Research Article

# Method Development and Validation for Simultaneous Estimation of Cabozantinib and Nivolumab in Rat Plasma by HPLC

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**ABSTRACT**

A simple, sensitive and rapid chromatographic method was developed and validated for simultaneous quantification of Cabozantinib and Nivolumab in rat plasma using Alectinib as internal standard. The samples were assayed by the Waters alliance e-2695 HPLC instrument using Symmetry C18 column (150x4.6mm, 3.5µ) under isocratic condition. Here the buffer was Triethyl amine at pH 2.5 adjusted with OPA. Mobile phase used was 0.1% TEA and Acetonitrile (70:30) with a flow rate of 1ml/min. The eluent was monitored at 219nm for simultaneous measurement of Cabozantinib and Nivolumab. The calibration curves were linear over the range of 1-20µg/ml of Cabozantinib and 0.1-2µg/ml of Nivolumab. The method was validated in terms of system suitability, selectivity, sensitivity, accuracy, precision, recovery, matrix effect, linearity and stability. The % RSD of peak areas of all measurements always less than 2. The proposed method was specific for the simultaneous determination of Cabozantinib and Nivolumab in rat plasma.

**Keywords:** HPLC, Cabozantinib, Nivolumab, Plasma.

## INTRODUCTION

Cabozantinib, sold under the brand-name Cabometyx and Cometriq, is a medication used to treat medullary thyroid cancer[1,2] and a second line treatment for renal cell carcinoma[3,4] among others. It is a small molecule inhibitor[5]of the tyrosine kinases c-Met[6] and VEGFR2, and also inhibits AXL and RET. It was discovered and developed by Exelixis[7] Inc. In 2012 cabozantinib in its capsule formulation was approved by the USFDA under the name Cometriq for treating patients with medullary thyroid cancer. The capsule form was approved in Europe for the same purpose in 2014. In April 2016 the USFDA[8] granted approval for marketing[9,10] the tablet formulation as a second line treatment for kidney cancer[11] and the same was approved in Europe in October of that year. Cabozantinib is used in two forms. A capsule form is used since 2012 to treat medullary thyroid cancer and a tablet form is used since 2016 as a second line treatment for renal cell carcinoma.

Nivolumab, marketed as Opdivo, is a medication used to treat cancer[12]. It is used as a first line treatment for inoperable or metastatic melanoma[13] in combination with ipilimumab[14] if the cancer does not have a mutation[15] in BRAF, as a second-line treatment following treatment with ipilimumab and if the cancer has a mutation in BRAF, with a BRAF inhibitor[16], as a second-line treatment for squamous non-small lung cancer, and as a second-line treatment for renal cell carcinoma. Nivolumab has recently been approved for small cell lung cancer[17]. It had not been tested in pregnant women but based on the mechanism of action and animal studies, is probably toxic to the fetus[18]; it is not known if it is secreted in breast milk[19]. Side effects include severe immune-related inflammation[20] of the lungs[21], colon, liver[22], kidneys[23], and thyroid[24] and there are effects on skin, central nervous system[25], the heart and the digestive system[26]. It is a human IgG4 anti-PD-1 monoclonal antibody[27]. Nivolumab works as a checkpoint inhibitor, blocking a signal that would have prevented activated T cells from attacking the cancer, thus allowing the immune system[28] to clear the cancer. It was discovered at Medarex[29], developed by Medarex and Ono Pharmaceutical, and brought to market by Bristol-Myers Squibb (which acquired Medarex in 2009) and Ono. FDA has approved Nivolumab for primary or metastatic rothelial carcinoma[30], the most common form of bladder cancer[31]. It can be prescribed for locally advanced or metastatic form of the condition that experience disease progression during or following platinum-containing chemotherapy[32] or have progression within 12 months of neoadjuvant treatment with platinum-containing chemotherapy.

Nivolumab was not tested in pregnant women but based on how the drug works and on animal studies, it is likely to cause harm to a baby; it is not known if nivolumab is secreted in breast milk. Nivolumab and other PD-1 inhibitors, appear to be effective in people with brain metastases[33] and auto immune disease[34].

## MATERIALS AND METHODS

### Chemicals and Reagents

Acetonitrile, Ortho Phosphoric acid (OPA) and water (HPLC grade), Triethyl amine (HPLC grade) were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Cabozantinib and Nivolumab as reference standards were procured from GLS Pharma, Hyderabad.

**Equipment**

Waters alliance-2695 chromatographic system consisting of quaternary pump, PDA detector- 2996 and chromatographic software Empower-2.0 was used.

