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A SIMPLE ASSAY METHODS DEVELOPMENT AND VALIDATION OF VALACYCLOVIR IN BLOOD, URINE AND PHARMACEUTICAL DOSAGE FORMS BY SPECTROPHOTOMETRY

Nabil A. F. Alhemiary[1]*, Ahmed N. Al-Hakemi [2]

[1] Department of Chemistry, College of Science & Arts – Sharurah, Najran University, Sharurah, Saudi Arabia.
[2] Department of Chemistry, College of Science, Qassim University, Buraidh, Saudi Arabia.
[1,2] Department of Chemistry ,College of Science , Ibb University, P. O. Box 70270, Ibb, Yemen.

Abstract:
Two rapid, simple, sensitive and selective spectrophotometric methods have been developed for the quantitative estimation of Valacyclovir hydrochloride in pharmaceutical formulations and different human body fluids (Blood and urine). The proposed methods were based on the reduction of the nitro group to amino group of the drug. The resulting amine was then subjected to proposed methods. Method A was based on the formation of oxidative coupling reaction between the corresponding drug and brucine – $NaIO_4$ to form violet colored chromogen at 543 nm. Method B was based on the oxidation followed by coupling with 3- methyl-2-benzothiazolinonehydrazone (MBTH) in presence of ferric chloride ($FeCl_3$) to form green colored chromogen at 644 nm. The procedures described were applied successfully to the determination of the compound in their dosage forms and body fluids. Beer's law is obeyed over the concentration ranges of 5–110 µg/mL and 3–45 µg/mL for brucine and MBTH, respectively, with correlation coefficients of 0.9968 and 0.9990, and molar absorptivity 0.4642×10^4 and 0.9761×10^4 L/mol. cm for method A and method B, respectively. The analytical parameters such as apparent limits of detection (LOD) and quantification (LOQ) were also reported for two methods.

Keywords: *Spectrophotometry; Oxidation reaction; Valacyclovir hydrochloride; Pharmaceutical.*

Corresponding author:

Nabil A. F. Alhemiary ,
Department of Chemistry,
College of Science & Arts – Sharurah,
Najran University, Sharurah,
Saudi Arabia.
E-mail: dralhemiary@gmail.com

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INTRODUCTION:

Valacyclovir hydrochloride, L-valine-2-[(2-amino-1, 6-dihydro-6-oxo-9-hipurin-9-yl) methoxy] ethyl ester hydrochloride, (Figure. 1), is the *L*-valyl ester prodrug of the antiviral drug acyclovir that exhibits activity against herpes simplex virus types, 1 (HSV-1) and 2 (HSV-2) and vericellazoster virus [1]. The mechanism of action of ganciclovir involves the highly selective inhibition of herpes virus DNA replication, via enhanced uptake in herpes virus-infected cells and phosphorylation by viral thymidine kinase. The substrate specificity of acyclovir triphosphate for viral, rather than cellular, DNA polymerase contributes to the specificity of the drug [2, 3]. Valacyclovir is converted rapidly and extensively to acyclovir as result of first-pass intestinal and hepatic metabolism through enzymatic hydrolysis [4] . The oral bioavailability of acyclovir is higher after administration of Valacyclovir relative to acyclovir itself. Literature survey reveals that few methods like high performance liquid chromatography [5-9] spectrofluorometric [10,11] voltammetry [12,13] and spectrophotometric methods [14-18].

To the best of our knowledge, few spectrophotometric methods for the determination of Valacyclovir in pharmaceutical formulations, body fluids and other additives were reported. The present study documents an accurate, sensitive, rapid, selective and reproducible visible spectrophotometric assay, which meets an accepted analytical validation. Spectrophotometry is the technique of choice today in the laboratories of research, hospitals and pharmaceutical industries due to its low cost and inherent simplicity.

