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Research Paper

Simple and Sensitive Spectrophotometric Determination of Ganciclovir Using Bromate-Bromide, M-Cresol Purple and Erioglaucine

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Two spectrophotometric methods, which are simple and sensitive, are described for the determination of ganciclovir (GNC) in bulk drug and in its formulations. The methods use bromate-bromide, m-cresol purple and erioglaucine as reagents. GNC is treated with a known excess of bromate-bromide mixture in acid medium and after the bromination reaction is ensured to be complete, the unreacted bromine is estimated by treating with a fixed amount of either m-cresol purple and measuring the absorbance at 540 nm (method A) or erioglaucine and measuring the absorbance at 630 nm (method B). In both methods, the amount of bromate that reacted corresponds to the drug content. The calibration graphs were found to be linear over 0.5-10 and 0.6-7.5 μg mL^{-1} for method A and method B, respectively, with corresponding molar absorptivity values of 1.86 $x10^5$ and 2.29 x 10^4 L mol^{-1} cm^{-1}. The Sandell sensitivity values, limits of detection (LOD) and quantification (LOQ) are also reported. Accuracy and precision of the assays were determined by computing the intra-day and inter-day variations at three different levels of GNC; the intra-day and inter-day RSD were < 2.66% and the accuracy was better than 2.49%. The methods were successfully applied to the determination of GNC in two different brands of tablets with good accuracy and precision and without detectable interference by excipients. The accuracy was further ascertained by placebo blank and synthetic mixture analyses and also by recovery experiments *via* standard-addition procedure.

Key Words: Ganciclovir; Determination; Titrimetry; Spectrophotometry; Pharmaceuticals

Introduction

Ganciclovir (GNC), chemically known as 2-amino-9-{[(1,3-dihydroxypropan-2-l)oxy]methyl}-6,9-dihydro-3*H*-purin-6-one (Fig. 1) is a nucleoside analogue widely used in the treatment of cytomegalovirus infections. It has proved to be effective against cytomegalovirus in immunocompromised patients, mainly in those with the acquired immunodeficiency syndrome (AIDS), congenital immunodeficiency or in individuals following organ transplantation [1, 2]. Various techniques have been developed for the determination of GNC in pharmaceuticals. It is official in the United States Pharmacopoeia [3], which describes a HPLC method for its determination in injections and in oral suspension.

The literature is enriched with several methods for the determination of GNC in pharmaceutical dosage forms including body fluids. The most extensively used technique for the quantitation of ganciclovir is HPLC but, most of the procedures using this technique are devoted to body fluids like plasma [4-14], plasma and tissues [15], serum [16] and blood samples [17]. HPLC has also been applied for the determination of GNC in eye drops [18]. GNC in bulk drug and in its formulations has been assayed by UV-spectrometry by measuring the absorbance of 0.1M HCL and 0.1M NaOH at 253 and 266 nm,

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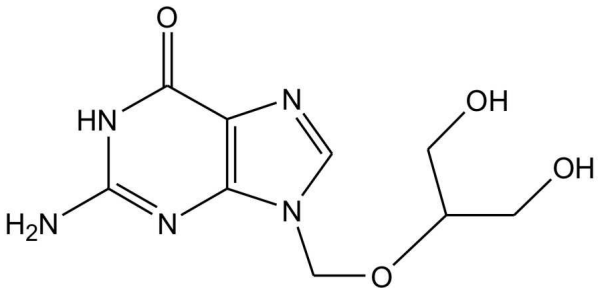


Fig. 1: Structure of Ganciclovir (GNC)

