*Journal of Pharmaceutical Research International*

***32(36): 18-27, 2020; Article no.JPRI.62926***
***ISSN: 2456-9119***
*(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)*

# Determination of $pK_a$ and Degradation Products of the Mutated BRAF Inhibitor Vemurafenib and UPLC Assay in Human Urine

**Senem Şanli[1*], Ayşe Özdemir[2], Gülşen Güven[3] and Süleyman Gökçe[1]**

*[1]Department of Chemistry, Faculty of Science and Arts, Uşak University, Uşak, Turkey.*
*[2]Division of Biochemisty, Faculty of Medicine, Uşak University, Uşak, Turkey.*
*[3]Department of Chemistry, Faculty of Science and Arts, Adnan Menderes University, Aydin, Turkey.*

***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors SŞ and SG designed the study, wrote the protocol and performed the statistical analysis. Authors AÖ and GG wrote the first draft of the manuscript and managed the analyses of the study. Authors SŞ and GG managed the literature searches and performed necessary corrections. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/JPRI/2020/v32i3630986
Editor(s):
(1) Dr. Jongwha Chang, University of Texas, USA.
Reviewers:
(1) A. Nurudeen Arowona, Kaduna Polytechnic, Nigeria.
(2) Salome Dini, Zhejiang University, China and Islamic Azad University, Iran.
Complete Peer review History: http://www.sdiarticle4.com/review-history/62926

***Original Research Article***

***Received 02 October 2020***
***Accepted 07 December 2020***
***Published 19 December 2020***

## ABSTRACT

In this study, the p$K_a$ value of vemurafenib was determined by the dependence of the retention factor on the pH of the mobile phase. The change of $pK_a$ in the different acetonitrile fraction ranging between 57.5 and 62.5% (v/v) was studied by using an LC-UV method. Additionally, a simple, reliable, and rapid UPLC method has been developed for the determination of vemurafenib in human urine. Efavirenz was used as an internal standart. For vemurafenib and efavirenz, selectivity factors were found as 1.204. The asymmetry and capacity factors were obtained as 1.100 and 7.532 for vemurafenib.

Vemurafenib was exposed to thermal, photolytic, hydrolytic, and oxidative stress conditions, and the stressed samples were detected by LC/MS/MS method.

*Keywords: Vemurafenib; UPLC; $pK_a$; degradation products; urine.*

**Corresponding author: E-mail: senemkamacisanli@gmail.com, senem.sanli@usak.edu.tr;*

## 1. INTRODUCTION

Malignant melanoma is the most thrusting type of skin cancer. It has been shown that somatic mutations in the β-rapidly accelerated fibrosarcoma (BRAF) gene are present in 66% of the melanoma cell lines. Vemurafenib (Fig. 1) is the first selective, potent and orally bioavailable inhibitor of the serine/threonine-protein kinase B-Raf protein encoded by the V600E mutated BRAF gene [1]. The drug was approved by the FDA in August, 2011 [2]. B-Raf is a frequently mutated protein kinase [3] and mutation of the gene is very common in melanoma [4,5]. Vemurafenib is chemically propane- 1-sulfonic acid {3-[5-(4-chlorophenyl)-1H-pyrrolo[2,3-b] pyridine-3-carbonyl]-2,4difluoro-phenyl}-amide with empirical formula is $C_{23}H_{18}ClF_2N_3O_3S$ and molecular weight of 489.9 [6]. The aqueous solubility of this drug is very low and independent of pH.

The absorption, metabolism, distribution, toxicity and excretion are important parameters of a drug molecule and its dissociation constant (p$K_a$) manages the distribution, elimination of substances, solubility and absorption. p$K_a$ values have lots of application such as solvent extraction, pharmaceutical development, ion transport and acid-base titration. Chromatographic retention, toxicity and pharmaceutical properties of organic acids and bases are influenced by acid-base properties [7]. In the literature, there is no study for the determination of p$K_a$ values of vemurafenib by using liquid chromatography (LC) in acetonitrile–water binary mixtures.

Since liquid chromatography commonly requires only a small amount of the compounds, it is used as a powerful technique for determining dissociation constants, and the studied samples need not to be pure, and that insufficient solubility in water is not a serious disadvantage. Measuring solute or titrant concentrations are not necessary, just only retention times are required for calculation in this method. Moreover, the calculation is simple and independent of the purity of solubility [8]. In the literature, there is only one reported p$K_a$ values are given for Vemurafenib as 7.9 and 11.1 but any details of the method and medium were not dedicated [9].