**Chromatographic conditions**

Chromatographic separation was carried out in isocratic mode at room temperature using Symmetry $C_{18}$ (150x4.6mm, 3.5µ) column. The mixture of 0.1% Triethyl amine and Acetonitrile 70:30 v/v at a flow rate of 1.0ml/min was used as a mobile phase. The injection volume was 10µl and eluents was monitored at 219nm using PDA detector. The run time was 8 min.

**Preparation of Standard and quality control samples**

Two separate stock solutions of Cabozantinib and Nivolumab were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as subject sample analysis. The stock solutions of Cabozantinib and Nivolumab were prepared in acetonitrile at a free base concentration of 10µg/ml of Cabozantinib and 1µg/ml of Nivolumab. Primary dilutions and working standard solutions were prepared from stock solutions using mobile phase as diluent. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention time of Cabozantinib and Nivolumab. Eight-point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Cabozantinib and Nivolumab. Calibration samples were made at concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15 and 20µg/ml of Cabozantinib and 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2µg/ml of Novolumab.

**Sample Preparation**

For sample preparation, 500µl of plasma sample, 500µl of acetonitrile and 500µl of internal standard, 500µl of standard stock and 1000µl of diluent to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 500rpm for 20min. Collect the supernatant solution in HPLC vial and lnject into the chromatogram.

**Validation of the Bioanalytical Method**

The method was validated by the determination of the following parameters: Specificity, linearity, range, recovery, accuracy, precision, lower limit of quantification (LLOQ) and stability studies according to the currently accepted US food drug administration (FDA) bioanalytic method validation guidance.

**Specificity**

Randomly selected six blank plasma samples, which were collected under controlled conditions, were carried through the protein precipitation procedure and chromatographed individually to determine the extent to which endogenous plasma components could contribute to interference with the analyte or the internal standard.

**System suitability**

The system suitability was assured by determining peak retention time, peak area, plate count and tailing factor for Cabozantinib and Nivolumab and Alectinib. The acceptance criteria for system suitability are CV<1%, asymmetry factor<2 and plate count>2000.

**Accuracy**

Accuracy of the method was determined at three different concentration levels. Mean and %RSD were calculated, and the results indicate that the method was accurate.

**Precision**

Precision of the data was reported in terms of Repeatability, intra-day precision, and inter-day precision. The results indicate that the method is precise.

**Linearity**

The linearity of the method was estimated by preparing calibration samples which were prepared by spiking appropriate amount of sample and organic solvent into control plasma to get 10µg/ml of Cabozantinib and 1µg/ml of Nivolumab.

**Sensitivity**

The sensitivity for simultaneous determination of Cabozantinib and Nivolumab was evaluated with respect to Alectinib peak. The LOD and LLOQ were determined by calculating the signal/noise ratio (S/N). According to FDA guidelines, the analyte response at the LLOQ should be atleast 5 times the response compared to blank response.

**LOD and LOQ**

The sensitivity of the proposed method for measurement of Cabozantinib and Nivolumab were estimated in terms of LOD and LOQ. The limit of detection and limit of quantification was determined according to ICH guidelines for the validation of analytical procedure. The formulae used were,

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

**Recovery**

Recovery was determined at three levels LQC, MQC and HQC. In extracted process samples are spiked with plasma and un extracted process samples are not spiked with plasma.

**Matrix Effect**

To evaluate matrix effect different lots of rat plasma were spiked with analyte concentration levels at LQC and HQC.

**Stability Studies**

***Freeze thaw stability***

Drug stability was determined after three freeze thaw cycles. Thre aliquots of each higher and lower concentrations was frozen for 24 hours and then thawed unassisted at room temperature, when thawed completely the samples were refrozen under the same conditions as before. Freeze and thaw cycles were repeated twice or more and anlysed on third cycle.

***Auto sampler stability***

Three aliquots of each low and high, low and medium concentrations were stored at 2-8°C temperature in auto sampler for 24 hours and then analyzed.

***Bench top stability***

Three aliquots of low, medium and high unprocessed QC samples were kept at room temperature for 24 hours. After 24 h the samples were analyzed.

***Long-term stability***

Three aliquots of low, medium and high QC samples were frozen at -20°C for 30days and the samples were analyzed.

***Stock solution stability***

The stability of stock solutions of both drug and internal standard were evaluated at room temperature for 6h.

## RESULTS AND DISCUSSION

To obtain the best chromatographic condition, different columns like $C_{18}$, $C_8$, and CN and mobile phases composed of tri ethyl amine of pH-2.5 with OPA and acetonitrile were tested. The best chromatographic separation occurred on Symmetry column with a mobile phase consisting of 0.1% TEA and Acetonitrile in 70:30 ratios at a flow rate of 1ml/min and UV detection at 219nm.

