DOI: https://doi.org/10.31351/vol35iss3pp151-158

Development and validation of a specific method for the detection and quantification of Bortezomib in the prepared Bortezomib bovine serum albumin nanoparticles nanoparticles and other pharmaceutical preparations

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Received 29/1/2025, Accepted 29/7/2025, Published 28/9/2026

Abstract

A new, simple, and precise analytical method for detecting Bortezomib is a selective proteasome inhibitor that acts as a potent anticancer drug. This method is developed and validated using a reverse phase High-performance liquid chromatography (HPLC) device with (C18,4.6×250mm) column, mobile phase composed of methanol: water (70:30), an isocratic flow rate of 1 ml/min, and UV detection at 270 nm. Using this method, the drug exhibited a retention time of 6.77 minutes and also proved to be precise and specific with an RSD% less than 2%, which complies with the requirement of ICH. It also shows linearity in both responses (area and height) in the range of 10-100 µg/ml with an R2 0.997 and 0.998, respectively, indicating a wide range for the Bortezomib measurement in different pharmaceutical formulations. The limit of detection (LOD) and Limit of quantification (LOQ) were also calculated to be 3.2 and 9.6 µg/ml, respectively, showing a low level for detection and quantification of the item. The accuracy is also determined to be 99.83%±0.77. In conclusion, the suggested method is validated according to ICH guidelines regarding precision, specificity, linearity, accuracy, and robustness.

Keywords: Detection, Bortezomib Quantification, Bortezomib, HPLC, validation, proteasome inhibitor.

Introduction

Bortezomib (BTZ) is a selective proteasome inhibitor; this class of drugs has cytotoxic activity by interrupting the ubiquitin-proteasome system. They prevent the degradation of proapoptotic cofactors, including cyclin-dependent kinase inhibitors that regulate the cell cycle. This accumulation of these factors promotes cell apoptosis, hence the cytotoxic activity. Bortezomib was approved mainly for the treatment of multiple myeloma. Still, recent research has been established to verify that this drug is effective in cytotoxic activity in 60 different human cancer cell lines such as lung, breast, pancreatic, prostate, head, and neck melanoma cancers. (1)

Chemically, Bortezomib is (1R)-3-methyl-1-[[(2S)-1-oxo-3- phenyl-2-[(pyrazinyl carbonyl) amino]propyl]amino]- butyl]boronic acid as shown in Fig 1. (2) BTZ was formulated as Bovine Serum Albumin (BSA) nanoparticles for better targeting effect since BSA nanoparticles have better penetration to the tumor cells by passive targeting (EPR) effect and active targeting since tumor cells suffer from overexpression of GP60 and SPARC receptors and these receptors bind with albumin. (3).

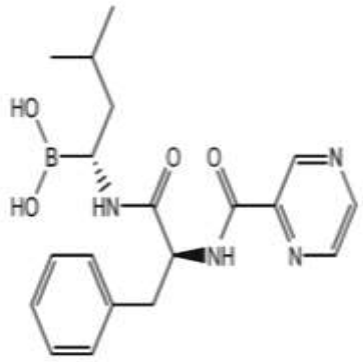

Figure 1. Chemical Structure of Bortezomib

Several analytical methods have been suggested for determining BTZ in pharmaceutical formulations using HPLC, including different detection methods such as UV, mass spectroscopy, and liquid chromatography. Still, these methods require tedious work, preparation, and complex identification techniques. For example, Kamalzadeh et al. developed and validated an HPLC method for detecting and quantifying BTZ enantiomers. Although it is helpful to separate these compounds, it requires a more complex mobile phase. Also, the results for this method showed high LOD and LOQ (52, 160 µg /ml), respectively. The high values of these qualities usually indicate the low sensitivity of the method to detect BTZ from pharmaceutical preparations.