To our knowledge, a few methods in the literatures include determination of vemurafenib in several matrices by LC [10,11], LC coupled to tandem mass spectrometry (LC-MS/MS) [12,13,14]. However, UPLC method has not been reported for the determination of vemurafenib in human urine. UPLC is a new liquid chromatography category that improves the overall interweave properties of speed, precision, and resolution, while preserving the practicality and principles of HPLC [15]. Short analysis times and faster separations can be done with high resolution and sensitivity due to using shorter columns in UPLC.

In this research a fast, simple, economical, accurate and precise, reproducible, and fully verfied UPLC method was developed with good detection limits for estimation of vemurafenib in urine samples. The stability tests were also conducted according to the International Conference on Harmonization (ICH) Guidelines (ICH Guideline (Q1AR) 2000, ICH Guideline (Q2A) (R1) [16,17]. By using the LC/ MS/ MS technique, the stability behavior of the

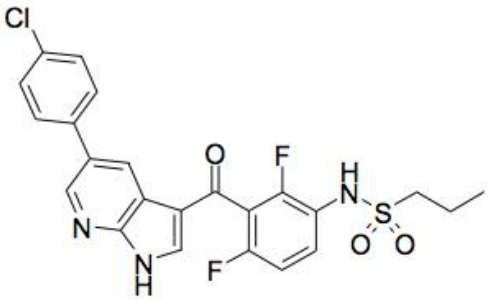
| **Compound** | **Chemical Structure** |
|---|---|
| Vemurafenib<br><br>N-{3-[5-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridin-3-carbonyl]-2,4-difluorophenyl}propane-1-sulfonamide<br><br>CAS No: 918504-65-1 |  <br>$C_{23}H_{18}ClF_2N_3O_3S$ MW : 489.9 g mol$^{-1}$ |

**Fig. 1. Chemical structure of vemurafenib**

vemurafenib was studied in the proposed procedure. For this purpose, stress tests are performed under acidic, basic, oxidative, thermal and photolytic conditions for the studied substance. Based on the m/z values and ionization particles, degradation mechanism can be proposed from LC/MS/MS method and products will be determined quantitatively.

On the other hand, this paper focuses on the determination of $pK_a$ values of vemurafenib several acetonitrile (ACN)-water mixtures, 57.5, 60 and 62.5% (v/v), by using high performance liquid chromatography technique.

## 2. EXPERIMANTEL

### 2.1 Chemicals and Reagents

All chemicals were analytical reagent-grade. Vemurafenib was kindly donated by Roche Pharm. Ind. (Istanbul, Turkey). Internal standard efavirenz (IS), were obtained from Sigma. Acetonitrile (organic modifier), potassium hydrogen phthalate (standard buffer) and sodium hydroxide, dimethylsulfoxide (DMSO) and formic acid were purchased from Merck (Darmstadt, Germany). Ortho-phosphoric acid (minimum 85%) was obtained from Riedel-de Haen Germany. Milli-Q system (Millipore, Bedford, MA, USA) was used to get ultrapure water.

### 2.2 Equipment

For p$K_a$ analysis, the Agilent Technologies 1260 Infinity LC system was used equipped with a G1311B quaternary pump, G1329B auto injector and G4212B diode array detector. X-Terra RP-18 (250 mm×4.60 mm ID×5 µm) column was used as stationary phase. Mettler Toledo MA 235 pH/ion analyzer (Schwerzenbach, Switzerland) was used for pH measurements of the mobile phase.

For human urine analysis, at on Agilent 1290 UPLC Infinty system with binary pump, online degasser, automatic injection system, column heater was used. Dedection of the solutions was done by using a diode array dedector. A Zorbax Eclipse plus C18 (2.1×50 mm ID ×1.8µ) column was used stationary phase.

The stability-indicating degradation behavior of the vemurafenib using LC/MS/MS was carried out on Agilent 6460 triple quad mass spectrometer. This instrument was equipped with an electrospray ionisation (ESI), operating in positive mode and configurated scan mode monitoring.

### 2.3 Chromatographic and Mass Spectrometric Procedure

For p$K_a$ studies, CAN: water combination at 57.5, 60.0, and 62.5% (v/v), containing 10 mM o-phosphoric acid were used as the mobile phases. Sodium hydroxide was used to adjust the pH of the mobile phase between 2.5 and 9.0. The flow rate was adjusted at 1.0 mL $min^{-1}$ and injection volume was 10 µL. The temperature was maintained at 25°C during the experiments. The optimal wavelength was chosen as 249 nm for vemurafenib.

The measurement of pH obtained is of great importance for the determination of p$K_a$. 0.05 mol/kg potassium hydrogen phthalate was used as a primary reference standard in order to measure the pH values of the mobile phases, dissolved in the ACN–water mixture in accordance with IUPAC rules [18]. Efavirenz was chosen as the internal standard for vemurafenib analysis.

