SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ABACAVIR AND DOLUTEGRAVIR BY UPLC METHOD

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INTRODUCTION

Antiretroviral therapy (ART) has evolved significantly over the last three decades since the development of the first nucleoside analogues NRTIs (nucleoside reverse transcriptase inhibitors). Since the arrival of triple therapy, the challenge of sustained and complete viral suppression has been solved for the majority of patients [1]. The major limiting factors for improving the long-term success of ART are tolerability and convenient administration of the pill burden [2]. The latest class of the antiretroviral drugs developed was integrase inhibitors (INI). Dolutegravir (fig. 1) is an integrase inhibitor, particularly focused on maintaining a favorable safety profile and a high-efficiency rate within a single-tablet regimen (STR). It improves resistance barrier and allowing co-formulation with an NRTI backbone. Dolutegravir has been compared against both other classes of Human Immunodeficiency Virus (HIV) antiretrovirals as well as other integrase nuclear strand inhibitors. In August 2013, Dolutegravir was approved by Food and Drug Administration (FDA) for its use in both patients who have never taken ART (ART-naïve) and patients who have taken ART (ART-experienced) [3-5]. It is predicted that very soon an STR containing dolutegravir, abacavir and lamivudine will become available.

Fig. 1: Chemical structure of dolutegravir

Abacavir (fig. 2) [6] is a synthetic analogue of the naturally occurring purine nucleoside, guanine and it is a type of NRTI (nucleoside analog reverse transcriptase inhibitor) with the HIV antiretroviral activity agent. It differs from other reverse transcriptase inhibitors (didanosine, lamivudine, stavudine, zalcitabine and zidovudine) in structure. It belongs to the class of a carbocyclic nucleosides analogue rather than a dideoxynucleoside analogue. It is converted by intracellular enzymes to the carbovir triphosphate, active metabolites. Abacavir is vigorous in vitro against HIV-1 and HIV-2. It is a poor inhibitor of cellular Deoxyribonucleic acid (DNA) polymerases α, β and γ. After oral administration of abacavir sulphate is rapidly absorbed and it is distributed extensively. An absolute bioavailability of abacavir sulphate is ~83%, which is not affected by food. In December 1998, abacavir is approved by FDA.

Fig. 2: Chemical structure of abacavir

Lamivudine (fig. 3) [7] is a drug in the same category of nucleoside reverse transcriptase inhibitors as abacavir. It is an analogue of cytidine and it can reduce both types of the reverse transcriptase of hepatitis B virus and HIV reverse transcriptase. Lamivudine administered orally, it is rapidly absorbed with a bioavailability of 80% to 87%. FDA approval granted in 1995 for lamivudine to use pediatric and adult based on increases in CD4 T-lymphocyte count [8] on a regimen of zidovudine and lamivudine compared with either drug alone or compared with a combination of zidovudine and zidovudine was initial approval [9]. Lamivudine is in combinations with several triple nucleoside analogues has been shown to lead to high virologic failure in previously untreated individuals [10-13].
In the literature, numerous methods were described to determine separately or in a combination of lamivudine, abacavir and dolutegravir with other drugs in pharmaceutical formulation [14-23]. Still, very few methods were reported to determine these drugs simultaneously in biological matrices by using high-performance liquid chromatography (HPLC), tetratrim and UV-visible spectrophotometer [24-35]. Literature survey results, till now no UPLC analytical work on the determination of this combination.

Our aim was to develop a simple, accurate, sensitive method for simultaneous determination of lamivudine, abacavir and dolutegravir in combined pharmaceutical dosage form by UPLC with UV detection, where simple mobile phase composition was used for chromatographic separation without any ion-pairing agent. Total retention time for analysis was short with a good resolution between these components. All these reasons make this new method really lucrative. This method was also validated for linearity, sensitivity, precision, accuracy, selectivity and degradation studies according to the International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Lamivudine, abacavir and dolutegravir were obtained from Pharmatrain (Kukatpally, Hyderabad). The chemicals and solvents used in this study were of analytical grade and HPLC grade, respectively. Potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate (K₂HPO₄), orthophosphoric acid and acetonitrile were obtained from Merck (Mumbai, India). Milli-Q-Water purification system manufactured by Millipore (USA) generated water having a resistivity of 18.2 MΩcm.

Equipment

Waters-AQCUITY UPLC consisted of binary solvent manager with part number: 186015001, sample manager with part number: 186015005, single column manager with part number: 186015007 and PDA detector with part number: 186015026 with Waters Empower 2 PC workation used for method development and validation.