On the other hand, Clemens et al. also developed an HPLC method. Although this method is highly sensitive with calibrated changes of 0.5-2500 pg but, the detection method was by tandem mass spectroscopy, which is a highly complicated and expensive device. This paper suggests a new, simple, validated method for detecting and quantifying BTZ in the formulated BTZ albumin nanoparticles. [4-8]

Materials and Methods

Method

The mobile phase is composed of methanol: water (70:30) % (HPLC grade, Chemlab), flow rate 1 ml/min, temperature 30 °C and the detection is done by UV detector at 270 nm, and the injection volume is 10 µL. The HPLC device is by Knuar, Germany; the software is claritychrome. Nova Atom C18 column, 4.6×250mm,5µm,120A, and Nova Z-Guard C18core 4.6×10mm, 5µm,120A were purchased from Anhui Wanyi Science and Technology, China.HPLC vials 1.5 ml with cracked lids were obtained from CKlabs company, China.

The preparation of a standard solution of BTZ

Ten milligrams of BTZ (Xian International Healthcare Factory, China) were dissolved in a 100 ml volumetric flask, then 80 ml of HPLC grade methanol was added to that flask, sonicated for 10 minutes to ensure complete dissolution of the drug, then complete the volume to 100 ml mark to obtain a solution of 100 µg /ml concentration and this is the stock solution. [9]

Preparation of standard samples

Different samples of BTZ solution with concentrations ranging from (10-100 µg /ml) were prepared, and each sample was filtered by a 0.45 µm syringe filter before being placed in HPLC vials to be analyzed, as shown in the following paragraphs.

Method optimization

The method selected to analyze BTZ was evaluated for validation parameters, including linearity, limit of detection, limit of quantification, specificity, repeatability, and robustness according to ICH guidelines Q2 and FDA guidance. [10-12]

Preparation of Bortezomib Bovine serum albumin nanoparticles (BTZ-BSA NP)

The preparation of BSA NP started by dissolving 200 mg of BSA in 5 ml distilled water and allowing it to be stirred for a few minutes on a magnetic stirrer at 600 rpm. 15 mg of BTZ is dissolved in 3.8 ml acetone and then added to the aqueous solution of BSA dropwise using a syringe pump with an injection speed of 1 ml/min. The addition of the organic phase results in the precipitation of the dissolved albumin to form albumin nanoparticles. [13]

Precision (repeatability)

Precision analysis was done by repeated analysis of the same standard solution of BTZ in methanol (100 µg /ml); six injections were made of the same solution to measure the repeatability of the method of analysis and the relative standard deviation percent (RSD%) of the responses was calculated. RSD is a measure of consistency that shows the precision of the results when the same sample is analyzed multiple times. [14]

$$RSD\% = \frac{Standard\ deviation}{mean} * 100\%$$

Specificity (selectivity)

The specificity validation is done by preparing different samples of BTZ with the only excipient that is used in this research, which is bovine serum albumin. Six samples were prepared to prove this method's specificity for BTZ only, three with the same BTZ concentration and different BSA concentrations. In comparison, the other three samples contain the same BSA concentration but different BTZ concentrations. The response for each analysis was recorded and examined. [15]

Linearity

Several dilutions from the stock solution were prepared to obtain solutions with concentrations ranging from 10-100 µg /ml. Each sample was analyzed by the HPLC device in triplicate, and then the calibration curve was obtained by plotting the response (Area and height) of the peak that appeared versus the concentration used. [16]

LOD and LOQ

LOQ is the lowest amount of the drug detected by this analytical method, while LOQ is the lowest concentration of this drug, which can be precisely and accurately measured. These two parameters can be obtained from the standard

deviation of the response and the slope of the calibration curve [17,18,19], as shown in the following equations;

LOD= 3.3 SD/ Slope

SD is the response standard deviation

LOQ = 10 SD/ slope

Robustness

The robustness was measured when small but deliberate changes were made to the analysis method, such as flow rate and the percentage of the organic phase to the water phase. The flow rates were (0.9, 1, and 1.1 ml/min) respectively, and the percentage of the organic phase to the water phase were (68:32, 70:30, and 72:28) respectively. These changes have been made, and the changes in the response were measured. Robustness analysis ensures that small changes in the method don't change the outcome profoundly. [20]

