Quantification of an antiviral drug (stavudine) by three procedures based on redox and complex formation using N-bromosuccinimide

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Three simple, sensitive and rapid methods are described for the determination of stavudine (STV) in bulk drug and in formulations using N-bromosuccinimide (NBS) as the oxidimetric reagent. In titrimetry, an aqueous solution of STV is titrated directly in HCl medium with NBS using methyl orange as indicator. Spectrophotometric methods involve the addition of a known excess of NBS to STV in HCl medium followed by estimation of the unreacted oxidant by two reaction schemes involving the use of iron(II) and thiocyanate (method A) or tiron (method B). In all the methods, quantification is based on the amount of NBS reacting with STV. Calculations in titrimetry are based on a 1:2 (STV:NBS) reaction stoichiometry and the method is applicable over 1-9 mg range. In spectrophotometric methods, the absorbance is found to decrease linearly with STV concentration. Beer’s law is obeyed over the ranges 0.5-4.0 and 1.5-18 µg mL$^{-1}$ for method A and method B, respectively. The calculated molar absorptivity values are 6.5×10$^4$ and 1.1×10$^4$ L mol$^{-1}$cm$^{-1}$ for method A and method B, respectively. The limits of detection (LOD) and quantification (LOQ) are also reported for both methods. The RSD values for intra-day and inter-day precision studies were <2.5 and 3.0%, respectively. The procedures were successfully applied for the determination of STV in pharmaceutical formulations with good recovery, good accuracy and precision, and without measurable interference by the excipients.

Keywords: Stavudine determination, Spectrophotometry, N-bromosuccinimide, Pharmaceuticals

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Stavudine (STV), chemically known as 2'-3'-didehydro-2'-3'-dideoxythymidine, is a nucleoside analog reverse transcriptase inhibitor (NARTI) active against HIV$^1$. STV is phosphorylated 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 which is indicated for HIV infections$^2$. Many techniques have been used for the determination of STV in pharmaceuticals and include HPLC with UV-detection$^3$-8, HPTLC$^9,10$, LC-MS$^{11}$, mass spectrophotometry$^{12}$ and UV-spectrophotometry$^{13}$. Many of the above procedures require time-consuming sample preparation and expensive instrumentation. Despite its considerable history and established versatility, no titrimetric method has yet been reported for the determination of STV. Only three visible spectrophotometric methods$^{14-16}$ are available in the literature, which suffer from one or the other disadvantage such as expensive chemical, heating/extraction step or poor sensitivity.

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 NBS as the oxidimetric reagent, and iron(II) and thiocyanate or tiron 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 was used for all absorbance measurements.

Reagents and materials
All chemicals were of analytical reagent grade and distilled water used to prepare solutions. N-bromosuccinimide (∼0.01 mol L$^{-1}$) solution was prepared by dissolving about 1.8 g of the compound (SRL Research Chemicals, India) in water with the aid of heat and diluted to one litre with water and standardized$^{17}$. The solution was stored in an amber coloured bottle and used for titrimetry. It was diluted appropriately to get 180 and 650 µg mL$^{-1}$ NBS for use in spectrophotometric method A and B, respectively. Hydrochloric acid (5 mol L$^{-1}$) was prepared by diluting concentrated hydrochloric acid (s.d. Fine Chem, Mumbai, India; sp. gr. 1.18) appropriately with water and used in titrimetry and
method A, and the 5 mol L\(^{-1}\) solution of acid was diluted further to get 1 mol L\(^{-1}\) for use in spectrophotometric method B. Ferrous ammonium sulphate, FAS (0.01 mol L\(^{-1}\)) was prepared by dissolving about 400 mg of the salt (S.d. Fine Chem, Mumbai, India) in 50 mL of water containing 1 mL of dil H\(_2\)SO\(_4\), and diluted to 100 mL with water, and standardized\(^{18}\) 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. Tiron solution (1.0%) was prepared by dissolving 1.0 g of the chemical (Loba Chemie, Mumbai, India) in 100 mL of water. Ammonium thiocyanate (3 mol L\(^{-1}\)) was prepared by dissolving 23 g of the chemical (S.d. Fine Chem, Ltd., Mumbai, India) in 100 mL water. Sodium acetate trihydrate and 70 mL of 1 mol L\(^{-1}\) sodium acetate trihydrate and 70 mL of 1 mol L\(^{-1}\) sodium acetate, buffer of pH 1.09 was prepared by mixing of 50 mL 1 mol L\(^{-1}\) sodium acetate trihydrate and 70 mL of 1 mol L\(^{-1}\) H\(_2\)SO\(_4\) and diluting to 250 mL with water. Pharmaceutical grade STV (99.8% pure) was received from Cipla India Ltd, as gift and was used as received. A stock standard solution containing 1 mg mL\(^{-1}\) STV was prepared by dissolving accurately weighed 250 mg of pure drug in water and diluting to the mark in a 250 mL calibrated flask. This was used for titrimetric work, and for spectrophotometric work, the same was diluted appropriately with water to get a working concentration of 10 µg mL\(^{-1}\) for method A and 60 µg mL\(^{-1}\) for method B.

