RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE COMBINATION OF IMIQUIMOD AND SALICYLIC ACID

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Received: 19 May 2020, Revised and Accepted: 14 Jul 2020

ABSTRACT

Objective: The present study was undertaken to develop and validate an RP-HPLC method for the combination of imiquimod and salicylic acid.

Methods: The method was carried out on Nucleodur C18 (250 mm × 4.6 mm I.D., 5 µm) using low-pressure gradient elution mode. The mobile phase was used as 30M potassium dihydrogen phosphate and acetonitrile (45:55) pH 6.5 adjusted using ortho-phosphoric acid. The concentration of solvents was 1-20 µg/ml and the volume of injection was 20 µl with the flow rate of 1.0 ml/min. The absorption maxima of salicylic acid and imiquimod were found 234 nm and 226 nm, respectively.

Results: The method was validated and showed the linearity greater than 0.99% and with precision (RSD%<1). The limit of detection (LOD) and limit of quantification (LOQ) of salicylic acid was found to be 0.09756 µg/ml and 0.2956 µg/ml, respectively, and imiquimod was found to be 0.044031 µg/ml and 0.13334 µg/ml, respectively.

Conclusion: The method developed in the present study was found to be sensitive, specific, and can be applied for the simultaneous estimation of imiquimod and salicylic acid.

Keywords: Imiquimod, Salicylic acid, Reverse-phase high-performance liquid chromatography (RP-HPLC), Validation, International council for harmonization (ICH)

INTRODUCTION

Imiquimod is chemically 1-(2-methyl propyl)-1H-imidazo[4, 5-c]quinolin-4-amine, which is a novel synthetic compound that is a member of the imidazoquinoline family of drugs [1]. The molecular formula of imiquimod is C14H13N3O2, which is a white crystalline powder and the molecular weight is 240.30g/mol. The solubility of imiquimod is freely soluble in oleic acid and lactic acid with melting point 292-294 °C and pKa value 2.7. Imiquimod is an immune response modifiers use in the treatment for external genital warts by the mechanism response modifier that acts as a toll-like receptor 7 agonists. It does not kill the viruses that cause warts directly, however, also used to treat skin problems of face and scalp that is called acitnic keratoses and also for various skin cancers called superficial basal cell carcinoma. In a present-day commercially available as Aldara® 5% imiquimod cream, approved by U. S. Food and Drug Administration ("FDA") in 1997 [2-4].

Salicylic acid (from latin salix, willow tree, from the bark of which the substance used to be obtained) is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. Salicylic acid has the formula C7H6(OH) COOH, where the OH group is ortho to the carbonyl group and is poorly soluble in water (2 g/l at 20 °C).

Aspirin (acetylsalicylic acid or ASA) can be prepared by the esterification of the phenolic hydroxyl group of salicylic acid with the acetyl group from acetic anhydride or acetyl chloride. The compound is white crystalline powder and the molecular weight is 138.12 g/mol. The solubility of salicylic acid is good in ether, CCl4, benzene, propanol, acetone, ethanol, oil of turpentine, toluene [5, 6]. It shows its anti-inflammatory action by suppressing the activity of cyclooxygenase (COX), an enzyme that is responsible for the production of pro-inflammatory mediators such as the prostaglandins [7]. Salicylic acid also has its anti-acne action [8]. Chemical structure of salicylic acid is given below:

Fig. 1: Chemical structure of imiquimod

Simultaneously steps were taken for these two drugs successfully. Therefore it was thought to develop an accurate and rapid RP-HPLC method for simultaneous estimation of the combination of imiquimod and salicylic acid. This method was validated as per the current International Conference on Harmonization (ICH) guideline [9-11].

MATERIALS AND METHODS

Salicylic acid was procured as a gift sample from SGPTC Pvt. Ltd and imiquimod drug was obtained as a gift sample from Glenmark Pharma, Mumbai India. All other chemicals like o’phosphoric acid, potassium dihydrogen phosphate, and disodium hydrogen

Fig. 2: Chemical structure of salicylic acid
within the acceptance criteria 100±10 %. In this method drug solution at the level of 80%, 100%

Recovery studies were carried out by [12, 13].

**Identification of standard drugs**
The identification of standard drugs was carried out by melting point study, infrared spectroscopic study, and solubility study.

**Rp-hplc method**

**Selection of detection wavelength**
A known concentration of 10 μg/ml of salicylic acid and imiquimod were prepared in methanol and scanned in the UV region 200-400 nm.

