

# Development of a validated HPLC method for simultaneous determination of olanzapine and aripiprazole in human plasma

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**ABSTRACT:** A simple and rapid HPLC method was developed and validated for simultaneous determination of two antipsychotic drugs, olanzapine and aripiprazole, in spiked human plasma. Optimization studies were performed using experimental design approach. The most important analysis parameters were selected using fractional factorial design and optimum levels of these parameters were determined with Box-Behnken design. Separation was achieved on a monolithic column (Rp-18, 100-4.6 mm) set at 35°C with a flow rate of 0.8 ml/min. Gradient elution was performed with a mobile phase consisting of phosphate buffer (pH 3.14, 20 mM) and acetonitrile. Retention times of olanzapine and aripiprazole were 2.34 and 6.90 minutes, respectively. The method was linear in the range of 0.125-50.0 µg/ml for olanzapine and 0.500-50.0 µg/ml for aripiprazole. The quantification limits for olanzapine and aripiprazole were found 0.069 and 0.498 µg/ml, respectively. The method was found accurate and precise based on recovery values, which were between 99-102% and relative standard deviations, which were lower than 2%. Robustness of the method and the stability of analytes were also investigated.

**KEYWORDS:** Olanzapine; aripiprazole; HPLC; determination; experimental design; human plasma.

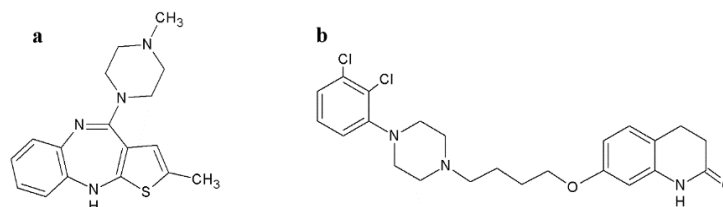
## 1. INTRODUCTION

Schizophrenia is a chronic and severe psychiatric condition characterized with psychosis, inactivity, lack of motivation and cognitive disorders [1]. Antipsychotic drugs are used for the treatment of schizophrenia as a first choice. Antipsychotic drugs are classified as typical and atypical antipsychotics. Atypical ones, which are developed recently, show their effects by blocking 5-HT<sub>2A</sub> receptors and they have less affinity to D<sub>2</sub> receptors compared to typical ones. Affinity profiles for other receptors depend on the drug. Drugs in the atypical group are much more effective at negative symptoms and cognitive disorders of schizophrenia and they are less likely to cause tardive dyskinesia and extrapyramidal side effects compared to typical antipsychotics. On the other hand, atypical ones have risk of developing obesity, high blood glucose and cholesterol levels, and metabolic syndrome due to high blood pressure [2].

Recently, the combined use of different antipsychotic drugs has been tried for treatment of drug-resistant schizophrenia patients. Combinations of antipsychotics with antidepressants; anticonvulsants with lithium; and the use of antipsychotics in combination with each other become a common practice in clinics although treatment guides suggest monotherapy [3]. One of this combination approaches is the addition of aripiprazole to olanzapine treatment to prevent dose-related side effects of olanzapine. Chemical structures of these molecules can be seen at Figure 1. Aripiprazole, which is an atypical antipsychotic drug approved in 2002 by FDA, is effective in positive and negative symptoms of schizophrenia. Its major advantages are lower risk for weight gain, increased lipid or prolactin levels, extrapyramidal side effects and sedation in comparison with typical and other atypical antipsychotics [4]. Another atypical antipsychotic, olanzapine has been used in the treatment of schizophrenia since 1996. It has advantages like lower risk for extrapyramidal side effects and minimal increase in prolactin levels. However, olanzapine has the risk for weight gain and elevated levels of insulin, leptin, and blood lipids in comparison with typical and other atypical antipsychotics [4].