Accuracy (recovery)

Accuracy is the closeness between the calculated value of a substance using the proposed method and the true value. It was examined by injecting three different solutions with concentrations 10, 20, and 30 µg/ml of BTZ in methanol, and then each concentration was calculated based on the response of both height and area using the calibration curves of BTZ using height and area, respectively. The measured values were then compared to the known concentrations to evaluate the method's accuracy, as shown in the following equation: [21,22]

$$Recovery\ \% = \frac{measured\ value(concentration)}{true\ value\ (concentration)} * 100\ \%$$

Results and Discussion

Chromatogram and retention time

After injecting BTZ standard solution 20 µg /ml and comparing it with a blank, a sharp and well-defined peak appeared with a retention time of 6.77 min, as shown in Figure 2.

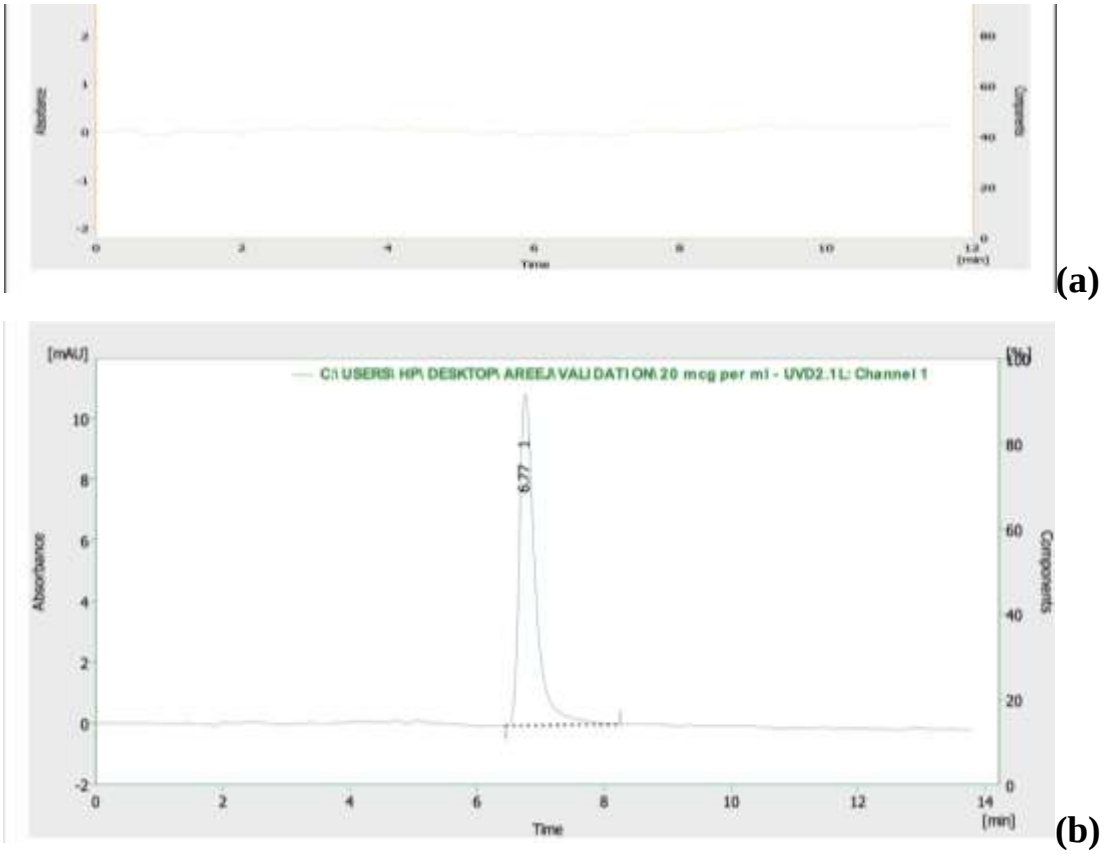

Figure 2. Chromatograms of the(a) blank (only the mobile phase) and (b)the standard of solution of BTZ (20 µg/ml) in methanol