Methods

**Titrimetry**

A 10 mL aliquot of standard drug solution containing 1-9 mg of STV was accurately measured into a 100 mL titration flask and the solution was acidified by adding 5 mL of 5 M-hydrochloric acid and titrated with 0.01 M NBS using one drop of methyl orange indicator to a colourless end point. An indicator blank was run and the amount of STV consumed in the blank titration was subtracted from that consumed in the sample titration. The drug content in the aliquot was calculated from the amount of NBS reacted with STV.

**Spectrophotometric 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 1 mL each of 5 mol L\(^{-1}\) H\(_2\)SO\(_4\) and NBS (180 µg mL\(^{-1}\)), the last being added using micro burette. The content was mixed and the flasks were let stand for 5 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 1 mL of 3 mol L\(^{-1}\) thiocyanate. The volume was diluted to the mark with water, mixed well and absorbance of each solution was measured at 470 nm against a water blank.

**Spectrophotometric method B**

Varying aliquots (0.25-3.0 mL) of standard STV solution (60 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 mol L\(^{-1}\) H\(_2\)SO\(_4\) before adding 1 mL of NBS (650 µg mL\(^{-1}\)) by means of micro burette. The content was mixed well and allowed to stand for 15 min with occasional shaking. To each flask was then added 1 mL of 1400 µg mL\(^{-1}\) FAS, and after 5 min, 1 mL each of 1.5 mol L\(^{-1}\) sodium acetate, buffer of pH 1.09 and 1% tiron were added and diluted to the mark with water. The absorbance of each solution was measured at 670 nm against water blank.

In either spectrophotometric 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 250 mg of STV was weighed into a 250 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 titrimetric method. The tablet/capsule extract (1 mg mL\(^{-1}\)) was diluted suitably with water to get working concentrations of 10 and 60 µg mL\(^{-1}\) for method A.
and method B, respectively before subjecting to analysis by spectrophotometric methods.

**Results and Discussion**

All the three methods described here are based on the oxidation reaction involving STV and NBS in hydrochloric acid medium. The titrimetric method is direct whereas the spectrophotometric methods are indirect and are based on the determination of residual NBS after having allowed the oxidation reaction to go to completion under the specified experimental conditions. The amount of NBS reacted corresponds to the drug content in all the methods.

**Method development**

**Titrimetry**

Reproducible and stoichiometric results were obtained when hydrochloric acid medium was employed as the reaction medium. The results were found to be unaffected when 4.5 to 5.5 mL of 5 mol L\(^{-1}\) acid was used in a total volume of 15 mL; and thus 5 mL of 5 mol L\(^{-1}\) hydrochloric acid was employed in the investigation. The reaction was found to be quantitative with a stoichiometry of 1:2 (STV: NBS) for the range investigated (1-9 mg).