respectively [19]. The methods are reported to be moderately sensitive with molar absorptivity values of 2.0 x 10^3. There are only three reports on the use of visible spectrophotometry for the assay of GNC in pharmaceuticals. Schelling *et al.* [20] have devised an enzyme kinetic method which involves the use of multiple reagents and scrupulously controlled experimental variables. The procedure also requires expensive radiolabelled substrates. Recently, Gouda [21] has reported the application of five σ and π-acceptors for the spectrophotometric determination of GNC in its formulations *via* charge-transfer complexation reaction. The methods have been found to be applicable over 5-225μg ml^{-1} ranges with limits of detection (LOD) values in the range of 0.36-2.45 μg ml^{-1}. In a very recent report, [22] Prakash *et al.* have described two procedures based on Schiff base formation using p-dimethylaminocinnamaldehyde and oxidative coupling reaction involving the use of iron(III) and 3-methyl-2-benzothiazolinone hydrazone, but the methods are poorly sensitive with linear dynamic range of 10-50 μg ml^{-1} and 50-250 μg ml^{-1} and also require heating at 40°C, respectively. The methods also employ expensive reagents. A flow injection chemiluminescence method has also been described by Wang *et al.* [23]. Though the method appears to be sensitive with a LOD of 2.35 μg ml^{-1} it requires a complicated and expensive flow-injection assembly as shown in Table 1.

In this paper, two spectrophotometric methods for the determination of GNC in pharmaceuticals based on bromination reaction using bromate-bromide mixture and by employing two dyes are described. The developed methods were optimized and validated as per the guidelines of the International Conference on Harmonization [24]. The methods are important alternatives to other analytical methods with clear advantages in terms of simplicity, cost effectiveness, speed and sensitivity.

EXPERIMENTAL

Apparatus

A Systronics model 106 digital spectrophotometer with 1-cm matched quartz cells was used for all absorbance measurements.

Reagents and Materials

All the reagents used were of analytical-reagent grade and distilled water was used throughout the investigation.

Bromate-bromide Mixture

A 1000 μg mL^{-1} $KBrO_3$ solution containing a large excess of KBr was prepared by dissolving 100 mg of $KBrO_3$ and 1 g of KBr in water and diluting to the mark in a 100 mL calibrated flask. This was diluted stepwise to get 40 and 18 μg mL^{-1} bromate solutions for use in method A and method B, respectively.

Hydrochloric Acid (0.1M, 1M and 5M): Concentrated acid (S.d. Fine Chem, Mumbai, India, Sp gr 1.18) was diluted appropriately with water to get 0.1 M,1M (method A) and 5 M (method B).

m-Cresol Purple (100 μg mL^{-1}): First, a 1000 μg mL^{-1} solution was prepared by dissolving 118 mg of dye (S.d. Fine Chem, India, dye content 85%) in 10 ml of 0.05M sodium hydroxide and diluted to the mark with water in a 100 mL calibrated flask and filtered. This was diluted 10-fold to obtain a working concentration of 100 μg mL^{-1}.

Erioglaucine (300 μg mL^{-1}): A 1000 μg mL^{-1} solution was first prepared by dissolving 111 mg of dye (S.d. Fine Chem, Mumbai, India, dye content 90%) in water and diluting to the mark in a 100 mL calibrated flask and filtered. The stock solution was diluted appropriately to get 300 μg mL^{-1} with water.

Pure GNC (Pharmaceutical grade) sample was kindly provided by S.d. Fine Chem. India Ltd., as a

Table 1: Comparison of the performance characteristics of the present spectrophotometric methods with the published methods

S.No.	Reagents used	Methodology	λ_{max} (nm)	Linear range	LOQ (μg mL^{-1})	Remarks	Ref.
1.	ATP, NADH, NAD	Enzymatic	340	-	-	Requires vigorous control of experimental variables and expensive radiolabelled substrates; multiple reagents required	22
2.	σ and π-donors	C-T complexation reaction	-	5-225 μg/mL	0.36-2.45 μg/mL	Less sensitive and multiple reagents used	23
3.	a) p-dimethylamino-cinnamaldehyde and MBTH b) Iron (III)- MBTH	Schiff base formation Oxidative coupling reaction	-	10-50 μg/mL	50-250 μg/mL	Poorly sensitive and expensive reagents used	24
4.	Ce(IV)-rhodamine in H2SO4 medium	Chemiluminescence reaction	-	5 x 10^{-8} mgL^{-1}	2.35 x 10^{-8} mgL^{-1}	Requires complicated and expensive flow-injection assembly	25
5	a) $KBrO_3$-KBr /HCl and m-cresol purple	Bromination of drug and determination of unreacted Br_2 with m-cresol purple	540	2-10 μg mL^{-1}	0.56	No organic solvents are used no heating or extraction step and ecofriendly chemicals used, simple instrument employed	Present work
	b) $KBrO_3$-KBr /HCl and erioglaucine	Bromination of drug and determination of unreacted Br_2 with erioglaucine	630	0.75-7.5 μg mL^{-1}	0.35		

gift and used as received. Two brands of tablets, namely, Ganguard-500 (Ranbaxy Lab., Ahmedabad, India) and Nataclovir-250 (Natco Pharma Ltd, Hyderabad, India) were purchased from the commercial sources.