For urine and LC-MS studies, acetonitrile-water (50/50 (v/v) includeing % 0.1 (v/v) formic acid was used as a mobile phase. The column teperature was kept at 30°C. For urine analysis, the system operates at 249 nm for vemurafenib and efavirenz (I.S). Triple-quadrapole mass spectrometer with an electrospray ionization source has been used in positive mode. A mobile phase composed of ACN-water mixture containing %0.1 formic acid was used to attaine good resolution, symmetrical peak shapes and shorter run time. Vemurafenib was monitored at m/z 490.0 → 383.0 and monitored at m/z 490.0 → 254.9. Table 1 summarises the MS operating parameters.

### 2.4 Preparation of Standard Solutions and Setting up a Calibration Plot

In case of p$K_a$ analysis, 100 µg/mL stock solutions of the working standards were prepared in mobile phase. All the dilutions for studied compounds were made with the mobile phase. Sodium hydroxide was prepared in water and used in pH adjustment.

**Table 1. Mass spectrometric parameters for the analysis of vemurafenib**

| | |
|---|---|
| Gas Temp (°C) | 325 |
| Gas Flow (L/min) | 11 |
| Nebulizer (Psi) | 45 |
| Sheat gas heater (°C) | 400 |
| Sheat gas flow | 12 |
| Capillary (V) | 3000 |
| Vcharging | 500 |
| Parent mass (m/z) | 490 |
| Product mass (m/z) | 383 |
| Dwell time (ms) | 45 |
| Collision energy (V) | 30 |
| Retention time (min) | 1.491 |
| Fragmentor voltage | 160 |

In case of urine analysis, vemurafenib was prepared in DMSO and efavirenz was prepared in MeOH at concentration of 100 µg $mL^{-1}$ and used in LC-MS analysis. All stocks were stored in the dark and kept at 4°C to minimize decomposition.

Calibration graph was setting up by plotting the ratio of the peak area to the drug concentration of IS. Appropriate amounts of stock solutions were spiked to urine in order to prepare calibration solutions. The concentration of vemurafenib (0.5, 1, 3, 5, 10 and 20 µg/mL) into 5 mL of blank human urine was used as calibration solutions. IS concentration was kept at a constant level of 10.0 µg/mL. The urine samples were prepared as described under Urine Anaysis part.

## 2.5 Analysis of Spiked Urine Samples

Urine samples were taken from healthly volunteers and used for the validation studies. Deionized water was used for 5-fold dilution of urine sample. 2 mL of diluted urine was transferred to a 110x30 mm plastic tube and 3 mL of methanol was added to this solution. Different amounts of vemurafenib were added to the human drug-free urine from healthy volunteers, while the internal standard concentration was fixed at 10.0 µg $mL^{-1}$. The spiked urines were vortexed and passed through a 0.45 µM filter. 20 µL of each sample was injected into the UPLC system.

Known amounts of each compound were added to urine samples collected from healthy volunteers in order to determine the intraday and interday precisions. The intra- and inter-day percentage relative standard deviations (RSD) were used for determination of precision values.

To provide additional control over the accuracy of this improved method, the recovered assays were performed by adding known amounts of pure drug to previously analyzed human urine sample. Two different concentrations of vemurafenib and a fixed level of an internal standard were added to the blank urine samples. The spiked urine samples were analyzed using the procedures described for the calibration standards. The recovery values were calculated by comparing the concentrations from spiked samples with the actual added concentration. Five repeated experiments were performed to calculate the average recovery of these compounds.

By using the standard deviation of response (s) and the slope of the calibration curve (m), limit of detection (LOD) and limit of quantification (LOQ) were calculated from the equations of LOD = 3.3 × s/m and LOQ = 10 × s/m.

## 2.6 Preparation of Stress Samples for Establishment of Stability-Indicating Assay

ICH guidelines recommend deterioration studies to establish the stability-determining feature of the analysis method [16]. Each sample was prepared in triplicate. Degradation studies consist of heat (at 75°C), acidic, alkaline hydrolysis, UV light and oxidation of the compounds.

For degradation of vemurafenib in thermal and photo-degradation conditions, it was carried out in solid state. Vemurafenib was put in a controlled-temperature oven at 75°C for 6 h. For acidic and oxidative degradation, vemurafenib was diluted with 0.1 N HCl and 3% - 30% $H_2O_2$ to achieve 10 µg $mL^{-1}$ vemurafenib. For stress

conditions, vemurafenib solutions was allowed to stand for 1 h under specified conditions. Alkaline hydrolysis of vemurafenib was conducted using 0.1 N NaOH for 3 h. Samples of solid drug substances for photolytic stress were irradiated with UV radiation at 254 nm for 6 hours and 24 hours. The products obtained under different stress conditions were determined by LC–MS.