Precision (repeatability)

After injection of the same solution containing 100 µg /ml of BTZ in methanol, a sharp and well-defined peak appeared with a retention time of 6.7 minutes, and the response was measured in mAu for both height and area. mAu is a milli absorbance unit that measures the responses of HPLC devices with UV detectors. It is proportional to the sample concentration as it passes through the device, as shown in "Table

1".RSD was less than 2% for both responses, which is within the acceptable limits of ICH guidelines, indicating that the method is valid regarding repeatability. Ghosh et al. also performs a validation process regarding precision with an RSD% of 0.2%. [23]

Table 1. The Results of repeated six injections of the same standard BTZ solution 100 µg /ml in methanol

Sample number	Retention time (min)	Response area(mAu)	Response height (mAu)
1	6.783	1210.02	70.1
2	6.800	1190	69.6
3	6.780	1205	69.9
4	6.780	1201	70.08
5	6.779	1198	69.8
6	6.798	1199	70.89
mean		1,200.5	70.061667
St deviation		6.774954	0.446247
%RSD		0.564%	0.637%

Linearity

Different concentrations of BTZ solution in methanol were injected, and the response of each solution was recorded (as shown in Table 2). As shown in "Figure 3", the retention time for each solution is similar. Still, they vary in the response regarding both area and height, which complies with the response of HPLC results that state they are proportionally related to the concentration used. The linearity of the responses is examined by plotting both height and area versus concentration (as shown in Figures 3 and 4, respectively). Both calibration curves are mentioned with their correlation coefficient, R^2, which is close to 1, meaning that the results for this method are linear for the concentration range used (10-100) µg /ml. This wide range allows the versatile and flexible measurement of BTZ in different pharmaceutical formulations with various doses and strengths. [24]

Table 2. The Results of BTZ Solutions at Various Concentrations in Methanol

Sample concentration (µg /ml)	Response area(mAu)	Response height (mAu)
20	184.031±0.95	10.873±1.19
30	331.603±0.53	20.072±1.007
40	454.310±1.13	28.134±0.87
60	661.564±0.51	41.130±2.42
70	784.229±0.68	48.206±0.71
80	923.056±2.74	57.179±1.27
90	1032.301±2.06	61.778±1.34
100	1212.842±1.24	70.688±1.322

