Development and validation of bexarotene by bioanalytical methods using liquid chromatography-tandem mass spectroscopy (LC-MS/MS)

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Abstract

Background: The aim of this study was to develop and validate accurate and precise UPLC method with tandem mass spectrometry (Waters) for the determination of bexarotene in human plasma using bexarotene D4 as internal standard (IS).

Results: The retention time of bexarotene was 2.75 ± 0.30 min. The method was validated with respect to system suitability, linearity, accuracy, precision, matrix effect, auto sampler carryover test, and recovery. Linearity was found to be 1.04 to 351.93 μg/mL. LOQQC, LQC, INTQC, MQC, and HQC were found to be 1.0550, 2.7800, 25.2700, 131.61, and 263.23 respectively. The mean percentage recovery was found to be 95.72%.

Conclusion: The bioanalytical method, a selective and sensitive liquid chromatography-mass spectrometry method to quantitate bexarotene in K 2EDTA human plasma over the concentration range 1.0440 to 351.9320 ng/mL, was successfully validated. This method is suitable for sample analysis to support bioequivalence/bioavailability and/or pharmacokinetic studies involving formulations of bexarotene.

Keywords: Bexarotene, Validation, Liquid chromatography, Mass spectrometer

Background

Bexarotene (brand name: Targretin) [1] is an antineoplastic (anticancer) agent approved by the US Food and Drug Administration (FDA) (in late 1999) and the European Medicines Agency (EMA) (early 2001) for use as a treatment for cutaneous T cell lymphoma (CTCL) (Fig. 1) [2, 3]. It is a third-generation retinoid. The retinoic acid receptors (RARs) regulate cell differentiation and proliferation whereas RXRs regulate apoptosis [4]. LC-MS-based method that utilized both RPLC and HILIC separations was carried out [1–4], followed by multivariate data analysis to discriminate the global urine profiles of BC patients and healthy controls [1, 5]. The purpose of this study was to identify a potential biomarker pattern in urine using metabonomics to aid non-invasive BC detection using complementary chromatographic techniques [6, 7].

Methods

A few methods are available in literature [3, 8–16]. A new bioanalytical LC-MS/MS method was performed on the LC-MS/MS (API 4000) [6, 7, 17], consisting of binary gradient pump UV detector (LC-20 AD) employed for analysis, and rheodyne injector with 20 μl fixed loop was used for the present study. Bexarotene was eluted with a flow rate of 1 ml/min using a mobile phase of acetonitrile: buffer 1(90:10, v/v). The retention time of bexarotene analyte is 2.75 ± 0.3 min.
Extraction procedure: acetone-M: 10 mM ammonium format as extraction solvent
Spiked plasma samples were vortexed to ensure complete mixing of contents; 50 μl of internal standard (1 μl/ml of bexarotene D4) solution was added into all respectively labeled empty RIA vials except blank. Five hundred microliters of plasma samples was added to the respective labeled RIA vials containing internal standard solution and vortexed. Two hundred microliters of buffer 1 was added to all the samples and vortexed. 2.5 ml of extraction solvent was added to all the samples and capped. Samples were vibramaxed at 2000 rpm for 10 min. Then, the samples were centrifuged at 3500 rpm for 5 min in a refrigerated centrifuge between 2 and 8 °C. Two milliliters of supernatant was transferred into respective labeled RIA vials. All the samples were dried at 40 °C and 15 psi using LV evaporator. The dried residues get reconstituted with 300 μl of mobile phase and vortexed. The phospholipid removal cartridges get conditioned with 1 ml of acetone-M followed by 1 ml of conditioning solution. The samples were loaded into cartridges and eluted into RIA vials. The samples were transferred into respective labeled auto injector vials and loaded into LC-MS/MS [3].

Results
Method validation
The method was validated according to ICH Guidelines Q2 (R1) with respect to system suitability, linearity, accuracy, precision, matrix effect, auto sampler carryover test, and recovery.

System suitability
Aqueous standard or extracted standard equivalent to middle level of CC standard concentration with internal standard was prepared. Six replicates from the same vial were injected into the chromatographic device. Mean, standard deviation, and percentage coefficient of variation for the retention time and area/area ratio were calculated.

Linearity
Different serial dilutions were repeated, and fresh aqueous standards (for CCs) were prepared. An appropriate regression model with minimal or no weighing (1/x or 1/x²) was used. The standards were run in the LC-MS/MS, and linearity was evaluated.