## 3. RESULTS

$pK_a$ value information is more important than other parameters because of the absorption, evaluation and bioavailability of the drugs in the body. Ionization value also helps in selecting the pH of the buffer to be used in the mobile phase [7,19].

Vemurafenib is a substituted azaindole; it also contains a sulfonamide moiety but shows little structural relationship to 'sulfonamide' drugs (see Fig. 1). Vemurafenib is achiral and does not show stereoisomerism. This compound belongs to the class of organic compounds known as phenylpyridines. Structure-based calculation and prediction of structural properties of Vemurafenib are available in web-base programs such as Chemaxon and given as 8.87 (amine) [20].

The retention factors were obtained over a pH range of 2.5–8.0 in order to determine the $pK_a$ of this drug using the LC method. As an example, in Fig. 2, the experimental data obtained at 57.5, 60 and 62.5 (v/v) acetonitrile –water binary mixture (k vs pH) are plotted for this drug, showing the sigmiodal curves. As can be clearly seen, ideal sigmoidal shape was obtained for vemurafenib.

For the urine analysis, human urine samples were taken fron non-drug healty users and stored at -20°C before analysis. The urine samples were collected over a 4-h period in plastic containers before analysis. Potentially interfering compounds need to be removed before analysis. Protein precipitation in urine samples was provided by of mobile phase addition. After thawing to ambient temperature, 1 mL of urine was mixed with 9 mL of acetonitrile in order to precipitate proteins from the specimen, vortexed for 3 min. For urine alaysis, efavirenz was used as an internal standard.

For vemurafenib and efavirenz, selectivity factors were found as 1.204. The asymmetry and capacity factors were obtained as 1.100 and 7.532 for vemurafenib.

The chromatogram is given in Fig. 3. Both compounds were separated by a sharp peak and symmetry over a period of 1.5 minutes. Calibration graphs were created for Vemurafenib. Calibration curve parameters are given in Table 2.

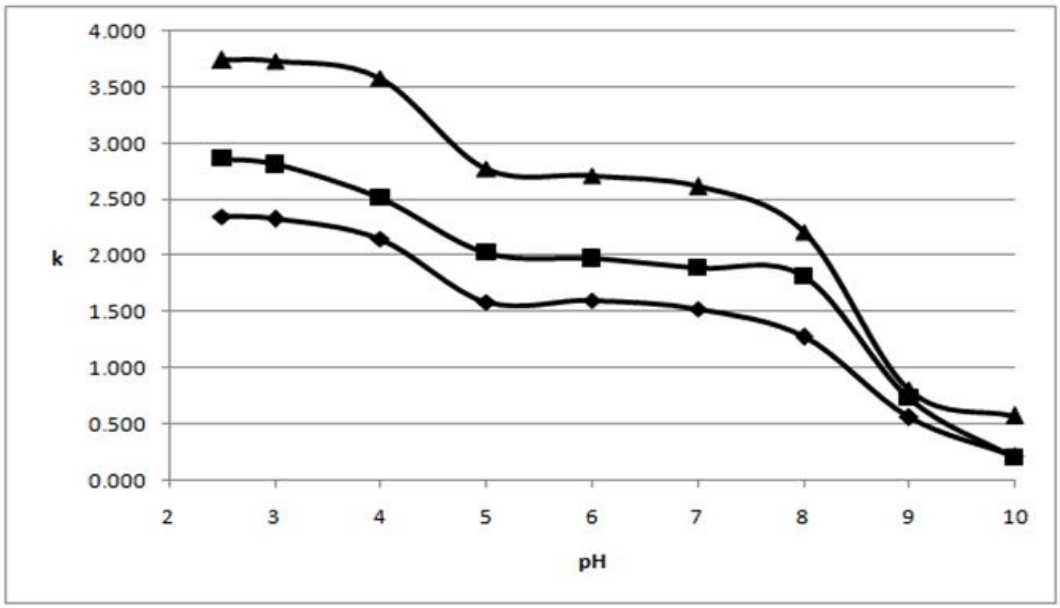



**Fig. 2. Plot of chromatographic retention factor, k, vs. the pH of mobile phase**
*♦ %62.5 ACN-water; ■ %60 ACN-water; ▲%57.5 ACN-water*

**Table 2. Statistical evaluation of the calibration data of vemurafenib by UPLC**

| Compound | Vemurafenib |
|---|---|
| Linearity Range (µg.mL$^{-1}$) | 0.5-20 |
| Slope | 0.1208 |
| Intercept | 0.0083 |
| Correlation Coefficient | 0.999 |
| Detection Limit (µg.mL$^{-1}$) | 0.933 |
| Quantitation Limit (µg.mL$^{-1}$) | 2.822 |
| Within-day Precision[a] (RSD%) | 0.272 |
| Between-day Precision[a] (RSD%) | 0.290 |