Ganciclovir Standard Solution

A 100 μg m L^{-1} standard drug solution was prepared by dissolving 25 mg of pharmaceutical grade GNC in 0.1M HCl, the volume was made upto 250 mL in a calibrated flask with 0.1M HCl. This solution was then diluted with 0.1M HCl to get 20 and 30 μg mL^{-1} solutions for use in method A and method B, respectively.

General Analytical Procedures

Method A (Using m-Cresol purple)

Different aliquots (0.25-5.0 mL) of 20 μg mL^{-1} GNC solution were accurately measured into a series of 10 mL calibrated flasks and the total volume was adjusted to 5 ml with water. To each flask were added 1 mL each of bromate-bromide solution (40 μg mL^{-1} w. r. t. $KBrO_3$) and 5 M hydrochloric acid. The content was mixed well and allowed to stand for 15 min with occasional shaking. Then, 1 mL of 100 μg mL^{-1} m-cresol purple solution was added to each flask and diluted to the mark with water. The absorbance of each solution was measured at 540 nm against a reagent blank after 5 min.

Method B (Using Erioglaucine)

Varying aliquots of standard GNC solution (0.2-2.5 mL) of 30 μg mL^{-1} were transferred into a series of 10 mL calibrated flasks by means of a micro burette, and the total volume was brought to 2.5 mL by adding 1 M HCl. To each flask, 5 mL of 1 M HCl and 1 mL of bromate-bromide solution (18 μg mL^{-1} w.r.t. $KBrO_3$) were added. After mixing the content, the flasks were allowed to stand for 15 min with occasional shaking. Then, 1 mL of 300 μg mL^{-1} erioglucin solution was added to each flask and

diluted to the mark with water. The absorbance was measured at 630 nm against a reagent blank after 5 min.

A calibration graph was prepared by plotting absorbance versus concentration of drug and the concentration of the unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

Procedure for Tablets

Ten tablets each containing 250 mg of GNC were weighed accurately and pulverized. An amount of tablet powder equivalent to 10 mg was transferred into a 100 mL volumetric flask. The content was shaken well with about 70 mL of 0.1M HCl for 20 min. The mixture was diluted to the mark with 0.1M HCl. It was filtered using Whatmann No 42 filter paper. First 10 mL portion of the filtrate was discarded and suitable aliquot was subjected to analysis following the procedure described after appropriate dilution with 0.1M HCl to get 20 and 30 μg mL^{-1} GNC and general procedures were applied to determine the drug content.

RESULTS AND DISCUSSION

Many dyes are irreversibly destroyed to colourless products by oxidizing agents in acid medium [25] and this observation has been exploited for the indirect spectrophotometric determination of some bioactive compounds [26-30]. In the proposed spectrophotometric methods, the ability of bromine to cause bromination of GNC and irreversibly destroy m-cresol purple and erioglaucine dyes to colourless products in acid medium has been used. Both spectrophotometric methods are based on the bromination of GNC by a measured excess of *in situ* generated bromine and subsequent determination of the unreacted bromine by treating with m-cresol purple or erioglaucine and measuring the absorbance at 540 nm or 630 nm (Fig. 2). In either method, the absorbance increased linearly with increasing concentration of GNC.

GNC, when added in increasing concentrations to a fixed concentration of *in situ* generated bromine, consumes the latter and there will be a concomitant decrease in its concentration. When a fixed concentration of either dye is added to decreasing concentrations of bromine, a concomitant increase in the concentration of dye is obtained. This is observed as a proportional increase in absorbance at the respective λ_{max} with increasing concentration of GNC (Fig. 3).