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There are many reports in the literature about the combined use of olanzapine and aripiprazole [5-10]. As an example, Duggal reported the first case about this combination [5]. He observed a significant improvement in negative symptoms by the addition of aripiprazole to the treatment of a schizophrenic patient who had a partial response to olanzapine alone. Chen *et al.* reported two successful cases about the reduction of obesity and metabolic disturbance during the treatment of olanzapine with the administration of aripiprazole [6]. In these case reports, it appears that the addition of aripiprazole to olanzapine treatment to avoid the dose-related side effects seems to give positive results. However, the mechanism of this effect has not been fully understood yet.



**Figure 1.** Chemical structures of olanzapine (a) and aripiprazole (b).

The quantification of antipsychotics in biological matrices is important for therapeutic and forensic purposes [11]. In literature, a few methods were reported for the determination of olanzapine in plasma or serum samples using HPLC with UV detection [12-14]. Similarly, there are a number of methods for the determination of aripiprazole in plasma or serum samples using HPLC with UV detection [15-17]. In addition, several LC-MS methods developed for the determination of various antipsychotics including aripiprazole and olanzapine in plasma or serum samples were reported [18-22]. According to our knowledge, there is no HPLC method for the co-analysis of these two active substances.

Experimental design is being widely used for optimizing analytical methods. In this approach multivariate data are fitted to a linear or quadratic function in order to provide information about a system. Statistical theory is used to choose the values of each factor to optimize investigated responses [23]. Experimental design has advantages such as valid results and also time, effort and resource saving when compared to traditional univariate procedures [24].

The aim of this study is to develop an effective, simple and rapid HPLC method for simultaneously quantification of olanzapine and aripiprazole. Experimental design was used during the method development for a faster and easier optimization process compared with the univariate procedures. The separation of analytes was performed on a monolithic column considering its superior performance for fast separations with low pressure. An internal standard was used to get improved precision and accuracy for the quantification. Finally, developed method was validated according to the International Conference on Harmonization guideline [25]. Considering the advantages of HPLC, the development of a HPLC method for the simultaneous analysis of these two antipsychotic drugs is considered an important contribution to the literature.

## 2. RESULTS

### 2.1. Method optimization

Initially some preliminary studies were performed using a 20.0 µg/ml olanzapine and 20.0 µg/ml aripiprazole solution. Acetonitrile was selected as organic solvent and carbamazepine as internal standard during these studies. It was decided to use gradient elution due to very different retention times of olanzapine and aripiprazole. Studies were performed using a Chromolith High Resolution (Rp-18, 100-4.6 mm) column.

The fractional factorial design were used to investigate the importance of factors on responses. pH, buffer concentration, initial and final percentage of acetonitrile in gradient program, gradient time, flow rate and column temperature were selected as factors. Factors and their levels at fractional factorial design were given in Table 1. Their effects on six different responses, retention factors and tailing factors of two analytes and resolutions of following peaks ( $K_{OLA}$ ,  $K_{ARI}$ ,  $T_{OLA}$ ,  $T_{ARI}$ ,  $R_1$ ,  $R_2$ ), were investigated. Experiments were performed in random order according to the plan defined by fractional factorial design. Experimental design and data analysis were done using Minitab 17. Coefficients of response model can be seen in Table 2. pH, buffer concentration and initial percent of acetonitrile were selected as most important factors because only the model terms with corresponding p-value less than 0.05 are significant at 95% confidence level and they have effect on more than one response. Optimum values of other factors were determined considering separations, retention times and peak morphologies of the analytes. 50% acetonitrile was chosen as final

percentage of acetonitrile in 5 minutes of gradient program and also flow rate and column temperature were set at 0.8 ml/min and 35°C, respectively.