[a] *Each value is the mean of five experiments*

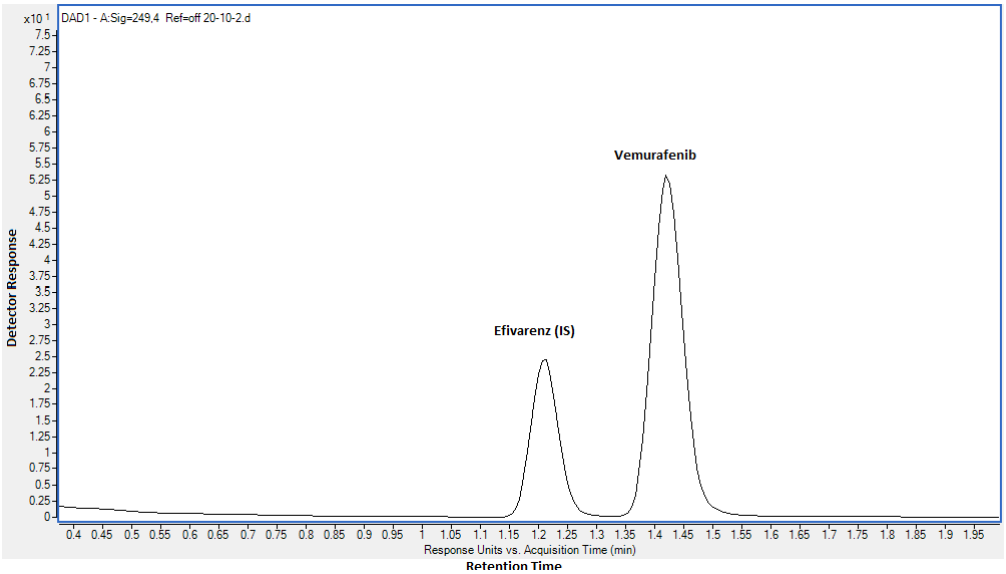



**Fig. 3. Chromatogram of standard mixture. experimental conditions as in 'chromatographic procedure'**

Within calibration curves, two different concentrations were prepared in both media and assayed with calibration curves to determine within-day and between day variability. The within-day and between-day precision, accuracy, and reproducibility were determined as the RSD% and mean value. The intraday variation was found between 0.268 and 0.239, RSD% values for urine calibration. Intermediate precision was determined by replicate analysis over 3-day period and 0.127 and 0.237, RSD% values.

To check the applicability of the method to biological materials, recovery studies for drug quantitation in human urine were performed using the calibration graph method, which reached the percentage recoveries determined for each analysis. Blank urine samples were spiked with studied drug. After five repeated experiments, the mean percentage recovery for urine sample was 97.924, with RSD% values of 0.5583. Typical chromatograms of urine sample (spiked with vemurafenib) is shown in Fig. 4. There are no extra peaks in the chromatograms of the urine samples.

## 3.1 Acid Induced Degradation Product

The drug was found to be highly stable to acidic degradation as compared to that of hydrochloric acid was used at 100°C for 30 min at 80°C for degradation. Major degradation products were seen 383, 214.9 and 136.9 m/z (Fig. 5a).

## 3.2 Base Induced Degradation Product

Vemurafenib was almost degradeted by NaOH. 1 M sodium hydroxide solution was used at 100°C for 30 min. Major degradation products

were seen 413, 214, 162.8 and 102 m/z (Fig. 5b).

### 3.3 Hydrogen Peroxide Induced Degradation Product

In 3% hydrogen peroxide at 100°C the drug was found to be stable to degradation as negligible degradation was seen after exposing drug to 30% hydrogen peroxide. degradation products were seen 413, 148.9 and 102 m/z (Fig. 5c).

### 3.4 Photochemical and Thermal Degradation Product

The drug was found to be stable against photochemical and thermal degradation.