Chromatographic conditions

The chromatographic analysis was performed in an isocratic elution mode for 8 min run time at ambient column temperature. The mobile phase consists of phosphate buffer (2.95 g of potassium dihydrogen phosphate and 5.45 g of dipotassium hydrogen phosphate in 1 l Milli-Q-Water) and adjusted pH to 3.0 with orthophosphoric acid-methanol (30:70%v/v), the flow rate of pump was set to 0.25 ml/min, Zodiac SIL RP C18 column (length 250 mm × 4.6 mm inner diameter, 3 μm particle size), the chromatogram was monitored with UV detector at 260 nm and injection volume was 5 μl. The mobile phase was used as diluent.

Methodology

Preparation of standard solution

The standard stock solution was prepared by taking accurately weighted 15 mg, 30 mg and 2.5 mg of lamivudine, abacavir and dolutegravir working standards into 10 ml clean and dried volumetric flask, add diluent let it be dissolved completely and using the same diluent make volume up to the mark. Take 1 ml of the above solution into 10 ml volumetric flask and add diluent to make it up to the mark. For the preparation of the standard solution, 1.6 ml of solution was taken from above stock solution into 10 ml volumetric flask and added diluent to make it up to the mark.

Preparation of sample solution

For the preparation of sample solution, 10 tablets were accurately weighed and crushed to obtain a fine powder. The quantity of powder equivalent to 15 mg of lamivudine, 30 mg of abacavir and 2.5 mg of dolutegravir was transferred to 10 ml volumetric flask and add diluent, let it be dissolved completely and using the same diluent make the volume up to the mark. Take 1 ml of the above solution into 10 ml volumetric flask and add diluent to make it up to the mark. The obtained solution was appropriately diluted with the mobile phase to get the final dilution of 0.024 mg/ml lamivudine, 0.048 mg/ml abacavir and 0.004 mg/ml dolutegravir.

RESULTS AND DISCUSSION

To optimize the RP-UPLC parameters, to reach a good resolution and peak tailing for lamivudine, abacavir and dolutegravir, many chromatographic parameters were tested. Several mobile phases of different ratios were analyzed to get good resolution, peak shape and to provide sufficient selectivity for the drugs. The phosphate buffer provided a higher sensitivity and selectivity than other buffers did. Using methanol and acetonitrile as organic components shown results of higher sensitivity, but varying the amounts of methanol and acetonitrile in the mobile phase affected the resolution, tailing factor, theoretical plates and run time. Varying the pH of the mobile phase resulted in poor peak shapes and poor resolution. So we introduced potassium dihydrogen phosphate and dipotassium hydrogen phosphate into the mobile phase to adjust the pH of the buffer to 3.0. The optimized mobile phase consisted of 2.95 g of potassium dihydrogen phosphate and 5.45 g of dipotassium hydrogen phosphate in 1 l Milli-Q-Water and adjusted pH to 3.0 with orthophosphoric acid-methanol (30:70%v/v). The column elution was monitored at 260 nm and the injection volume was 5 μl. The column oven temperature was maintained at 25 °C (ambient). The Zodiac SIL RP C18 (4.6 mm × 250 mm with a particle size of 3 μm) was used with a constant flow rate of 0.25 ml/min in isocratic mode. Retention times (Rt) of the drugs were evaluated for lamivudine, abacavir and dolutegravir. The theoretical plate numbers (N) were calculated for the principal peak and its degradation product. The theoretical plate numbers (N) were 27100, 2247 and 3175 respectively, in all the analytical runs. The standard and sample chromatograms were shown in fig. 4 and 5.

System suitability test

Before sample analysis, the chromatographic parameters used in this analysis must confirm the system suitability parameters within the limits. The retention time (Rt), tailing factor (T) and theoretical plate number (N) for the principal peak and its degradation product were evaluated for lamivudine, abacavir and dolutegravir. The tailing factors were 1.23, 1.25 and 1.29 for lamivudine, abacavir and dolutegravir, respectively. The theoretical plate numbers (N) were 2755, 2190 and 2693 respectively. The retention times (Rt) of the drugs were 1.763 min, 2.247 min and 3.175 min respectively, in all the analytical runs. The standard and sample suitability parameters (table 1) satisfied the USP guidelines and ICH guidelines.

| Parameters          | Lamivudine | Abacavir | Dolutegravir |
|---------------------|------------|----------|--------------|
| Retention time      | 1.763      | 2.247    | 3.175        |
| USP plate count     | 2755       | 2190     | 2693         |
| USP tailing         | 1.23       | 1.25     | 1.29         |
| Standard area       | 935905     | 185063   | 27100        |
Assay of pharmaceutical formulation

The proposed method was effectively applied to find lamivudine, abacavir and dolutegravir in their tablet dosage form. The results obtained (table 2) were comparable with the corresponding labelled amounts.