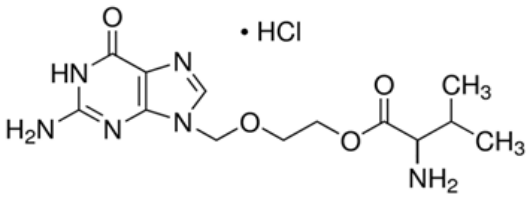

Fig 1: Chemical structures of Valacyclovir hydrochloride.

EXPERIMENTAL:

Apparatus

A GENESYS 10S UV-Vis a double-beam spectrophotometer (Thermo Spectronic, USA) with a fixed slit width (1.8nm) connected to an IBM computer loaded with Thermo Spectronic Vision Lite version 4 software and 1cm quartz cell were used for the registration and treatment of absorption spectra.

Materials and Reagents

All chemicals and reagents used were of analytical grade and water used was always double distilled water. Valacyclovir hydrochloride (Sigma- Aldirch, USA) was obtained and used as received; its purity was > 98%.

3- Methyl-2-benzothiazolinone hydrazine (MBTH) (0.2%w/v): Prepared by dissolving 200 mg in 100 mL distilled water.

$FeCl_3$ (0.5% w/v): Prepared by dissolving 500 mg (Merck) in 100 mL distilled water.

Brucine solution (0.2%w/v): prepared by dissolving 200 mg of Brucine (Sigma –Aldirch) in 100 mL distilled water.

$NaIO_4$ solution (0.2%w/v): prepared by dissolving 200 mg of sodium metaperiodate (BDH) in100 mL distilled water and standardized iodometrically.

H_2SO_4 solution (2.3 mol/L): Prepared by diluting 6.38 ml of 18 mol/L H_2SO_4 to 100 mLwith distilled water.

Valterx® tablets (Glaxo SmithKine) and Valciver® tablets (Cipla Pharmaceuticals, India) were labeled to contain 500 mg VCV per tablet.

Reduction of Nitro Group in Valacyclovir [19]

100 mg of valacyclovir pure or equivalent tablet powder was accurately weighed and dissolved in 20 mL of ethanol. This solution was treated with 10 ml of 5 M HCl and 0.5 g of Zinc powder was added in the portions, while shaking and refluxed at 80°C for 10 min. The solution was filtered using a Whitman filter paper 41 to remove the insoluble matter and the volume was made up to 100 mL with ethanol to get the concentration 1000 µg/mL.

Preparation Working Standard Solution

The resulting amine from the above solution 10 mL was taken into 100 mL volumetric flask and made up to the mark with methanol to get the concentration 100 µg/mL and dilution was carried out to the further working standards.

Construction of Calibration Curves

Method A

Aliquots of VCV ranging from 5-110 µg/mL were transferred into 10 mL volumetric flasks. To this, 2.5 mL of Brucine solution 2 mL Sodium metaperiodate solution and 2 mLof (1.2 mol/L) sulphuric acid were added to each flask. The flasks were shaken thoroughly and placed in a boiling water bath for about 15 min. The reaction mixture was then cooled to room temperature and total volume was adjusted to 10 mL with distilled water. The absorbance of each solution was measured at 543 nm against a reagent blank.

Method B

Aliquots of valacyclovir ranging from 2-45 µg/mL were transferred into a series of 10 mL volumetric flasks. To each flask 1 ml of aqueous solution of ferric chloride (0.5% w/v), 1.0 ml aqueous solution of MBTH (0.2% w/v) were added followed by dil HCl. The final volume was made up to 10 mL with distilled water. The absorbance of the green colored species formed was measured at 644 nm against reagent blank.

RESULTS AND DISCUSSION:

Absorption Spectra

Method A

Brucine (2, 3-dimethoxystrychnidin–10 one) under acidic conditions has been reported to be an effective reagent for the spectrophotometric determination of NO^{-3}, NO^{-2} and Ce^{+4}. Sodium periodate (IO^{-4}) is an efficient oxidant and is color stabilizer. This investigation was concerned with the development of a spectrophotometric method for the routine determination of valacyclovir using brucine–IO^{-4}. The violet color λ_{max} of 543 nm (Figure. 2) developed was stable for 10 h.