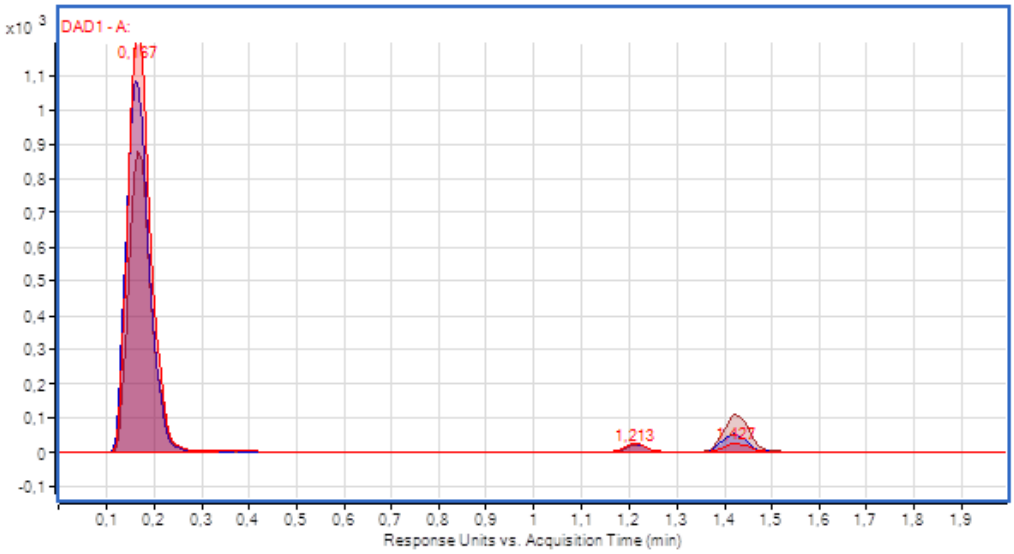



**Fig. 4. Chromatogram for urine samples (spiked with vemurafenib)**

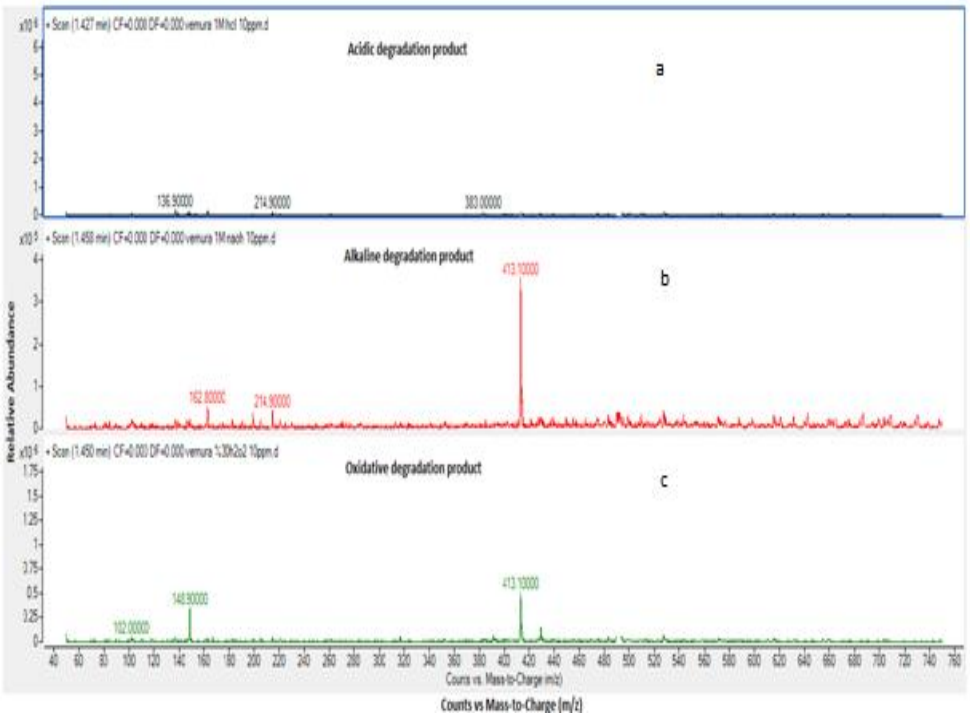
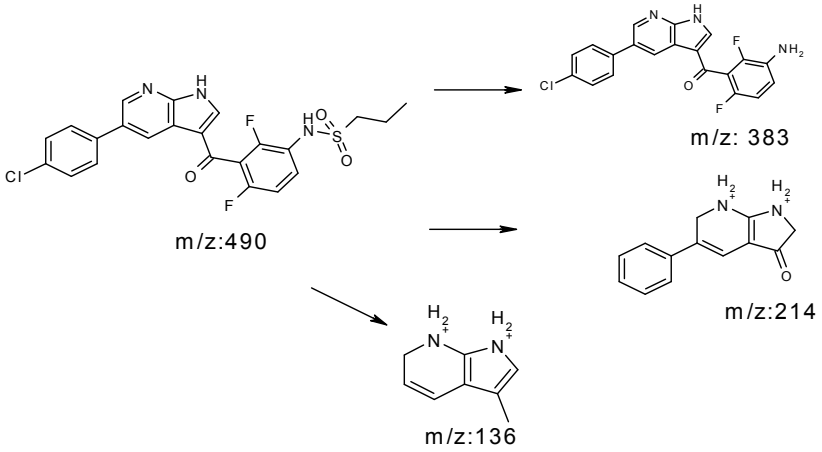



