Research Paper

Quantification of an Antiviral Drug(Stavudine) using Bromate-Bromide as an Agent based on Redox and Complexation Reactions

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Two simple, sensitive and rapid methods are described for the determination of stavudine (STV) in bulk drug and in formulations using bromate-bromide as the oxidimetric reagent. The methods are based on the oxidation of STV by known amount *in situ* generated bromine followed by determination of unreacted bromine by two different reaction schemes. In both procedures, the residual bromine is reduced by an excess of iron(II), and the resulting iron(III) is complexed with thiocyanate and measured at 470 nm (method A) or with tyron at pH 1.09 and measured at 670 nm (method B). In both methods, the absorbance is found to decrease linearly with STV concentration. Beer's law is obeyed over the ranges 0.5-4.0 and 2-30 µg mL^{-1} for method A and method B, respectively. The calculated molar absorptivity values are 5.6×10^4 and 5.2×10^3 L $mol^{-1}cm^{-1}$ for method A and method B, respectively. The methods were successfully applied to the determination of STV in formulations and the results tallied well with the label claim and the results were statistically compared with those of a reference method by applying the Student's t-test and F-test. No interference was observed from the concomitant substances normally added to preparations. The accuracy and validity of the methods were further ascertained by performing recovery experiments *via* standard-addition method.

Key Words: Stavudine Determination; Spectrophotometry; Bromate-Bromide; Pharmaceuticals

Introduction

Stavudine (STV), chemically known as 2^1-3^1- didehydro -2^1-3^1- dideoxythymidine (Fig. 1), is a nucleoside analog reverse transcriptase inhibitor (NARTI) active against HIV [1]. STV is phosporylated by cellular kinases into active triphosphate. Stavudine triphosphate inhibits the reverse transcriptase by competing with natural substrate, thymidine triphosphate. It also causes termination of DNA synthesis by incorporating into it. STV is the fourth antiretroviral drug in the market and is indicated for HIV infections[2]. Various techniques have been used for the determination of STV in biological matrices. The drug in blood plasma has been determined by high performance liquid chromatography (HPLC),[3-5] and liquid chromatography-mass spectrometry (LC-MS)[6]; blood serum by LC-MS[7,8], capillary electrophoresis[9] and micellar electrokinetic chromatography (MEKC)[10]; in human cells *in vivo* by radio immuno assay (RIA) technique[11] and other biological materials by LC-MS[12,13]. Many methods have been reported for the determination of STV in pharmaceuticals and they include HPLC with UV-detection[14-19], HPTLC[20,21], LC-MS[22] and mass spectrophoto-metry[23]. All these methods require time-consuming sample preparation and expensive instrumentation. The HPLC methods[14-19] are either poorly sensitive or applicable to multi-component dosage forms, and face the problem of short column life. An

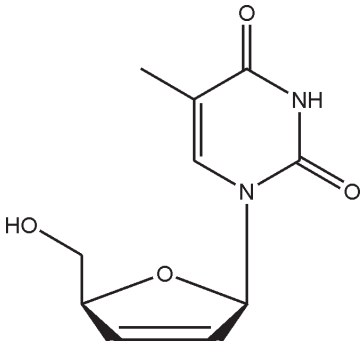

Figure 1: *Structure of stavudine*

UV-spectrophotometric method[24] has also been described for the determination of STV in pharmaceutical preparations.

Visible spectrophotometry, because of its simplicity, speed, sensitivity, reasonable accuracy and precision, and cost-effectiveness, continues to be the most preferred method for routine analytical work. There are three reports on the visible spectrophotometric determination of STV. Sharma *et al.*[25] have used three reaction schemes using permanganate-Fast Green FCF, permanganate/periodate-MBTH and iron(III) chloride-ferricyanide as reagents for the assay of STV. The same authors [26] applied NBS-celestine blue, cobalt thiocyanate and ammonium molybdate as chromogenic agents. Sensitive determination of STV based on oxidative-

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coupling reaction involving the use of iron (III) chloride and 3-methyl-benzothiazolinone hydrazone (MBTH) was developed by Sankar *et al*.[27]. But most of the reported methods suffer from one or the other disadvantage such as poor sensitivity, heating or extraction step and/or use of expensive chemical and organic solvent (Table 1).

