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VALIDATED CAPILLARY ZONE ELECTROPHORESIS METHOD FOR THE DETERMINATION OF ACUTE NEUROTOXIN AZIDE IN AN ANTIRETROVIRAL DRUG

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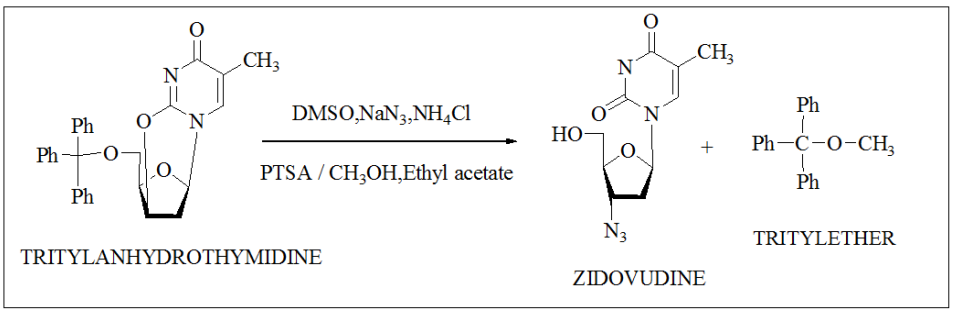
ABSTRACT
A rapid and sensitive capillary zone electrophoresis method was developed, optimized and validated for determination of acute neurotoxin azide, in an antiretroviral drug - Zidovudine. Electrophoresis was carried out in an aqueous run electrolyte containing 7.5 mM of sodium chromate, 5 mM of boric acid and 0.1 mM of cetyltrimethylammonium bromide. Separation of azide was achieved using a bare fused silica capillary had an effective length of 56 cm and an internal diameter of 50 µm with an extended light path detection window. Capillary was thermostated at 25° C and -12 µA current applied across on it. Nanolitre volumes of standard and sample solutions injected hydrodynamically by applying 50 mbar pressure for 45 sec. Analyte was monitored at a wavelength, signal: 460 nm and reference: 275 nm. The detector response found to be linear for azide in the concentration range of 0.09 µg/ml to 1.5 µg/ml. LOD and LOQ values of azide are found to be 0.09 µg/mL and 0.28 µg/mL respectively. RSD for replicate (n=6) injections of standard solution containing 1.0 µg/mL of azide was < 5% and for precision in intra-day (n=6) and inter-day (n=6) experiments was within 5%. Accuracy for the azide is 94.3%. The proposed method is specific, accurate, sensitive, rugged and precise for routine analysis of azide in quality control laboratories.

KEYWORDS: Antiretroviral, Azide, Capillary zone electrophoresis, Indirect UV detection, Zidovudine.

INTRODUCTION
3′-Azido-3′-deoxythymidine is the chemical name of zidovudine, it is also called as azidothymidine and its molecular formula is $C_{10}H_{13}N_5O_4$. Zidovudine is an antiretroviral medication used to prevent and treat Human Immunodeficiency Virus (HIV) / Acquired immunodeficiency syndrome (AIDS). Zidovudine is a HIV nucleoside analog reverse transcriptase inhibitor (NRTI). It works by inhibiting the enzyme called reverse transcriptase, that Human Immunodeficiency Virus uses to make its DNA and therefore decreases replication of the virus. Zidovudine drug prevents Human Immunodeficiency Virus transmission, from mother to unborn child.[1] Zidovudine is a dideoxynucleoside compound in which an azido group has replaced the 3'-hydroxy group on the sugar moiety, this modification prevents the formation of the 5' to 3' phosphodiester linkage which is crucial for the viral DNA growth.

Synthesis has shown in below reaction.



Organic and inorganic azides have shown versatile chemical reactivities for the synthesis of nitrogen containing compounds. Organic azides are especially used for 1, 3-dipolar cycloaddition with alkynes in biology and pharmaceutical industry. Organic azides are used for the synthesis of heterocyclic compounds such as five and six membered ring compounds pyrroles and pyridines which are highly utilize in the manufacturing of active pharma ingredients as reagents or raw materials.