Selectivity/specificity
This is to check whether there is an interference in peak. Two sets of six normal lots of plasma and one hemolyzed were taken. The aqueous LLOQ dilution was prepared and was spiked in one set of six normal lots of plasma, one hemolyzed lot to achieve LLOQ concentration for analyte, and the specificity sample was processed. The internal standard dilution was prepared, and only 50 μl of internal standard dilution was added to another set of six normal lots of plasma, one hemolyzed was processed for specificity samples. Selectivity samples were prepared in the presence of both analyte and internal standard using the six normal blank plasmas and one hemolyzed.

Precision and accuracy
The precision was determined by calculating percentage %CV at each concentration level of QC sample, and the accuracy was determined by calculating the percentage of nominal value at each concentration level of QC samples.

Ruggedness
One P&A batch was performed by employing the same instrument with different analysts and alternatively performed on different instruments of same make.

Recovery
The overall mean recovery, SD, and %CV were calculated. The recovery experiment was carried out by inject the six replicates of unextracted low, medium, and high QC samples, along with freshly processed CC set and QCs (6 LOC, 6 MQC, and 6 HQ).

Stability
Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used, do not affect the concentration of the analyte. The stability tests conducted in method validation are as follows:

1. Stock solution stability: short-term stock solution stability, long-term stock solution stability
2. Stability in biological matrix: bench top stability, freeze-thaw stability, long-term stability, blood stability

Fig. 1 Chemical structure of bexarotene. Formula: C24H28O2. Molar mass: 348.478 g/mol
Method development
The LCMS/MS procedure was optimized for the estimation of bexarotene with the mobile phase of acetonitrile: buffer 1 (90:10, v/v); the optimum flowrate was 1 ml/min with a column oven temperature and autosampler temperature of 40 °C and 10 °C respectively. Retention time of analyte is 2.75 ± 0.3 min, and IS is 2.73 ± 0.3 min.

Specificity and selectivity
Selectivity was evaluated by analyzing a total of nine lots on the instrument [17] obtained from independent sources (Table 1). No significant interferences were observed at the retention times of analyte and internal standard (see Table 3).

Signal-to-noise (S/N) ratio
The signal-to-noise ratio was determined for bexarotene at LLOQ concentrations in nine independent lots of K2EDTA human normal plasma including one lot of hemolyzed plasma, one lot of heparin plasma, and one lot of lipemic plasma [8] demonstrating acceptable S/N intensity.

Carryover test
Carryover is calculated as the percentage peak area observed in a processed blank plasma injected immediately after a processed ULOQ calibration standard, which was used from PA-01 batch sample analysis. No significant carryover was observed for bexarotene and internal standard (see in Table 2).

Matrix effect and matrix factor
Matrix factor and matrix effect were calculated, and results are given in Tables 3 and 4.

Linearity
Linearity established [9] by preparing an eight-point standard calibration curve in K2EDTA human plasma covering the bexarotene concentration ranges from 1.0440 to 351.9320 μg/mL using bexarotene D4 as internal standard. The calibration curve was shown to be linear for bexarotene as shown in Fig. 2; the results are seen in Table 5.

Weighting scheme
The absolute values of residuals of the back-calculated bexarotene calibration standards for the curve were tabulated, and the sum of the absolute values of the residuals was calculated for each weighting factor. The weighting factor of 1/X² provided the least sum value

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**Table 1** Specificity and selectivity for bexarotene and internal standard (MTR-BA-LC-MS/MS-05)