**Table 1.** Factor and levels for fractional factorial design and Box-Behnken design.

| Experimental design         | Factors                     | Levels |     |     |
|-----------------------------|-----------------------------|--------|-----|-----|
|                             |                             | -1     | 0   | +1  |
| Fractional factorial design | pH                          | 3.0    | 4.5 | 6.0 |
|                             | Buffer concentration        | 10     | 20  | 30  |
|                             | Initial percent of ACN      | 20     | 25  | 30  |
|                             | Final percent of ACN        | 40     | 45  | 50  |
|                             | Gradient time               | 3      | 4   | 5   |
|                             | Flow rate                   | 0.8    | 0.9 | 1.0 |
|                             | Temperature                 | 25     | 30  | 35  |
| Box-Behnken design          | pH (x1)                     | 3.0    | 4.0 | 5.0 |
|                             | Buffer concentration (x2)   | 10     | 15  | 20  |
|                             | Initial percent of CAN (x3) | 20     | 25  | 30  |

ACN: Acetonitrile

**Table 2.** Calculated coefficients for fractional factorial design.

| Coefficients           | K <sub>OLA</sub> | K <sub>ARI</sub> | T <sub>OLA</sub> | T <sub>ARI</sub> | R <sub>1</sub> | R <sub>2</sub> |
|------------------------|------------------|------------------|------------------|------------------|----------------|----------------|
| Constant               | 2.325*           | 8.311*           | 1.5713*          | 1.2484*          | 15.95*         | 15.00*         |
| pH                     | 1.643*           | 3.952*           | 0.1457           | 0.0358           | -13.72*        | 8.55*          |
| Buffer concentration   | -0.248*          | -0.729           | -0.2124*         | -0.0277          | 0.38           | 0.84           |
| Initial percent of ACN | -0.415*          | 0.667            | 0.1012           | 0.0214           | -2.52*         | 3.40*          |
| Final percent of ACN   | -0.110           | -1.504*          | -0.0404          | 0.0110           | -0.63          | -0.23          |
| Gradient time          | 0.107            | 0.311            | -0.0175          | 0.0073           | 0.98           | 0.94           |
| Flow rate              | 0.059            | 0.467            | 0.2033*          | 0.0101           | -0.71          | -0.45          |
| Temperature            | 0.098            | -0.961           | -0.0028          | -0.0300          | -0.43          | -2.32          |

K<sub>OLA</sub>: Retention factor of olanzapine; K<sub>ARI</sub>: Retention factor of aripiprazole; T<sub>OLA</sub>: Tailing factor of olanzapine; T<sub>ARI</sub>: Tailing factor of aripiprazole; R<sub>1</sub>: Resolution between olanzapine and carbamazepine; R<sub>2</sub>: Resolution between carbamazepine and aripiprazole, ACN: Acetonitrile. \* Coefficients significant for p-value < 0.05.

Box-Behnken design was used to optimize the selected factors. Factors and their levels at Box-Behnken design were given in Table 1. After performing appropriate experiments in random order according to the plan defined by Box Behnken design, data analysis were done using Minitab 17. As seen in Table 3, effects of all three factors were found to be significant on most responses. Derringer's desirability function was used to optimize four responses (retention factors and tailing factors of two analytes) simultaneously. Each response was transformed to a desirability function using equations given below (Equation 1-3). Equation 1, 2 or 3 were selected depending on the target value for the response which is a maximum or minimum value or located between a lower and upper limit, respectively. Overall desirability (D) was calculated by geometrical mean of individual desirability functions. Desirability functions have a value between 0, for a completely undesirable response, and 1, for a fully desired response. Then, calculations were made using overall desirability as only response. Coefficients of response model for overall desirability function can be seen in Table 3.

$$d = \begin{cases} 0 & \text{if } Y_i \leq L_i \\ \left[ \frac{Y_i - L_i}{T_i - L_i} \right]^s & \text{if } L_i < Y_i \leq T_i \\ 0 & \text{if } Y_i \geq T_i = U_i \end{cases} \quad (1)$$

$$d = \begin{cases} 1 & \text{if } Y_i \leq T_i = L_i \\ \left[ \frac{Y_i - U_i}{T_i - U_i} \right]^s & \text{if } U_i < Y_i \leq T_i \\ 0 & \text{if } Y_i \geq U_i \end{cases} \quad (2)$$

$$d = \begin{cases} \left[ \frac{Y_i - L_i}{T_i - L_i} \right]^s & \text{if } L_i \leq Y_i \leq T_i \\ \left[ \frac{Y_i - U_i}{T_i - U_i} \right]^t & \text{if } T_i < Y_i < U_i \\ 0 & \text{if } Y_i < L_i \text{ or } Y_i > U_i \end{cases} \quad (3)$$

$L_i$  is lower value,  $U_i$  is upper value and  $T_i$  is target value that is desired for the response  $Y_i$ .  $s$  and  $t$  are the weights and when they are equal to 1, the desirability function is linear [26].