**Spectrophotometry**

The methods are based on the oxidation of STV by a known excess of NBS in hydrochloric acid medium, reducing the unreacted oxidant by iron (II) and subsequent determination of iron (III) by thiocyanate method\(^{19}\) or by tiron method of Vector Potter and Armstrong\(^{21}\) and modified by Keshavayya *et al*\(^{20}\). When a fixed concentration of NBS is made to react with increasing concentration of STV, there occurs a concomitant fall in the former’s concentration. When the unreacted NBS 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 and iron(III)-tiron 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 NBS and subsequent reduction of the residual oxidant by iron (II) were optimized. Considering 5.5 \(\mu\)g mL\(^{-1}\) as the upper limit of iron that could be determined by thiocyanate method, 18 \(\mu\)g mL\(^{-1}\) NBS was found to produce it from 38.7 \(\mu\)g mL\(^{-1}\) FAS. However, slightly higher concentration (40 \(\mu\)g mL\(^{-1}\)) FAS was used to ensure a quantitative reaction in method A. Similarly in method B, fixing 18 \(\mu\)g mL\(^{-1}\) as the upper limit of iron that could be determined by tiron method, 140 \(\mu\)g mL\(^{-1}\) FAS and 65 \(\mu\)g mL\(^{-1}\) NBS were used. One mL of 5 mol L\(^{-1}\) 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 NBS and complexation of iron (III) with thiocyanate. However, the formation of iron(III)-tiron complex(1:1) is \(pH\) dependent and 1 mL of 1 mol L\(^{-1}\) HCl in a total volume of \(\sim\)5 was used to cause oxidation of drug by NBS and the latter’s reduction by iron(II), and later the \(pH\) was raised to \(\sim\)1.0 by adding 1.0 mL of 1.5 mol L\(^{-1}\) 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 5-15 min but the reduction of NBS by iron(II) and subsequent complexation of iron(III) with thiocyanate or tiron was instantaneous.

**Analytical parameters**

A linear relation is found between absorbance and concentration in the ranges, 0.5-4.0 and 1.5-18 \(\mu\)g mL\(^{-1}\) STV for method A and method B, respectively. The calibration graphs are described by the equation:

\[
Y = 0.6064 + (-0.1212) X \quad \text{--- method A (r= -0.9994)} \\
Y = 0.5887 + (0.0302) X \quad \text{--- method A (r= -0.9996)}
\]

(where \(Y\) = absorbance, \(X\) = concentration in \(\mu\)g mL\(^{-1}\) and \(r\) = regression coefficient) obtained by the method of least squares. Molar absorptivity values are calculated to be \(6.5 \times 10^4\) and \(1.1 \times 10^4\) L mol\(^{-1}\) cm\(^{-1}\) for method A and method B, respectively and the limits of detection and quantification are found to be 0.07, 0.21 and 0.21, 0.64 \(\mu\)g mL\(^{-1}\), for method A and method B, respectively and these values demonstrate the high sensitivity of the methods.

**Method validation**

**Evaluation of accuracy and precision**

Intra-day precision was assessed from the results of seven replicate analyses on pure STV solution containing 5 mg, 2.0 \(\mu\)g mL\(^{-1}\) and 10.0 \(\mu\)g mL\(^{-1}\) STV by titrimetry, method A and method B, respectively, and the RSD values were calculated to be 0.66, 0.97 and 1.18% which speak of the high repeatability of the methods. The methods were also found to be
highly accurate with the corresponding relative errors of 0.2, 1.5 and 1.5%. The inter-day relative standard deviations (n=5) were less than 3%.

Application
The proposed methods were successfully applied for the determination of STV in one brand of tablets and two brands of capsules each containing 30 or 40 mg of STV. Percent STV found relative to label claim ranged from 98.62 to 102.1 with standard deviations of 0.39-1.46. The performance of the proposed methods was compared with that of a reference method13 (uv-spectrophotometry) by applying the 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 indicating that the proposed methods are as accurate and precise as the reference method. The accuracy of the proposed method was further checked by performing recovery experiments. The mean recovery and relative standard deviation (n=3) were in the range of 97.56-105.3 and 0.72-1.8%, respectively. These results also suggested that there is no interference from the common excipients present in dosage forms.

Conclusions
Three new methods have been developed and appropriately validated for the assay of STV. The titrimetric method is the first ever proposed for STV and is applicable over a microscale. Both spectrophotometric methods are based 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.

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