the specificity of absorption maxima of all dilutions in increasing linear order.

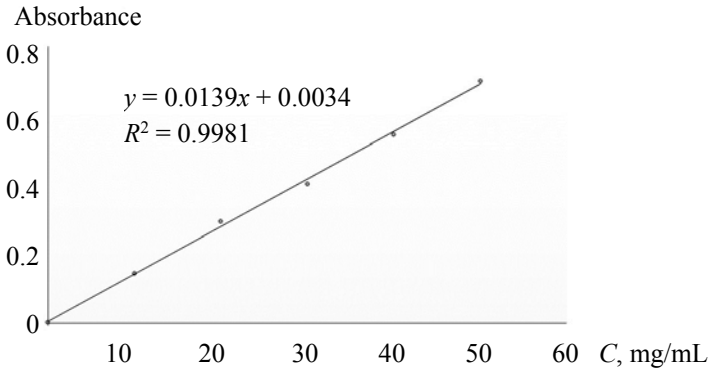

Fig. 2. The calibration curve for the ripasudil hydrochloride hydrate.

TABLE 1. Linearity Studies of Ripasudil Hydrochloride Hydrate

Concentration, μg/mL	Absorbance* ± SD (n=6)	%RSD
0	0	0
10	0.1443 ± 0.0602	0.417434
20	0.2957 ± 0.0223	0.075363
30	0.4043 ± 0.0358	0.088491
40	0.5489 ± 0.0308	0.05605
50	0.7055 ± 0.0498	0.070588

*Average of six estimations.

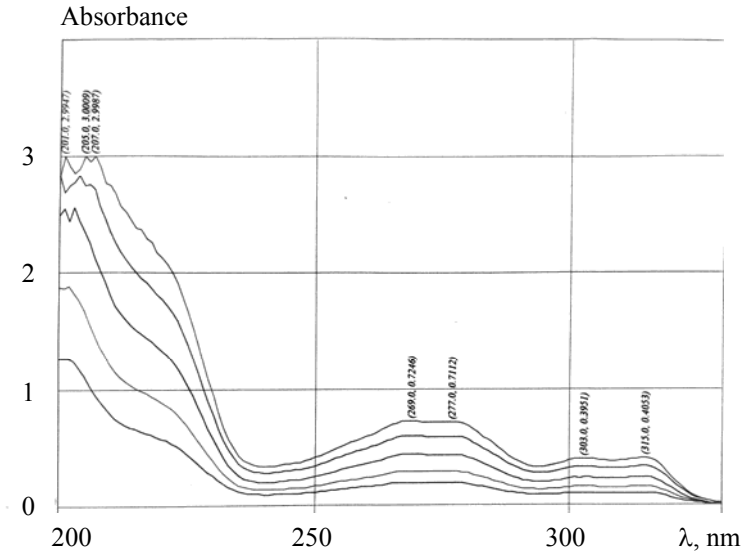

Fig. 3. UV scan of ripasudil hydrochloride hydrate dilutions (10 to 50 µg/mL).

The closeness of a sequence of individual analyte readings performed repeatedly on the same sample determines the precision of an analytical procedure. A relative standard deviation (%RSD) was used to compute it. Ideally, the value of %RSD should be less than 2%. Low, medium, and high concentrations are commonly used to test repeatability. The type of analysis determines the acceptance criteria. Intermediate precision depicts the differences in day-to-day analysis caused by various analysts, instruments, and other associated factors. The % RSD for absorbance values of 5, 10, and 20 µg/mL ripasudil hydrochloride hydrate at three different time periods, i.e., 0, 4, and 8 h within a day, was achieved to be 0.042, 1.568, and 0.356% whereas on three different days (interday) values 0.483, 0.714, and 0.581% were found. The data for both the repeatability and intermediate precision are compiled in Table 2, which were realized to be within the specified limits (<2%), as described in the ICH guidelines.

TABLE 2. Intraday Precision (Repeatability) and Interday Precision (Intermediate Precision) Studies

Concentration, µg/mL	Time, h	Intraday precision (n = 3)			Interday precision (n = 3)		
		Absorbance measured	Absorbance (mean ± SD)	% RSD	Absorbance measured	Absorbance (mean ± SD)	% RSD
5	0 4 8	0.2351 0.235 0.2352	0.2351 ± 0.0001	0.0425	0.2539 0.253 0.2554	0.2541 ± 0.0012	0.48308
10	0 4 8	0.2618 0.2679 0.2698	0.2665 ± 0.0042	1.5683	0.3262 0.3274 0.3229	0.3255 ± 0.0023	0.71433
20	0 4 8	0.4239 0.4226 0.4209	0.4225 ± 0.0015	0.356	0.4698 0.4752 0.4720	0.4723 ± 0.0027	0.581478

The percentage mean recovery of ripasudil hydrochloride hydrate was found to be 99.61, 98.105, 99.55, 99.5, and 99.43%, respectively, for 80, 90, 100, 110, and 120% of the added drug. The data for the recovery studies are compiled in Table 3. The average percentage recovery of the drug was found to be 98.10±0.853 to 99.61±0.517 which lies within acceptable limits of mean percentage recovery with %RSD value less than 2%, which indicates good accuracy.