Method B

The formation of green colored complex was employed in the quantitative detection of valacyclovir with MBTH in presence of ferric chloride. However when MBTH was initially mixed with VCV and then with oxidizing agent, a green colored compound was produced with maximum absorbance in the visible range at 644 nm and shown in (Figure. 3). MBTH loses two electrons and one proton due to oxidation with Fe (II), forming an electrophilic intermediate, which is the active coupling species. The electrophilic intermediate and the analyte under goes electrophilic reaction with the formation of colored product and the elimination of one molecule of water.

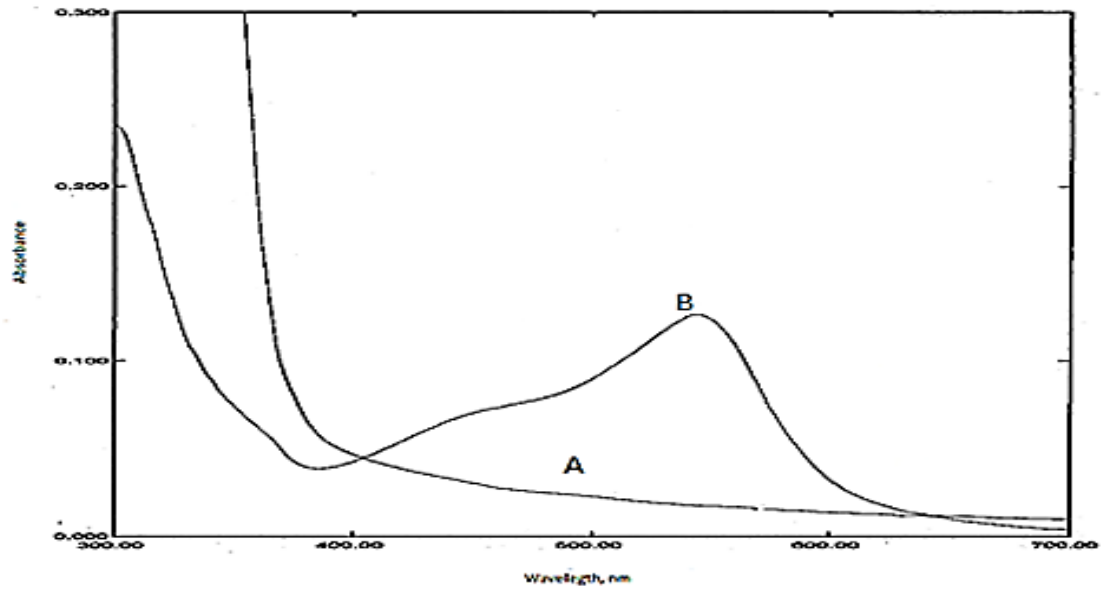

Fig 2: Absorption spectrum of (A) blank (B) brucine with15 µg/mL Valacyclovir.