Optimization Parameters

Preliminary experiments were performed to fix the upper limits of the dye concentrations that could be measured spectrophotometrically, and these were found to be 10 μg mL^{-1} and 30 μg mL^{-1} for m-cresol purple and erioglaucine, respectively.

A bromate concentration of 4.0 μg mL^{-1} was found to irreversibly destroy the red colour of 10 μg mL^{-1} m-cresol purple whereas 1.8 μg mL^{-1} oxidant

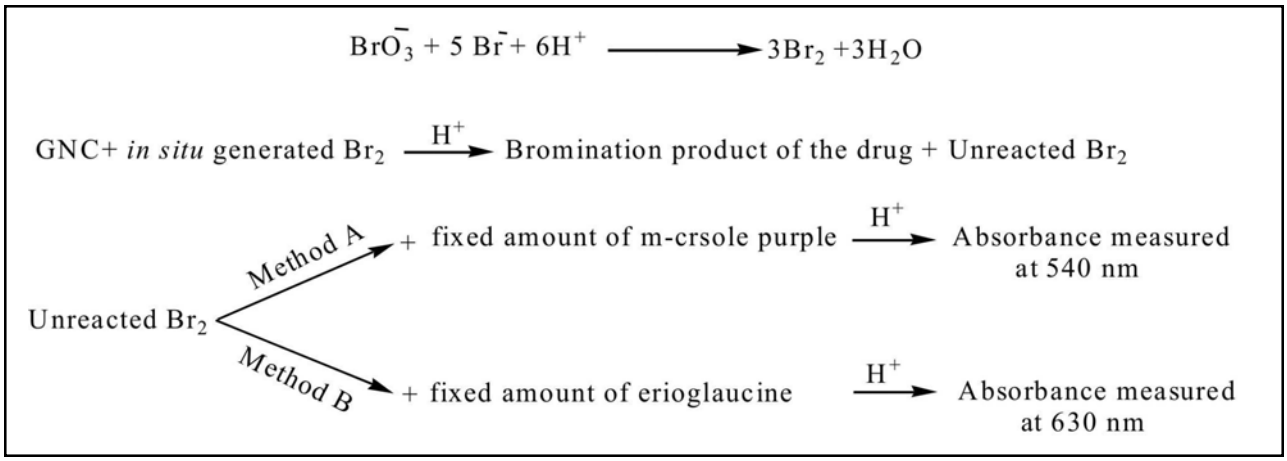


Fig. 2: Reaction scheme

was required to bleach the green colour due to 30 µg mL^{-1} erioglacine in acid medium. Hence, different concentrations of GNC were reacted with 1.0 mL of 40 µg mL^{-1} bromate in method A and 1 mL of 18 µg mL^{-1} oxidant in method B in the presence large excess of bromide and in acid medium followed by the determination of the residual bromine as described under the respective procedures.

Hydrochloric acid was the medium of choice for the bromination of GNC by bromine as well as the latter's determination employing the dyes. The absorbance of the dyes was not affected in 0.625-3.125 and 0.042-0.50 M hydrochloric acid concentration for method A and method B, respectively. However, since 1.11 M and 0.56 M hydrochloric acid in a total volume of about 8.0 and 9.0 mL for method A and method B, respectively, were found sufficient to cause bromination of drug in a reasonable time of 15 min, the same concentration was maintained for the determination of unreacted bromine with the dyes. The specified acid concentrations for bromination reaction were found not critical. The bromination reaction were to be complete in 15 min for both method A and method B, respectively, and contact times up to 60 min had no effect on the absorbance of the dyes. A contact time of 5 min in both methods was necessary for the bleaching of the dye colour by the residual bromine. The absorbance of either dye solution even in the presence of the brominated drug product was found to be stable for one day.