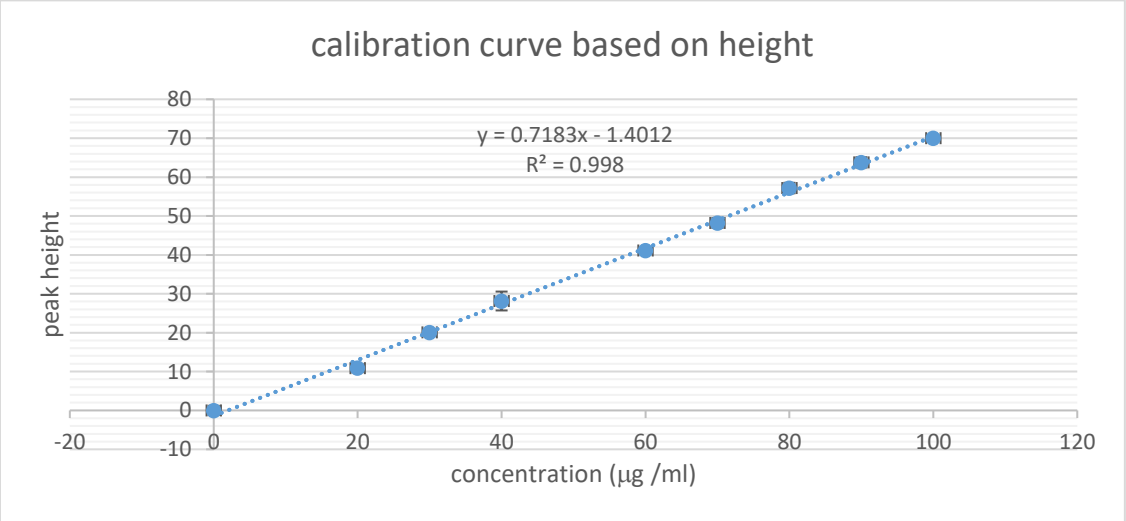

Figure 3. The calibration curve of BTZ peak height response versus concentration

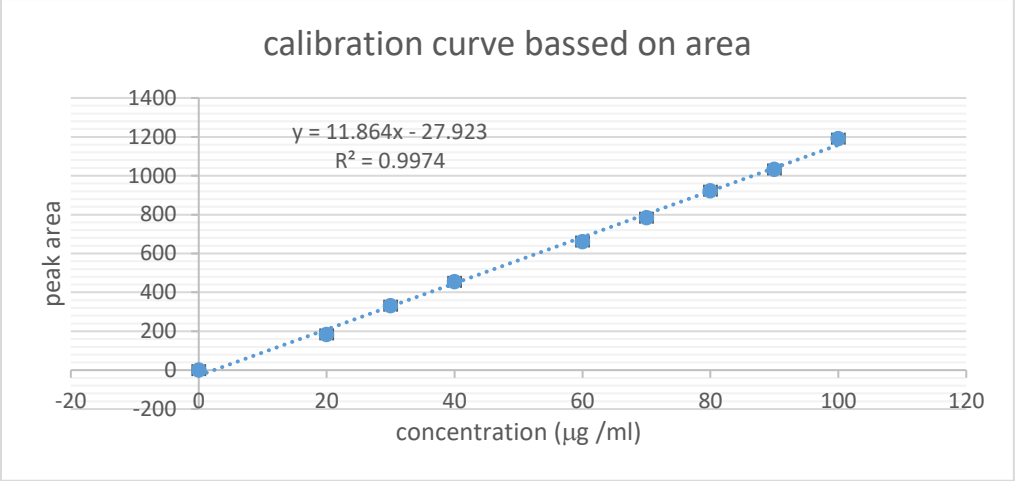

Figure 4. The calibration curve of BTZ peak area response versus concentration

LOD and LOQ

LOD and LOQ were calculated based on the equations of the calibration curves mentioned earlier, and the results are 3.2 µg /ml and 9.4 µg /ml, respectively. These numbers suggest that the lowest concentration that could be detected using this method is 3.2 µg /ml, while the lowest concentration to be quantified in a pharmaceutical preparation is 9.6 µg /ml, which is three times LOD. The low LOD and LOQ values are key parameters for measuring the method's sensitivity and analytical performance. In this sense, low LOD explains the technique's capability to detect even very low concentrations of BTZ with confidence. In contrast, low LOQ affirms that it can accurately quantify the least concentration of the drug with acceptable precision and accuracy. Such high sensitivity is crucial in pharmaceutical analysis, where determining trace levels of active ingredients and impurities is a primary requirement to guarantee the quality and safety of the final product. Furthermore, low LOD and LOQ values indicate that the method can be used for routine quality control and stability study formulations with low drug content. [25]

Specificity

This method was found to be specific for the detection and quantification of BTZ, not the excipients present in the formula. It was done by preparing two sets of solutions. The first set consists of three standard solutions with three different BTZ concentrations (100,150 and 200 µg/ml), but the BSA concentration remains the same in these three solutions. The second set of solutions has different BSA concentrations (100,200 and 300 µg /ml) while maintaining the BTZ concentration (100 µg /ml). The components of each solution and the responses of area and height were recorded for each solution, as shown in Table 3. The retention time for each solution was the same, which indicates the specificity of the isolation of BTZ in the column. On the other hand, the responses in area and height increased when the BTZ concentration increased, while the responses remained the same when the BTZ concentration was the same. These findings suggest the specificity of the method to identify and quantify BTZ in the solutions, not the excipient present, which is BSA. [26]