The objective of this investigation was to devise simple, rapid, sensitive and economically viable procedures that could be used to determine STV in bulk drug and pharmaceutical dosage forms. The methods rely on the use of bromate-bromide as the oxidimetric reagent, and iron(II) and thiocyanate or tyron as the subsidiary reagents. The proposed methods have been demonstrated to be superior to the reported methods with respect to speed, simplicity, sensitivity, cost-effectiveness and eco-friendliness.

Experimental Procedure

Apparatus

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

Reagents and Materials

All chemicals were of analytical reagent grade and distilled water used to prepare solutions.

Bromate-bromide mixture (20 and 150 µg mL^{-1} in $KBrO_3$). A stock standard solution equivalent to 1000 µg mL^{-1} $KBrO_3$ and a large excess of KBr was first prepared by dissolving accurately weighed 100 mg of $KBrO_3$ and 1g of KBr in water and diluting to the mark with water in a 100 mL calibrated flask. This was diluted stepwise to obtain working concentrations containing 20 and 150 µg mL^{-1} $KBrO_3$ for use in method A, and method B, respectively.

Hydrochloric acid: Concentrated hydrochloric acid (S.D. Fine Chem, Mumbai, India; sp. gr. 1.18) was diluted appropriately with water to get 5 M for method A and diluted further to get 1 M for use in method B.

Ferrous ammonium sulphate, FAS (400 and 1400 µg mL^{-1}): A stock solution equivalent to 0.01 M FAS was prepared by dissolving about 400 mg of the salt (S.d. Fine Chem, Mumbai, India) in 50 mL of water containg 1mL of dil H_2SO_4, and diluted to 100 mL with water, and standardized[28] using pure potassium dichromate. The stock solution was then diluted appropriately with water to get 400 and 1400 µg mL^{-1} FAS for method A and method B, respectively.

Tyron (1.0%): About 1.0 g of Tyron (Loba Chemie, Mumbai, India) was dissolved in 100 mL of water.

Ammonium thiocyanate (3 M): Prepared by dissolving 23g of the chemical (S.d. Fine Chem. Ltd., Mumbai, India) in 100 mL water.

Sodium acetate tri hydrate (1.5 M): Prepared by dissolving 24.5 g of the chemical (S.d. Fine Chem. Ltd., Mumbai, India) in 100 mL water.

Buffer of pH 1.09: Prepared by mixing of 50 mL 1 M sodium acetate and 70 mL of 1 M HCl and diluting to 250 mL with distilled water.

Standard drug solution: Pharmaceutical grade STV (99.8% pure) was received from Cipla India Ltd, as gift

Table 1: Comparison of performance characteristics of proposed methods with the existing spectrophotometric methods

Sl. No.	Reagent*	λ_{max}, nm	Linear range, µg mL^{-1}	ϵ, L $mol^{-1}cm^{-1}$	Remarks	Ref
1.	(a) NBS-celestine blue	540	0.7-6.0	1.6×10^4		25
	(b) Cobalt thiocyante	610	1.5-15.0	7.7×10^3	Involves extraction step with organic solvent, less sensitive	
	(c) Ammonium molybdate	700	11-150	1.0×10^3	Requires heating; least sensitive.	
2.	Iron (III)-MBTH				Longer contact time, expensive chemical	26
3.	(a) $KMnO_4$-Fast green FCF	640	1-8	1.28×10^4		27
	(b) KMnO4/NaIO4-MBTH	620	0.6-6.0	2.02×10^4	Longer contact time, expensive chemical	
	(c) Iron (III)-ferricyanide.	740	9.0-75.0	1.24×10^3	Less sensitive	
4.	(a) Bromate-bromide/thiocyanate	470	0.5-4.0	5.6×10^4	Wide linear dynamic range, highly sensitive	Present method
	(b) Bromate- bromide/tyron	670	2-30	5.2×10^3	No heating/extraction, Shorter reaction time, wide linear dynamic range and sensitive.	