Method validation

According to the international conference on harmonization (ICH) guideline, ICH Q2(R1) [33], this method was validated.

Linearity

Calibration plots for the analytes were prepared with standard stock solutions to yield the concentration ranges of 15-75 µg/ml for lamivudine, 30-150 µg/ml for abacavir, 2.5-12.5 µg/ml for dolutegravir into the UPLC system. In between the ranges given above, five concentrations were taken and triplicate injection of each concentration was performed.

Calibration curves were plotted between analyte concentrations versus that analyte area. Linearity regression analysis of the data gave correlation coefficient value, slope and intercept. For concentration between 15 µg/ml and 150 µg/ml, the calibration curves were linear. By the values of the correlation coefficients (R), the linearity of the calibration curves was validated. The correlation coefficient was 0.999 for these three drugs. The results of the linearity experiment were listed in table 3. Linearity graphs were shown in fig. 6, 7 and 8.

| Parameters                              | Lamivudine | Abacavir | Dolutegravir |
|-----------------------------------------|------------|----------|--------------|
| Concentration range(µg/ml)              | 15-75      | 30-150   | 2.5-12.5     |
| Correlation coefficient                 | 0.999      | 0.999    | 0.999        |
| Intercept                               | 1397       | 21826    | 10988        |
| Slope                                   | 14378      | 6610     | 59803        |
Accuracy/Recovery

Accuracy was performed using a standard addition technique by recovery studies. The pre-analyzed samples were spiked with extra 50%, 100%, and 150% of each standard lamivudine, abacavir and dolutegravir and by using the proposed method mixtures were analyzed. The recovery studies were conducted in triplicate. The proposed method afforded a recovery of 98.60–101.69% after the additional standard drug solution was spiked with the presciently analyzed test solutions. The recovery percentages were in the ranges from 98.96 to 100.92 %, from 99.80 to 101.69 % and from 99.60 to 100.34% respectively. The values of the recovery (%) were shown in table 4, which indicates the accuracy of the proposed method.
For the precision, repeatability expressed the same chromate-

Dolutegravir

Abacavir

Lamivudine

Drug

Robustness of the method was performed by making slight
deliberate changes in the analytical methodology like flow rate and

Limit of detection (LOD) and limit of quantification (LOQ)

The lowest amount of analyte in the drug, which can be detected, but
not necessarily quantified, indicates the limit of detection (LOD). The
lowest amount of analyte in the drug, which can be quantitatively
determined with suitable precision and accuracy indicates the limit of
quantification (LOQ). The limit of quantification (LOQ) and limit of
detection (LOD) were determined based on the slope and the standard
deviation of the response using the signal-to-noise ratio (S/N) as per
ICH guidelines Q2(R1) 2005. The LODs for lamivudine, abacavir and
dolutegravir were found to be 0.021, 0.330 and 0.038 µg/ml and the
LOQs were 0.056, 1.320 and 0.095 µg/ml, respectively (table 6).

Robustness

Robustness of the method was performed by making slight
deliberate changes in the analytical methodology like flow rate and
solvent ratio. It was observed that this method did not significantly
affect in system suitability parameters like USP tailing factor,
theoretical plates and resolution, which confirmed that the
developed UPLC method is robust (table 7).