Azide is available as sodium azide in pure form and is a white crystalline solid. It is readily soluble in water to yield azide anion (N_3^-). Sodium azide is used in the process of manufacturing of zidovudine. Azidation takes place at 3' position on the sugar moiety of tritylanhydrothymidine when reaction with sodium azide in the presence of ammonium chloride. Azide is acute neurotoxic in nature.[2-7] and azide particularly affects organs that undergo high rates of respiration, such as the heart and the brain. The toxicity of azide is comparable to that of alkali cyanide. Sodium azide contact with acids causes the liberation of hydrazoic acid (HN_3) gas[8] which is extremely toxic than salt. Fatal doses occur with exposure of 10 µg/g[9] but exposure to small doses can cause eye and skin irritation. So it requires a sensitive method to estimate trace levels in pharmaceutical drug substances.

Numerous methods have been reported for the quantification of azide ion in various samples by using Spectrophotometry[4,10,11] High performance liquid chromatograpy[5] (HPLC), Gas chromatography[12] (GC), Ion chromatography[13,14] (IC) and Capillary electrophoresis[15,16] (CE) techniques. Iron(III), cerium(IV), ammonium nitrate and ferric perchlorate reagents were used for the determination of azide in spectrophotometric methods. Precolumn derivatisation applied with pentafluorobenzyl bromide reagent to form pentafluorobenzyl-azide derivative in HPLC method. GC method also used precolumn derivatisation with propionic anhydride reagent to form propionyl azide. Supressed conductivity detection applied in Ion chromatography methods. Pre-column derivatisation with 3, 5-Dinitrobenzoyl chloride and direct detection by using inorganic anion buffer of pH 7.7 were used for the detection of azide in capillary electrophoresis methods. Inorganic anion buffer of pH 7.7 is a readymade buffer supplied by Agilent technologies. But in chromatography techniques, there is sample matrix interference at the retention time of azide peak and chromatography techniques required long run time to elute the sample matrix from the column.[17]

In this study acute neurotoxin azide was determined by capillary zone electrophoresis using sodium chromate as a probe in the buffer electrolyte. Reported method has more sensitive than previously reported capillary electrophoresis methods. Separation was achieved on bare fused silica capillary with buffer electrolyte consists 7.5 mM of sodium chromate anhydrous, 5 mM of boric acid and 0.1 mM of Cetyltrimethylammonium bromide. Advantage of the proposed method is non-interference from the high concentrated sample matrix even after complete dissolution in diluent.

MATERIALS AND METHODS

Materials

Zidovudine drug substance and its related substances procured from Aurobindo pharma Ltd. Sodium azide standard, sodium chromate anhydrous, boric acid, cetyltrimethylammonium bromide, sodium hydroxide, HPLC grade acetonitrile and acetic acid were purchased from Merck India. Bare fused silica capillary (Part No: G1600-61232) procured from Agilent technologies India. Milli-Q grade (Millipore, MA, USA) water was used throughout experiments. 0.45m PVDF membrane filters were procured from Merck Life Science.

Instrumentation

Capillary electrophoresis (CE) instrument is 3D HPCE instrument model G1600A (Agilent technologies, Waldbronn, Germany) equipped with diode array detector (DAD), and data handling software is agilent Chemstation.

Run electrolyte, standard and sample solutions preparation

Run electrolyte was prepared by dissolving about 440 mg of sodium chromate anhydrous, 77 mg of boric acid and 10 mg of cetyltrimethylammonium bromide in 250 ml of water, pH adjusted to 7.5 with diluted acetic acid or with dilute sodium hydroxide solution. A degassed mixture of water and acetonitrile in the ratio of 70:30 v/v containing sodium hydroxide of about 0.002M is used as diluent. The azide stock solution was prepared by dissolving 155 mg of sodium azide into a 100 mL clean dry volumetric flask with diluent. The standard solution of azide at about 1 µg/mL was prepared from sodium azide stock solution and the sample solution at about 100 mg/mL of zidovudine was prepared in the diluent. All solutions were filtered through 0.45 µm membrane filter.