Table 2: Sensitivity and regression parameters for spectrophotometric methods

Parameter	Method A	Method B
λmax, nm	540	630
Linear range, µg mL^{-1}	0.5-10	0.6 -7.5
Molar absorptivity(ε), L mol^{-1} cm^{-1}	$1.99x10^4$	$2.7x10^4$
Sandell sensitivity*, µg cm^{-2}	0.0139	0.0103
Limit of detection (LOD), µg mL^{-1}	0.12	0.06
Limit of quantification (LOQ), µg mL^{-1}	0.37	0.18
Regression equation, Y**		
Intercept (a)	–0.035	–0.035
Slope (b)	0.0645	0.1021
Standard deviation of a (S_a)	0.0157	0.0104
Standard deviation of b (S_b)	0.017	0.025
Regression coefficient (r)	0.9980	0.9975

*Limit of determination as the weight in µg per mL of solution, which corresponds to an absorbance of A = 0.001 measured in a cuvette of cross-sectional area 1 cm^2 and l = 1 cm; **Y=a+bX, Where Y is the absorbance, X is concentration in µg mL^{-1}, a is intercept, b is slope

Method Validation

Analytical Parameters of Spectrophotometric Methods

A linear correlation was found between absorbance at λ_{max} and concentration of GNC in the ranges given in Table 2. The graphs are described by the regression equation:

$$Y = a + bX$$

(where, Y = absorbance of 1-cm layer of solution; a = intercept; b = slope and X = concentration in µg mL^{-1}). Regression analysis of the Beer's law data using the method of least squares was made to evaluate the slope (b), intercept (a) and correlation coefficient (r) for each system and the values are presented in Table 2. A plot of absorbance and concentration, yielded straight lines with slopes equal to 0.0645 and 0.1021 for method A and method B, respectively, further establishing the linear relation between the two variables. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell sensitivity values [29] of all the three methods are also given in Table 2. The limits of detection (LOD) and quantitation (LOQ) calculated according to ICH guidelines [23] using the formulae:

LOD = 3.3 S/b and LOQ = 10 S/b, (where, S is the standard deviation of blank absorbance values, and b is the slope of the calibration plot) are also presented in Table 2. The high values of ε and low values of Sandell sensitivity and LOD indicate the high sensitivity of the proposed methods.

Accuracy and Precision of the Methods

To compute the accuracy and precision, the assays described under "general procedures" were repeated seven times within the day to determine the repeatability (intra-day precision) and five times on different days to determine the intermediate precision (inter-day precision) of the methods. These assays were performed on three levels of analyte. The results of this study are summarized in Table 3. The percentage relative standard deviation (%RSD) values were ≤ 2.66% (intra-day) and ≤ 2.49% (inter-day) indicating high precision of the methods. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and taken concentrations for GNC. Bias {bias % = [(Concentration found-known concentration) x 100/ known concentration]} was calculated at each concentration and these results are also presented in Table 3. Percent relative error (%RE) values of ≤ 2.99% demonstrates the high accuracy of the proposed methods.

Selectivity

The proposed methods were tested for selectivity by placebo blank and synthetic mixture analyses. A placebo blank containing talc (250 mg), starch (300 mg), lactose (30 mg), calcium carbonate (50 mg), calcium dihydrogen orthophosphate (20 mg), methyl cellulose (40 mg), sodium alginate (70 mg) and magnesium stearate (100 mg) was extracted with 0.1M HCl and solution made as described under "analysis of dosage forms". A convenient aliquot of solution was subjected to analysis by spectrophotometry (method A and method B) according to the recommended procedures. In all the cases, there was no interference by the inactive ingredients.

A separate test was performed by applying the proposed methods to the determination of GNC in a synthetic mixture. To the placebo blank of similar composition, 10 mg of GNC was added, homogenized and the solution of the synthetic mixture was prepared as done under "analysis of tablet". Synthetic mixture solution (100 μg mL^{-1} in GNC) was appropriately diluted to get 20 and 30 μg mL^{-1} solutions, and analysed by method A and method B, separately, and the corresponding percentage recoveries of GNC were calculated to be 96.38±1.85 and 97.33±2.33. The results demonstrated the accuracy as well as the precision of the proposed methods. These results complement the findings of the placebo blank analysis with respect to selectivity.