| Plasma lot ID     | Specificity (blank) | Selectivity (spiked LLOQ) | % interference in blank | Area ratio | S/N ratio (≥ 5) |
|-------------------|---------------------|---------------------------|-------------------------|------------|-----------------|
|                   | Analyte IS peak     | Analyte IS peak           | Analyte IS peak         | Analyte IS | Analyte IS      |
| MAT-C-0293-III    | 1805 2581           | 30,895 1,392,710         | 58.424                  | 0.1335     | 0.0160          |
| MAT-C-0577-III    | 1274 930            | 31,179 1,861,940         | 40.861                  | 0.0499     | 0.0167          |
| MAT-C-0578-III    | 1070 1798           | 29,447 1,823,809         | 36.336                  | 0.0986     | 0.0161          |
| MAT-C-0579-III    | 513 1694            | 27,120 1,813,246         | 18.916                  | 0.0934     | 0.0150          |
| MAT-C-0580-III    | 425 506             | 29,375 1,868,739         | 14.468                  | 0.0271     | 0.0157          |
| MAT-C-0586-III    | 1528 1132           | 25,471 1,774,320         | 59.990                  | 0.0638     | 0.0144          |
| MAT-C-0544-I(H)   | 1147 745            | 28,883 1,769,483         | 39.712                  | 0.0421     | 0.0163          |
| MAT-6188-II(L)    | 1396 2263           | 28,664 1,815,377         | 48.702                  | 0.1247     | 0.0158          |
| MAT-6198-IX (heparin) | 453 1361       | 27,446 1,825,500         | 16.505                  | 0.0746     | 0.0150          |
| MAT-C-0577-III    | 1274 930            | 31,179 1,861,940         | 40.861                  | 0.0499     | 0.0167          |
| MAT-C-0578-III    | 1070 1798           | 29,447 1,823,809         | 36.336                  | 0.0986     | 0.0161          |
| MAT-C-0579-III    | 513 1694            | 27,120 1,813,246         | 18.916                  | 0.0934     | 0.0150          |
| MAT-C-0580-III    | 425 506             | 29,375 1,868,739         | 14.468                  | 0.0271     | 0.0157          |
| MAT-C-0586-III    | 1528 1132           | 25,471 1,774,320         | 59.990                  | 0.0638     | 0.0144          |
| MAT-C-0544-I(H)   | 1147 745            | 28,883 1,769,483         | 39.712                  | 0.0421     | 0.0163          |
| MAT-6188-II(L)    | 1396 2263           | 28,664 1,815,377         | 48.702                  | 0.1247     | 0.0158          |
| MAT-6198-I (heparin) | 453 1361       | 27,446 1,825,500         | 16.505                  | 0.0746     | 0.0150          |

| Mean              | 3.71016            | 0.07863                  | 0.01567                 | SD        | 0.000731        |
|                   | %CV                | 4.67                     |                         | %CV       | 4.67            |

**Table 2** Carryover test

| Sample ID         | Analyte peak area | IS peak area |
|-------------------|-------------------|--------------|
| Extracted blank   | 0                 | 645          |
| Extracted LLOQ+IS | 6166              | 456,817      |
| Extracted ULOQ+IS | 1,955,374         | 413,370      |
| Extracted blank I | 0                 | 504          |
| Extracted blank II | 0              | 419          |
| Average of extracted blank | 0 | 462 |
| % carry over      | 0.00              | − 0.04       |
with residuals of calibration curve standards. Hence, $1/X^2$ was selected to use for this validation. See the results in Table 6.

### Table 3 Matrix effect and matrix factor for bexarotene at LQC level

| Plasma lot ID | Aqueous sample | Spiked sample | Matrix factor of analyte | Matrix factor of IS | IS normalized matrix factor | Area ratio Aqueous sample | Spiked sample |
|---------------|----------------|---------------|----------------------------|---------------------|-----------------------------|----------------------------|---------------|
| MAT6223-I     | 48,348         | 1,336,922     | 45,987                     | 1,317,311           | 1.08                        | 1.03                       | 1.05          | 0.0362 | 0.0349 |
| MAT6224-I     | 42,001         | 1,271,968     | 42,437                     | 1,277,296           | 0.99                        | 1.00                       | 0.99          | 0.0333 | 0.0332 |
| MAT6225-I     | 43,318         | 1,289,768     | 40,402                     | 1,257,917           | 0.95                        | 0.98                       | 0.97          | 0.0336 | 0.0321 |
| MAT6220-I     | 37,838         | 1,240,867     | 42,187                     | 1,239,881           | 0.99                        | 0.97                       | 1.02          | 0.0305 | 0.0340 |
| MAT6204-I     | 41,861         | 1,246,249     | 43,795                     | 1,222,111           | 1.03                        | 0.96                       | 1.07          | 0.0336 | 0.0358 |
| MAT6205-I     | 42,559         | 1,278,563     | 41,373                     | 1,221,142           | 0.97                        | 0.96                       | 1.01          | 0.0333 | 0.0339 |
| MATC-0544-XII(H) | 43,332  | 1,289,828     | 1.02                       | 1.01               | 1.01                        |                            |               | 0.0336 |       |
| MAT6188-IX (L) | 42,395         | 1,282,621     | 0.99                       | 1.00               | 0.99                        |                            |               | 0.0331 |       |
| MAT6198-(X)-heparin Plasma | 43,054   | 1,277,420     | 1.01                       | 1.00               | 1.01                        |                            |               | 0.0337 |       |
| Mean          | 42,654.16      | 1,277,389.5   | 1.003                      | 0.990              | 1.01333                     | 0.033                      | 0.033         |       |       |
| SD            | 0.037          | 0.023         | 0.03082                    |                     |                            |                            |               |       |       |
| %CV           | 3.76           | 2.42          | 3.04                       |                     |                            |                            |               |       |       |

Sensitivity
The sensitivity for bexarotene at LLOQ level in $K_2$EDTA human plasma determined based on the analysis of six replicates of LLOQ (1.0440 ng/mL) samples was prepared and analyzed against calibration curve standards. See the results in Table 7.