**Fig. 5. Typical LC/MSMS spectra of drugs under drastic stressed conditions**

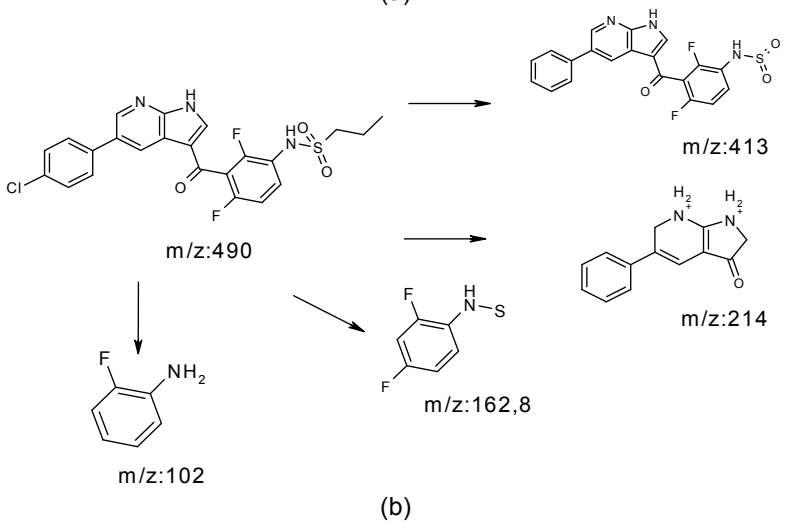
## 4. DISCUSSION

Knowing the various physico-chemical parameters of the pharmaceutically active compounds, such as $pK_a$, solubility and maximum wavelength, is important before developing the LC method. In this study, the retention factors were calculated for each mobile phase composition and pH studied. For this purpose, the effect of the pH of the mobile phase on the retention factors (*k*) was investigated and necessary values of vemurafenib were calculated using NLREG 4.0 software from *k*/pH [21]. Typical sigmoidal curve was obtained for vemurafenib. $pK_a$ values of Vemurafenib in studied ACN-Water Binary Mixtures are 7.96, 8.01 and 8.04, respectively. The obtained values of retention factors for the neutral and ionic forms of vemurafenib in 57.5 ± 0.08, 60 ± 0.09 and 62.5 ± 0.10(*v/v*) ACN-water binary mixtures calculated by NLREG program.

LC–MS analysis was carried out for degradation of vemurafenib using a 6460 Triple Q-Quadrupole Mass Spectrometer with suitable volatile formic acid (%0.1) as mobile phase. Satisfactory separation of degradation product was achieved using a $C_{18}$ column. Vemurafenib was stable under photochemical and termal degradation. For acidic and alkaline and hydrogen peroxide conditions, the degradation product formed shows the *m*/*z* of 413, 214, 162.8, 102 and 148.9. Possible structure of degradation products of vemurafenib for acidic, alkaline and hydrogen peroxide conditions were given in Fig 6a, 6b and 6c respectively.




(a)




(b)

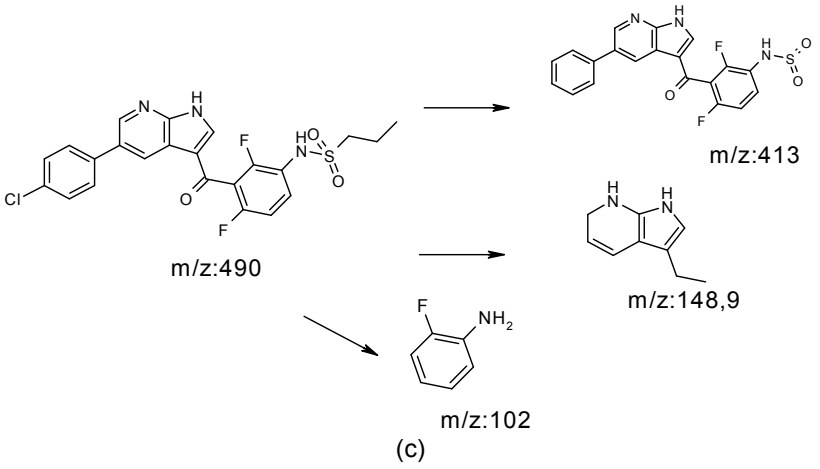



**Fig. 6. Structure of degradation product of vemurafenib**

## 5. CONCLUSION

In this paper a sensitive, specific, accurate, validated and well- defined stability indicating first UPLC method for the determination of vemurafenib in urine sample. The method was completely validated showing satisfactory data for all the method validation parameters tested. Also the important parameter p$K_a$ was determined by means of the RP-LC method.