Table 4: Accuracy results of lamivudine, abacavir and dolutegravir

| Drug name | % concentration | Area | Amount added (mg) | Amount found (mg) | % recovery | mean recovery |
|-----------|-----------------|------|-------------------|------------------|------------|--------------|
| Lamivudine | 50% | 573733 | 7.5 | 7.42 | 98.96 | 99.9 |
|           | 100% | 1158357 | 15 | 14.98 | 99.9 | 100.92 |
|           | 150% | 175375 | 22.5 | 22.70 | 101.00 | |
| Abacavir  | 50% | 732134 | 15 | 15.2 | 101.52 | 101.00 |
|           | 100% | 1557348 | 30 | 29.94 | 99.8 | 100.92 |
|           | 150% | 2380289 | 45 | 45.76 | 101.69 | 100.92 |
| Dolutegravir | 50% | 541198 | 1.25 | 1.25 | 100.34 | 99.98 |
|           | 100% | 1074405 | 2.5 | 2.49 | 99.6 | 100.92 |
|           | 150% | 1618551 | 3.75 | 3.75 | 100.02 | 100.92 |

Data of n=3 replicates

Table 5: Precision and inter-day precision results for lamivudine, abacavir and dolutegravir

| Injection | Precision | Inter-day precision |
|-----------|-----------|---------------------|
|           | Lamivudine | Abacavir | Dolutegravir | Lamivudine | Abacavir | Dolutegravir |
| Injection 1 | 641219 | 831356 | 654221 | 637987 | 828667 | 652517 |
| Injection 2 | 641645 | 831763 | 654574 | 638983 | 829544 | 654557 |
| Injection 3 | 642197 | 832877 | 655600 | 639198 | 829935 | 654622 |
| Injection 4 | 643020 | 833975 | 656731 | 639852 | 830731 | 654726 |
| Injection 5 | 644273 | 835545 | 657468 | 639951 | 830995 | 655234 |
| Injection 6 | 642460 | 833403 | 656718 | 640553 | 831033 | 655761 |
| Average    | 642469.0 | 833153.2 | 655885.3 | 639420.6 | 830151.0 | 654697.9 |
| Standard deviation | 1083.8 | 1529.5 | 1302.6 | 899.8 | 942.9 | 1104.5 |
| % RSD      | 0.17 | 0.18 | 0.20 | 0.14 | 0.11 | 0.17 |

Data of n= 6 replicates

Table 6: LOD and LOQ values of lamivudine, abacavir and dolutegravir

| Drug      | LOD concentration (µg/ml) | LOQ concentration (µg/ml) |
|-----------|---------------------------|---------------------------|
| Lamivudine | 0.021                     | 0.056                     |
| Abacavir  | 0.330                     | 1.32                      |
| Dolutegravir | 0.038                 | 0.095                     |

Table 7: Robustness study for the UPLC method

| Drug      | Parameter | Retention time | Peak area | USP plate count | USP tailing |
|-----------|-----------|----------------|-----------|----------------|-------------|
| Lamivudine | Flow1     | 1.950          | 712143    | 2504            | 1.26        |
|           | Flow2     | 1.607          | 731317    | 2563            | 1.25        |
|           | Low Organic | 1.666       | 582337    | 2537            | 1.24        |
|           | High Organic | 1.550     | 712143    | 2504            | 1.26        |
| Abacavir  | Flow1     | 2.475          | 928580    | 2698            | 1.32        |
|           | Flow2     | 2.039          | 757879    | 2904            | 1.29        |
|           | Low Organic | 2.485       | 761420    | 2229            | 1.33        |
|           | High Organic | 2.375     | 928580    | 2698            | 1.32        |
| Dolutegravir | Flow1     | 3.488          | 731317    | 2809            | 1.38        |
|           | Flow2     | 2.877          | 596086    | 2421            | 1.34        |
|           | Low Organic | 4.705       | 595173    | 3060            | 1.44        |
|           | High Organic | 3.988      | 731317    | 2809            | 1.38        |
Degradation studies

According to stability testing of new drug substances and products, a guideline of ICH desires that to clarify the inherent stability characteristics of the active component stress testing was implemented. The aim of this work was to carry out the stress degradation studies on the lamivudine, abacavir and dolutegravir using the proposed method.

Formulation drug products were exposed to thermal stress, oxidative stress, photolytic, hydrolytic stress under acidic medium and basic medium. An ideal stability indicating method, quantifies the standard drug alone and also resolves its degradation products. So described different types of stress used were thermal, oxidative, photolytic, acidic and basic hydrolysis. Some unknown degradant peaks were observed in the acidic, basic, peroxide, photolytic and thermal studies. But based on the peak purity, no degradant peaks were reported at the retention time (RT) of lamivudine, abacavir and dolutegravir. Therefore, the drugs were stable up to the specified period of 12 h when the proposed method is used, or they are susceptible to acids, alkali, hydrogen peroxide, photolytic and thermal.