Intra-batch precision and accuracy of bexarotene
See the results in Table 8.

### Ruggedness
Accuracy, assay precision, and accuracy value for ruggedness batch (PA-03) were determined by

### Table 4 Matrix effect and matrix factor for bexarotene at LQC level (MTR-BA-LC-MS-MS-05)

| Plasma lot ID | Aqueous sample | Spiked sample | Matrix factor of analyte | Matrix factor of IS | IS normalized matrix factor | Area ratio Aqueous sample | Spiked sample |
|---------------|----------------|---------------|----------------------------|---------------------|-----------------------------|----------------------------|---------------|
| MAT-6223-I    | 48,348         | 1,336,922     | 45,987                     | 1,317,311           | 1.08                        | 1.03                       | 1.05          | 0.036 | 0.03  |
| MAT-6224-I    | 42,001         | 1,271,968     | 42,437                     | 1,277,296           | 0.99                        | 1.00                       | 0.99          | 0.033 | 0.03  |
| MAT-6225-I    | 43,318         | 1,289,768     | 40,402                     | 1,257,917           | 0.95                        | 0.98                       | 0.97          | 0.033 | 0.03  |
| MAT-6220-I    | 37,838         | 1,240,867     | 42,187                     | 1,239,881           | 0.99                        | 0.97                       | 1.02          | 0.030 | 0.03  |
| MAT-6204-I    | 41,861         | 1,246,249     | 43,795                     | 1,222,111           | 1.03                        | 0.96                       | 1.07          | 0.033 | 0.03  |
| MAT-6205-I    | 42,559         | 1,278,563     | 41,373                     | 1,221,142           | 0.97                        | 0.96                       | 1.01          | 0.033 | 0.03  |
| MAT-C-0544-XII(H) | 43,332   | 1,289,828     | 1.02                       | 1.01               | 1.01                        |                            |               | 0.03  |       |
| MAT-6188-IX (L) | 42,395         | 1,282,621     | 0.99                       | 1.00               | 0.99                        |                            |               | 0.03  |       |
| MAT-6198-(X)-heparin Plasma | 43,054  | 1,277,420     | 1.01                       | 1.00               | 1.01                        |                            |               | 0.03  |       |
| Mean          | 42,654.16      | 1,277,389.5   | 1.003                      | 0.990              | 1.01333                     | 0.033                      | 0.033         |       |       |
| SD            | 0.037          | 0.023         | 0.03082                    |                     |                            |                            |               |       |       |
| %CV           | 3.76           | 2.42          | 3.04                       |                     |                            |                            |               |       |       |
Table 5 Back-calculated concentrations of bexarotene for calibration curve standards

| Standard ID | A      | B    | C    | D    | E    | F    | G    | H    | Slope | Intercept | \( r^2 \) |
|-------------|--------|------|------|------|------|------|------|------|-------|------------|-----------|
| Actual concentration (ng/mL) | 1.044  | 2.776 | 10.096 | 25.240 | 59.390 | 131.97 | 263.95 | 351.93 |       |            |          |
| PA–01       | 1.019  | 2.959 | 9.955 | 25.592 | 56.512 | 134.04 | 255.36 | 359.85 | 0.013  | –0.0002   | 0.998     |
| PA–02       | 1.052  | 2.722 | 9.990 | 25.479 | 57.677 | 132.92 | 272.27 | 352.68 | 0.013  | 0.0011    | 0.999     |
| Mean        | 1.0362 | 2.8407| 9.9728 | 25.535 | 57.095 | 133.48 | 263.81 | 356.27 |       |            |          |
| SD          | 0.02319| 0.167301| 0.024607 | 0.079974 | 0.823921 | 0.787929 | 11.956327 | 5.072289 |       |            |          |
| %CV         | 2.24   | 5.89  | 0.25  | 0.31  | 1.44  | 0.59  | 4.53  | 1.42  |       |            |          |
| %Nominal    | 99.25  | 102.33 | 98.78  | 101.17 | 96.14  | 101.14 | 99.95  | 101.23 |       |            |          |

Table 6 Weighting scheme

| Weighting—1/X² | Absolute values of residuals | Weighting—1/X | Absolute values of residuals |
|----------------|------------------------------|---------------|------------------------------|
| 1.83           | 3.01                         | 5.23          | 4.99                         |
| 0.10           | 0.03                         | 1.67          | 1.45                         |
| 4.85           | 4.60                         | 4.66          | 4.94                         |
| 0.17           | 0.44                         | 1.61          | 1.34                         |
| Sum            | 20.12                        | 20.80         |