Robustness and Ruggedness

The robustness of the methods was evaluated by making small incremental changes in the volume of acid and contact time, and the effect of the changes was studied on the absorbance. The changes had

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

Method	GNC taken*	Intra-day accuracy and precision (n=7)			Inter-day accuracy and precision (n=5)		
		GNC found* ±SD	%RE	%RSD	GNC found*±SD	%RE	%RSD
A	6.0	6.11±0.09	1.80	1.44	6.10±0.09	1.53	2.49
	8.0	8.04±0.12	0.44	1.54	8.02±0.15	0.29	2.84
	10.0	9.93±0.24	0.71	2.37	9.98±0.23	0.18	2.30
B	4.5	4.40±0.04	2.31	2.66	4.39±0.05	2.49	2.50
	6.0	5.93±0.05	1.13	1.59	5.93±0.06	1.14	2.44
	7.5	7.32±0.07	2.42	2.48	7.34±0.05	2.16	2.99

*The values are in μg mL^{-1} for method A and method B; %RE. Percent relative error, %RSD. relative standard deviation and SD. standard deviation respectively, at the 95% confidence level

negligible influence on the results as revealed by small intermediate precision values expressed as % RSD ($\leq$ 2.68%). Method ruggedness was expressed as the RSD of the same procedure applied by four different analysts as well as using three different cuvettes, the inter-analysts RSD were within 2.15% whereas the inter-cuvettes RSD for the same GNC concentrations were within 2.20% suggesting that the developed methods were rugged. The results are shown in Table 4.

Table 4: Method robustness and ruggedness expressed as intermediate precision (% RSD)

Method	GNC taken[#]	Robustness		Ruggedness	
		Parameter altered			
		Volume of HCl[*] (%RSD)	Reaction time[**]	Inter-analysts, (%RSD) (n=4)	Inter-cuvettes, (%RSD) (n=4)
A	6.0	1.18	2.74	2.15	2.20
B	3.0	2.02	2.02	2.09	1.88

[#]The values are in µg mL^{-1} for method A and method B. [*]Volumes of HCl added in method A were 1.8, 2.0 and 2.2 mL and method B were 4.8, 5.0, and 5.2 [**]In method A and method B reaction time studied were 14, 15, and 16 min

Application to Formulations

The proposed methods were applied to the determination of GNC in two representative tablets Ganguard-500 and Nataclovir-250 purchased from local stores and containing other inactive ingredients. The results in Table 5 show that the methods are successful for the determination of GNC and that the excipients in the dosage forms did not interfere. The results obtained (Table 5) were statistically compared with the official USP method [3]. The same batch tablets were analysed by the official USP method. The method describes that the samples was chromatographed on a column (4.6 mm x 10 cm) containing packing L_1 with a mobile phase consisting of 1.04 g of monobasic ammonium phosphate and 2.0 g of phosphoric acid in 1 liter water, at a flow rate of 1.2 mL min^{-1} and the UV-detection being set at 254 nm. The results obtained by the proposed methods agreed well with those of reference method and with the label claim. When the results were statistically compared with those of the reference method by applying the Student's t-test for accuracy and F-test for precision, the calculated Student's t-value and F-value [30] at 95% confidence level did not exceed the tabulated values of 2.77 and 6.39, respectively, for four degrees of freedom. Hence, no significant difference exists between the proposed methods and the reference method with respect to accuracy and precision.