RESULTS AND DISCUSSION

Method development

Capillary zone electrophoresis is an effective technique for the analysis of small inorganic anions such as chloride, sulphate, nitrate, azide, etc. The separation of inorganic anions was achieved based on their electrophoretic mobilities, resulted by their charge to mass ratio. Capillary zone electrophoresis is widely used as a separation technique in various fields. Literature is available for the determination of azide by using different techniques.[4,6,11,13-16] A rapid and sensitive Capillary zone electrophoresis method using Indirect UV detection has been developed and validated for the determination of azide in the zidovudine drug substance. Azide is a non-chromophoric ion, which can be overcome by using indirect UV detection[18], for this chromate, phthalate, benzoate, dipicolinic acid are used

as anionic probes[19, 20] in run electrolyte. The electrophoretic mobility of the probe must closely match that of the ions being detected, to get high sensitivity and sharp peaks.

Development was started with potassium hydrogen phthalate as run electrolyte, which is having high buffering capacity resulting poor response for azide. Response was not improved with sodium benzoate and dipicolinic acid (pyridine-2,6-dicarboxylic acid) buffers since their electrophoretic mobilities are far away that of the azide ion. Chromate was chosen as a probe because of its electrophoretic mobility $-81.1\times10^{-9}m^2V^{-1}s^{-1}$ is close to azide ion electrophoretic mobility $-71.9\times10^{-9}m^2V^{-1}s^{-1}$[21] and also chromate has given less baseline noise, optimal peak shape and sensitivity for azide ion compared to other probes. Ionized silanol (SiO^-) groups present on the capillary inner walls give longer migration times for anions in negative electric fields, due to the reverse direction of electroosmotic flow (EOF). The addition of a cationic surfactant[22,23] to the run electrolyte in a concentration less than its critical micelle concentration (CMC), can direct the EOF in the direction of anions migration, results in shorter analysis time. Tetrabutylammonium bromide, tetrabutylammonium hydroxide and cetyltrimethylammonium bromide were used as a cationic surfactant to reverse the EOF. At low concentrations of tetrabutylammonium bromide and tetrabutylammonium hydroxide (0.25mM) in run electrolytes, azide eluted at longer migration times. Cetyltrimethylammonium bromide is suitable for the reversal of EOF with less migration time. Boric acid was introduced to strengthen the run electrolyte ultimately to get a stable baseline.

Optimised conditions

Run electrolyte containing 7.5 mM of sodium chromate anhydrous, 5 mM of boric acid, and 0.1 mM of Cetyltrimethylammonium bromide is used for the separation. The separation was carried out on a bare fused silica capillary has a total length of 64.5 cm, an effective length of 56 cm and an internal diameter of 50 µm, with extended light path (150 µm) detection window. Capillary preconditioned with run electrolyte for 3 min between runs, and a constant current having negative polarity of 12 µA with 0.5 min ramp, applied across the capillary. Constant current was chosen to get reproducible migration times during the analysis. Capillary maintained at 25°C. Data acquisition time was 10 min and data collected at Signal: 460 nm and Reference: 275 nm. Sodium azide standard and samples were introduced hydrodynamically into the capillary, by applying 50 mbar pressure for 45 sec. This method has good sample stacking and provides a sharp peak for the analyte.

All calculations related to quantitative analysis were performed with an external standard method, by measuring peak areas of azide in standard and sample solutions.

Capillary conditioning

Rinse the new capillary with 1M sodium hydroxide for 3 min followed by water for 5 min, then precondition with 0.1M sodium hydroxide for 5 min, followed by water for 5 min. Condition the capillary with CTAB at high concentration for about 30 min.

Precondition the capillary with run electrolyte for about 30 min before start the analysis. Flush the capillary for 3 min with run electrolyte between the runs.

METHOD VALIDATION

The optimized method has been validated according to ICH guidelines[24] to prove its performance characteristics, thereby verifying its suitability and reliability for monitoring the azide in zidovudine drug substance during routine analysis. The validation parameters studied were selectivity, sensitivity, linearity, precision (system precision, method precision, and intermediate precision) and accuracy. The results obtained from the experiments are summarized in the next paragraphs.

System suitability

System suitability has been demonstrated by injecting six replicates of standard solution consists of 1 µg/mL of azide diluted from a stock solution containing 1.55 mg/mL of sodium azide into the CE system. The %RSD of corrected area of azide for six replicate injections was found to be < 5. Results were tabulated in Table 1. Migration time reproducibility for azide peak is < 2%.

Table 1: % RSD for standard solution in three different days.