**Table 3.** Calculated coefficients for Box-Behnken design.

| Coefficients | $K_{OLA}$ | $K_{ARI}$ | $T_{OLA}$ | $T_{ARI}$ | D        |
|--------------|-----------|-----------|-----------|-----------|----------|
| b0           | 1.2522*   | 4.3957*   | 1.8903*   | 1.3132*   | 0.5339*  |
| b1           | 0.8782*   | 0.7478*   | 0.2241*   | 0.0973*   | -0.0469  |
| b2           | -0.0322   | -0.0320*  | -0.1058*  | 0.0009    | 0.0631*  |
| b3           | -0.4534*  | -0.4653*  | -0.0588   | -0.0321   | 0.0244   |
| b11          | 0.1903*   | 0.4767*   | -0.1976*  | -0.0441   | -0.1092* |
| b22          | 0.0724*   | -0.0037   | 0.2111    | -0.0661*  | 0.0311   |
| b33          | 0.0969*   | -0.1025*  | -0.0447   | 0.0576    | -0.0597  |
| b12          | -0.0061   | -0.0184   | -0.0382   | -0.0541   | 0.0640   |
| b13          | -0.2610*  | 0.0749*   | 0.0540    | -0.0759*  | 0.1789*  |
| b23          | 0.0161    | -0.0025   | -0.0079   | -0.0049   | -0.0443  |
| $R^2_D$      | 0.9990    | 0.9995    | 0.9223    | 0.9354    | 0.8617   |
| Adj. $R^2_D$ | 0.9973    | 0.9985    | 0.7823    | 0.8192    | 0.8510   |

$K_{OLA}$ : Retention factor of olanzapine;  $K_{ARI}$ : Retention factor of aripiprazole;  $T_{OLA}$ : Tailing factor of olanzapine;  $T_{ARI}$ : Tailing factor of aripiprazole; D: Overall desirability,  $R^2_D$ : Determination coefficient, Adj.  $R^2_D$ : Adjusted determination coefficient.  
\* Coefficients significant for p-value < 0.05.

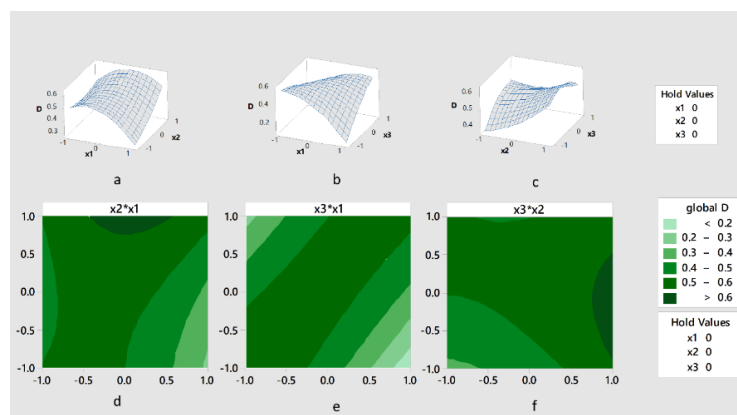
In order to find overall desirability's determination coefficient ( $R^2_D$ ), predicted responses, predicted individual desirability functions and predicted overall desirability functions were calculated. Equations 4 and 5 were used to calculate  $R^2_D$  and adjusted  $R^2_D$  values.

$$R^2_D = 1 - \frac{SSE}{SSTO} = 1 - \frac{\sum (D - D_{pred})^2}{\sum (D - \bar{D})^2} \quad (1)$$

