Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **38** (4): (2019)

Received: November 16, 2018
Accepted: January 16, 2019

Development and Validation of a RP-HPLC Method for Vemurafenib in Human Urine

Gülşen GÜVEN *

Department of Chemistry, Faculty of Arts and Sciences,
Aydın Adnan Menderes University, Aydın, Turkey

***SUMMARY*. Vemurafenib (propane-1-sulfonic acid{3-[5-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridine-3-carbonyl]-2,4difluoro-phenyl}-amide) is a BRAF enzyme inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of late stage melanoma. Reversed phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the determination of vemurafenib and erlotinib was used as an internal standard. Erlotinib (IS) and vemurafenib were separated by using a X-Terra RP-18 column (250 x 4.60 mm, ID 5 μm) and detected by a DAD detector set at 249 nm. The mobile phase consisted of a mixture of acetonitrile:water 60:40 (v/v) at a flow rate of 1.0 mL/min. Isocratic separation was performed in less than 7 min. The calibration was found to be linear in the range 0.2-10 mg/L. Inter-day and intra-day precision was less than 0.72 %. This analytical method was successfully applied in spiked samples of human urine.**

RESUMEN. Vemurafenib (ácido propano-1-sulfónico{3-[5-(4-clorofenil)-1H-pirrolo[2,3-b] piridina-3-carbonil]-2,4difluoro-fenil}-amida) es una inhibidor enzimático del BRAF aprobado por la Administración de Drogas y Alimentos de los Estados Unidos (FDA) para el tratamiento del melanoma en etapa tardía. El método de cromatografía líquida de alto rendimiento de fase inversa (RP-HPLC) fue desarrollado y validado para la determinación de vemurafenib y se utilizó erlotinib como estándar interno. Erlotinib (IS) y vemurafenib se separaron mediante el uso de una columna X-Terra RP-18 (250 x 4,60 mm, ID 5 μm) y se detectaron mediante un detector DAD configurado a 249 nm. La fase móvil consistió en una mezcla de acetonitrilo: agua 60:40 (v/v) a un caudal de 1,0 mL/min. La separación isocrática se realizó en menos de 7 min. Se encontró que la calibración era lineal en el rango de 0.2-10 mg/L. La precisión interdía e intradía fue inferior al 0,72%. Este método analítico se aplicó con éxito en muestras enriquecidas de orina humana.

INTRODUCTION

Mutated BRAF is a new target for the treatment of advanced melanoma and provides an important opportunity for new therapies. Vemurafenib, a drug which is indicated as an option for first-line therapy in patients with unresectable or metastatic melanoma with the BRAF V600E mutation, was approved by the FDA in August 2011. Vemurafenib is an ATP competitive small molecule, which act as a BRAF (V600E) kinase inhibitor that binds to V600E and inhibits oncogenic BRAF. This drug inactivates this protein, thereby inhibiting downstream proliferation and signalling, resulting in cancer cell apoptosis. Vemurafenib is the first selective inhibitor of mutated BRAF and has proven effective in the treatment of melanoma in many clinical trials [1-5].

High performance liquid chromatography (HPLC) is a versatile separation technique that separates analytes by passing them through a column packed with micrometer sized particles. HPLC is the most common used method among all analytical separation techniques. The reason for the fact that the method is so widespread, sensitivity is readily adaptable to the correct quantitative determinations, non-volatile species or easily degradable species with temperature. In the HPLC method, finding the best chromatographic separation conditions is the most impor-

KEY WORDS: human urine, liquid chromatography, vemurafenib.

* Author to whom correspondence should be addressed. *E-mail:* gguven@adu.edu.tr

ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

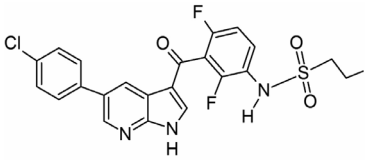

Figure 1. Chemical structure of vemurafenib.

tant aim of optimization studies. Various parameters such as column type, mobile phase composition, column temperature, flow rate, pH should be optimized simultaneously in order to develop the method in HPLC.

The literature survey revealed that few reversed phase liquid chromatography and liquid chromatography –mass spectrometry (LC-MS/MS) methods have been reported for the analysis of vemurafenib in human plasma and pharmaceutical dosage form [6-14].

The aim of this work that development of a new LC method with good selectivity, sensitivity, simple, rapid and fully validated for the analysis of vemurafenib (Fig. 1). There are no publication about the optimization the co-effects of mobile phase composition and pH on vemurafenib detection. Moreover, in the publications so far, vemurafenib has been studied in plasma samples intensively, but there are no publications for vemurafenib detection in urine specimens.