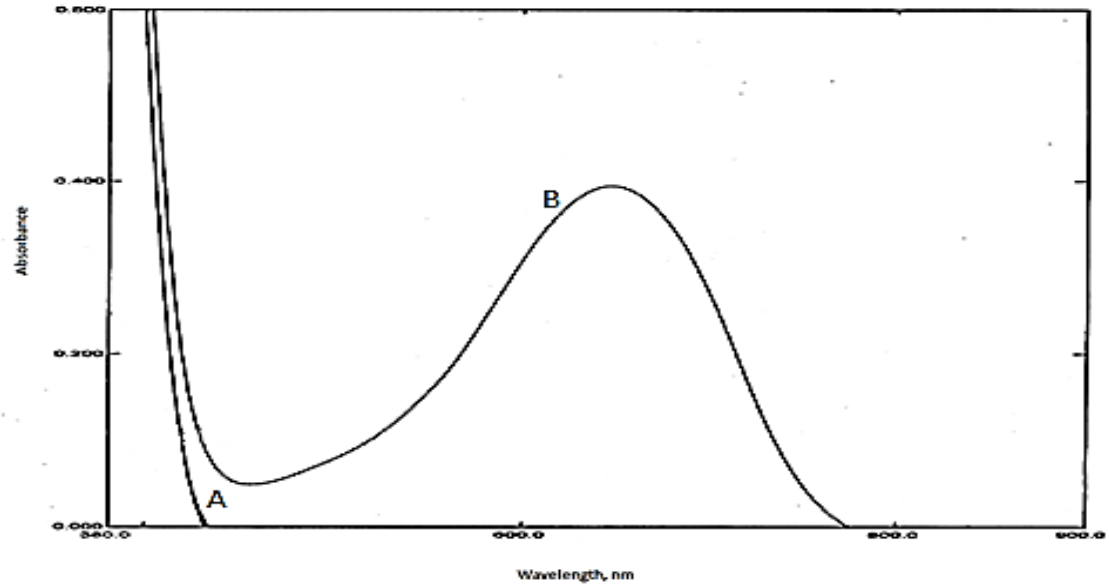

Fig 3: Absorption spectrum of (A) blank (B) MBTH with10 µg/mL Valacyclovir.

Optimum Reaction Conditions (Method A):
The optimum conditions for the method was established by varying one parameter at a time and keeping the others fixed and observing the effect produced on the absorbance of colored species and incorporated in the procedure.

Effect of Reagent Concentration
The effect of reagent was studied by measuring the absorbance of solution containing a fixed concentration of valacyclovir (15 µg/mL) and varied amount of the respective reagent. Maximum color intensity of the complex was achieved with 2.5 mL and 2 mL of 0.2% w/v Brucine and NaIO4 reagent solutions respectively for valacyclovir. Although a larger volume of the reagent had no pronounced effect on the absorbance of the formed complex.

Effect of Time and Temperature
The reaction of valacyclovir depends on time, maximum absorbance intensity was observed after 8.0 min for valacyclovir at room temperature. The reaction of valacyclovir was studied at different temperatures (25–100ºC) the values of maximum absorbance of the oxidative coupling product was almost constant at 25–80ºC for valacyclovir, further temperature decreases the absorbance.

Effect of Acid
The optimum sulphuric acid strength of the 2 mL of diluted reaction mixture for maximum color development with minimum blank color was 1.0-1.5 M.

Sequence of Reagents Added
The following order gives the maximum absorbance and stability i.e. Drug-reagent -acid.

Optimum Reaction Conditions (Method B)
The optimizations of the methods were carefully studied to achieve complete reaction formation, highest sensitivity and a maximum absorbance. Reaction conditions of oxidative coupling complex found by studying with preliminary experiments.

Effect of Reagent
The addition of 1mLof aqueous Ferric chloride (0.5%w/v) solution and 1mLof MBTH reagent (0.2%w/v) ware sufficient to obtain the maximum and reproducible absorbance for 10.0 µg/mL valacyclovir. Smaller amounts resulted in complete complex formation. Increased concentration had no effect on complex formation, although absorbance increased slightly owing to the reagent background used.

Effect of Reaction Time and Stability of Colored Species
The optimum reaction time was investigated from 0.5 to 4.0 min by following the color development at ambient temperature (25±2ºC). Complete color intensity was attained after 2.0 min of mixing for complex. Rising the temperature up to 30ºC has no effect on the absorbance of the formed complex, whereas above 30ºC, the absorbance start to decay. The absorbance remains stable for at least 3 h.

Effect of Acid
The reaction product, green color found to flocculate with in 20–30 min of color development. To delay the flocculation, acid added before dilution. Hydrochloric acid found to give more stable color and reproducible results.

Effect of Order in Which Reagents Added
After fixing all other parameters, a few of the experiments were performed to ascertain the influence of the order in which reagents were added. The following order: Drug-reagent –acid gave maximum absorbance and stability

The Nature of The Reaction Product
Job's method of continuous variations using equimolar concentrations of the drug (base form) and reagents (2.8 $x10^{-5}$ M in method A and 4.2 $x10^{-5}$ M in method B). The results indicated that 1:1 (drug/reagent) complex formed in all the methods. Five solutions containing valacyclovir and the reagent Brucine or MBTH in various molar ratios, with a total volume of 10 mL in all the methods were prepared. The absorbance of solutions ware subsequently measured at 543 nm in method A and 644 nm in method B. The graphs of the results obtained (Figure. 4) gave a maximum at a molar ratio of X_{max} =0.5 in all the methods which indicated the formation of a 1:1 complex between valacyclovir and Brucine or MBTH reagents.