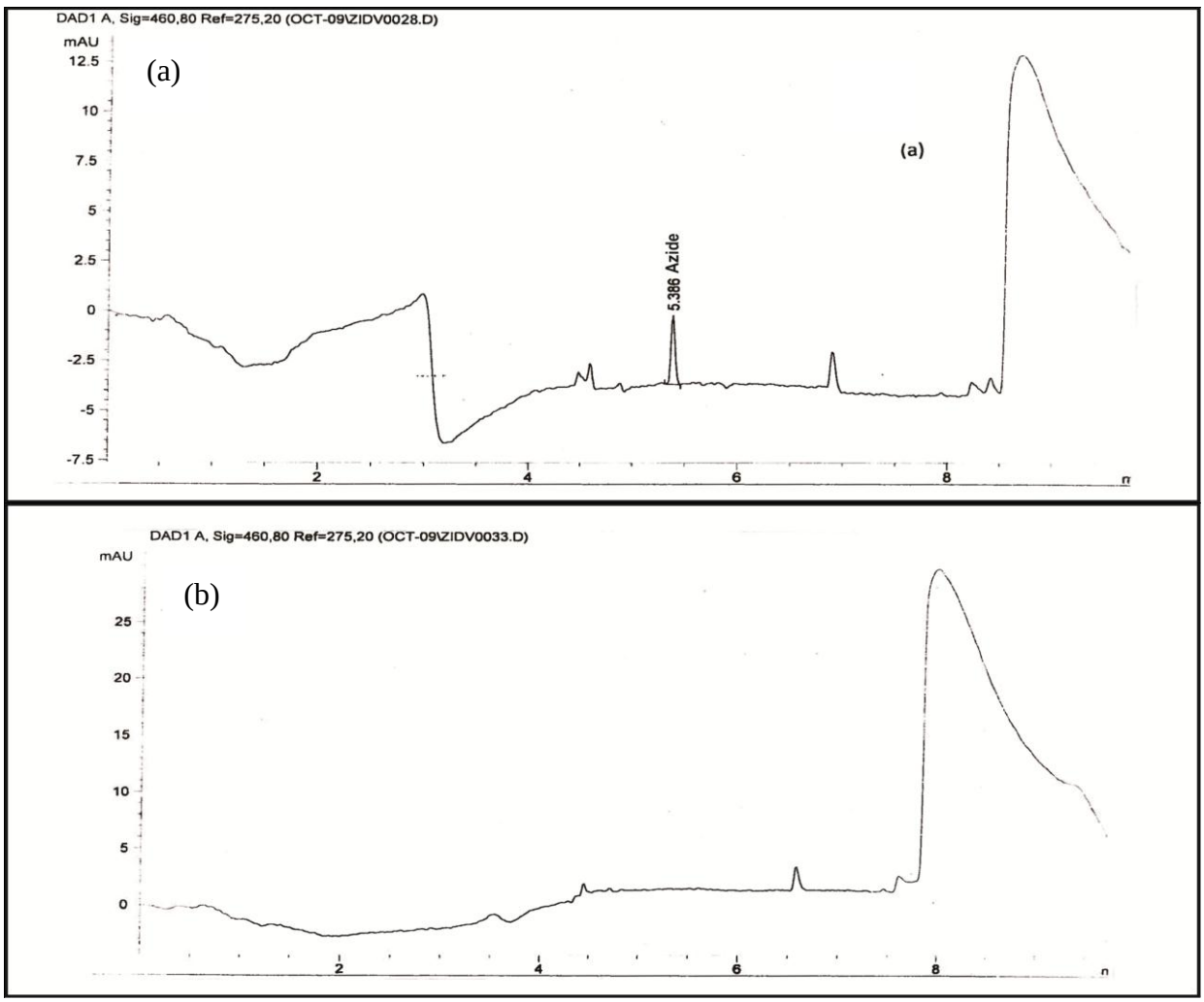
S.No.	Day-1	Day-2	Day-3
	Area count ($\times10^{-2}$) mAU		
1	2.93489	2.98708	2.82829
2	2.66613	2.92821	2.78187
3	2.72207	2.95900	2.80096
4	2.78022	2.93245	2.91805
5	2.79310	3.05643	2.82275
6	2.85831	3.12777	2.80051
Average	2.79245	2.99849	2.82541
SD	0.096	0.079	0.048
% RSD	3.4	2.6	1.7

Specificity

The specificity of the proposed method was performed in the presence of zidovudine and its related substances. Azide was not found in the sample, and therefore the sample was spiked with azide at a level of 10 µg/g with respect to sample, along with other known impurities of zidovudine. The absence of interference in the presence of all related substances of Zidovudine with azide has been demonstrated by % RSD, between the specificity and method precision values. Results were tabulated in Table 2. Figure1 shows electropherograms of the azide standard solution, blank solution, sample, sample spiked with azide along with other known impurities of zidovudine.