*MBTH. 3-methylbenzothiazolinone hydrazone; NBS. N-bromosuccinimide.

and was used as received. A stock standard solution containing 500 µg mL^{-1} STV was prepared by dissolving accurately weighed 50 mg of pure drug in water and diluting to the mark in a 100 mL calibrated flask. This was diluted appropriately with water to get a working concentration of 10 µg mL^{-1} STV for method A and 100 µg mL^{-1} STV for method B.

Procedures

Spectrophotometry using Iron(II) and Thiocyanate (method A)

Different aliquots (0.5—4.0 mL) of standard 10 µg mL^{-1} STV solution were accurately measured and transferred into a series of 10 mL calibrated flasks by means of a micro burette and the total volume was adjusted to 4.0 mL by adding water. To each flask was added 1mL each of 5 M HCl and bromate-bromide reagent (20 µg mL^{-1} in $KBrO_3$), the last being added using microburette. The flasks were stoppered, then content was mixed and the flasks were let stand for 10 min. Then, 1 mL of 400 µg mL^{-1} FAS was added to each flask (micro burette), and again the flasks were let stand for 5 min followed by the addition of 1 mL of 3 M thiocyanate. The volume was diluted to the mark with water, mixed well and absorbance of each solution was measured at 470 nm against water blank.

Spectrophotometry using Iron(II) and Tyron (Method B)

Varying aliquots (0.2—3.0 mL) of standard STV solution (100 µg mL^{-1}) were accurately measured into a series of 10 mL calibrated flasks by means of a micro burette and the total volume was brought to 3 mL by adding water. The solution in each flask was acidified by adding 1 mL of 1 M HCl before adding 1mL of bromate-bromide reagent (150 µg mL^{-1} in $KBrO_3$)by means of micro burette. The flasks were stoppered, then content was mixed well and allowed to stand for 5 min with occasional shaking. To each flask was then added 1mL of 1400 µg mL^{-1} FAS, and after 5 min, 1 mL each of 1.5 M sodium acetate, buffer of pH 1.09 and 1% tyron were added and diluted to the mark with water. The absorbance of each solution was measured at 670 nm against water blank.

In either method, a standard graph was prepared by plotting the decreasing absorbance values versus concentration of STV. The concentration of the unknown was read from the standard graph or computed from the respective regression equation derived using the Beer's law data.

Procedure for Tablets/Capsules

Twenty tablets/contents of capsules were weighed and ground into a fine powder. An amount of powder equivalent to 50 mg of STV was weighed into a 100 mL calibrated flask, 60 mL of water added and the mixture shaken for 20 min; then the volume was made up to the mark with water, mixed well and filtered using a Whatman No. 42 filter paper. First 10 mL portion of the filtrate was discarded and a suitable aliquot was then subjected to analysis by taking a convenient aliquot using the procedure described earlier.

Results and Discussion

The proposed methods are indirect and are based on the determination of residual bromine after allowing the reaction between STV and *in situ* generated bromine to go to completion, and rely on two different well-known reaction schemes.

Method Development

The methods are based on the oxidation of STV by a known excess of *in situ* generated bromine in hydrochloric acid medium, reducing the unreacted oxidant by iron (II) and subsequent determination of iron (III) by thiocyanate method [29] or by tyron method of Potter and Armstrong [30] and modified by Keshavayya *et al*. [31]. When a fixed concentration of bromine is made to react with increasing concentration of STV, there occurs a concomitant fall in the former's concentration. When the unreacted oxidant is reduced by a fixed concentration of iron(II), there will be a proportional decrease in the concentration of iron (III). This is observed as a proportional decrease in the absorbance of iron (III) – thiocyanate complex or iron(III)-tyron complex on increasing the concentration of STV, which formed the basis for the determination of drug.