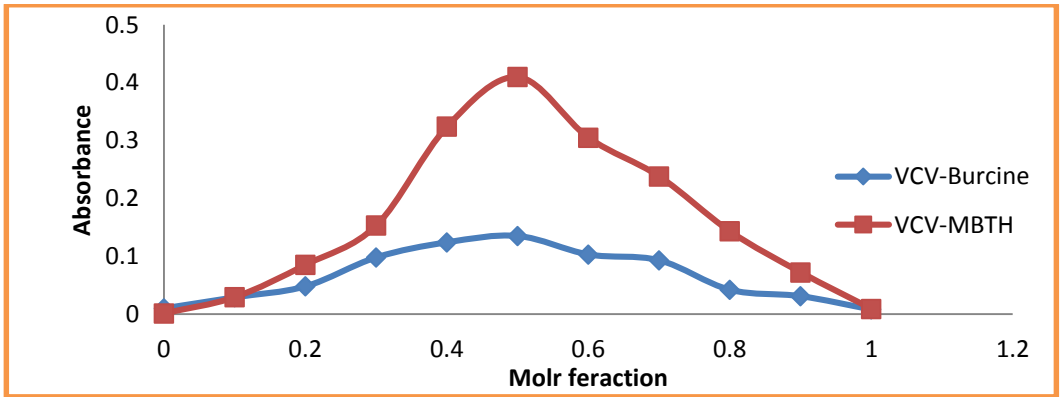

Fig 4: Job's continuous-variations plots

Effects of Interference

In pharmaceutical analysis, it is important to test the selectivity towards the excipients and fillers added to the pharmaceutical preparations. Several species which can occur in the real samples together with drug were investigated. The level of interference was considered acceptable. Commonly, encountered excipients such as talc, starch, glucose etc. did not interfere in the determination. In order to apply the proposed method to the analysis of pharmaceutical formulations, the influence of commonly used excipients starch, lactose, glucose, sugar, talc, sodium chloride, titanium dioxide and magnesium sterate and additives was studied by preparing by mixing known amount (20 mg) of PRX with various amounts of the common excipients: starch (50 gm), glucose (10 gm), lactose (10 gm), acacia (10 mg), talc (5 mg), and magnesium stearate (10 mg). These laboratory-prepared samples were analyzed by the proposed method applying the general recommended procedure. The recovery values were 97.01 -101.62 ± 0.15– 1.06%, (Table 1). These data confirmed the absence of interference from any of the common excipients with the determination of VCV by the proposed method.

Method Validation

Linearity and Sensitivity

At the established experimental conditions, standard calibration curves for Valacyclovir with Burcine and MBTH reagents were constructed by plotting absorbance verses concentration. The linear regression curves were obtained in the Beer's law range of 5.0-110 and 2.0-45 µg/mL with correlation coefficient 0.9968 and 0.9990 in each case respectively. Regression characteristics including slope, intercept, correlation coefficient and also the molar absorptivity values for each proposed method are given in (Table 1). The detection limit (LOD) and quantification limit (LOQ) were calculated by using the following equations:

$$LOD = \frac{3.3 \times \sigma}{S} \& LOQ = \frac{10 \times \sigma}{S}$$

Where, σ is the standard deviation of seven replicate determinations under the same conditions as for the sample in the absence of the analyte and S is the slope of the calibration graph. The LOD values were calculated to be 0.995 and 0.366 µg/mL respectively (Table 2).

Table 1: Analysis of valacyclovir in presence of common excipients by the proposed method.