Table 3. Results for the specificity of the method

Sample ID	Sample components	Retention time (min)	Response area (mAu)	Response height (mAu)
A	100 µg /ml BTZ:100 µg /ml BSA	6.693±0.08	1212.842±2.4	73.391±1.23
B	100 µg /ml BTZ:200 µg /ml BSA	6.72±0.05	1131.701±3.5	70.792±0.96
C	100 µg /ml BTZ:300 µg /ml BSA	6.717± 0.11	1183.877±2.2	69.654±0.87
D	150 µg /ml BTZ:100 µg /ml BSA	6.698 ±0.07	1649.603±5.4	106.210±1.45
E	200 µg /ml BTZ: 100 µg/ml BSA	6.700 ±0.05	2161.916±6.5	130.654±2.3

Robustness

Modifying method conditions, such as the flow rate and mobile phase composition, led to slight variations in retention time and minimal changes in peak area and height responses. (as shown in Tables 4 and 5). Increasing the flow rate to 1.1 ml/min resulted in fastening of the appearance of the peak while decreasing the flow rate to 0.9 ml/min resulted in slowing the appearance of the peak, which is reasonable because the peak is an indication of the separation of BTZ in a specific retention time with no noticeable change in the response of area and height since the concentration of BTZ in the sample didn't change.

Altering the composition of the mobile phase affects the retention time, as it changes the polarity of the mobile phase, thereby modifying the separation conditions within the column and influencing the elution of BTZ from other components in the solution. Sample G is the standard sample with the optimized conditions. [27]

Table 4. Results of the Robustness (changing the flow rate)

Sample ID	Condition of the separation process change	Retention time (min)	Response area (mAu)	Response height (mAu)
F	Flow rate 0.9 ml/min	7.283±0.2	1454.262±5.3	72.618±1.35
G	Flow rate 1 ml/min and	6.633±0.34	1434.277±8.3	74.757±3.4
H	Flow rate 1.1 ml/min	5.983±0.28	1323.873±6.7	76.668±2.8

Table 5. Results of the Robustness (changing the mobile phase composition)

Sample ID	Condition of the separation process change	Retention time (min)	Response area (mAu)	Response height (mAu)
I	Organic: water (70:30)	6.633±	1434.277±8.3	74.757±3.4
J	Less organic (68:32)	7.317 ±	1506.003±2.4	70.510±0.9
K	More organic (72:28)	6.083 ±	1518.416±7.2	88.915±2.6

Accuracy

Recovery was 99.2%, 99.6% , and 100.7% for BTZ from the three solutions with concentrations of 10, 20 and 30µg/mL, respectively. The RSD was 0.502%, significantly less than the suggested limit of 2% by ICH norms. These findings indicate that the developed method can be considered sufficiently accurate and reliable for determining the BTZ in various concentrations. Thereby confirming its suitability for the determination of BTZ in pharmaceutical preparations. The excellent recovery and the low variability indicate the method's reliability, not only for routine quality control purposes but also in research work. Demurtas et al. performed an HPLC validation for budesonide to obtain an accuracy ranging from (97-100.41) %, which also complies with ICH guidelines. [28]

Table 6. Summary of the chromatographic conditions and results for this method

The criteria	The optimal condition
Mobile phase	Methanol: Water (70:30)
Flow rate	1 ml/min
UV detection	270 nm
Injection volume	10 µL
Temperature	30°C
Calibration curve based on area	y = 11.864x - 27.923
The correlation coefficient of the calibration curve based on area	R^2 = 0.997
Calibration curve based on height	y = 0.7183x - 1.4012
The correlation coefficient of the calibration curve based on height	R^2 = 0.998
LOD	3.2 µg/ml
LOQ	9.6 µg/ml