Vemurafenib's behavior under various stress conditions was studied; The degradation products were determined by LC-MS / MS. The drug was stable in thermal and photolytic conditions. Degradation products were well separated from the drug substance which demonstrates the stability of the method. The information presented here could be very useful for quality monitoring of bulk samples and can also be used to check the quality of the drug during stability studies.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENTS

The authors are thankful for the financial aid received from the Scientific Research Projects Unit (BAP-2016/MF004) of Usak University. We gratefully acknowledge to Dr. Jose Luis Beltran from the University of Barcelona for kindly providing the NLREG 4.0 program.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Eggermont AM, Robert C. New drugs in melanoma: It's a whole new world. Eur. J. Cancer. 2011;47:2150-2157.
2. Heakal Y, Kester M, Savage S. Vemurafenib (PLX4032): An orally available inhibitor of mutated BRAF for the treatment of metastatic melanoma. Ann. Pharmacother. 2011;45:1399-1405.
3. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, et al., Patterns of somatic mutation in human cancer genomes. Nature. 2007;446:153-158.
4. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, Roth JA, Albelda SM, Davies H, Cox C, Brignell G, Stephens P, Futreal PA, Wooster R, Stratton MR, Weber BL. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res. 2002;62:6997-7000.
5. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks

E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, et al., Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949-954.
6. Available:www.chemblink.com/products/vemurafenib
7. Sanli S, Akmese B, Sanli N and Ozkan SA. Method for determination of $pK_a$ values of some anticancer agents and their assay. Chromatographia. 2013;76(21-22):1467-1475.
8. Erdemgil FZ, Şanli S, Şanli N, Özkan G, Barbosa J, Guiteras J, Beltrán JL. Determination of p$K_a$ values of some hydroxylated benzoic acids in methanol-water binary mixtures by LC methodology and potentiometry. Talanta. 2007;72(2): 489-496.
9. Malin Strömqvist, Master's Thesis, Development of quantitative methods for the determination of vemurafenib and its metabolites in human plasma, Linköping University Department of Physics, Chemistry and Biology; 2014.
10. Zheng Y, Thomas-Schoemann A, Sakji L, Boudou-Rouquette P, Dupin N, Mortier L, Vidal M, Goldwasser F, Blanchet B. An HPLC-UV method for the simultaneous quantification of vemurafenib and erlotinib in plasma from cancer patients. J. Chromatogr B. 2013;928:93–97.
11. Chhabda PJ, Balaji M, Srinivasarao V, Appa Rao KM. Development and validation of a new simple and stability indicating RP-HPLC method for the determination of vemurafenib in presence of degradant products. Der Pharma Chemica. 2013;5: 189-198.
12. Sparidans RW, Durmus S, Schinkel AH, Schellens JHM, Beijnen JH. Oral availability and brain penetration of the B-RAF$^{V600E}$ ınhibitor vemurafenib can be enhanced by the P-Glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) ınhibitor elacridar. J. Chromatogr. B. 2012;889:144–147.
13. Nijenhuis CM, Rosing H, Schellens JHM, Beijnen JH. Development and validation of a high-performance liquid chromatography–tandem mass spectrometry assay quantifying vemurafenib in human plasma. J. Pharm. Biomed.Anal. 2014;88:630–635.
14. Alvarez JC, Funck-Brentano E, Abe E, Etting I, Saiag P, Knapp A. A LC/MS/MS micro-method for human plasma quantification of vemurafenib. Application to treated melanoma patients. J. Pharm. Biomed. Anal. 2014;97:29–32.
15. Gumustas M, Kurbanoglu S, Uslu B, Ozkan SA. UPLC versus HPLC on drug analysis: advantageous, applications and their validation parameters. Chromatographia. 2013;76:1365–1427.
16. ICH Guideline (Q1AR) Stability testing of new drug substances and products International Conference on harmonization IFPMA, Geneva; 2000.
17. ICH Guideline (Q2A) (R1) Validation of analytical procedures: Text and methodology; 2005.
18. Rondinini S, Mussini PR, Mussini T. Reference value standard for pH measurements in organic solvents and water-organic solvent mixtures of moderate to high permittivities. Pure and Applied Chemistry. 1987;59:1549–1560.
19. Gumustas M, Sanli S, Sanli N, Ozkan SA. Determination of pK(a) values of some antihypertensive drugs by liquid chromatography and simultaneous assay of lercanidipine and enalapril in their binary mixtures. Talanta. 2010;82:1528–1537.
20. Available:https://chemaxon.com/products/chemicalize
21. NLREG Version 4.0. P.H. Sherrod; 1991. Available:http://www.sandh.com/Sherrod (Accessed 17 May 2018).



*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://www.sdiarticle4.com/review-history/62926*