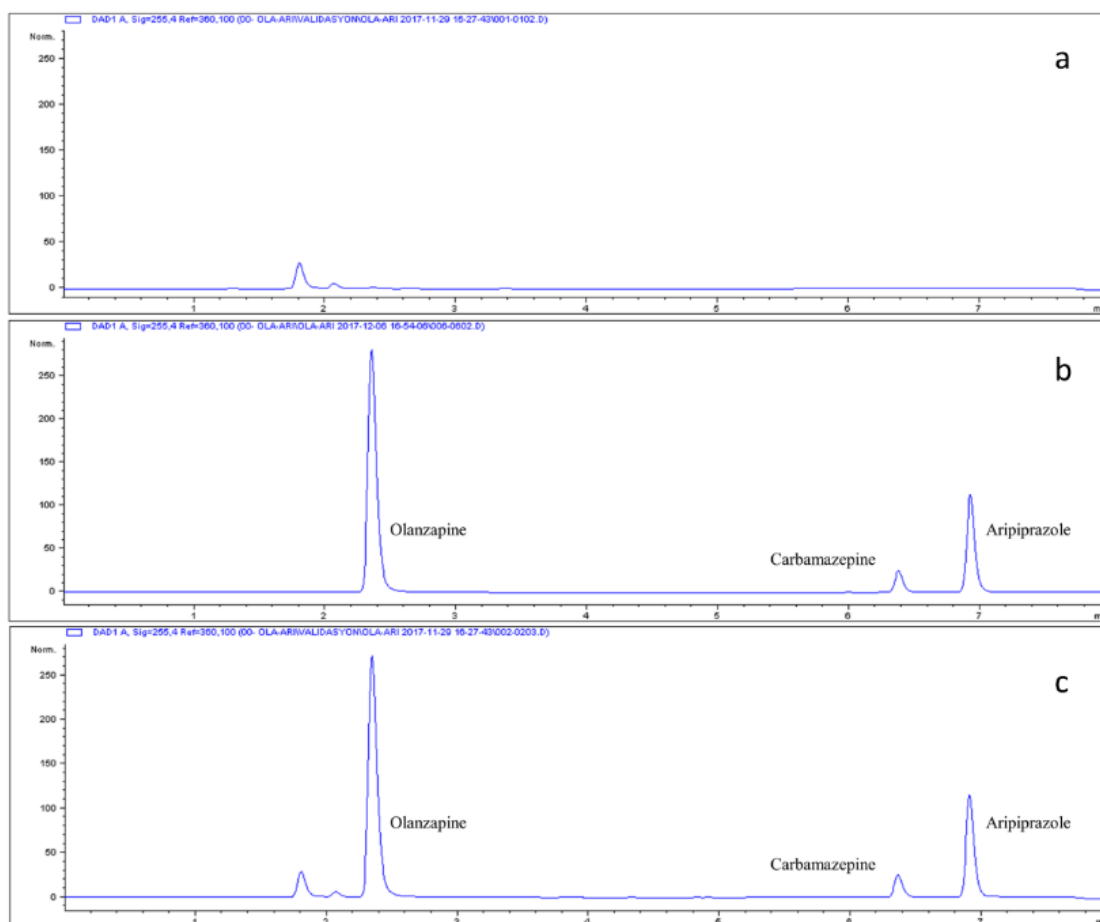
$$Adj. R^2_D = 1 - \frac{SSE}{SSTO} = 1 - \frac{\sum (D - D_{pred})^2 / (N - 2)}{\sum (D - \bar{D})^2 / (N - 1)} \quad (2)$$

SSE is the sum of squares due to error, SSTO is the total sum of squares,  $D_{pred}$  is the predicted D value for each case and  $\bar{D}$  is the mean of D values. High  $R^2_D$  and adjusted  $R^2_D$  values, which were found as 0.8617 and 0.8510, respectively, indicate the goodness-of-fit for this model.