MATERIAL METHODS

Chemicals and reagents

Vemurafenib standard (99.5%) was kindly donated by Roche F. Hoffmann-LaRoche Ltd (Basel, Switzerland). Erlotinib (IS) was supplied from Nobel (Istanbul, Turkey). HPLC grade acetonitrile, dimethyl sulfoxide (DMSO), phosphoric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). HPLC grade water was prepared by through a 0.45 µM Millipore filter (Millipore Company, USA).

Apparatus

The LC system used for method development consisted of an Agilent Technologies 1260 Infinity LC system equipped with a quaternary pump-G1311B, auto injector-G1329B and diode array detector-G4212B. The detection was carried out at 249 nm. An X-Terra RP-18 (4.60 × 250 mm I.D., 5 µm) column was used as stationary phase. The pH of mobile phase was measured by a digital pH meter WTW 315i (Weilheim, Germany).

Chromatographic conditions

The mobil phase ratio was optimazed in isocratic conditions. Three different mobile phase ratios of acetonitrile-water (57.5, 60.0, and 62.5%, v/v) were used to investigate the effect of mobile phase composition on chromatographic separation. Each mobile phase containing 12.5 mM *o*-phosphoric acid was adjusted to different pH values from 7.0 to 10.0 by adding of 1 M NaOH. Chromatographic measurements were done at 30 °C with an eluent flow rate of 1 mL/min. The detection was performed at 249 nm. Three injections (10 µL) of each solution were injected into the HPLC system.

Preparation of standard solution

Stock solution of vemurafenib and erlotinib were prepared in DMSO, to give 0.1 mg/mL concentration. Working solutions of vemurafenib for calibration standards were prepared at five concentrations (0.2, 0.5, 1.0, 5.0, and 10.0 µg/mL) by dilution in mobile phase of the stock solution. The prepared stock solution was stored at 4 °C.

Calibration standards were prepared in the concentration range of 0.2-10.0 µg/mL for the determination vemurafenib in urine samples. The concentration of IS (erlotinib) was fixed at a constant level of 2 µg/mL. Appropriate volumes of stock standard solution of analyte and IS were adjusted with mobile phase in 10 mL volumetric flasks. 10 µL of standard solution was injected into the chromatographic system. The chromatogram at 249 nm was selected for monitoring of vemurafenib. The calibration curves were constructed by plotting the ratio of the peak area against the drug concentration.

Preparation of human urine samples

Human urine sample was obtained from healthy volunteers. One mL of urine sample was diluted 1:5 with deionized water. Two mL of diluted urine sample was transferred to a centrifuge tube, 3 mL of acetonitrile (IS) was added and known amount of vemurafenib was spiked. This solution was diluted to 10 mL with mobile phase, mixed in a vortex for 1 min and was filtered through a 0.45 µm membrane filter. The total dilution factor of urine sample was 1:25 fold.

RESULTS AND DISCUSSION

Chromatographic conditions were optimized for RP-LC determination of vemurafenib. The in-

fluence of pH on the mobile phase, organic solvent composition and column temperature were examined to provide good resolution of the analyte. The different mobile phase ratios (57.5, 60.0 and 62.5 % v/v) of acetonitrile-water were used to develop an efficient and reproducible method. pH values between 7.0 and 10.0 were tested and pH 9.0 was selected as shorter analysis time. Four column temperatures (25, 30, 40, and 50 °C) were tested and 30 °C was selected because of shorter analysis time. The optimal separation condition was achieved when mobile phase is 12.5 mM phosphate buffer containing acetonitrile (60:40 v/v) at pH 9.0, producing well resolved and symmetrical peak shapes without any tailings. X-Terra RP-18 (250 × 4.60 mm, ID 5 µm) column was used for separation and DAD detector set at 249 nm.

Erlotinib was selected as an internal standard because it is an EGF receptor tyrosine kinase inhibitor used in non small lung cancer treatment. Additionally, it was observed that shorter retention times with better peak shape and resolution was found with the investigated compound.

System suitability tests are an important part of the development of the LC method. The USP Pharmacopoeia recommends that these tests can be performed before the analysis [15]. These tests include parameters such as asymmetry factor, retention time, plate number, capacity factor, selectivity and RSD % of peak height or area for repeated injections. The theoretical plate numbers (N) were 24350 for erlotinib (IS) and 22857 for vemurafenib. Asymmetry factors of 0.912 and 0.862 were obtained for erlotinib (IS) and vemurafenib, respectively. The selectivity factor and resolution were obtained as 1.708 and 8.588 for vemurafenib, respectively. The retention times of erlotinib and vemurafenib were 5.17 and 6.69, respectively. In the proposed HPLC method, approximately 7 min is sufficient for the separation of the compounds (Fig. 2).

The USP requirements were met with the results obtained from the system suitability tests. The linearity of the calibration curve was shown by least squares linear regression analysis. Three replicate injections of the standard solutions were made and the peak area of the chromatogram was plotted against the concentration of studied drug to obtain the corresponding calibration graph.