**Selection of mobile phase and optimization of chromatographic condition**
Initially, the mobile phase was tried with methanol: water, acetonitrile: water, acetonitrile: water: methanol and then finally with phosphate buffer: acetonitrile: at various pH conditions. The following optimized parameters were used as a final method for the simultaneous estimation of salicylic acid and imiquimod.

**Chromatographic condition**
In the optimized parameters, the stationary phase was Nucleodur C18 (250 nm × 4.6 mm ID, 5 μm) using low-pressure gradient elution mode. 30M potassium dihydrogen phosphate and acetonitrile (45:55) pH 6.5 were used as mobile phase and pH were adjusted using orthophosphoric acid. The concentration of solvents was 1-20 μg/ml and the volume of injection was 20 μl with the flow rate of 1.0 ml/min. The absorption maxima of salicylic acid and imiquimod were set in 234 nm and 226 nm, respectively.

**Standard stock solution preparation (100 μg/ml)**
100 μg/ml solution was prepared using 10 mg of salicylic acid 1 mg of imiquimod and about 1 ml of diluent was added and sonicate to dissolve to make the standard solution.

**Preparation of calibration curve**
Different concentration was prepared 0.1 μg/ml to 1 μg/ml for imiquimod and 1 μg/ml to 10 μg/ml for salicylic acid from the standard stock solution, respectively.

**Validation of RP-HPLC method**

**Linearity**
Different concentrations of both the drugs were prepared for linearity i.e. salicylic acid 1-10 μg/ml and imiquimod 0.1-1 μg/ml [12, 13].

**Accuracy**
Recovery studies were carried out by the addition of standard drug solution at the level of 80%, 100%, and 120% to the preanalyzed sample. Results of the recovery study were found to be within the acceptance criteria 100±10 %. In this method, the known concentration of standard drug was added to the assay sample [14, 15].

**Precision**
Different concentrations of both the drugs were used for intra-day and inter-day variation for six times in the same day. The concentration was 5 μg/ml of salicylic acid and 0.5 μg/ml imiquimod, and then % RSD was calculated [16, 17].

**Limit of detection and limit of quantification**
The limit of detection can be calculated using the following equation as per ICH guidelines [18].

\[ \text{LOD} = 3.3 \times \frac{N}{S} \]

Where N is the standard deviation of the intercepts of the drug, and S is the slope of the corresponding calibration curve.

The limit of quantification can be calculated using the following equation as per ICH guidelines.

\[ \text{LOQ} = 10 \times \frac{N}{S} \]

Where N is the standard deviation of the intercepts of the drug, and S is the slope of the corresponding calibration curve [19].

**Robustness**
Same samples of salicylic acid and imiquimod concentration 5μg/ml and 0.5μg/ml respectively by considered variation in the method parameters i.e. change in wavelength or change in flow rate, change in pH of the mobile phase. The change in the responses of salicylic acid and imiquimod were noted in terms of %RSD [20].

**Specificity**
The specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients. A volume of 20μl of individual ingredients and excipients solution was injected and the chromatogram was recorded [21].

**Ruggedness**
The ruggedness was studied by analyzing the same samples of salicylic acid and imiquimod concentration 5μg/ml and 0.5μg/ml, respectively by changing the analyst. The change in the responses of salicylic acid and imiquimod were noted in terms of %RSD [22].

**RESULTS AND DISCUSSION**

**Identification of imiquimod and salicylic acid**
Salicylic acid and imiquimod were observed for organoleptic properties like physical appearance, odor, and melting point. The drugs were identified with the help of UV and FTIR and exhibited absorption maxima was 234 nm for salicylic acid and 226 nm for imiquimod when methanol was used as a solvent as mentioned in the literature (fig. 3).

![Fig. 3: UV spectrum of salicylic acid (A) and imiquimod (B) in methanol](image)

**(A)**

**(B)**
Method validation

Linearity

The linearity of both the drugs was found within acceptance criteria. The correlation coefficient ($r^2$) obtained was calculated and it was found to be greater than 0.99 for salicylic acid and imiquimod is given in the below table, which is well within the acceptance criteria. Good linearity with the coefficient of correlation 0.99 indicated that the proposed method was linear within the range of 1 to 10 µg/ml, 0.1 to 1 µg/ml, respectively for salicylic acid and imiquimod.