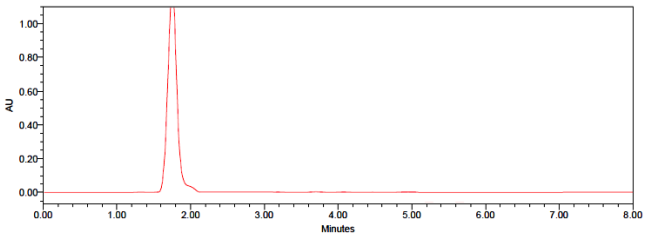


**Figure 1:** Chromatogram of blank rat plasma

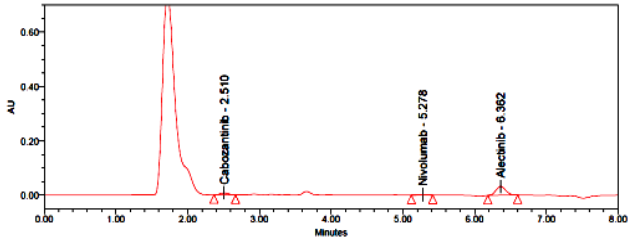


**Figure 2:** Blank plasma spiked with analytes and IS

The influence of both organic modifier and concentration was carefully studied. Increasing the organic concentration not only improves peak shape and decreasing run time. Decreasing organic concentration resulted in excessive tailing of eluting peaks and long run time.

**Validation of the proposed method**

***Linearity of calibration curves and lower limit of quantification***

Linearity of the method was found to be in the concentration range of 1-20µg/ml of Cabozantinib and 0.1-2µg/ml of Nivolumab. The linearity graph was plotted between concentrations vs. area. The results of linearity were shown in table 1 and 2.

***Specificity and Selectivity***

The plasma obtained from six different rats after administration of the drugs Cabozantinib and Nivolumab were analyzed.

**Table 1:** Assay parameters and regression characteristics of Cabozantinib and Nivolumab

| Parameter | Cabozantinib | Nivolumab |
|---|---|---|
| Linearity range | 1-20µg/ml | 0.1-2µg/ml |
| Detection limit | 0.1 | 0.01 |
| Quantification limit | 1 | 0.1 |
| Regression Equation Y* | | |
| Number of experiments | 8 | 8 |
| Slope | 0.135607 | 0.66601 |
| Intercept | 0.00305 | 0.00710 |
| Corelation Coefficient | 0.99914 | 0.99901 |

***Recovery***

The absolute recoveries of the studied drugs were determined by comparing the peak area ratio of the QC sample spiked in rat plasma and defined in three runs with those of post extracted plasma blanks with the known amount of analytes.

**Table 2:** Recovery studies of Cabozantinib and Nivolumab

| Concentration | Cabozantinib (% Recovery) | Nivolumab (% Recovery) |
|---|---|---|
| LQC | 97.5 | 98.28 |
| MQC | 98.64 | 98.65 |
| HQC | 98.54 | 99.52 |

***Precision and Accuracy***

The precision and accuracy of the method were assessed by analyzing six replicate samples at low, medium and high concentration levels. Precision and accuracy were determined by %CV. The %CV and accuracy results were found to be within the acceptable limits.

**Table 3:** Intra and Inter day precision and accuracy for Cabozantinib

| Nominal Conc. (µg/ml) | Within run | | | Between run | | |
|---|---|---|---|---|---|---|
| | Mean (µg/ml) | Precision (%CV) | Accuracy | Mean (µg/ml) | Precision (%CV) | Accuracy |
| 0.546 | 0.551 | 0.67 | 98.67 | 0.525 | 0.71 | 96.53 |
| 5.067 | 5.117 | 0.11 | 97.26 | 5.167 | 0.12 | 98.62 |
| 10.547 | 10.684 | 0.15 | 95.21 | 10.258 | 0.16 | 98.75 |
| 15.267 | 15.275 | 0.11 | 98.32 | 15.285 | 0.1 | 99.31 |