Optimal conditions were found using Minitab 17 response optimizer and confirmed with the surface and counter plot graphics (Figure 2). From these results, pH 3.14, 20 mM buffer concentration and 20% initial acetonitrile concentration were predicted as optimal. Finally, an analysis was performed at theoretically optimal conditions (Figure 3c) and appropriate retention times and peak symmetries with good resolution were obtained.



**Figure 2.** Counter plot and surface plot graphics: (a and d)  $D(\text{desirability})=f(\text{buffer concentration } (x_2), \text{ pH of the mobile phase } (x_1))$ ; (b and e)  $D(\text{desirability})=f(\text{initial percent of acetonitrile } (x_3), \text{ pH of the mobile phase } (x_1))$ ; (c and f)  $D(\text{desirability})=f(\text{initial percent of acetonitrile } (x_3) \text{ and buffer concentration } (x_2))$ .



**Figure 3.** Chromatograms of blank plasma (a), standard solution (containing 48.0 µg/ml olanzapine, 48.0 µg/ml aripiprazole and 10.0 µg/ml carbamazepine (b), quality control solution (containing 48.0 µg/ml olanzapine, 48.0 µg/ml aripiprazole and 10.0 µg/ml carbamazepine. Mobile phase: phosphate buffer (pH 3.14 and 20 mM) (A) and acetonitrile (B); flow rate 0.8 ml/min; column temperature 35°C; detection wavelength 255 nm.

## 2.2. Method validation

System suitability was assessed by seven replicated analyses of quality control solution at middle concentration. The system suitability parameters were given in Table 4. All parameters were found acceptable as seen in the table.

The developed method was validated according to the recommendations of International Conference on Harmonization guideline Q2R1 [25]. Specificity, linearity, accuracy and precision, detection and quantitation limits and robustness of the developed method were investigated. Besides, the stability of analytes was shown.

The specificity of the method was assessed by comparing the chromatograms of blank plasma and quality control solutions (Figure 3a, 3c). No interfering peaks were observed in retention times of analytes at the chromatogram of blank plasma (Figure 3a). The matrix effect was assessed by comparing responses of quality control solutions with standard solutions as references (Figure 3b). Peak area ratios for quality control solutions versus standard solutions were found between 99 and 102%. Thus, no matrix effect was observed.

In order to investigate the linearity of the method, the calibration curves were constructed using different concentrations ranging from 0.125 to 50.0 µg/ml for both of the analytes. The method was linear in the range of 0.125-50.0 µg/ml for olanzapine and 0.500-50.0 µg/ml for aripiprazole. Signal to noise ratio of 3:1 and 10:1 were used to estimate the detection and quantitation limits, respectively. Obtained calibration equations, coefficients of determination ( $R^2$ ), the limits of detection and quantitation are summarized in Table 5.

**Table 4.** System suitability parameters (6.00 µg/ml Olanzapine and 6.00 µg/ml Aripiprazole, n=7)

| Parameters                | Observed value          |                        | Requested value |
|---------------------------|-------------------------|------------------------|-----------------|
|                           | Olanzapine              | Aripiprazole           |                 |
| Retention time (min)      | 2.34                    | 6.90                   | -               |
| Retention factor (k)      | 0.88                    | 4.53                   | 2-10            |
| Tailing factor (T)        | 1.40                    | 1.18                   | ≤2              |
| Resolution (Rs)           | 33.48 (R <sub>1</sub> ) | 4.85 (R <sub>2</sub> ) | >2              |
| RSD (%) of retention time | 0.06                    | 0.02                   | <1              |
| RSD (%) of peak area      | 0.12                    | 0.54                   | <1              |

R<sub>1</sub>: Resolution between olanzapine and carbamazepine, R<sub>2</sub>: Resolution between carbamazepine and aripiprazole, RSD: Relative standard deviation.