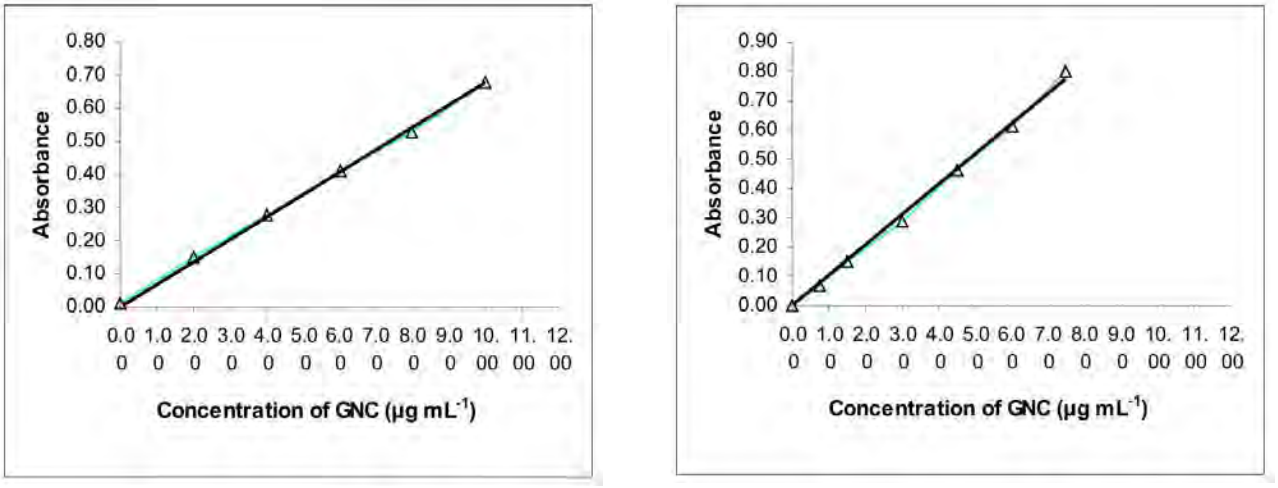
Recovery Study

To further assess the accuracy of the methods, recovery experiments were performed by applying the standard-addition technique. The test was done

Table 5: Results of analysis of tablets by the proposed methods and statistical comparison of the results with the reference method

Tablet brand name	Nominal amount(mg/tablet)	Found[*] (Percent of label claim ± SD)		
		Reference method	Proposed methods	
			Method A	Method B
Ganguard-500	500	100.5±0.73	99.52±0.58 t = 0.36 F = 1.58	99.14±0.54 t = 0.47 F = 1.82
Nataclovir-250	250	102.3±1.40	101.3±1.30 t = 1.17 F = 1.16	100.8±1.02 t = 1.48 F = 1.88

[*]Average of five determinations; Tabulated t = value at the 95% confidence level is 2.77. Tabulated F = value at the 95% confidence level is 6.39



Method-A **Method-B**

Fig. 3: Linearity graph

by spiking the pre-analysed tablet powder with pure GNC at three different levels (50, 100 and 150 % of the content present in the tablet powder taken) and the total was found by the proposed methods. Each test was repeated three times. In all the cases, the recovery percentage values ranged between 98.52 and 104.7% with relative standard deviation in the range 1.05-2.36%. Closeness of the results to 100 % showed the fairly good accuracy of the methods. The results are shown in Table 6.

Conclusions

Two useful methods for the determination of GNC using bromate-bromide mixture, m-cresol purple and erioglaucine have been developed and validated according to ICH guidelines. The proposed spectrophotometric methods do not require any expensive equipment and specialized technicians when compared alongside HPLC and bioassay. The proposed methods are one of the most sensitive ever reported for GNC and are much simpler than the existing spectrophotometric methods with respect to optimum conditions. They do not involve stringent experimental conditions unlike the reported methods [4-14]. They rely on the use of simple and inexpensive chemicals. An additional advantage of the methods is that the measurement is made at longer wavelengths where the interferences from the co-formulated substances is far less than that at shorter wavelengths employed in most reported methods. These

Table 6: Results of recovery study using standard addition method

Tablet studied	Method A				Method B			
	GNC in tablet extract, µg mL^{-1}	Pure GNC added, µg mL^{-1}	Total GNC found, µg mL^{-1}	Pure GNC recovered (Percent±SD*)	GNC in tablet extract, µg mL^{-1}	Pure GNC added, µg mL^{-1}	Total GNC found, µg mL^{-1}	Pure GNC recovered (Percent±SD*)
Ganguard-500	3.98	2.0	6.09	101.52±1.09	2.99	1.50	4.38	100.1±1.06
	3.98	4.0	8.02	100.3±2.15	2.99	3.00	5.93	104.7±1.05
	3.98	6.0	9.85	98.52±2.23	2.99	4.50	7.30	102.6±1.08
Nataclovir-250	3.99	2.0	6.12	102.5±2.36	3.00	1.50	4.52	99.05±1.15
	3.99	4.0	8.01	101.1±2.40	3.00	3.00	5.98	101.3±1.82
	3.99	6.0	9.94	99.7±2.16	3.00	4.50	7.28	100.7±2.16

*Mean value of three determinations

advantages coupled with good accuracy and precision make the methods highly suitable for routine use in laboratories where the modern and expensive instruments are not available.

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