Table 7 Sensitivity

| Parameters                              | LLOQ  |
|-----------------------------------------|-------|
| Actual concentration (ng/mL)           | 1.0440|
| PA-01 (MTR-BA-LC-MS/MS-19)             | 1.0198|
| PA-02 (MTR-BA-LC-MS/MS-19)             | 1.0526|
| PA-03 (MTR-BA-LC-MS/MS-05)             | 1.0710|
| Mean                                    | 1.04780|
| SD                                      | 0.025935|

Fig. 2 Calibration curve
### Table 8 Intra-batch precision and accuracy of bexarotene

| QC ID | LOQQC | LQC | INTQC | MQC   | HQC   |
|-------|-------|-----|-------|-------|-------|
| Actual concentration (ng/mL) | 1.0500 | 2.7800 | 25.2700 | 131.6160 | 263.2320 |
| Calculated concentration (ng/mL) (MTR-BA-LC-MS/MS-19) PA–01 (06 Apr 2015) | 1.1639 | 2.9471 | 25.8272 | 135.3234 | 277.1292 |
| | 1.1622 | 2.8307 | 26.0325 | 128.5281 | 276.5622 |
| | 1.0975 | 2.9013 | 25.3157 | 131.3295 | 272.8455 |
| | 1.0866 | 2.8823 | 25.3351 | 132.7338 | 267.9314 |
| | 1.1758 | 3.0348 | 26.1218 | 136.8898 | 264.0381 |
| | 1.1043 | 2.9858 | 26.5157 | 133.2429 | 277.9904 |
| Mean | 1.13172 | 2.93033 | 25.85800 | 133.00792 | 272.74947 |
| SD | 0.039664 | 0.073957 | 0.469270 | 2.947768 | 5.662107 |
| %CV | 3.50 | 2.52 | 1.81 | 2.22 | 2.08 |
| %Nominal | 107.78 | 105.41 | 102.33 | 101.06 | 103.62 |
| Calculated concentration (ng/mL) (MTR-BA-LC-MS/MS-19) PA–02 (07 Apr 2015) | 1.0709 | 2.7864 | 26.1883 | 134.5485 | 270.4602 |
| | 1.0005 | 2.8122 | 25.8356 | 134.5475 | 274.3989 |
| | 0.8813 | 2.8064 | 25.3888 | 138.3150 | 266.9063 |
| | 1.0456 | 2.8642 | 25.3942 | 133.0021 | 272.3938 |
| | 1.1056 | 2.9071 | 25.5199 | 132.3109 | 266.4461 |
| | 1.0615 | 2.8626 | 25.6117 | 136.5209 | 273.7517 |
| Mean | 1.02757 | 2.83982 | 25.65642 | 134.87415 | 270.72617 |
| SD | 0.079461 | 0.045549 | 0.308551 | 2.228847 | 3.417745 |
| %CV | 7.73 | 1.60 | 1.20 | 1.65 | 1.26 |
| %Nominal | 97.86 | 102.15 | 101.53 | 102.48 | 102.85 |

### Table 9 Ruggedness (MTR-BA-LC-MS/MS-05)

| Standard | A | B | C | D | E | F | G | H |
|----------|---|---|---|---|---|---|---|---|
| Actual conc (ng/mL) | 1.0440 | 2.7760 | 10.0960 | 25.2400 | 59.3900 | 131.9760 | 263.9500 | 351.9320 |
| Calculated conc (ng/mL) | 1.0710 | 2.5793 | 10.0606 | 25.4973 | 62.1811 | 131.0732 | 264.5648 | 350.4726 |
| %nominal | 102.59 | 92.91 | 99.65 | 101.02 | 104.70 | 99.32 | 100.23 | 99.59 |