Table 1: Linearity of salicylic acid

| Conc. (µg/ml) | Area1       | Area2       | Area3       | Mean        | SD           | %RSD        |
|--------------|-------------|-------------|-------------|-------------|--------------|-------------|
| 1            | 55346       | 55051       | 55866       | 55421       | 412.6439     | 0.744562    |
| 2            | 94156       | 94907       | 94550       | 94537.67    | 375.6519     | 0.397357    |
| 3            | 129106      | 125734      | 124812      | 126550.7    | 2260.491     | 1.786234    |
| 4            | 162548      | 165085      | 167974      | 165202.3    | 2714.902     | 1.64338     |
| 5            | 187989      | 189886      | 190641      | 190925.6    | 3099.256     | 1.625703    |
| 6            | 230434      | 236106      | 231690      | 232743.3    | 2979.099     | 1.279993    |
| 7            | 267238      | 268739      | 264993      | 266990      | 1885.274     | 0.706121    |
| 8            | 304641      | 308556      | 307314      | 306837      | 2000.613     | 0.652012    |
| 9            | 342474      | 341383      | 348660      | 344712.3    | 3924.53      | 1.14028     |
| 10           | 383247      | 388043      | 386039      | 3924.53     | 2493.371     | 0.645885    |

*Conc. = concentration, mean±SD, n=3, SD = standard deviation, RSD = relative standard deviation

Table 2: Linearity of imiquimod

| Conc. (µg/ml) | Area1         | Area 2         | Area 3         | Mean           | SD           | %RSD        |
|--------------|---------------|---------------|---------------|---------------|--------------|-------------|
| 0.1          | 100795        | 100900        | 100854        | 100849.7      | 52.63396     | 0.52191     |
| 0.2          | 165045        | 165650        | 164071        | 16492.2       | 796.6536     | 0.483049    |
| 0.3          | 225478        | 220799        | 227558        | 22461.7       | 3461.78      | 1.541229    |
| 0.4          | 260053        | 267975        | 267947        | 267398.3      | 697.723      | 0.267035    |
| 0.5          | 330441        | 336627        | 332784        | 333284        | 3123.163     | 0.937088    |
| 0.6          | 395254        | 394840        | 397691        | 395928.3      | 1540.485     | 0.389082    |
| 0.7          | 468121        | 469870        | 463485        | 467158.7      | 3299.488     | 0.706288    |
| 0.8          | 522375        | 524480        | 525548        | 524134.3      | 1614.496     | 0.308031    |
| 0.9          | 595412        | 595953        | 595245        | 595536.7      | 370.0977    | 0.062145    |
| 1            | 655536        | 652236        | 651043        | 652940        | 2325.349     | 0.356135    |

*Conc. = concentration, mean±SD, n=3, SD = standard deviation, RSD = relative standard deviation
Table 3: Accuracy results of salicylic acid and imiquimod

| Conc. level | Area        | Amount added | Amount recovered | % Recovery | SD     | %RSD  |
|------------|-------------|--------------|------------------|------------|--------|-------|
| 80%        | 167045      | 4 µg/ml      | 4.12             | 103.055    | 2172.9 | 1.3165|
|            | 163972      | 4 µg/ml      | 4.03             | 100.932    | 101.049|       |
|            | 164141      | 4 µg/ml      | 4.04             | 101.049    |        |       |
| 100%       | 183748      | 5 µg/ml      | 4.58             | 91.674     | 882.13 | 0.4775|
|            | 185458      | 5 µg/ml      | 4.63             | 92.619     |        |       |
|            | 1894979     | 5 µg/ml      | 4.61             | 92.354     |        |       |
| 120%       | 215834      | 6 µg/ml      | 5.47             | 91.170     | 1362.2 | 0.6275|
|            | 216921      | 6 µg/ml      | 5.50             | 91.671     |        |       |
|            | 218541      | 6 µg/ml      | 5.54             | 92.417     |        |       |

| Conc. level | Area        | Amount added | Amount recovered | % Recovery | SD     | %RSD  |
|------------|-------------|--------------|------------------|------------|--------|-------|
| 80%        | 261795      | 0.4 µg/ml    | 0.369            | 92.39      | 685.89 | 0.2619|
|            | 260825      | 0.4 µg/ml    | 0.367            | 91.99      |        |       |
|            | 263171      | 0.4 µg/ml    | 0.371            | 92.95      |        |       |
| 100%       | 319439      | 0.5 µg/ml    | 0.463            | 92.67      | 1936.4 | 0.6028|
|            | 321026      | 0.5 µg/ml    | 0.465            | 93.18      |        |       |
|            | 323292      | 0.5 µg/ml    | 0.469            | 93.92      |        |       |
| 120%       | 375617      | 0.6 µg/ml    | 0.555            | 92.45      | 1236.2 | 0.3289|
|            | 377201      | 0.6 µg/ml    | 0.557            | 92.88      |        |       |
|            | 374765      | 0.6 µg/ml    | 0.553            | 92.22      |        |       |

*Conc. = concentration, mean±SD, n=3, SD = standard deviation, RSD = relative standard deviation
Accuracy
The results indicate that the recoveries are well within the acceptance range of 80–120% (table 3). The chromatogram of salicylic acid and imiquimod drugs at level 80% was shown in fig. 8.