**Table 5.** Linearity data.

| Analytes     | Range (µg/ml)    | Regression equation   | R <sup>2</sup> | LOD (µg/ml) | LOQ (µg/ml) |
|--------------|------------------|-----------------------|----------------|-------------|-------------|
| Olanzapine   | 0.125-50.0 (n=9) | y = 0.2367 x + 0.0136 | 0.9999         | 0.021       | 0.069       |
| Aripiprazole | 0.500-50.0 (n=7) | y = 0.0878 x - 0.0087 | 0.9998         | 0.150       | 0.498       |

R<sup>2</sup>: Coefficient of determination, LOD: Limit of detection, LOQ: Limit of quantitation.

The accuracy, intra and interday precision of the developed method were evaluated by seven replicated analyses of quality control solutions. As seen in Table 6, accuracy and precision were expressed as relative error and relative standard deviation, respectively. The method was found accurate based on recovery values, which were between 99-102%. The low relative standard deviations (lower than 2%) indicate a good method precision.

**Table 6.** Accuracy and precision data.

| Added Concentration (µg/ml) | Intraday (n=7) |         | Interday (n=21) |         |        |
|-----------------------------|----------------|---------|-----------------|---------|--------|
|                             | Recovery (%)   | RSD (%) | Recovery (%)    | RSD (%) |        |
| Olanzapine                  | 0.75           | 101.75  | 1.0418          | 101.91  | 1.1200 |
|                             | 6.00           | 99.51   | 0.1921          | 99.11   | 0.3424 |
|                             | 48.0           | 99.76   | 0.2424          | 100.84  | 0.7911 |
| Aripiprazole                | 0.75           | 101.46  | 1.6715          | 101.41  | 1.3772 |
|                             | 6.00           | 99.54   | 0.6981          | 99.40   | 1.1331 |
|                             | 48.0           | 101.73  | 0.6056          | 101.88  | 0.5532 |

RSD: Relative standard deviation.

The robustness of the method was investigated performing analyses under intentionally modified chromatographic conditions. Small changes were made in buffer concentration, pH and flow rate of mobile phase and column temperature. Recoveries were calculated in these new conditions. Recoveries which are between 98 and 102% and small changes in retention times indicate the robustness of the method (Table 7).

**Table 7.** Robustness data.

| Parameter                 |      | Olanzapine   |                      | Aripiprazole |                      |
|---------------------------|------|--------------|----------------------|--------------|----------------------|
|                           |      | Recovery (%) | Retention time (min) | Recovery (%) | Retention time (min) |
| Buffer concentration (mM) | 18   | 99.18        | 2.376                | 100.86       | 6.936                |
|                           | 22   | 98.30        | 2.359                | 99.00        | 6.913                |
| pH                        | 3.04 | 98.51        | 2.328                | 100.55       | 6.929                |
|                           | 3.24 | 100.49       | 2.422                | 101.37       | 6.940                |
| Flow rate (ml/min)        | 0.7  | 98.74        | 2.687                | 100.44       | 7.513                |
|                           | 0.9  | 100.81       | 2.115                | 100.12       | 6.465                |
| Column temperature (°C)   | 33   | 100.71       | 2.366                | 101.93       | 6.956                |
|                           | 37   | 98.83        | 2.380                | 101.00       | 6.907                |

The stability of analytes were also investigated using quality control solutions which were stored at room temperature for 24 hours or at -20°C for two weeks or were exposed to three freeze thaw cycles for this purpose. The analytes were stable under all conditions according to calculated recoveries seen in Table 8.

**Table 8.** Stability data.

| Added Concentration (µg/ml) | Short-term stability (24 h, room temperature) |         | Long-term stability (2 weeks, -20°C) |         | Freeze-thaw stability (3 cycles) |         |        |
|-----------------------------|---|---------|--------------------------------------|---------|----------------------------------|---------|--------|
|                             | Recovery (%)                                  | RSD (%) | Recovery (%)                         | RSD (%) | Recovery (%)                     | RSD (%) |        |
| Olanzapine                  | 0.75  | 98.67   | 0.4041                               | 97.01   | 0.1332                           | 99.88   | 0.3915 |
|                             | 6.00  | 98.58   | 0.2273                               | 98.78   | 0.1991                           | 98.34   | 0.2763 |
|                             | 48.0  | 101.24  | 0.1914                               | 97.26   | 0.7940                           | 100.74  | 0.2102 |
| Aripiprazole                | 0.75  | 99.31   | 0.7195                               | 100.27  | 0.3934                           | 98.97   | 0.5995 |
|                             | 6.00  | 98.83   | 0.1464                               | 96.44   | 0.1523                           | 99.49   | 0.2952 |
|                             | 48.0  | 99.61   | 0.0771                               | 91.53   | 0.8782                           | 98.82   | 0.1616 |