### Table 10 Precision

| QC ID | LOQQC | LQC | INTQC | MQC   | HQC   |
|-------|-------|-----|-------|-------|-------|
| Actual concentration (ng/mL) | 1.0500 | 2.7800 | 25.2700 | 131.6160 | 263.2320 |
| Calculated concentration (ng/mL) | 1.2696 | 2.6583 | 28.1908 | 140.8541 | 270.9386 |
| | 0.9477 | 2.6411 | 26.3654 | 142.1994 | 276.2971 |
| | 0.9881 | 2.6797 | 27.1780 | 140.3503 | 277.4336 |
| | 1.0373 | 2.7942 | 27.6385 | 138.9731 | 275.7356 |
| | 1.0366 | 2.6154 | 27.3704 | 141.6173 | 269.9140 |
| | 1.0515 | 2.8529 | 27.9700 | 141.5019 | 269.4408 |
| Mean | 1.05513 | 2.70693 | 27.45218 | 140.91602 | 273.29328 |
| SD | 0.111988 | 0.094595 | 0.649825 | 1.147251 | 3.575913 |
| CV | 10.61 | 3.49 | 2.37 | 0.81 | 1.31 |
| Nominal | 100.49 | 97.37 | 108.64 | 107.07 | 103.82 |
analyzing six replicates each of LOQQC, LQC, INTQC, MQC, and HQC samples using different instrument (MTR-BA-LC-MS/MS-05) of the same make and model (UPLC with Triple Quad API 4000), different analytical column (BAC-0644), and different analyst (Table 9).

Intercept = 0.0019, Slope = 0.0118, \( r^2 = 0.9996 \)

**Precision**
The precision of the assay was measured by the percentage coefficient of variation over the concentration range of LOQQC, LQC, INTQC, MQC, and HQC samples of bexarotene during the course of partial validation. See the results in Table 10.

**Recovery of bexarotene and IS**
The recovery of bexarotene was determined by comparing the detector response of analyte at three distinct levels of extracted low-, medium-, and high-quality control samples of PA-01 with detector response obtained from unextracted aqueous quality control samples at low, medium, and high level respectively. See the results in Tables 11 and 12.

IS recovery = 95.72%

**Stability**

*Freeze-thaw stability*
Six replicates of bexarotene samples at LQC and HQC concentration in K\(_2\)EDTA human plasma were analyzed after four freeze-thaw (FT4) cycles. See the results in Table 13.

*Bench top stability*
Bench top stability of bexarotene in K\(_2\)EDTA human plasma was evaluated at room temperature. Six replicates of LQC and HQC samples were processed after keeping the samples on bench for about 12.30 h. See the results in Table 14.

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**Table 11 Recovery of bexarotene**

| Quality control sample ID | Aqueous analyte area | Extracted analyte area |
|---------------------------|----------------------|------------------------|
| LQC                       | 16,201               | 17,052                 |
|                           | 16,045               | 16,462                 |
|                           | 20,992               | 16,952                 |
|                           | 20,215               | 16,941                 |
|                           | 19,423               | 17,689                 |
| Mean                      | 18,762               | 17,666                 |
| % recovery                | 94.16                |                        |
| MQC                       | 766,900              | 764,709                |
|                           | 766,878              | 748,447                |
|                           | 756,525              | 743,886                |
|                           | 769,926              | 744,175                |
|                           | 766,882              | 741,445                |
|                           | 757,276              | 753,506                |
| Mean                      | 764,065              | 749,361                |
| % recovery                | 98.08                |                        |
| HQC                       | 1,548,386            | 1,398,325              |
|                           | 1,543,021            | 1,373,844              |
|                           | 1,562,977            | 1,421,433              |
|                           | 1,573,624            | 1,379,191              |
|                           | 1,557,258            | 1,372,197              |
|                           | 1,542,212            | 1,352,389              |
| Mean                      | 1,554,580            | 1,382,897              |
| % recovery                | 88.96                |                        |

**Recovery result**

|                      | LQC | MQC | HQC | Mean |
|----------------------|-----|-----|-----|------|
| Recovery             | 94.16| 98.08| 88.96| 93.73|
| SD                   | 4.57|     |     |      |
| %CV                  | 4.88|     |     |      |

**Table 12 Recovery of internal standard**

| Quality control Sample ID | Aqueous IS area | Extracted IS area |
|---------------------------|-----------------|-------------------|
| LQC                       | 412,999         | 430,053           |
|                           | 412,341         | 432,371           |
|                           | 406,330         | 434,330           |
|                           | 409,620         | 436,925           |
|                           | 412,767         | 511,773           |
|                           | 405,852         | 440,319           |
| Mean                      | 431,367.8333    | 412,910.0556      |
| MQC                       | 463,632         | 417,498           |
|                           | 456,101         | 430,227           |
|                           | 465,696         | 418,482           |
|                           | 465,204         | 414,215           |
|                           | 465,835         | 400,164           |
|                           | 462,232         | 417,807           |
| Mean                      | 431,367.8333    | 412,910.0556      |
| HQC                       | 421,616         | 372,759           |
|                           | 423,021         | 366,983           |
|                           | 416,624         | 384,868           |
|                           | 413,104         | 380,280           |
|                           | 427,069         | 383,931           |
|                           | 424,578         | 359,396           |
| Mean                      | 431,367.8333    | 412,910.0556      |
**Auto sampler stability for bexarotene**

Six replicates of LQC and HQC samples were processed and kept stored in auto sampler at 10 °C for 96.80 h [11]. See the results in Tables 15 and 16.