Precision
The %RSD for the area of six replicate injections was found to be within the specified limits i.e. for salicylic acid, it was 0.742393% and imiquimod it was observed 0.995204%. Whereas the % RSD for intra and inter-day precision of salicylic acid and imiquimod were observed below 2% [18-20]. The low values of % RSD indicate that the method is precise. % RSD for interday precision and intraday precision was given below that was found to be within the specified limits.

Limit of detection and limit of quantitation
The LOD was found to be 0.09756 µg/ml and 0.044031 µg/ml for salicylic acid and imiquimod respectively and LOQ was found to be 0.2956 µg/ml and 0.13334 µg/ml salicylic acid and imiquimod respectively which showed that sensitivity of the method was high.

Robustness
The Percentage of RSD should not be more than 2. The %RSD obtained for change of flow rate, change in wavelength, and pH variation in the mobile phase was found to be below 2, which was within the acceptance criteria. Hence the method was robust.

Ruggedness
The results were found within a specified limit. % RSD was less than 2. Data are tabulated in table 9 and fig. 10.

Specificity
Specificity of salicylic acid and imiquimod are shown in fig. 11 and table 10. All the data were within limits. %RSD of salicylic acid in specificity was observed 0.186425% and for imiquimod, it was 0.384456%.

Table 4: Repeatability of salicylic acid and imiquimod

| S. No. | Salicylic acid Conc.(µg/ml) | Area | Imiquimod Conc.(µg/ml) | Area |
|--------|-----------------------------|------|------------------------|------|
| 1      | 5                           | 180729 | 0.5                    | 312844 |
| 2      | 5                           | 180281 | 0.5                    | 318685 |
| 3      | 5                           | 181842 | 0.5                    | 311867 |
| 4      | 5                           | 182989 | 0.5                    | 310441 |
| 5      | 5                           | 183886 | 0.5                    | 316627 |
| 6      | 5                           | 182048 | 0.5                    | 312784 |
| Mean   |                             | 18196.25| Mean                   | 313874.7 |
| Standard Deviation | 1350.877 | Standard Deviation | 3123.692 |
| %RSD   |                             | 0.742393| %RSD                   | 0.995204 |

*Mean for six independent analysis, mean±SD, n=6, Conc. = concentration, RSD =relative standard deviation

Table 5: Intraday and intraday precision of salicylic acid and imiquimod

| S. No. | Salicylic acid Conc.(µg/ml) | Area | Imiquimod Conc.(µg/ml) | Area |
|--------|-----------------------------|------|------------------------|------|
| 1      | 5                           | 193388 | 0.5                    | 371303 |
| 2      | 5                           | 194165 | 0.5                    | 366835 |
| 3      | 5                           | 195142 | 0.5                    | 369995 |
| 4      | 5                           | 194057 | 0.5                    | 370772 |
| 5      | 5                           | 195544 | 0.5                    | 368306 |
| 6      | 5                           | 194999 | 0.5                    | 369934 |
| Mean   |                             | 194749.2| Mean                   | 313874.7 |
| Standard Deviation | 582.2053 | Standard Deviation | 3123.692 |
| %RSD   |                             | 0.298951| %RSD                   | 0.995204 |
| Interday precision | Area | Imiquimod Conc.(µg/ml) |
| 1      | 5                           | 192990 | 1                      | 5     |
| 2      | 5                           | 193563 | 2                      | 5     |
| 3      | 5                           | 193229 | 3                      | 5     |
| 4      | 5                           | 193825 | 4                      | 5     |
| 5      | 5                           | 193199 | 5                      | 5     |
| 6      | 5                           | 193807 | 6                      | 5     |
| Mean   |                             | 193435.5| Mean                   | 369890.8 |
| Standard Deviation | 347.2819 | Standard Deviation | 2046.481 |
| %RSD   |                             | 0.179534| %RSD                   | 0.553266 |

*Mean for six independent analysis, mean±SD, n=6, RSD =relative standard deviation

Table 6: Limit of detection and quantitation

| API         | Concentration | Intercept | Slope mean | LOD | LOQ |
|-------------|---------------|-----------|------------|-----|-----|
| Salicylic acid |                | 18267     | 19774      | 17719 | 1064.14112 | 35992.3333 | 0.09756 µg/ml | 0.2956 µg/ml |
| Imiquimod     |                | 34160     | 34149      | 35575 | 820.144499 | 61466.3333 | 0.0944031 | 0.13334 µg/ml |