The constructed calibration curve was linear in the range of 0.2-10.0 µg/mL. The regression equations of the plots were calculated by least

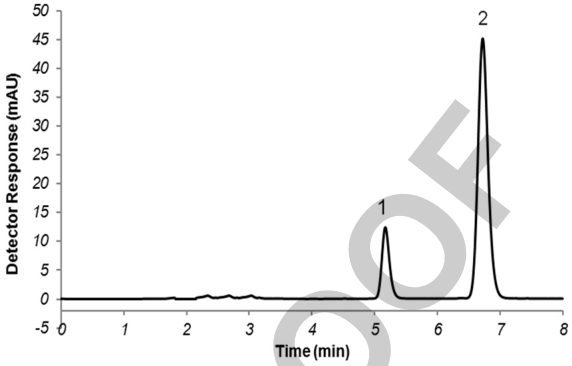

Figure 2. Optimized chromatogram of proposed method. (**1**) 2 µg/mL erlotinib and (**2**) 10 µg/mL vemurafenib.

Compounds	Vemurafenib
Retention time (min)	6.69
Linearity range (µg/mL)	0.2-10.0
Slope	0.492
Intercept	0.0119
Correlation coefficient	0.9999
Detection limit (µg/mL)	0.0482
Quantitation limit (µg/mL)	0.146

Table 1. Statistical evalution of the calibration data of vemurafenib by RP-HPLC.

square regression method. The assay showed that correlation coefficient was higher than 0.999 (Table 1).

The limit of detection (LOD) and limit of quantification (LOQ) values were determined by the following equations by the help of the standard deviation of response (*s*) and the slope (*m*) of the corresponding calibration curve: *LOD = 3.3 s/m and LOQ = 10 s/m*.

Calculated LOD and LOQ values were found to be 0.0482 and 0.146 µg/mL, respectively. These results indicate that the method provided adequate sensitivity. This LOQ value for vemurafenib is lower than previously reported with HPLC-UV methods [6-7] but it is fairly close to that reported with LC-MS/MS [8-14].

Repeated analyses of standard solutions in the mobile phase were performed to demonstrate the precision and reproducibility of the method. Intra-day reproducibility was determined by repeated analysis of standard solutions containing vemurafenib at two different concentrations (0.5 and 5.0 µg/mL). The inter-

Intra-day assay		
Concentration (µg/mL)	0.500	5.000
Calculated mean ± SD	0.495 ± 0.017	4.960 ± 0.018
Recovery	99.106	99.194
RSD %	1.451	0.374
Inter-day assay		
Concentration (µg/mL)	0.500	5.000
Calculated mean ± SD	0.5035 ± 0.008	4.904 ± 0.035
Recovery	100.704	98.085
RSD %	1.648	0.720

Table 2. Summary of repeatability (intra-day) and reproducibility (inter-day) precision data.

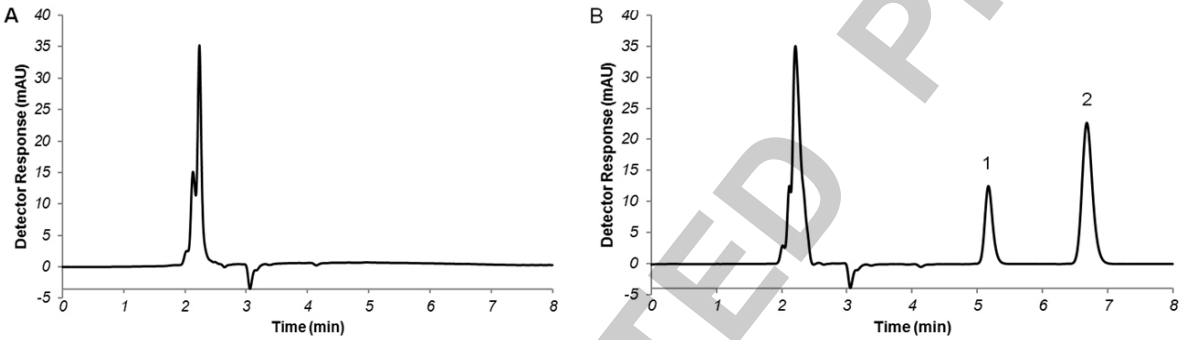

Figure 3. Chromatograms. (**A**) Blank human urine at 249 nm; (**B**) Human urine spiked with 2 µg/mL erlotinib (**1**) and 5 µg/mL vemurafenib (**2**).