Excipients	Recovery%*± SD
Starch	99.91±0.15
Glucose	100.23±1.03
Lactose	101.62±0.35
Acacia	98.62±0.18
Talc	101.58±1.06
Magnesium stearate	97.01±0.58

*Average of three determinations.

Table 2: Optimum conditions and analytical parameters

Parameters	VCV-Butcine complexes	VCV-MBTH complexes
λmax (nm)	543	644
Linearity range µg/mL	5.0 - 110	2.0 - 45
Molar absorptivity L/mol.cm	4642.80	9761.90
Slope (b)	0.0095	0.0283
Intercept (a)	0.0235	0.0213
Correlation coefficient	0.9968	0.9990
LOD µg/mL	0.995	0.366
LOQ µg/mL	3.010	1.109

A = b x ± a, where a = intercept, b = slope, and x = concentration (µg/mL)

Table 3: Evaluation of intra-day and inter-day precision and accuracy.

Method	Valacyclovir taken µg/mL	Intra-day (n=5)			Inter-day (n=5)		
		Found [a] µg/mL	%RSD [b]	% RE [c]	Found µg/mL	%RSD [b]	% RE
Method A	5	5.031	1.23	0.628	4.991	0.772	0.176
	10	10.056	0.852	0.556	10.003	0.117	0.028
	15	14.985	0.616	0.100	14.999	0.211	0.008
Method B	2	2.006	0.885	0.320	2.012	1.29	0.610
	8	7.987	0.976	0.160	8.023	0.647	0.293
	15	14.999	0.696	0.007	15.015	0.507	0.097

[a]Mean value of five determinations; [b] Relative standard deviation (%); [c]Relative error (%).

Accuracy and Precision

In order to determine the accuracy and precision of the proposed methods, solutions containing four different concentration of valacyclovir were prepared and analyzed in five replicates. The relative standard deviation as precision and percentage relative error (Err%) as accuracy of the suggested methods were calculated at 95% confidence levels, and can be considered satisfactory. Precision was carried out by five determinations at four different concentrations in these spectrophotometric methods. The percentage relative error was calculated according to the following equation. Err% = [(Found – Added) /Added] x100. The inter-day and intra-day precision and accuracy results are shown in (Table 3). The analytical results for accuracy and precision show that the methods proposed have good repeatability and reproducibility.

Robustness and Ruggedness

To evaluate the robustness of the methods, two important experimental variables volume of reagent and reaction time, were slightly altered and the effect of this change on the absorbance of the charge transfer complexes was studied. The results of this study are presented in (Table 4) and indicated that the proposed methods are robust. Methods ruggedness was evaluated by performing the analysis following the recommended procedures by two different analysts and on three different spectrophotometers by the same analyst. From the %RSD values presented in (Table 4), one can conclude that the proposed methods are rugged.

Applications to Analysis of Tablets

The proposed methods were applied to the determination of Valacyclovir in tablets and capsules (Table 5). The results obtained were statistically compared with those of the official method [20] by applying the Students t-test for accuracy and F-test for precision. The official method describes a UV spectrophotometric Valacyclovir of solution in 0.1 M HCl shows maximum absorbance at 255 nm. As can be seen from the Table 5, the calculated t-test and F-value at 95 % confidence level did not exceed the tabulated values of 2.78 and 6.39, respectively, for four degrees of freedom. The results indicated that there is no difference between the proposed methods and the official method with respect to accuracy and precision.

Analysis of Recovery of Valacyclovir from Blood and Urine Samples

These proposed methods were also applied to the determinations of Valacyclovir in blood and urine samples. The blood and urine samples were prepared for the analysis of recovery of Valacyclovir with these proposed methods. The blood and urine samples were obtained from healthy volunteers. Blood sample (5 mL) and urine sample (5 mL) collected from healthy volunteers who had not taken any medicine during a proceeding week were incubated at 30°C for 1h and centrifuged at 3000 r/min for 15 min. The blood and urine samples were added respectively according to the proposed procedure. The results were presented in (Table 6). High accuracy and good recoveries were obtained, which indicates that the proposed methods can be successfully applied to recover Valacyclovir in the blood and urine samples.