*mean±SD, n=3, LOD =limit of detection, LOQ =limit of quantitation
Table 7: Robustness of salicylic acid and imiquimod at different flow rates and column temperature

| Conc. (µg/ml) | Flow rate 0.690 ml/min | Flow rate 0.700 ml/min | Flow rate 0.710 ml/min |
|--------------|-------------------------|-------------------------|-------------------------|
|              | Salicylic acid         | Imiquimod               | Salicylic acid         | Imiquimod               | Salicylic acid         | Imiquimod               |
| 5, 0.5       | 185355                 | 336750                  | 185716                 | 331298                  | 179209                 | 322635                  |
| 5, 0.5       | 186035                 | 331907                  | 185978                 | 327514                  | 180088                 | 319692                  |
| Mean         | 184881                 | 331433                  | 185234                 | 332858                  | 181071                 | 323943                  |
| SD           | 185690.333             | 333363.333              | 185642.7              | 330556.7              | 180996                 | 322090                  |
| %RSD         | 0.54889634             | 0.8826704               | 0.263284               | 0.831339               | 0.518676               | 0.675983                |

Table 8: Robustness of salicylic acid and imiquimod at different mobile phase ratio and different wavelength

| Conc. (µg/ml) | Buffer: ACN 49.9:50.1 | Buffer: ACN 50.1:49.9 | Buffer: ACN 50:50 |
|--------------|------------------------|------------------------|-------------------|
|              | Salicylic acid         | Imiquimod               | Salicylic acid         | Imiquimod               | Salicylic acid         | Imiquimod               |
| 5, 0.5       | 192990                 | 371471                  | 193807              | 370423                  | 189534                 | 366900                  |
| 5, 0.5       | 194999                 | 369934                  | 194057              | 370772                  | 194165                 | 366835                  |
| 5, 0.5       | 195544                 | 368306                  | 189264              | 368613                  | 193229                 | 369038                  |
| Mean         | 194511                 | 369903.7               | 192376              | 369936                  | 192309.3               | 367591                  |
| SD           | 1345.11598             | 1582.718               | 2697.968             | 1158.964               | 2448.648               | 1253.56                 |
| %RSD         | 0.69153723             | 0.427873               | 1.402445             | 0.313282               | 1.237286               | 0.34102                 |

Table 9: Ruggedness study by analyst 1 and analyst 2

| Conc. (µg/ml) | Analyst 1 | Analyst 2 |
|--------------|-----------|-----------|
|              | Salicylic acid | Imiquimod | Salicylic acid | Imiquimod |
| 5, 0.5       | 193925     | 371153      | 20.3497       | 346815    |
| 5, 0.5       | 194588     | 371303      | 198780       | 384112    |
| Mean         | 193199     | 371146      | 196176       | 380535    |
| SD           | 193870.6667 | 371200.6667 | 199484.3333 | 383820.6667 |
| %RSD         | 0.358808865 | 0.023893371 | 1.860283181 | 0.820772054 |
Fig. 10: Chromatogram of salicylic acid and imiquimod by analyst 1 (A) and analyst 2 (B)

Table 10: Specificity data of salicylic acid and imiquimod

| Conc.(µg/ml) | Specificty | Salicylic acid | Imiquimod |
|--------------|------------|----------------|-----------|
|              | Area       |                |           |
| 5, 0.5       | 189068     | 336047         |           |
| 5, 0.5       | 188871     | 335741         |           |
| 5, 0.5       | 189556     | 333678         |           |
| Mean         | 189165     | 335155.3       |           |
| SD           | 352.6514   | 1288.524       |           |
| %RSD         | 0.186425   | 0.384456       |           |

*Conc. = concentration, mean±SD, n= 3, SD = standard deviation, %RSD = relative standard deviation

CONCLUSION

The RP-HPLC method was carried out on Nucleodur C18 (250 mm × 4.6 mm I.D., 5 µm) using low-pressure gradient elution mode. The limit of detection (LOD) and limit of quantification (LOQ) of salicylic acid was found to be 0.09756 µg/ml and 0.2956 µg/ml, respectively, and imiquimod was found to be 0.044031 µg/ml and 0.13334 µg/ml, respectively. The developed and validated method for imiquimod and salicylic acid was found to be sensitive, specific, and accurate and can be effectively applied for the simultaneous estimation of imiquimod and salicylic acid.

ACKNOWLEDGMENT

The Author is highly grateful to Dr. R. K Abhilashi, Chairman Abhilashi Group of Institutions, Mandi, India, for providing necessary facilities to carry out the research.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the author has contributed equally.

CONFLICT OF INTERESTS

Declared none

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