**Long-term stock solution stability for bexarotene**

Stock solution bexarotene with concentration of 975.6417 μg/mL was kept in the refrigerator for 14 days [12]. A fresh stock of 986.1296 μg/mL was prepared on the day of analysis. Both stocks were diluted to LQC and HQC equivalent concentration of 0.1317 μg/mL and 0.1331 μg/mL for stored and fresh stock respectively. The area ratios of stability stock solution at LQC and HQC level were compared against freshly prepared stock solution LQC and HQC level. See the results in Tables 17 and 18.

**Limit of detection**

From LLOQ sample (1.0540 ng/mL), four different lower concentrations (0.8440, 0.6340, 0.4240, and 0.2120 ng/mL) including five times the lower concentration (LOD dilution) were prepared, and six replicates of these samples were analyzed. So the selected LLOQ (approx. 1.0540 ng/mL) was more suitable to quantify bexarotene in plasma using LC-MS/MS.

**Reinjection reproducibility** [14–16]

CC standards, LQC, and HQC samples of PA-02 were reinjected after 08.95 h. Percentage nominal for LQC

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### Table 13 Freeze-thaw stability for bexarotene (at −70 °C ± 15 °C and −30 °C ± 10)

| QC ID | −70 °C ± 15 °C LQC FT4 | −30 °C ± 10 °C LQC FT4 |
|-------|-------------------------|-------------------------|
|       | Actual concentration (ng/mL) | 2.7800 | 2.7800 |
|       | Calculated concentrations (ng/mL) | 2.7739 | 2.7739 |
|       |                                            | 2.7193 | 2.7193 |
|       |                                            | 2.9684 | 2.9684 |
|       |                                            | 2.6266 | 2.6266 |
|       |                                            | 2.9136 | 2.9136 |
|       |                                            | 2.8143 | 2.8143 |
| Mean  |                                             | 2.80268 | 2.81788 |
| SD    |                                             | 0.125448 | 0.120119 |
| %CV   |                                             | 4.48 | 4.26 |
| %nominal |                                         | 100.82 | 101.36 |

### Table 14 Bench top stability for bexarotene

| Stability hours | 12.30 h | 96.80 h |
|-----------------|---------|---------|
| QC ID           | LQC (stability) | HQC (stability) | LQC (stability) | HQC (stability) |
| Actual concentration (ng/mL) | 2.7800 | 263.2320 | 2.7800 | 263.2320 |
| Calculated concentration (ng/mL) | 2.7480 | 272.6737 | 2.8654 | 274.3981 |
|                                            | 2.7554 | 268.0237 | 2.8602 | 273.6167 |
|                                            | 2.8146 | 268.2582 | 2.8930 | 272.6483 |
|                                            | 2.8589 | 258.8974 | 2.9033 | 266.1408 |
|                                            | 2.8595 | 263.2458 | 2.7949 | 270.6783 |
|                                            | 2.7803 | 266.8280 | 2.7693 | 267.6223 |
| Mean  |                                             | 2.80278 | 266.32113 | 2.84768 | 270.85075 |
| SD    |                                             | 0.049512 | 4.729323 | 0.053940 | 3.349675 |
| %CV   |                                             | 1.77 | 1.78 | 1.89 | 1.24 |
| %nominal |                                         | 100.82 | 101.17 | 102.43 | 102.89 |
and HQC for bexarotene was 98.99 and 100.30%, respectively. The percentage CV for LQC and HQC for bexarotene was 3.66 and 1.97%, respectively. See the results in Table 19.

### Conclusion

Bioanalytical method is developed and validated as per ICH guidelines for the estimation of bexarotene in human plasma by using LC-MS/MS. The mobile phase was selected after trying various combinations of polar solvents. The proportion of solvents and variation of buffers were found to be quite critical as slight variation in it adversely affected the resolution of peaks. Considering all the facts, the validation parameter was finally fixed for this method. The bioanalytical method, a selective and sensitive liquid chromatography-mass spectrometry method to quantify bexarotene in K2EDTA human plasma over the concentration range from 1.0440 to 351.9320 ng/mL, was successfully validated. This method is suitable for sample analysis to support bioequivalence/bioavailability and/or pharmacokinetic studies involving formulations of bexarotene.