The terms robustness and ruggedness describe an analytical method's ability to stay largely unaffected by modifications in process parameters (mobile phase composition, column age, column temperature, etc.) and impactful external conditions (temperature, air humidity, etc.) throughout normal use, as well as recognize its reliability. The mean absorbance values of sample solutions with a concentration of 20 µg/mL were

analyzed by two different analysts and at two different temperatures (20 and 30°C) and are compiled in Table 4. The %RSD values of sample solutions by two different analysts and at two different temperatures were found to be less than 2%, which validated the robustness of the analytical method.

TABLE 3. Recovery/Accuracy Studies of Five Different Concentrations of Ripasudil Hydrochloride Hydrate by the Proposed Method

Preanalyzed sample solution, µg/mL	% of drug added	Amount of drug added, µg/mL	Average amount recovered, µg/mL (n = 3)	% Recovery (mean ± SD)	%RSD
20	80	16	35.86	99.61 ± 0.517	0.0051
	90	18	37.28	98.10 ± 0.853	0.0086
	100	20	39.82	99.55 ± 0.949	0.0095
	110	22	41.79	99.50 ± 0.605	0.0060
	120	24	43.75	99.43 ± 0.305	0.0030

TABLE 4. Robustness Studies of Ripasudil Hydrochloride Hydrate

	%Assay (30°C)	%Assay (20°C)		%Assay (30°C)	%Assay (20°C)
Analyst 1	0.4048	0.3224	Analyst 2	0.4045	20°C
	0.4046	0.3251		0.4047	0.3244
	0.4044	0.3244		0.4046	0.3242
	0.4041	0.3246		0.4048	0.3223
	0.4039	0.3248		0.4044	0.3245
	0.4040	0.3246		0.4041	0.3248
Mean	0.4043	0.3243	Mean	0.4045	0.3247
SD	0.000358	0.000968	SD	0.000248	0.3242
%RSD	0.09	0.30	%RSD	0.06	0.000931

The LOD is the minimum concentration at which the technique can detect (but not estimate) the analyte. It is also known as the minimum concentration that can be effectively differentiated from background noise. The LOD of ripasudil hydrochloride hydrate was found to be 0.0785 µg/mL, which showed the high sensitivity of the analytical method. The complete data for the calculation of LOD is mentioned in Table 5. The LOQ is the minimum concentration of the analyte that can be reliably quantified by the method. A suitable precision and trueness should be shown for something to be considered reliable. The LOQ of ripasudil hydrochloride hydrate was found to be 0.2355 µg/mL, which showed the high sensitivity of the analytical method. The complete data for the calculation of LOD is mentioned in Table 5.

TABLE 5. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Concentration, µg/mL	SD	LOD, µg/mL	LOQ, µg/mL
0.4048 0.4046 0.4044 0.4041 0.4039 0.4040 0.4045 0.4047 0.4046 0.4048	0.000331	0.0785	0.2355

For application of the proposed method for pharmaceutical formulation 1 mL of ripasudil hydrochloride hydrate eye drop solution (commercially marketed as Ripatec and manufactured by Ajanta Pharma Ltd.) label claim 4.896 mg of ripasudil hydrochloride hydrate equivalent to 4.0 mg of ripasudil was placed in a 10-mL volumetric flask and the volume was taken up to the mark with HPLC-grade distilled water for commercial formulation analysis. Furthermore, 1 mL of the aforesaid dilution was transferred to a 50-mL volumetric flask and the capacity was filled up to the mark with HPLC-grade distilled water to achieve a concentration of 20 μg/mL. A UV spectrophotometer was used to scan it within the range 200–400 nm. At 278 nm, the spectrum was captured [16]. The content of ripasudil hydrochloride hydrate in marketed product is determined by the proposed method: amount found (mean ± SD, mg) 4.868 ± 0.056, potency 99.345%, %RSD 0.0115.

Conclusions. The current analytical method has been validated in accordance with the ICH Q2(R1) guideline and meets certain acceptance requirements. The analytical approach was found to be precise, linear, accurate, robust, and stable, according to the findings. The current work is to our knowledge the first UV spectroscopic method for determining the concentration of ripasudil hydrochloride hydrate in pharmaceutical formulations. This technique can be easily utilized for regular quality control investigations and other research and development tasks.

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