**Table 4:** Intra and Inter day precision and accuracy for Nivolumab

| Nominal Conc. (µg/ml) | Within run | | | Between run | | |
|---|---|---|---|---|---|---|
| | Mean (µg/ml) | Precision (%CV) | Accuracy | Mean (µg/ml) | Precision (%CV) | Accuracy |
| 0.0576 | 0.0562 | 3.2 | 98.63 | 0.0538 | 4.65 | 96.85 |
| 0.514 | 0.524 | 3.59 | 97.45 | 0.518 | 3.38 | 98.32 |
| 1.056 | 1.067 | 0.47 | 98.62 | 1.047 | 0.64 | 99.17 |
| 1.548 | 1.559 | 0.33 | 94.28 | 1.541 | 0.51 | 97.42 |

### Stability

The stability of the drugs Cabozantinib and Nivolumab in rat plasma was assessed by analyzing six replicate samples at the low and high concentration levels at ambient temperature over 24h. The measured concentrations of the drugs in these samples sitting at room temperature for 24h were compared with that obtained with the corresponding sample. Freshly prepared and proceed immediately. The results indicate the stability of the studied drug in rat plasma over three freeze thaw cycles. Also the studied drug showed the stability in rat plasma when stored at -60°C for wet extract stability when compared with the freshly prepared sample. The results are obtained are tabulated in 5, 6.

**Table 5**: Stability data for Cabozantinib

| Stability Condition | Concentration (µg/ml) | | %CV | |
|---|---|---|---|---|
| | LQC | HQC | LQC | HQC |
| Bench top | 5.01 | 15.01 | 0.89 | 1.05 |
| Wet extract | 5.02 | 15.11 | 1.14 | 0.56 |
| Freeze-Thaw | 5.00 | 15.08 | 1.24 | 0.69 |
| Auto sampler | 4.99 | 15.14 | 0.96 | 1.02 |

**Table 6**: Stability data for Nivolumab

| Stability Condition | Concentration (µg/ml) | | %CV | |
|---|---|---|---|---|
| | LQC | HQC | LQC | HQC |
| Bench top | 0.51 | 1.51 | 1.55 | 0.81 |
| Wet extract | 0.50 | 1.49 | 1.28 | 0.77 |
| Freeze-Thaw | 0.49 | 1.52 | 0.89 | 0.51 |
| Auto sampler | 0.52 | 1.56 | 1.65 | 0.57 |

## CONCLUSION

The method developed is a simple, rapid, accurate and reliable procedure for the analysis of Cabozantinib and Nivolumab in rat plasma, meeting all requirements for the validation of an analytical methodology. It is adequate to monitor patients receiving therapeutic dosage of the drugs. An attempt has been made to understand and explain the bio analytical method development point view. Some of the method and how is validation carried out were described in different situation encountered in the study sample analysis has been reported in this article. These various essential development and validation characteristics for bioanalytical methodology have been discussed with a view to improving the standard and acceptance in this area of research.

## REFERENCES


1. Stamakakos M, Paraskeva P, Stefanaki C, Katsaronis P, Lazaris A, Safioleas K, Kontzoglou K, Medullary thyroid carcinoma, The third most common thyroid cancer reviewed. Oncol Lett. 2 (1), 2011, 49-53.
2. Dionigi G, Bianchi V, Rovera F, et al. Medullary thyroid carcinoma, surgical treatment advances, Expert rev anticancer ther. 7 (6), 2007, 877-85.
3. Rini BL, Rathmell WK, Godley P, Renal cell carcinoma,CurrOpinOncol. 20 (3), 2008, 300-6.
4. Cohen, Herbert T, McGovern, Francis J, Renal-cell carcinoma, New Engalnd journal of Medicine. 353 (23), 2005, 2477-90.
5. Arkin MR, Wells JA. Small –molecule inhibitors of protein-protein interactions, progressing towards the dream, Nature reviews drug discovery. 3 (4), 2004, 301-17.
6. Bhise SB, Nalawade AD, Wadhawa H, Role of protein tyrosine kinase inhibitors in cancer therapeutics, Indian journal of Biochemistry& Biophysics. 41 (6), 2004, 273-80.