Various parameters associated with the oxidation of STV by the oxidant and subsequent reduction of the residual oxidant by iron (II) were optimized. Considering 5.5 µg mL^{-1} as the upper limit of iron that could be determined by thiocyanate method, 2 µg mL^{-1} bromate in the presence of a large excess of bromide was found to produce it from 400 µg mL^{-1} FAS in method A. Similarly in method B, fixing 18 µg mL^{-1} as the upper limit of iron that could be determined by tyron method, 140 µg mL^{-1} FAS and 15 µg mL^{-1} bromate with excess of bromide were used. One mL of 5 M HCl in a total volume of 6 mL was used for the oxidation step and the same quantity of acid was used for the reduction of oxidant and complexation of iron (III) with thiocyanate in method A. However, the formation of iron(III)-tyron complex(1:1) is pH dependent [31] and 1 mL of 1 M HCl in a total volume of ~5 was used to cause oxidation of drug by residual bromine and the latter's reduction by iron(II), and later the pH was raised to ~1.0 by adding 1.0 mL of 1.5 M sodium acetate solution. To ensure an optimum pH for the complex formation reaction, 1 mL

of buffer of pH 1.09 was also added. The oxidation of STV was complete in 5-10 min but the reduction of oxidant by iron(II) and subsequent complexation of iron(III) with thiocyanate or tyron was instantaneous.

Analytical Parameters

A linear relation is found between absorbance and concentration in the ranges given in Table 2. The calibration graphs are described by the equation:

$$Y = a + b X$$

(where Y = absorbance, a = intercept, b = slope and X = concentration in μg mL^{-1}) obtained by the method of least squares. Correlation coefficients, intercepts and slopes for the calibration data are also presented in Table 2. Sensitivity parameters such as molar absorptivity and Sandell sensitivity values, and the limits of detection and quantification calculated according to ICH guidelines [32] are also compiled in Table 2, and demonstrate the high sensitivity of the methods.

Method Validation

Evaluation of Accuracy and Precision: Intra-day and inter-day precision were assessed from the results of seven replicate analyses on pure drug solution. The mean values and relative standard deviation (RSD) values for seven replicate analyses at three different concentration levels were calculated. The accuracy of the methods was determined by calculating the percentage deviation observed in the analysis of pure drug solution and expressed as the relative error (RE). To determine the inter-day precision, analysis was performed over a period of five days preparing all solutions afresh each day. Table 3 summarizes the intra-day precision and accuracy data for the determination STV by the proposed methods.

Application

Table 4 gives the results of assay and reveals that there is close agreement between the results obtained by the proposed methods and the label claim. The results were also compared statistically with those obtained by a reference method by applying Student's t-test for accuracy and F-test for precision. At the 95% confidence level, the calculated t- and F-values did not exceed the tabulated values (t = 2.77 and F = 6.39), suggesting that the proposed methods are as accurate and precise as the reference method.

Accuracy and validity of the methods were further ascertained by performing recovery experiments *via* standard addition technique. To a fixed and known amount of STV in tablet/capsule powder (pre-analysed), pure drug was added at three levels and the total was found by the proposed methods. Each test was repeated three times. The recovery of pure STV added to tablet/capsule powder indicating that commonly encountered tablet excipients and additives such as talk, starch, lactose, sodium alginate, magnesium stearate, calcium gluconate and calcium dihydrogen orthophosphate did not interfere in the assay procedures.

Table 2: Analytical and regression parameters of spectro-photometric methods

Parameter	Method A	Method B
λ_{max}, nm	470	670
Beer's law limits, μg mL^{-1}	0.5-4.0	2-30
Molar absorptivity, L mol^{-1} cm^{-1}	5.6×10^4	5.2×10^3
Sandell sensitivity, μg cm^{-2}	0.004	0.043
Limit of detection, μg mL^{-1}	0.09	0.62
Limit of quantification, μg mL^{-1}	0.28	1.88
Regression equation, Y*		
Intercept (a)	0.497	0.409
Slope (b)	-0.089	-0.0103
Correlation coefficient, (r)	-0.9965	-0.9949
S_a	0.079	0.011
S_b	0.020	0.001

*Y = a+bX, where Y is the absorbance and X concentration in μg mL^{-1} S_a. Standard deviation of intercept; S_b. Standard deviation of slope.