Table 4: Robustness and ruggedness.

Method	Valacyclovir taken µg/mL	Method robustness		Method ruggedness	
		Parameters altered			
		Reagent volume [a,b], mL RSD%(n=3)	Reaction time [c,d] RSD%,(n=3)	Inter-analysts RSD%, (n=3)	Inter-cuvettes RSD%, (n=3)
Method A	5	0.274	0.260	0.464	0.356
	10	0.169	0.165	0.235	0.467
Method B	2	0.935	0.505	0.493	0.229
	15	0.071	0.108	0.064	0.034

[a] In methods A, the volume of reagent was 2, 2.5 and 3 mL. [c] The reaction time was 12, 15 and 17min.
[b] In methods B, the volume of reagent was 8, 1.0 and 1.2 mL. [d] The reaction time was 4, 5 and 6 min.

Table 5: Results of analysis of tablets by the proposed methods.

Tablet brand name	Labeled amount mg/tablet	Method	Amount taken (µg/mL)	Amount found* (µg/mL)	%Recovery ±SD*	T-test**	F-test***
Valterx	500	Method A	5	5.06	101.2± 0.59	1.23	2.41
			10	9.94	99.4±1.09		
		Method B	2	2.01	100.5± 1.35	1.86	2.93
			10	9.91	99.1± 0.84		
		Official method	10	10.11	101.10± 0.83	-	-
Valciver	500	Method A	5	5.13	102.6±0.67	1.41	1.84
			10	10.16	101.6± 1.11		
		Method B	2	1.98	99.0±0.52	1.07	2.46
			10	9.95	99.5± 1.35		
		Official method	10	10.08	100.8 ± 1.05	-	-

*Mean value of five determinations.
**Tabulated t-value at the 95% confidence level is 2.78.
***Tabulated F-value at the 95% confidence level is 6.39.

Table 6: The recovery of Valacyclovir in blood and urine.

Sample	Method A			Method B		
	Amount added µg/mL	Amount found µg/mL	% Recovery ± RSD	Amount added µg/mL	Amount found µg/mL	% Recovery ± RSD
Blood	5	5.09	101.8±0.35	4	3. 97	99.3±0.51
	10	10.17	101.7±0.11	10	10.20	102.0±0.27
Urine	5	4.94	98.8±0.72	4	4.02	100.5±0.46
	10	9.97	99.7±0.81	10	9.89	98.9±0.69

Table 7: Results of recovery study.

Tablets studied	Valacyclovir in tablets µg/mL	Pure Valacyclovir added µg/mL	Total found µg/mL	%Recovery* ±SD
Valterx	10	5	15.22	101.47 ± 0.62
		10	19.85	99.25±1.04
		15	24.89	99.56 ± 0.54
Valciver	16	8	24.19	100.79±0.86
		16	32.70	102.18±1.23
		24	40.76	101.98±0.88

*Mean value of three determinations.

Recovery Study

To further ascertain the accuracy of the proposed methods, recovery experiment was performed via standard addition technique. To a fixed and known amount of Valacyclovir in tablet powder (preanalyzed), pure Valacyclovir was added at three concentration levels (50, 100, and 150% of the level present in the tablet), and the total was measured by the proposed methods. The determination with each concentration was repeated three times, and the results of this study presented in (Table 7) indicated that the various excipients present in the formulations did not interfere in the assay, thereby further confirming the accuracy of the methods.

CONCLUSION:

Two rapid, simple, sensitive and selective spectrophotometric methods have been developed for the quantitative estimation of Valacyclovir in pharmaceutical formulations and different human body fluids (blood and urine). The proposed methods were based on the reduction of the nitro group to amino group of the drug. The methods were developed and validated as per the current ICH guidelines. The results showed that the proposed procedures compared favorably with reference method are satisfactory sensitive, accurate and precise. The optical characteristics such as Beer's law limits, molar absorptivity, and various statistical data were reported. The results of the analysis for the two methods have been validated statistically.

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