**Table 16** Auto sampler stability for internal standard

| Stability hours | QC ID | CS (IS area) | Stability samples (IS area) |
|-----------------|-------|--------------|-----------------------------|
| 0 h             | LQC   | 314,806      | 326,389                     |
|                 |       | 371,920      | 288,982                     |
|                 |       | 313,781      | 303,778                     |
|                 |       | 307,364      | 301,846                     |
|                 |       | 368,512      | 314,200                     |
|                 |       | 289,032      | 327,489                     |
| 96.80 h         | HQC   | 296,546      | 311,897                     |
|                 |       | 293,776      | 307,720                     |
|                 |       | 363,070      | 288,166                     |
|                 |       | 320,306      | 313,358                     |
|                 |       | 315,974      | 351,347                     |
|                 |       | 335,867      | 297,954                     |
| Mean            |       | 324,246.1667 | 311,093.8333                |
| % stability     |       | 95.94        |                             |

### Abbreviations

FDA: US Food and Drug Administration; RXR: The retinoid X receptor; EMA: European Medicines Agency; CTCL: Cutaneous T cell lymphoma; RPLC: Reverse phase liquid chromatography; HILIC: Hydrophilic interaction liquid chromatography; LC-MS: Liquid chromatography and mass spectroscopy; RARs: Retinoic acid receptors; IS: Internal standard; HPLC: High performance liquid chromatography; RIA: Radioimmunoassay; ICH: International Council for Harmonisation; LLOQ: Lower limit of quantification; ULOQ: Upper limit of quantification

**Table 17** Long-term stock solution stability for analyte LQC

| S. No. | Solution 1 (14 days) | Solution 3 (0 day) |
|--------|----------------------|---------------------|
|        | Analyte area | IS area | Area ratio | Analyte area | IS area | Area ratio |
| 1      | 47,688       | 1,336,789 | 0.0357    | 41,817      | 1,175,778 | 0.0356    |
| 2      | 48,589       | 1,346,976 | 0.0361    | 41,215      | 1,151,561 | 0.0358    |
| 3      | 48,301       | 1,361,402 | 0.0355    | 41,798      | 1,116,568 | 0.0374    |
| 4      | 45,066       | 1,366,924 | 0.0330    | 41,422      | 1,148,997 | 0.0361    |
| 5      | 47,950       | 1,385,003 | 0.0346    | 39,561      | 1,164,781 | 0.0340    |
| 6      | 48,638       | 1,388,752 | 0.0350    | 41,488      | 1,142,573 | 0.0363    |
| Mean   |             | 0.03498    |            | Mean        | 0.03587    |

**Table 18** Long-term stock solution stability for internal standard LQC

| S. No. | Solution 2 (14 days) | Solution 3 (0 day) |
|--------|----------------------|---------------------|
|        | Analyte area | IS area | Area ratio | Analyte area | IS area | Area ratio |
| 1      | 58,926       | 1,650,164 | 28.00401  | 41,817      | 1,175,778 | 28.11723 |
| 2      | 58,933       | 1,642,596 | 27.87226  | 41,215      | 1,151,561 | 27.94034 |
| 3      | 58,486       | 1,695,246 | 28.98550  | 41,798      | 1,116,568 | 26.71343 |
| 4      | 55,830       | 1,678,648 | 30.06713  | 41,422      | 1,148,997 | 27.73881 |
| 5      | 59,683       | 1,697,852 | 28.44783  | 39,561      | 1,164,781 | 29.44266 |
| 6      | 62,675       | 1,732,273 | 27.63898  | 41,488      | 1,142,573 | 27.53984 |
| Mean   |             | 28.50262   |            | Mean        | 27.91538   |

**Table 19** Reinjection reproducibility for bexarotene

| Batch ID | Reinjection reproducibility |
|----------|----------------------------|
| PA-02    | LQC | HQC | LQC | HQC |
| PA-02 samples | 2.7800 | 263.2320 | 2.7800 | 263.2320 |
| Re injected samples (08.95 h) | 2.7800 | 263.2320 | 2.7800 | 263.2320 |
| Actual concentrations (ng/mL) | 2.7864 | 263.2320 | 2.7800 | 263.2320 |
| Calculated concentration (ng/mL) | 2.7800 | 263.2320 | 2.7800 | 263.2320 |
| SD | 0.045549 | 3.417745 | 0.100850 | 5.206446 |
| %CV | 1.60 | 1.26 | 3.66 | 1.97 |
| % nominal | 102.15 | 102.85 | 98.99 | 100.30 |
| Ratio of means | 0.97 | 0.98 |
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