Conclusions

Two new methods have been developed and appropriately validated for the assay of STV. Both methods are based

Table 3: Evaluation of accuracy and precision

Method	STV taken μg mL^{-1}	STV found** μg mL^{-1}	Range, μg mL^{-1}	RE %	SD μg mL^{-1}	SDM μg mL^{-1}	RSD, %
A	1.0	0.98	0.06	2.00	0.011	0.004	1.12
	2.0	1.98	0.08	1.0	0.031	0.012	1.57
	3.0	2.97	0.20	1.00	0.055	0.021	1.85
B	10	9.95	0.07	0.50	0.066	0.025	0.66
	20	19.96	0.06	0.20	0.085	0.032	0.43
	30	29.85	0.05	0.50	0.102	0.039	0.34

** Mean value of seven replicate determinations

RE. Relative error; SD. Standard deviation; SDM. Standard deviation of mean; RSD. Relative standard deviation; at the 95% confidence level and six degrees of freedom.

Table 4: Results of determination of Stavudine in capsules and statistical comparison with the reference method

Formulation#	Nominal amount, mg	% found* ± SD		
		Reference method[24]	Method A	Method B
	30	100.02 ± 0.51	99.5 ± 0.69	100.8 ± 0.56
			t = 1.84	t = 1.77
Virostav[a]			F = 1.83	F = 1.21
(Tablet)		99.8 ± 0.56	99.2 ± 1.01	100.2 ± 1.02
			t = 1.21	t = 0.80
			F = 3.25	F = 3.32
	30	102.3 ± 0.95	101.8 ± 1.02	100.6 ± 1.05
			t = 0.80	t = 2.69
Stavir[b]			F = 1.15	F = 1.22
(capsule)	40	101.2 ± 0.62	100.2 ± 0.91	101.9 ± 1.33
			t = 0.86	t = 1.13
			F = 2.64	F = 4.60

*Mean value of five determinations
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Tabulated t-value at 95% confidence level is 2.77
Tabulated F-value at 95% confidence level is 6.39.

Table 5: Results of recovery experiments by standard addition method

Formulation studied	Method A				Method B			
	STV in preparation, µg mL^{-1}	STV added, µg mL^{-1}	Total found, µg mL^{-1}	Pure STV Recovered*, %	STV in preparation, µg mL^{-1}	STV added, µg mL^{-1}	Total found, µg mL^{-1}	Pure STV, recovered*, %
Virostav-tablet (30 mg)	1.00	1.0	1.98	97.6	10.08	5.0	15.15	101.3
	1.00	2.0	3.06	102.8	10.08	10.0	20.30	102.2
	1.00	3.0	4.01	100.3	10.08	15.0	24.90	98.8
Stavir capsule (40 mg)	1.00	1.0	1.99	99.3	10.19	5.0	15.17	99.6
	1.00	2.0	2.94	97.2	10.19	10.0	20.44	102.5
	1.00	3.0	4.08	102.5	10.19	15.0	25.39	101.3

*Mean value of three determinations

on well-characterised complexation reactions and the thiocyanate method is the most sensitive ever reported for STV in terms of wide linear dynamic concentration range and molar absorptivity. An additional advantage of the methods is that the absorbance is measured at longer wavelengths where the interference from excipients is far less than at shorter wavelengths. The methods should therefore find ready application in pharmaceutical industrial quality control.