7. Mc Carthy, Alice A. Exelixis Integrated drug discovery and development platform for human therapeutics, Chemistry& biology. 12 (4), 2005, 407-408.
8. Karki L, Review of FDA law related to Pharmaceuticals, The hatch-waxman act, regulatory amendments and implications for drug patent enforcement, Journal of the patent & Trademark office society. 87, 2005, 602-620.
9. Hunt, Shelby D. The nature and scope of marketing, Journal of Marketing. 40 (3), 1976, 17-28.
10. Bagozzi, Richard P. Marketing as exchange, Journal of Marketing. 39 (4), 1975, 32-39.
11. Cheungpasitporn W, Thongprayoon C, O Corragain OA, Edmonds PJ, Ungprasert P, Kittanamongkolchai W, Erickson SB. The risk of kidney cancer in patients with kidne stones, A systematic review and meta-analysis. QJM. 108, 2014, 205-12.
12. WHO Drug information. Vol.26, 2012, No. 2.
13. Fiddler IJ. Melanoma Metastasis, Cancer Control. 2 (5), 1995, 398-404.
14. Ribas, Antoni, Hodi, F Stephen, Callahan, Margaret, Konto, Cyril, Wolchok, Jedd. Hepatotoxicity with combination of vemurafenib and ipilimumab, N Engl j med. 368 (14), 2013, 1365-6.
15. Peck JR, Barreau G, Heath SC. Imperfect genes, Fisherian mutation and the evolution of sex, Genetics. 145 (4), 1997, 1171-99.
16. Sithanandam G, Kolch W, Duh FM, Rapp UR. Complete coding swquence of a human B-raf cDNA and detection of B-raf protein kinase with isozyme specific antibodies. Oncogene. 5 (12), 1990, 1775-80.
17. Nutting C, Horwich A, Fisher C, Parsons C, Deamaley DP. Small –cell carcinoma of the prostate, Journal of the royal society of medicine. 90(6), 1997, 340-1.
18. Ghosh, Shampa, Raghunath, Manchala, Sinha, Jitendra Kumar. 2017. Fetus, Encyclopedia of animal cognition and behavior, Springer international publishing. p, 1-5.
19. Hauck FR, Thompson JM, Tanabe KO, Moon RY, Vennemann MM. Breastfeeding and reduced risk of sudden infant death syndrome, a meta-analysis, Pediatrics. 128 (1), 2011, 103-10.
20. Rather, L J. Disturbance of function, The legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus, Bull N Y Acad Med. 47 (3), 1971, 3.3-322.
21. Arakawa H, Niimi H, Kurihara Y, Nakajima Y, Webb WR. Expiratory high-resolution CT: Diagnostic value in diffuse lung diseases, Americal journal of Roentgenology. 175 (6), 2000, 1537-1543.
22. Elias H, Bengelsdorf H. The structure of the liver in Vertebrates, Cells tissues Organs. 14 (4), 1952, 297-337.
23. Al- kahtani M A, Zuleta C, Caviedes-Vidal E, Garland Jr T. Kidney mass and relative medullary thickness of rodents in relation to habitat, body size, and phylogeny, Physiological and Biochemical Zoology. 77 (3), 2004, 346-365.
24. Page C, Cuvelier P, Biet A, Boute P, Laude M, Strunski V. Thyroid tubercle of Zuckerkandil, anatomical and surgical experience from 79 thyroidectomies, The journal of Laryngology and Otology. 123 (7), 2009, 768-71.
25. HuijzenR, Nieuwenhuys J, Voogd C van. 2007. The human central nervous system, Berlin. Springer. p-3.
26. Kong F, Singh RP. Disintegration of solid foods in human stomach, J Foods sci. 73 (5), 2008, R67-80.
27. Gelboin, Harry V, Krausz, Dristopher W, Gonzalez, Frank J, Yang, Tian J. Inhibitory monoclonal antibodies to humab cytochrome P450 enzymes, a new avenue for drug discovery, Trends in Pharmacological Sciences. 20 (11), 1999, 432-8.
28. O Byme KJ, Dalgleish AG. Chronic immune activation and inflammation as the cause of malignancy, British journal of Cancer. 85 (4), 2001, 473-83.
29. Breakthrough of the year 2013, Cancer immunotherapy, Science 20 December Vol. 342 no. 6165, p, 1432-1433.
30. Colin P, Koenig P, Ouzzance A, Berthon N, Villers A, Biserte J, Roupret M. Environmental factors involved in carcinogenesis of urothelial cell carcinomas of the upper urinary tract, BJU international. 104 (10), 2009, 1436-40.
31. Di Stasi SM, Riedl C. Updates in intravesical electromotive drug administration of mitomycin-C for non-muscle invasive bladder cancer, World journal of urology. 27(3), 2009, 325-30.
32. Corrie PG. Cytotoxic chemotherapy: clinical aspects. Medicine. 36 (1), 2008, 24-28.
33. Wen PY, Loeffler JS. Management of brain metastases, Oncology. 13 (7), 941-54, 1999, 957-61, discussion 961-2,9.
34. Katz U, Shoenfeld Y, Zandman-Goddard G. Update on intravenous immunoglobulins (IVIg) mechanisms of action and off-lable use in autoimmune diseases, Current pharmaceutical design. 17 (29), 2011, 3166-75.


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