Conclusion

Using HPLC to detect and quantify BTZ in pharmaceutical preparations is essential, especially when preparing BSA nanoparticles. This detection method is reproducible , reliable , and suitable for this purpose. It is also simple and doesn't require a lot of complications in the preparation of the mobile phase or the detection device since the mobile phase only contains water and methanol,

and a UV detector does the detection. This method could also detect and quantify BTZ in a wide range in a linear manner ranging from 10-100 μg/ml. In conclusion, this method is suitable for measuring BTZ in albumin nanoparticles and could be used in other pharmaceutical preparations.

Acknowledgment

We would like to extend our heartful gratitude to the dedicated and supportive staff of the College of Pharmacy/University of Baghdad for their assistance and guidance in completing this work. We also want to express our deepest gratitude to the College of Pharmacy/University of Kufa staff, especially the Department of Pharmaceutics, for allowing us to perform this validation and analysis in the pharmaceutics laboratory. We also would like to present a special thanks to Assistant Prof. Dr. Sarmad Al-Edresi for his guidance and help in setting up and developing this analytical method.

Conflicts of Interest

There is no conflict of interest.

Funding

No funds are provided by any organization.

Ethics Statements

No animal or human studies were performed to establish this work.

Author Contribution

A.A. contributed to the design, analysis, interpretation, drafting, and writing of the manuscript. M.M. contributed to the revision and proofreading the manuscript (supervisor).

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تطوير والتحقق من طريقة كشف وتحديد كمية البورتيزوميب باستخدام جهاز كروماتوغرافيا السائل عالي الأداء داخل جسيمات الالبومين النانوية المحضرة من ألبومين مصل البقر

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الخلاصة

طريقة تحليلية جديدة وبسيطة ودقيقة للكشف عن البورتيزوميب، الذي يعد مثبطًا انتقائيًا للبروتوزوم ويعمل كدواء مضاد قوي للسرطان. تم تطوير هذه الطريقة والتحقق من صحتها باستخدام جهاز كروماتوغرافيا السائل عالي الأداء (HPLC) مع عمود C18 (250×4.6 مم)، والطور المتنقل يتكون من ميثانول: ماء (٧٠:٣٠)، بمعدل تدفق ثابت قدره ١ مل/دقيقة وكشف بالأشعة فوق البنفسجية عند ٢٧٠ نانومتر. تم العثور على وقت الانحسار لهذا الدواء ليكون ٦,٧٧ دقيقة باستخدام هذه الطريقة. كما ثبت أنها دقيقة ومحددة مع نسبة انحراف نسبي (RSD%) أقل من ٢٪، مما يتوافق مع متطلبات معايير ICH. كما أظهرت الطريقة استجابة خطية ممتازة في نطاق ١٠-١٠٠ ميكروغرام/مل مع معامل ارتباط R^2 قدره ٠,٩٩٧ و٠,٩٩٨ للمساحة والارتفاع على التوالي، مما يشير إلى نطاق واسع لقياس البورتيزوميب في التركيبات الدوائية المختلفة. تم أيضًا حساب اقل تركيز يمكن الكشف عنه (LOD) واقل تركيز يمكن حسابه (LOQ)ليكونا ٣,٢ و ٩,٦ ميكروغرام/مل على التوالي، مما يظهر مستوى منخفض للكشف وقياس الكمية. كما بلغت نسبة الدقة (الاسترجاع) ٩٩,٨٣٪ ±٠,٧٧ وهذا ما يؤكد موثوقية الطريقة .في الختام، تم التحقق من صحة الطريقة المقترحة وفقًا لإرشادات ICH فيما يتعلق بالدقة، التحديد، الخطية، الاسترجاع والصلابة.

الكلمات المفتاحية: جسيمات الالبومين النانوية، بورتيزوميب، الكشف وتحديد الكمية ، جهاز الكروموتوغرافيا السائل عالي الأداء.