Table 2: %RSD of Azide - Method precision and specificity clubbed together.

S.No.	Sample	Azide (µg/g)
1	Method precision-1	8.73
2	Method precision-2	8.20
3	Method precision-3	8.70
4	Method precision-4	8.84
5	Method precision-5	8.11
6	Method precision-6	7.91
7	Specificity-1	9.83
8	Specificity-2	9.52
9	Specificity-3	9.67
Method precision	Mean	8.41
	SD	0.39
	%RSD	4.6
Specificity	Mean	9.67
	SD	0.16
	%RSD	1.7
Overall Mean		8.83
Overall SD		0.71
Overall %RSD		8.0



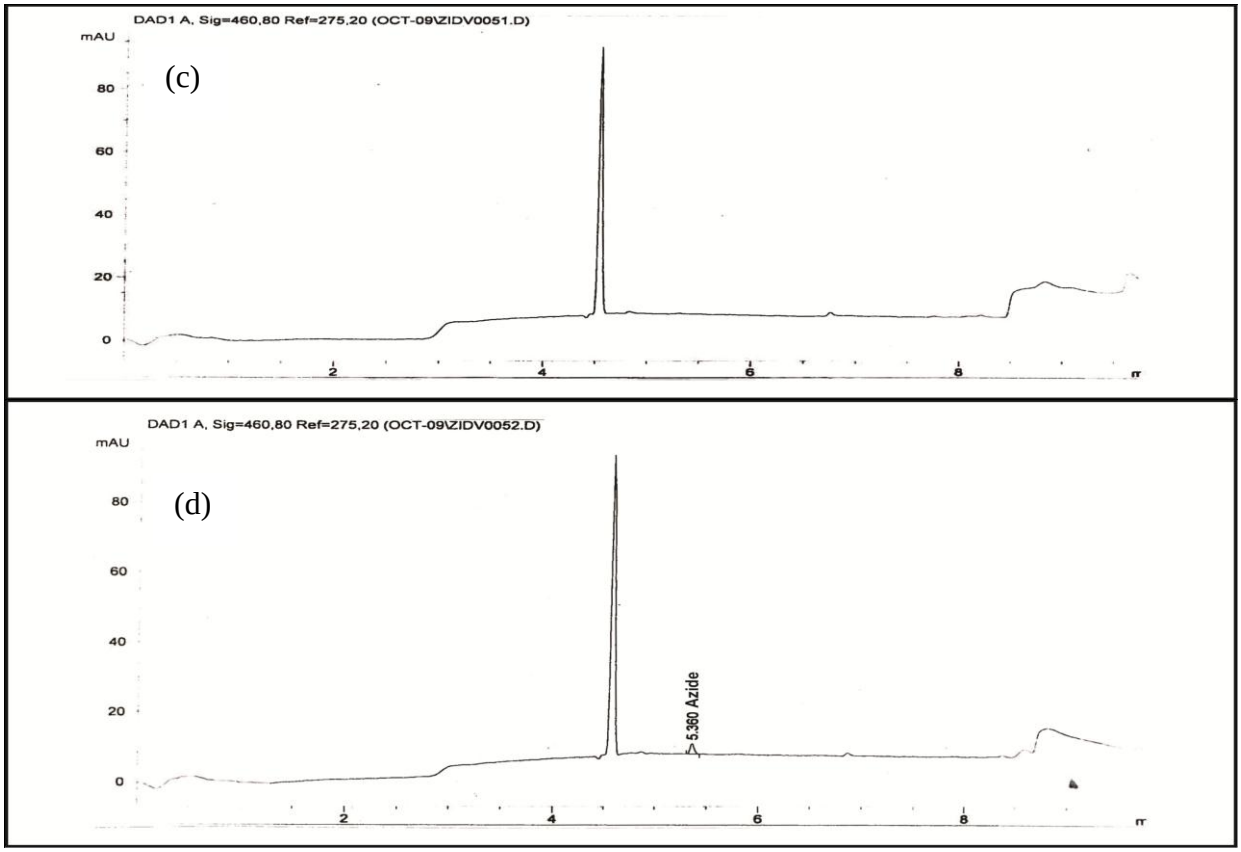


Fig. 1: Electropherograms of Azide standard solution (a), Diluent (b), Sample solution (c) and Spiked with known related substancs of zidovudine including azide (d).

Sensitivity

Limit of detection (LOD) and limit of quantitation (LOQ) are representing the sensitivity of an analytical method. The limit of detection (LOD) and limit of quantification (LOQ) were predicted using slope (S) and residual standard deviation (SD) obtained from a linear curve at lower concentration levels ranging between 0.3µg/ml and 1.5 µg/ml. The predicted limit of detection and quantification was found to be 0.9µg/g and 2.8 µg/g, respectively and each predicted level was prepared and verified for precision by analyzing six replicate measurements. The percentage relative standard deviation for six replicate measurements at predicting LOD and LOQ concentration levels was found to be 17.5 and 4.6 respectively. Results were tabulated in Table 3.

Table 3: The Statistical Evaluation of Linearity Data and Determination of LOD and LOQ.

Statistical parameter	Result
Concentration range (µg/ml)	0.300 – 1.499
Slope	2.753
Intercept	-0.03901
STEYX	0.07674
Correlation coefficient	0.9985
Limit of detection (µg/g)	0.9
Limit of quantification(µg/g)	2.8
Precision for LOD (%R.S.D)	17.5
Precision for LOQ (%R.S.D)	4.6

Accuracy

Accuracy was determined by the standard addition method in which known amount of azide was spiked to the sample solution. The experiments were performed in triplicate at 5 µg/g, 10 µg/g and 15 µg/g level. Average percent recovery obtained during the accuracy experiment was 94.3. Results were tabulated in Table 4.