Spiked urine concentration (µg/mL)	**1.000**	**5.000**
Calculated mean ± SD	1.020 ± 0.017	5.075 ± 0.072
Recovery	102.041	101.491
RSD %	0.227	1.419

Table 3. Results of the determination of vemurafenib in the presence of human urine.

day reproducibility of the method was determined by repeated analysis of standard solutions at two different concentration levels on three different days. Repeatability and reproducibility were expressed by mean recovery and RSD and the results are given in Table 2. The results show that there is no significant difference for the assay, as tested by inter- and intra-days. For this reason, all the results indicate that the method has good precision.

This proposed test was successfully applied for the determination of vemurafenib in the urine media. For this, urine samples were collected from healthy people, and were spiked with vemurafenib and erlotinib. Before the test, the urine samples were diluted as 25-fold. Chromatograms of blank and spiked urine sample are shown in Fig. 3. The urine matrix does not interfere with the determination of the vemurafenib.

The accuracy of the method for the analysis of urine samples was determined by recovery experiments. The recoveries and RSDs were calculated for five individual spiked urine samples, containing 1.0 and 5.0 µg/mL vemurafenib (Table 3). Very satisfactory results were obtained for vemurafenib. The peak areas were used to establish the internal standard. The recovery values for vemurafenib was calculated between 100.1 and 102.33%, by using the concentration ranges of (in triplicate) 1.0-5.0 µg/mL (in the presence of urine).

CONCLUSION

A new, simple, validated and rapid (about 7.5 min) HPLC method was developed for vemurafenib. An acceptable chromatographic resolution is obtained between vemurafenib and erlotinib. Inter-day and intra-day tests were found to be as similar.

This developed method could be easily applied to urine samples without pretreatment. It also observed that, there was no interference from the urine matrix for the determination of the vemurafenib. It can be concluded from these results that, this method can be used for routine analysis of vemurafenib in quality control and clinical monitoring purposes.

REFERENCES

1. Ascierto, P.A., J.M. Kirkwood, J.J. Grob, E. Simeone, A.M. Grimaldi, M. Maio, *et al*. (2012) J. *Transl*. *Med*. **10**(85): 1-9.
2. Chapman, P.B., A. Hauschild, C. Robert, J.B Haanen, P. Ascierto, J. Larkin, *et al*. (2011) *N*. *Engl*. *J*. *Med*. **364**(26): 2507-16.
3. Kim, K.B., K.T. Flaherty, P.B. Chapman, J.A. Sosman, A. Ribas, G.A. McArthur, *et al*. (2011) *J*. *Clin*. *Oncol*. **29**(15): 8519.
4. Trunzer, K., A.C. Pavlick, L. Schuchter, R. Gonzalez, G.A. McArthur, T.E. Hutson, *et al*. (2013) *J*. *Clin*. *Oncol*. **31**(14): 1767-74.
5. Shelledy, L. & D. Roman (2015) *J*. *Adv*. *Prac*. *Oncol*. **6**(4): 361-5.
6. Zheng, Y., A. Thomas-Schoemann, L. Sakji, P. Boudou-Rouquette, N. Dupin, L. Mortier, *et al*. (2013) *J*. *Chromatogr*. *B* **928**: 93-7.
7. Chhabda, P.J., M. Balaji, V. Srinivasarao & K.M.Ch. Appa Rao (2013) *Der Pharma Chem*. **5**(5): 189-98.
8. Rousset, M., K. Titier, S. Bouchet, C. Dutriaux, A. Pham-Ledard, S. Prey, *et al*. (2017) *Clin*. *Chim*. *Acta* **470**: 8-13.
9. Sparidans, R.W., S. Durmus, A.H. Schinkel, J.H.M. Schellens & J.H. Beijnen (2012) *J*. *Chromatogr*. *B* **889**: 144-7.
10. Nijenhuis, C.M., H. Rosing, J.H.M. Schellens & J.H. Beijnen (2014) *J*. *Pharm*. *Biomed*. **88**: 630-5.
11. Alvarez, J.C., E. Funck-Brentano, E. Abe, I. Etting, P. Saiag & A. Knapp (2014) *J*. *Pharmaceut*. *Biomed*. **97**: 29-32.
12. Vikingsson, S., M. Strömqvist, A. Svedberg, J. Hansson, V. Höiom & H. Gréen (2016) *Biomed*. *Chromatogr*. **30**(8): 1234-9.
13. Bihan, K., C. Sauzay, L. Goldwirt, F. Charbonnier-Beaupel, J.-S. Hulot, C. Funck-Brentano, *et al*. (2015) *Ther*. *Drug Monit*. **37**: 132-6.
14. Herbrink, M., N. de Vries, H. Rosing, A.D.R Huitema, B. Nuijen, J.H.M. Schellens, *et al*. (2016) *Ther*. *Drug Monit*. **38**(6): 649-56.
15. United States Pharmacopoeia, USP 32, National Formulary 27 (2009) *General Chapter 1092: The dissolution procedure: Development and Validation;* United States Pharmacopeial Convention: Rockville, MD.