Hydrolytic degradation under acidic conditions

Pipette 3 ml from standard stock solution containing 0.15 mg/ml, 0.3 mg/ml and 0.025 mg/ml of lamivudine, abacavir and dolutegravir into a 10 ml flask and added 1.0 ml of 0.1N HCl. Then, the volumetric flask was kept at room temperature (RT) for 6 h and then neutralized with 0.1N NaOH and added with diluents up to the mark. By using 0.45-micron syringe filters, filtered the solution and placed in vials. The results showed multiple peaks for the degradation products. The degradations percentage of the drugs observed were 5.00%, 8.03% and 20.69% (table 8), here no degradant peaks were observed at a retention time (RT) of lamivudine, abacavir and dolutegravir.

Hydrolytic degradation under alkaline conditions

Pipette 3 ml from a standard stock solution containing 0.15 mg/ml, 0.3 mg/ml and 0.025 mg/ml of lamivudine, abacavir and dolutegravir into a 10 ml flask and added 1 ml of 0.1N NaOH. Then the volumetric flask was kept at room temperature (RT) for 6 h and then neutralized with 0.1N HCl and filled with diluents up to the mark. By using 0.45-micron syringe filters, filtered the solution and placed in vials. The results showed multiple peaks for the degradation products. The degradations percentage of the drugs observed were 5.00%, 8.03% and 20.69% (table 8), here no degradant peaks were observed at a retention time (RT) of lamivudine, abacavir and dolutegravir.

Table 8: Degradation results of lamivudine, abacavir and dolutegravir

| Type of degradation | Lamivudine | Abacavir | Dolutegravir |
|---------------------|------------|----------|--------------|
| Sample area         | % recovered| % of degradation | % recovered | % of degradation | % recovered | % of degradation |
| Acid                | 889116     | 95.00    | 5.00         | 179391       | 91.97       | 8.03          | 214934       | 79.31       | 20.69       |
| Alkali              | 801257     | 85.61    | 14.39        | 168274       | 86.27       | 13.73        | 225846       | 83.34       | 16.66       |
| Thermal             | 786258     | 84.01    | 15.99        | 185487       | 95.09       | 4.91         | 254892       | 82.89       | 17.11       |
| Oxidative           | 842575     | 90.03    | 9.97         | 160578       | 82.32       | 17.68        | 224635       | 94.06       | 5.94        |
| Photolytic          | 852547     | 89.96    | 11.04        | 162587       | 83.35       | 16.65        | 221578       | 81.76       | 18.24       |

This method is specific for the determination of lamivudine, abacavir and dolutegravir with no interference and with good linearity, accuracy and precision. We achieved good separation for selected drugs. In addition, this separation technique uses simple, low cost and short runtime. The chromatographic conditions of this method were optimized for a short 8 min run time in RP-UPLC. It is an excellent method for the quantification of lamivudine, abacavir and dolutegravir in their pharmaceutical dosage forms.

At present, only HPLC methods were available in this combination. No UPLC methods were found till date.

CONCLUSION

The proposed RP-UPLC method for determination of lamivudine, abacavir and dolutegravir was developed and validated in pharmaceutical formulations. The described method adapted the use of an economical and easily available mobile phase, stationary phase, convenient and easy extraction procedures. The method was sensitive enough to detect low concentration of 0.021 µg/ml, 0.330 µg/ml and 0.038 µg/ml for lamivudine, abacavir and dolutegravir respectively. Recovery of selected drugs from spiked control samples were >99% by using this method. A stability-indicating RP-UPLC method for the estimation of selected drugs in their solid dosage forms was established and validated in accordance with the ICH guidelines.

ABBREVIATION

ART: Antiretroviral therapy; INI: Integrase Inhibitors; STR: Single tablet regimen; NRTIs: Nucleoside Reverse Transcriptase Inhibitors; UV Detector: Ultraviolet Detector; RSD: Relative standard deviation; HPLC: High-Performance Liquid Chromatography; ICH: International Conference on Harmonization; SD: Standard deviation; PDA: Photo diode array; LOD: Limit of detection; LOQ: Limit of quantitation; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; HIV: Human Immunodeficiency Virus; UPLC: Ultra Performance Liquid Chromatography; USP: United States Pharmacopeia; RT: Retention time; RT: Room temperature.

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AUTHOR CONTRIBUTION

Corresponding author and first author proposed the design of the study. The first author drafted the manuscript and carried out the all
The authors declare that they have no conflict of interest. All the authors have contributed equally. The authors read and approved the final manuscript.

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