Table 4.

S.No.	Amount added (µg/g)	Amount found (µg/g)	% Recovery	Statistical analysis	
50% level					
1	4.982	5.058	101.5	Mean	96.9
2	4.990	4.715	94.5	SD	4.0
3	4.993	4.752	94.6	%RSD	4.1
100% level					
4	9.975	9.182	92.1	Mean	91.9
5	9.968	9.320	93.5	SD	1.8
6	9.937	8.943	90.0	%RSD	1.9
150% level					
7	14.909	13.871	93.0	Mean	94.2
8	14.924	14.031	94.0	SD	1.3
9	14.931	14.278	95.6	%RSD	1.4
		Overall Mean	**94.3**		
		SD	**3.2**		
		%RSD	**3.4**		

Precision

Method precision was evaluated from six sample solutions prepared by spiked with azide at about 10 µg/g level. Precision was expressed as percent relative standard deviation (%RSD) of content of azide. Percent relative standard deviation obtained from experimental data was 4.8.

The intermediate precision of the method (ruggedness) was performed in the same way as described in method precision, however, by employing different analyst on another day using another lot of capillaries. The content of azide was determined in each preparation, and the percentage relative standard deviation for six replicate measurements was found to be 3.2. Results were tabulated in Table 5.

Table 5.

Sample ID	Azide (µg/g)	
	Method precision	**Intermediate Precision**
Sample-1	8.73	9.83
Sample-2	8.20	9.52
Sample-3	8.70	9.67
Sample-4	8.84	9.18
Sample-5	8.11	9.32
Sample-6	7.91	8.94
Mean	8.41	9.41
Mean SD	0.4	0.3
%RSD	4.8	3.2
95% Confidence interval(CI)	± 0.4	± 0.006

CONCLUSION

A capillary zone electrophoresis method has been developed, optimized and validated for the determination of acute neurotoxin azide in an antiretroviradrug-zidovudine. The results obtained from validation experiments proved that the optimized capillary zone electrophoresis method was specific, sensitive, linear, precise accurate and rugged for the determination of acute neurotoxin azide in zidovudine drug substance and can be used in the routine.

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