RSD: Relative standard deviation.

### 3. CONCLUSION

In this study, a HPLC method for the simultaneous determination of olanzapine and aripiprazole was developed and validated. Proposed method is the first reported HPLC method that enables the analysis of these two drugs together, in the literature. For a fast and easy optimization, experimental design was employed. Analytes and internal standard were separated in 8 minutes using a monolithic column at optimal conditions. The method was validated in terms of specificity, linearity, accuracy and precision, detection and quantitation limits and robustness. All studies were carried out using spiked human plasma samples to demonstrate the applicability of the method to biological samples. The limits of quantitation for olanzapine and aripiprazole were found 0.069 µg/ml and 0.498 µg/ml, respectively. These results demonstrated that the proposed method is suitable for the accurate quantification of the analytes in human plasma at low concentrations. The proposed fast and simple method can be used safely in the routine analysis of olanzapine and aripiprazole.

### 4. MATERIALS AND METHODS

#### 4.1. Materials

Olanzapine and carbamazepine were purchased from Sigma-Aldrich (Germany). Aripiprazole was obtained from Santa Cruz Biotechnology. All solvents were HPLC grade from Merck (Germany). Potassium dihydrogen phosphate was from Merck (Germany). Sodium acetate trihydrate, phosphoric acid and acetic acid were from VWR Chemicals.

## 4.2. Preparation of standard solutions and quality control solutions

Stock solutions of olanzapine (1.0 mg/ml), aripiprazole (0.5 mg/ml) and carbamazepine (1.0 mg/ml) were prepared in methanol individually and stored at -20°C. Aliquots of olanzapine and aripiprazole stock solutions were mixed and diluted serially with methanol. Carbamazepine stock solution was also diluted with methanol separately. For investigating matrix effect, standard solutions were prepared by diluting these solutions with water.

Calibration solutions were prepared by spiking serially diluted solutions of analytes and internal standard into drug-free human plasma. Plasma proteins were precipitated with acetonitrile at a ratio of 1 volume sample to 2 volumes acetonitrile. All solutions were vortexed and centrifuged, then clear supernatants were separated and filtered before analysis. At last, calibration standards containing 0.125, 0.250, 0.500, 1.00, 2.00, 4.00, 10.0, 25.0, 50.0 µg/ml olanzapine and aripiprazole were obtained.

Quality control solutions were prepared in the same way as calibration solutions but at different concentrations. Low, middle and high levels of quality control solutions were containing 0.750, 6.00 and 48.0 µg/ml olanzapine and aripiprazole.

## 4.3. Instrumentation and chromatographic conditions

An HPLC system (Agilent 1290 Infinity) was used during method development. This system was consisted of a binary pump, a degasser, an autosampler, a thermostated column compartment, and a diode array detector. A monolithic column (Chromolith High Resolution, Rp-18, 100-4.6 mm) set at 35°C and the mobile phase consisted of phosphate buffer (pH 3.14 and 20 mM) (A) and acetonitrile (B) was used for separation. A gradient program that is linear from 20 to 50% B (in 5 minutes) was employed. Run time was 8 minutes and the column was equilibrated to the starting conditions for 2 minutes. Flow rate was 0.8 ml/min. Carbamazepine was used as internal standard. Detection was performed with diode array detector at 255 nm.

## 4.4. Software

Experimental design study and validation calculations were performed using Microsoft Excel, Minitab 17 and GraphPad Prism 6 programs.

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