References

1. The Merck Index, 12th Edn., Merck and Co. Inc, White House Station. N J, pp 1502 (1994)
2. Current Index of Medical Specialities (CIMS)-Updated Prescribers Hand Book,(CMP Medica India Pvt Ltd, Bangalore, India) CIMS-90, July-Oct 2005 **382**
3. V Benzy, P Morin, P Courbe, G Leleu and L Agrofoglio *J Chromatogr B* **821** (2005) 132
4. C P W G M Verweij-van Wissen, R E Aarnouste and D M Burger *J Chromatogr B* **816** (2005) 121
5. J Contrerar, H Ma Gonsalez, R Menendez and M Lopez, *J Chromatogr B* **801** (2004) 199
6. J L Wiesner, F C W Sutherland, M J Smit, G H Van Essen, H K L Hunot, K J Swart and A F Hundt *J Chromatogr B* **773** (2002) 129
7. B Fan, M G Bertlett and J T Stewart *Biomed Chromatogr* **16** (2002) 383
8. R L S Raices, M C Salvadosi, R C Estrela, F R de Aquino Neto and G Suarez-Kurtz *Rapid Commu. Mass Spectro* **17** (2003) 1611
9. E A Pereira, G A Micke and M F M Tavarer *J Chromatogr A* **1091** (2005)169
10. B Fan and J T Stewart *J Liq Chromatogr Rel Technol* **25** (2002) 937
11. T T Tran, B L Robbins, F H Pinkerton, B Ferrua, J Grassi and A Fridland *Antiviral Res* **58** (2003)125
12. S Compain, D Schlemmer, M Levi, A Pruvost, C Goujard, J Grassi and H Benech *J Mass Spectr* **40** (2005) 9
13. Y Huang, E Zurlinden, E Lin, X Ki, J Tokumoto, J Golden, A Murr, J Engstorm and J Conte *J Chromatogr B* **799** (2004) 51
14. N L Rezk, R R Tidwell and A D M Kashuba *J Chromatogr B* **791** (2003) 137
15. N R Pai and A S Desai *Indian Drugs* **40** (2003) 111
16. M Sablon, J Contreras, O Diaz, R Perez, H Velez and C Rodriguez-Tanty *Acta Pharm Bonae* **23** (2004) 81
17. M Zhang and J Cen *Zhongguo Yiyao Gougye Zaghi* **34** (2003) 402
18. P Djurdjevic, A Laban, S Markovic and M Jelikik-Stankov *Anal Lett* **37** (2004) 2649

19. A Dunge, N Sharda, B Singh and S Singh *J Pharm Biomed Anal* **37** (2005) 1115
20. S BWankhede, K R Gupta and S G Wadodkar *Indian J Phar Sci* **67** (2005) 96
21. N Kaul, H Argawal, A R Paradkar and K R Mahadik *J Chromatogr Sci* **43** (2005) 406
22. A Volosov, C Alexandor,L Ting and S J Soldin *Clin Biochem* **35** (2002) 99
23. S J Soldin *PCT Int Appl* (2004) 40
24. D G Sankar, M V V N Reddy, J M R Kumar and T K Murthy *Aian J Chem* **14** (2002) 433
25. C S N Sarma, C K Sastri and C P S Sastry *Aian J Chem* **14** (2002) 683
26. C S N Sarma, C K Sastri and C S P Sastry *Acta Ciencia Indica Chem* **28** (2002) 221
27. D G Sankar, M V V N Reddy, J M R Kumar and T K Murthy *Indian J Pharm Sci* **64** (2002) 504
28. J Bassett, R C Denney, G H Jeffery and J Mendham Vogel's Text Book of Quantitative Inorganic Analysis Longman Group Ltd. England (1978) 376
29. E B Sandell B Colorimetric Determination of Traces of Metals, 3rd edn., Interscience Publishers Inc., New York, (1959) 524
30. R G Vector Potter and C E Armsbong *Anal Chem* **20** (1948) 1208
31. J Keshavayya, B N Achar, K Basavaiah and M V Krishna Swamy *J Inst Chem(India)* **60** (1988) 142
32. "Text on Validation of Analytical Procedures", ICH Harmonized Tripartite Guideline **6** 1994